THE ROLE OF INFLAMMATION IN LIPID METABOLISM

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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 3 mei 2012
klokke 16.15 uur

door

Janna Alida van Diepen

geboren te Wageningen
in 1983

PROMOTIECOMMISSIE

Promotor:	Prof. Dr. L.M. Havekes Prof. Dr. J.A. Romijn Prof. Dr. P.C.N. Rensen
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The work described in this thesis was performed at the department of Endocrinology and Metabolic Diseases at the Leiden University Medical Center, Leiden, The Netherlands.

The research described in this thesis was financially supported by the Netherlands Organization for Scientific Research (NWO 917.76.301) and the Dutch Diabetes Research Foundation (2005.01.003).

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged

ISBN: 978-94-6182-090-7

The printing of this thesis was financially supported by:
The Dutch Diabetes Research Foundation
J.E. Jurriaanse Stichting
Novo Nordisk B.V.
Boehringer Ingelheim
Greiner Bio-One

Layout & printing: Off Page, www.offpage.nl

TABLE OF CONTENTS

1

GENERAL INTRODUCTION

TRIGLYCERIDE METABOLISM

Lipids and lipoproteins

Triglycerides (TG) and cholesterol are the most common lipids in our diet and essential for the human body. TG are the main source of energy, and can be stored in white adipose tissue (WAT), used for ATP production in skeletal muscle and heart, and used for generating heat in brown adipose tissue (BAT). Cholesterol is essential, as a crucial component of cellular membranes and as precursor for steroid hormone synthesis. Since lipids are hydrophobic, they are transported in the blood as constituents of soluble particles called lipoproteins. These lipoproteins are composed of a lipid-rich core containing TG and esterified cholesterol, surrounded by a amphiphilic monolayer of phospholipids (PL), unesterified free cholesterol and one or more apolipoproteins. The latter facilitate formation of the lipoproteins and modulate the activity of proteins (i.e. enzymes and transfer factors) involved in lipoprotein remodeling in the circulation. Lipoproteins are subdivided into different classes based on their density, namely (from lowest to highest density) chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). Both chylomicrons and VLDL are consist mainly of TG and are therefore called TG-rich lipoproteins. The metabolism of TG-rich lipoproteins will be discussed in more detail in the following section and is schematically depicted in Figure 1.

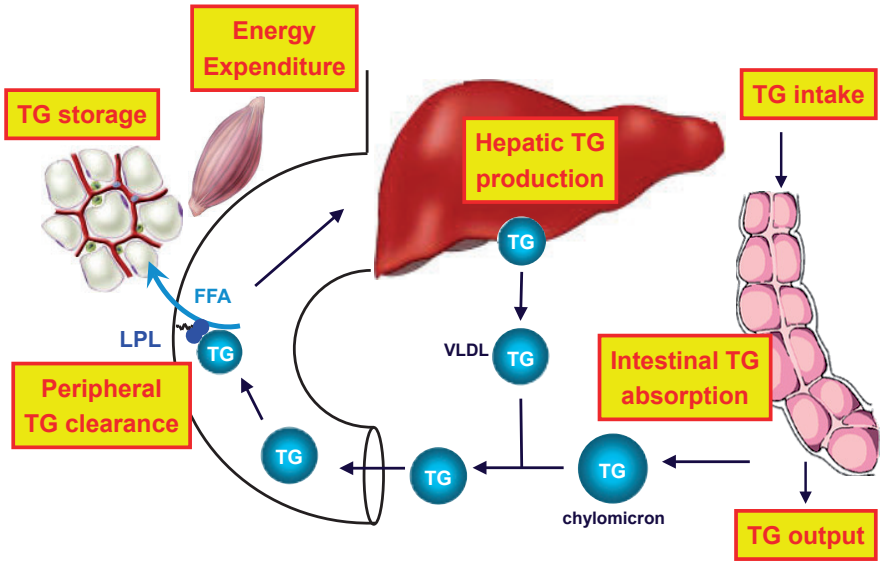

Figure 1. Schematic representation of TG-rich lipoprotein metabolism. See text for explanation.

TG-rich lipoprotein metabolism

In the intestine, dietary TG are lipolyzed by pancreatic lipases into 2-monoacylglycerol and fatty acids (FA), both of which are subsequently taken up by the intestinal cells (enterocytes) where the lipolysis products are re-synthesized into TG.[1] Enterocytes synthesize apoB that is lipidated in the endoplasmatisch reticulum (ER) by the microsomal TG transfer protein (MTP), resulting in the formation of a small apoB containing particle. After fusion with larger protein-free lipid droplets, a prechylomicron is formed which is transported towards the Golgi and secreted via exocytosis.[2] Chylomicrons are secreted into the lymph from which they are transported via the blood for storage (adipose tissue), or energy supply (skeletal muscle and heart)[3] or heat generation (brown adipose tissue).[4]

When no food is entering the intestine (during fasting), the liver is the organ that ensures a supply of TG by secretion of TG-rich VLDL particles. Assembly of VLDL in hepatocytes, the most predominant cell type in the liver, involves a similar pathway as chylomicron synthesis including MTP-mediated transfer of TG to apoB and fusion of the generated particle with other lipid droplets, resulting in the formation of mature VLDL that is released into the circulation.[5,6] The assembly of VLDL is highly dependent on the availability of TG within the hepatocyte. The main direct sources for VLDL-TG are 1) plasma-derived FA that are released by the adipose tissue mainly in the fasted state and are esterified in the hepatocyte into TG, 2) cytosolic TG that previously accumulated in the hepatocyte and 3) *de novo* synthesized FA that is used for *de novo* synthesis of TG within hepatocytes. FA synthase (FAS) and stearyl-CoA desaturase-1 (SCD-1) represent the most important genes involved in the *de novo* synthesis of FA and TG, respectively.

In the circulation, TG that are derived from chylomicrons and VLDL are lipolyzed by capillary-bound lipoprotein lipase (LPL) into glycerol and FA,, the latter being subsequently taken up by underlying tissues including WAT, skeletal muscle, heart, and BAT. In the postprandial state (after a meal), LPL expression is highest in WAT, thereby directing most of the chylomicron-derived TG into adipose tissue for storage.[7,8] In the fasted state, LPL expression relatively increases in skeletal muscle as compared to WAT thereby ensuring VLDL-derived FA as energy source.[9] LPL activity in heart is less regulated by the feeding status[10] and regulation of LPL in BAT is currently unknown. Through hydrolysis of TG, and concomitant uptake of FA by the peripheral tissues, the TG-rich lipoproteins reduce in size and become residual CM remnants and VLDL remnants (i.e. IDL and LDL). These are taken up by the liver through specific receptors such as the LDL receptor and LDLr-related protein (LRP). [11]

OBESITY AND ASSOCIATED METABOLIC DISTURBANCES

When energy intake exceeds energy expenditure (i.e. positive energy balance), TG is stored in the body as WAT. A prolonged positive energy balance will thus lead to overweight and obesity. In fact, world-wide obesity is reaching epidemic proportions

due to a sedentary lifestyle combined with a caloric-rich diet. The adipose tissue has the largest capacity to store energy in the form of TG, by increasing both adipocyte hypertrophy (increase in cell size) as well as hyperplasia (increase in cell number). However, since expandability of adipose tissue is limited, excess TG is redirected towards non-adipose tissues such as skeletal muscle and liver, both of which are able to store TG to a more limited extent.[12] In fact, obesity is frequently accompanied by excess fat accumulation (steatosis) in liver[13] and muscle,[14] which has been linked to the development of metabolic disturbances such as insulin resistance, ultimately resulting in the onset of type 2 diabetes.[15-17] In addition to insulin resistance, the excess TG availability leads to an increased circulation of TG in the plasma which is called hypertriglyceridemia and an important risk factor for the development of atherosclerosis and, ultimaltely, cardiovascular diseases (CVD).

Obviously, obesity and associated metabolic disorders including insulin resistance and atherosclerosis could at least partly be prevented by reducing food intake and increasing regular physical activity levels. Unfortunately, these lifestyle changes have proven to be very difficult to accomplish which urges the development of therapeutic strategies combating obesity and its associated disorders.

METABOLIC INFLAMMATION

Inflammatory pathways have evolved as important mediators that may link obesity to metabolic disturbances. Obesity is accompanied by low-grade systemic inflammation, which is characterized by increased presence of cytokines and other markers of inflammation in the circulation.[18, 19]

Both white adipose tissue and the liver are largely involved in the onset and development of metabolic inflammation. The expansion of the adipose tissue during development of obesity coincides with the influx of macrophages and secretion of pro-inflammatory cytokines in adipose tissue of obese mice and humans.[20, 21] The initial trigger for macrophage infiltration in adipose tissue is suggested to be the secretion of pro-inflammatory cytokines by adipocytes upon expansion[22] that attract and activate infiltrating macrophages, as adipocyte size is correlated to the amount of macrophage infiltration in adipose tissue.[21] In addition to adipose tissue, HFD and obesity also induce inflammation in liver, as reflected by increased markers of hepatic inflammation (SAA and CRP) in obese subjects and increased hepatic NF-κB activity in mice fed a HFD.[23, 24]

The obesity-associated low-grade inflammation appears to be causally involved in the onset and development of metabolic disturbances such as insulin resistance and atherosclerosis. Pro-inflammatory cytokines such as IL-1β and TNFα that are secreted by the liver and adipose tissue can directly induce insulin resistance by interfering with insulin signaling pathways[25, 26] and induce formation of atherosclerotic plaques.[27, 28]

The transcription and activation of these pro-inflammatory cytokines is regulated by activation of other proteins, such as NF-κB or NLRP3 inflammasome-activated

caspase-1. NF-κB is a transcription factor that regulates expression of several pro-inflammatory cytokines including IL-1β and TNFα.[29] The NLRP3 inflammasome caspase-1 is a protein-complex that is crucial for activation of the pro-inflammatory IL-1β. The exact role of NF-κB and the NLRP3 inflammasome in metabolic inflammation in adipose tissue and liver remains to be determined, as well as its contribution to metabolic disturbances. This may reveal new potential therapeutic targets in the treatment of obesity-associated insulin resistance and atherosclerosis.

OUTLINE OF THE THESIS

Albeit that metabolic inflammation plays a key role in the development of insulin resistance and atherosclerosis, no current therapies exist that target inflammation in the treatment of these metabolic diseases. Better understanding of the interaction between inflammatory pathways and metabolic disturbances will help to develop new strategies to treat patients at risk for type 2 diabetes and CVD. The research described in this thesis was performed to gain more insight into the effect of various inflammatory pathways on the development of hyperlipidemia, insulin resistance and atherosclerosis.

The first part of this thesis focuses on the relation between inflammation and hyperlipidemia, which are both risk factors for the development of atherosclerosis. In **chapter 2**, current knowledge on the role of lipids and inflammatory processes in the development of atherosclerosis is described, as well as the interaction between lipid metabolism and inflammation that may aggravate the development of atherosclerosis. The effects of lipid-lowering drugs on inflammatory processes, as well as the effects of the anti-inflammatory drugs on lipid metabolism are discussed. In **chapter 3**, the role of the anti-inflammatory drug aspirin on lipid metabolism was evaluated. Specifically, the mechanism by which aspirin reduces plasma lipid levels was investigated in the hypertriglyceridemic APOC1 mouse model, by studying the effects of aspirin on VLDL-triglyceride metabolism.

The liver is an organ that plays an important role in triglyceride metabolism as well as inflammation. The aim of the study described in **chapter 4** was to specifically determine the effect of hepatic NF-κB activity on the development of hypertriglyceridemia, by evaluating whether transgenic activation of IKK-β only in the hepatocytes of E3L mice affected VLDL-triglyceride metabolism directly. Whether the hepatic NF-κB activity affects the development of atherosclerosis was subsequently explored in **chapter 5**.

Toll-like receptor 4 (TLR4) is a receptor of the immune system that activates NF-κB signaling. Endotoxin, a component of Gram-negative bacteria and a well known ligand for TLR4, can induce hyperlipidemia during infection by increasing hepatic VLDL-TG production. Upon high-fat feeding, saturated FA are believed to activate TLR4/ NF-κB signaling in the liver, suggesting that TLR4 might be an important link between obesity-induced hepatic inflammation and hyperlipidemia. Therfore, the aim of **chapter 6** was to investigated whether absence of TLR4 could reduce VLDL-TG production in high-fat fed mice.

The second part of this thesis describes studies exploring the role of the inflammasome-mediated caspase-1 activity in obesity and insulin resistance. The inflammasome complex is another part of the innate immune system that activates caspase-1 that is responsible for the activation of the pro-inflammatory cytokines IL-18 and IL-1ß. These two pro-inflammatory cytokines have been shown to be involved the development of obesity and insulin resistance. In **chapter 7**, we questioned whether absence of components of the inflammasome complex would affect HFD-induced obesity, adipose tissue inflammation and insulin resistance. Since absence of inflammasome-mediated caspase-1 markedly reduced HFD-induced obesity and insulin resistance, the mechanism by which absence of caspase-1 reduces adipose tissue mass was investigated in **chapter 8**, by evaluating TG-rich lipoprotein metabolism in caspase-1 deficient and wild-type mice.

Finally, the results from these studies and its implications are discussed in **chapter 9.**

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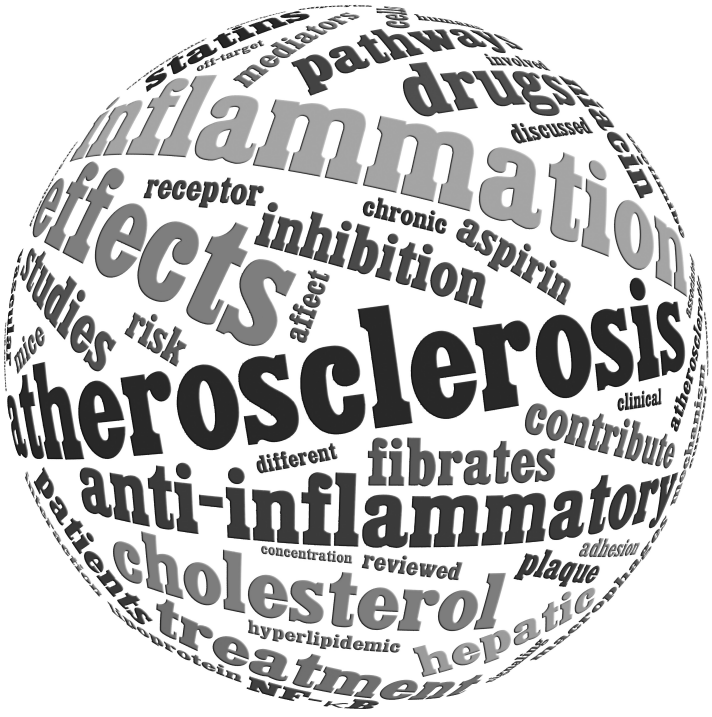

INTERPLAY BETWEEN LIPID METABOLISM AND INFLAMMATION – TWO TARGETS IN THE TREATMENT OF ATHEROSCLEROSIS

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Submitted

ABSTRACT

Hyperlipidemia and inflammation are well known risk factors for the development of atherosclerosis and both have been shown to play a causal role in the progression of atherosclerotic plaques. In recent years, a strong interplay between lipid metabolism and inflammation has been discovered, which can explain why lipid-lowering drugs and anti-inflammatory drugs reduce atherosclerosis beyond expectations based on their primary mode of action. In this review, we summarize the off-target effects of lipid-lowering drugs (*i.e.* statins, fibrates, niacin and ezetimibe) on inflammatory processes and those of inflammation-lowering drugs (*i.e.* salicylates, anti-TNF and IL-1ra) on lipid metabolism, and discuss the added value of these off-target effects in the use of these drug classes for the treatment of atherosclerosis.

1. INTRODUCTION

Both hyperlipidemia and inflammation are well known risk factors for the development of atherosclerosis and have been shown to play a causal role in the progression of atherosclerotic plaques.[1] In the current review, we will describe the role of lipids and inflammation in the development of atherosclerosis and the interaction between lipid metabolism and inflammation that influences the development of atherosclerosis. We will discuss the current knowledge about the off-target effects of lipid-lowering drugs on inflammatory processes, as well as the off-target effects of anti-inflammatory drugs on lipid metabolism, whereby the mechanisms underlying these effects have been mainly derived from experimental studies.

2. LIPIDS AND ATHEROSCLEROSIS

The prevalence of hyperlipidemia and cardiovascular disease (CVD) increases as a consequence of increased nutrient intake in the western societies, where the average diet contains a high percentage fat and cholesterol.[2] Hyperlipidemia is an important risk factor for the development of atherosclerosis and is characterized by increased plasma cholesterol and triglyceride (TG) levels. These lipids are carried by 'atherogenic' lipoproteins (chylomicrons, VLDL, their remnant lipoproteins and LDL) that can enter the arterial wall and accumulate inside, thereby causing lipid deposition and initiating early atherosclerosis.[3] LDL is prone to oxidative modifications by for example reactive oxygen species (ROS) resulting in oxidized LDL (oxLDL) that can induce endothelial cell activation in the vessel wall.[4] OxLDL can also be taken up by macrophage scavenger receptors such as scavenger receptor A (SRA) or CD36, turning macrophages into foam cells, thereby contributing to the formation of the so called 'foamy atherosclerotic plaques'.[5] Hyperlipidemia is frequently accompanied by decreased plasma levels of HDL, which is then called "dyslipidemia". HDL is considered to be atheroprotective mainly by increasing reverse cholesterol transport, a process whereby HDL acts as cholesterol acceptor and transports cholesterol from atherosclerotic lesions back to the liver for excretion into the bile.[6] Therefore, lipid-lowering therapy is the main treatment strategy for combating atherosclerosis, while HDL-raising is an evolving additional strategy.

3. INFLAMMATION AND ATHEROSCLEROSIS

Atherosclerosis is increasingly being considered as an inflammatory disease, since inflammatory processes play a key role in various stages of plaque development. These include the activation of endothelial cells (ECs) leading to expression of adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 that attract inflammatory cells (*e.g.* neutrophils, T-cells and monocytes) into the early atherosclerotic lesion. Within the plaque, smooth muscle cells (SMCs) and ECs secrete proinflammatory mediators that stimulate monocyte differentiation into macrophages. These macrophages further develop into foam

cells upon uptake of oxLDL and locally amplify the inflammatory response, thereby attracting more immune cells and inducing migration of SMCs into the plaque.[7-9]

Nuclear factor-kappaB (NF-κB) is a transcription factor that plays a key role in the development of atherosclerosis. NF-κB is expressed by multiple cell types in the atherosclerotic plaque such as ECs, SMCs and immune cells, in which it regulates expression of adhesion molecules (*e.g.* ICAM-1 and VCAM-1), chemokines (*e.g.* macrophage chemoattractant protein (MCP)-1), and proinflammatory cytokines (*e.g.* TNFα, IFN-γ, IL-1ß and IL-6), all of which play an important role during the various stages of plaque formation.[10, 11] In addition, NF-κB regulates the expression of several factors involved in apoptosis and cell proliferation.[10]

The inflammatory stimuli that contribute to the progression of atherosclerosis can have a local origin, as described above, or a non-vascular origin. Non-vascular sources of inflammation that are risk factors for atherosclerosis include chronic infection, inflammatory diseases (e.g. rheumatoid arthritis (RA)) and diet-induced inflammation, of which the latter will be described in paragraph 4.1.

4. INTERACTION BETWEEN LIPIDS AND INFLAMMATION

Although hyperlipidemia and inflammation are individual risk factors for the development of atherosclerosis, interaction between lipid metabolism and inflammatory pathways has been reported in multiple organs with different pathways involved, which will be described below.

4.1 Lipids affect inflammation

Diet-induced inflammation is a form of low-grade chronic inflammation that is caused by a nutrient overload, although the exact mechanisms underlying the development of this so called 'metabolic' inflammation are still under investigation. Cholesterol feeding elevates circulating markers of inflammation such as C-reactive protein (CRP) and serum amyloid A (SAA) in lean subjects.[12] In addition, obese humans are characterized by systemic inflammation as reflected by increased circulating CRP and cytokine levels.[13, 14]

Mechanistic studies in animals show that diets high in (saturated) fatty acids (FA) and/or cholesterol are accompanied by inflammatory processes in adipose tissue[15] and liver.[16, 17] Upon expansion of the adipose tissue, resident and infiltrating macrophages start to secrete inflammatory mediators (*e.g.* cytokines) that induce a low-grade systemic inflammation.[15, 18] In liver, high-fat feeding increases hepatic NF-κB activity and expression of proinflammatory cytokines.[19] A high cholesterol diet even more potently induces hepatic inflammation as reflected by increased macrophage content and expression of MCP-1, CD68 and proinflammatory cytokines as well as increased plasma SAA and E-selectin levels.[16, 17]

The exact mechanism(s) by which FA and cholesterol induce local inflammatory responses are not fully clear. Part of the mechanism may involve the increased translocation

of LPS from the gut into the circulation upon HFD feeding, thereby causing 'metabolic endotoxemia'.[20, 21] In addition, saturated FA are also believed to directly increase NF-κB signaling via activation of Toll-like receptors (TLRs).[22, 23] Studies in hyperlipidemic mouse models suggest that dietary cholesterol induces hepatic inflammation via a different mechanism. Dietary cholesterol increases circulating levels of (V)LDL and TG-rich remnants, which can be modified by oxidation and taken up by Kupffer cells via scavenger receptors thereby triggering an inflammatory response in the liver.[24]

The activation of local, non-vascular, inflammatory pathways by FA and cholesterol may aggravate the development of atherosclerosis, most likely via increased circulation of proinflammatory mediators. We recently showed that transgenic activation of hepatic NF-κB signaling contributes to the formation of atherosclerotic lesions.[25] In addition, increase in plasma SAA levels after cholesterol feeding has been shown to associate with atherosclerosis independent of plasma lipid levels.[26]

Besides induction of hepatic and adipose tissue inflammation, FA and cholesterol can similarly and simultaneously induce vascular inflammation.[27] Comparable to Kupffer cells in the liver, infiltrated monocytes in the vessel wall can take up modified oxLDL that activates or amplifies local inflammatory pathways such as the NF-κB pathway. *Ex vivo* studies have additionally shown that circulating monocytes can be activated by TG-rich lipoprotein remnants, most likely by direct interaction between TG-rich lipoproteins and monocytes and uptake of FA,[28] which might contribute to endothelial activation and monocyte adhesion to the vascular wall.

Interestingly, the cholesterol-induced hepatic and vascular inflammation appears reversible by reducing the cholesterol content of the diet, as reflected by a decreased hepatic and aortic NF-κB activity, decreased plasma SAA levels and a reduction in macrophage content in the atherosclerotic plaque.[29] Vascular inflammation may also be reduced by HDL that possesses anti-inflammatory capacities and reduces VCAM-1 and ICAM-1 expression in the aortic wall of cholesterol-fed rabbits.[30] However, since the potential of HDL as an anti-atherogenic therapy has already extensively been reviewed by others,[31, 32] it is not discussed in the current review.

4.2 Inflammatory mediators affect lipid metabolism

Although many studies focused on the effects of lipids on inflammatory pathways, only few studies specifically investigated the effects of metabolic inflammation on lipid metabolism. Interestingly, observations in patients with acute inflammation reveal that bacterial and viral infection is accompanied by hypertriglyceridemia.[33, 34] Most of the current knowledge on the mechanistic relation between inflammation and lipid metabolism comes from rodent and *in vitro* studies that investigated the effect of infection and acute inflammation on lipids and lipoprotein metabolism, which has been extensively reviewed.[35, 36] Changes in circulating lipoproteins during inflammation play a vital role in host defense during infection, however, similar changes in lipoproteins upon prolonged chronic inflammation will contribute to the development of atherosclerosis.

Lipopolysaccharide (LPS; or 'endotoxin') administration in rats induces a hyperlipidemic response, which is caused primarily by an increase of VLDL-TG.[37] Similarly, administration of the proatherogenic cytokines TNFα, IL-1ß or IL-6, increased plasma VLDL-TG levels,[35, 38] thereby inducing an atherogenic lipoprotein profile. The increased plasma VLDL-TG is mainly caused by increased VLDL-TG production, as related to an increased hepatic lipogenesis[39, 40] but also attributed by reduced VLDL-TG clearance via inhibition of lipoprotein lipase (LPL) activity.[37, 41] Although inflammation clearly increases plasma TG levels in humans and animal models, the effects on plasma cholesterol levels are less clear and differ between species.[35] While in humans both LPS injection[42] and infection[43, 44] reduces plasma cholesterol levels, due to a reduction in LDL and mainly HDL levels, in mice, LPS injection increases total cholesterol levels[45] and infection has no effect on plasma cholesterol levels.[46] In addition to increasing VLDL-TG and reducing HDL-C, infection and inflammation can contribute to a proatherogenic lipid profile by increasing oxidation of LDL.[47]

It remains to be established whether low-grade systemic inflammation, observed in atherosclerosis, has similar effects on lipid metabolism as high-grade acute inflammation, evoked by high doses of LPS and cytokines. We recently showed that genetic activation of NF-κB activity specifically in hepatocytes, at levels comparable to those induced by metabolic inflammation, increased VLDL-TG production[48] and aggravated atherosclerosis,[25] suggesting that metabolic inflammation can have similar adverse effects on lipoprotein metabolism as acute inflammation. Future experimental studies in rodents with a relatively mild tissue-specific increase of specific inflammatory molecules could be useful for further delineating the direct effects of metabolically relevant inflammatory pathways on lipid metabolism.

4.3 Interaction of inflammation and lipid metabolism in the progression of atherosclerosis

Taken together, it is evident that lipids, such as (saturated) FA and cholesterol, can induce inflammatory processes. *Vice versa*, acute inflammatory stimuli, such as LPS and cytokines as well as low-grade hepatic NF-κB activity, can induce proatherogenic changes in lipoprotein metabolism. Therefore, the effect of lipids on inflammation could likely explain the exponential rather than linear relationship between plasma cholesterol levels and severity of atherosclerosis that is observed in animal models.[16] On the other hand, this suggests that patients who use lipid-lowering drugs such as statins, fibrates and niacin that are currently used for the treatment of atherosclerosis will benefit from the off-target effects of these drugs with respect to reducing inflammation. Indeed, recent data provide evidence that these lipid-lowering drugs reduce atherosclerosis beyond their lipid-lowering activities, at least partly by reducing inflammatory signaling pathways.

In the remainder of this review, we will summarize current knowledge on the effects of lipid-lowering drugs on inflammatory processes (either directly or indirectly) and those of inflammation-lowering drugs on lipid metabolism, and discuss the added value of these off-target effects in the treatment of atherosclerosis.

5. EFFECT OF LIPID-LOWERING DRUGS ON INFLAMMATION

Lipid-lowering drugs are principally prescribed to reduce hyperlipidemia and thus atherosclerosis, thereby reducing the risk of cardiovascular events. Interestingly, most of these lipid-lowering drugs are able to reduce atherosclerosis beyond their lipid-lowering capacities. Experimental studies in rodents have revealed that several targets of lipid-lowering drugs, such as nuclear receptors or key regulators of lipid metabolism, do interact with inflammatory pathways and thereby reduce inflammation. This is suggested to be the mechanism by which lipid-lowering drugs have additional beneficial 'pleiotropic' effects on atherosclerosis and other CVD as seen in clinical trials. The mechanisms by which the currently used lipid-lowering strategies reduce inflammatory parameters are discussed below and summarized in table 1.

5.1 Statins

Statins are the most widely used lipid-lowering drugs that reduce LDL-cholesterol levels by competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting step in cholesterol biosynthesis. The inhibition of cholesterol synthesis induces compensatory hepatic expression of the LDL receptor (LDLr) and decrease in hepatic VLDL-production, thereby reducing plasma (V)LDL-cholesterol and -TG levels.[49] In addition, statins modestly increase plasma HDL levels by reducing hepatic expression as well as plasma concentration and activity of CETP.[50]

Statins very effectively reduce atherosclerosis and cardiovascular events in hypercholesterolemic subjects. Interestingly, the reduction in cardiovascular events in clinical trials can not be explained solely by the reduction in cholesterol levels.[51, 52] In addition to lowering lipids, statins have anti-inflammatory effects that contribute to their atheroprotective properties. Results from large clinical trials showed that statins reduce systemic markers of inflammation such as CRP and SAA.[53]

Statins may reduce inflammation secondary to reducing plasma cholesterol, since plasma cholesterol levels are related to tissue-specific inflammation[17] and a reduction in plasma cholesterol *per se* reduces markers of systemic inflammation such as SAA, hepatic NF-κB activity and macrophage content in the plaque,[29, 54] as discussed in paragraph 4.1. However, statistical analysis suggested that the reduction in plasma CRP levels upon statin treatment occurs partly independent of LDL-cholesterol levels.[55, 56] This finding has been strengthened by experimental studies in rodents where statins exert anti-inflammatory activity independent[57] or even in the absence[58, 59] of the lipid-lowering effects of statins. Mechanistic studies in the hyperlipidemic APOE*3-Leiden mouse model that responds to statins in lowering cholesterol similar to humans, revealed that statin treatment induced additional anti-inflammatory activities in liver and vasculature compared to a similar reduction in cholesterol levels by low-cholesterol feeding.[60]

The mechanisms by which statins reduce inflammation independent of lipid-lowering have mainly been investigated *in vitro*, which has been summarized by Schonbeck *et al.*[61] Statins appear to reduce multiple inflammatory pathways which can be attributed by secondary effects that are caused by HMG-CoA reductase inhibition.

HMG-CoA reductase controls the mevalonate pathway that not only results in synthesis of cholesterol, but also of isoprenoids that are required for the function of small GTPases (*e.g.* Rho, Rac, Rab, Ras, Rheb) that affect multiple signaling pathways.[62] Incubation of cultured ECs, SMCs and monocytes/macrophages with statins indeed revealed that statins directly reduce many different inflammatory pathways. Statins downregulated inflammatory-modulating transcription factors such as NF-κB and activator protein (AP)-1[63] and reduced expression of MCP-1[64] and proinflammatory cytokines such as IL-1ß, IL-6 and TNFα.[61, 65] In addition, statins reduce expression of adhesion molecules in primary human monocytes[66] and reduce monocyte-EC interactions *in vitro*.[67] Besides being powerful inhibitors of cholesterol synthesis, statins thus also appear powerful direct inhibitors of inflammatory pathways.

It remains to be established whether statins reduce all of these inflammatory pathways in humans, as relatively high concentrations of statins have been used for these mechanistic experiments *in vitro*. Importantly, i*n vivo* animal studies revealed that a high dosage of statin is an important determinant for its anti-inflammatory effects. High-dose statin treatment in mice reduced vascular monocyte adhesion and reduced the expression of inflammatory markers in both the liver (*i.e.* SAA and fibrinogen) as well as in the vessel wall (*i.e.* VCAM-1, MCP-1 and TNFα),[60] while low-dose statin treatment only reduced vascular monocyte adhesion and VCAM-1 expression.[68] Interestingly, monocytes from statin-treated hypercholesterolemic patients showed a dose-dependent reduction in the expression of adhesion molecules[66] and a study in patients with acute myocardial infarction (that activates the inflammatory response) confirmed that statin treatment dose-dependently reduced circulating CRP and TNFα levels.[69]

It is interesting that inhibition of HMG-CoA reductase by statins has direct inhibitory effects on cholesterol synthesis as well as inflammatory pathways. The fact that both cholesterol and isoprenoids are synthesized from the same precursors by the mevalonate pathway emphasizes the close relation between lipid and inflammatory pathways and clarifies why statins reduce atherosclerosis to a higher extent than expected based on their lipid-lowering ability.

5.2 Fibrates

Fibrates are the first line of drugs that are currently used for the treatment of hypertriglyceridemia. Fibrates are synthetic ligands for peroxisome proliferator-activated receptor α (PPARα), the nuclear receptor that activates genes involved in lipid metabolism upon activation. PPARα activation potently reduces plasma TG levels as a result of increased VLDL-TG clearance. This increased clearance is due to enhanced LPL activity by 1) upregulation of LPL expression and 2) upregulation of apolipoprotein AV (apoAV) and downregulation of apoCIII expression.[70, 71] Plasma VLDL and LDL levels may further be reduced by upregulation of hepatic LDLr expression.[72] Fibrates additionally increase HDL-cholesterol levels by hepatic upregulation of apoAI, leading to an increased HDL production, as well as downregulating both hepatic expression as well as plasma concentration and activity of CETP, that further increases

HDL-cholesterol.[73] Altogether, fibrates reduce plasma VLDL-TG and LDL-cholesterol levels and increases HDL-cholesterol levels, contributing to an anti-atherogenic lipid profile.

Indeed, fibrates have shown to reduce atherosclerosis and CVD in hyperlipidemic rodent models[74] and in dyslipidemic patients at risk for CVD.[75, 76] Interestingly, similar to statin treatment, fibrate treatment reduced atherosclerosis in patients to a greater extent than expected based on solely its lipid-lowering effects.[77] Kooistra *et al.*[78] showed in hyperlipidemic APOE*3-Leiden mice that fenofibrate reduces atherosclerosis beyond plasma cholesterol-lowering alone. This could, at least partly, be ascribed to the anti-inflammatory activities of fibrates. Indeed, not only in animal models, but also in dyslipidemic patients, fibrate treatment reduces inflammatory markers such as CRP and IL-6.[79, 80]

The reduction in systemic inflammation upon fibrate treatment may be secondary to local changes in lipid metabolism that affect inflammatory pathways. PPARα activation increases FA oxidation in adipose tissue, thereby reducing adipocyte hypertrophy, and increases adipocyte differentiation[81], processes that have been associated with reduced macrophage activation and gene expression of inflammatory markers in adipose tissue.[82] Similarly, activation of PPARα increases hepatic FA oxidation, thereby reducing hepatic steatosis and lipid peroxidation, that prevents the development of hepatic inflammation.[83] In line with this, fibrates reduce hepatic markers of inflammation and the macrophage content of the liver.[84, 85]

Although fibrates may (partly) reduce systemic inflammation secondary to local changes in lipid metabolism, many studies reveal that the main mechanism by which fibrates reduce inflammation is probably the direct inhibitory action of PPARα on NF-κB signaling. Activation of PPARα reduces inflammatory signaling via direct physical interaction, and thereby inhibition, of NF-κB[79] as well as by indirect inhibition of NF-κB via induction of IkB expression.[86] In the liver, this may explain why PPARα activation suppresses hepatic inflammatory gene expression independent of its effects on hepatic lipid content.[87] Altogether, fibrates thus reduce systemic inflammation, either via local changes in lipid metabolism in adipocytes and hepatocytes or via direct interaction of PPARα with NF-κB in peripheral tissues.

Besides adipocytes and hepatocytes, PPARα is expressed in multiple cell types involved in atherosclerosis development such as ECs, SMCs, and monocytes/macrophages, and co-localizes with these cells in atherosclerotic lesions.[88] It is therefore conceivable that most of the anti-inflammatory effects of fibrates in the arterial wall are mediated by direct inhibition of NF-κB by PPARα. Reduced NF-κB activity in the plaque directly reduces expression of VCAM-1 and ICAM-1,[89] thereby diminishing recruitment and infiltration of inflammatory macrophages to the plaques.[78] The PPARα mediated reduction in vascular NF-κB activity therefore likely contributes to the atherosclerosis-reducing effect of fibrates.

The direct interaction between two key transcription factors in the regulation of lipid- and inflammatory pathways, PPARα and NF-κB respectively, reveals another

close intracellular interaction between lipid metabolism and inflammation and explains why fibrates reduce atherosclerosis beyond expectations.

5.3 Niacin

Niacin (nicotinic acid or vitamin B3) has been used as a lipid-lowering drug for many years, although the G protein-coupled receptor (GPR) 109A that is responsible for its effects has only been elucidated a decade ago.[90-92] By activating GPR109A in adipocytes, niacin reduces the activity of hormone sensitive lipase (HSL) that acutely reduces the free FA (FFA) flux from adipose tissue to the liver. As hepatic VLDL-TG production is highly dependent on the influx of FFA from the circulation, this is commonly proposed as the main mechanism by which niacin reduces plasma (V) LDL levels. In addition, niacin reduces diacylglycerol acyltransferase-2 (DGAT2) activity in hepatocytes, thereby decreasing TG synthesis and apoB secretion [93]. Besides lowering (V)LDL, niacin is the most effective drug currently on the market to raise plasma HDL levels[94] and therefore gains increasing interest despite its adverse side effects (*i.e.* flushing and gastrointestinal disorders). The raise in plasma HDL levels upon niacin treatment is dependent on the presence of CETP and can be attributed to a reduced hepatic expression and plasma concentration and activity of CETP.[95, 96] This effect on CETP may be secondary to the reduction in hepatic TG and cholesterol content that has been observed upon niacin treatment.[96] Niacin thus effectively reduces the proatherogenic plasma lipid profile by decreasing (V)LDL and increasing HDL levels.

Niacin reduces atherosclerosis in patients[97, 98] and in various mouse models.[99-101] In addition to lowering lipids, niacin has anti-inflammatory effects that are likely to contribute to its atheroprotective properties. Similar to statin and fibrate treatment, niacin reduces plasma CRP levels in patients at risk for CVDs.[102]

Part of the anti-inflammatory effects of niacin may be secondary to the improved lipoprotein profile. Niacin reduces plasma (V)LDL that is prone for oxidative modulation, which likely reduces hepatic and vascular uptake of oxLDL by macrophages as described in paragraph 4.1. Niacin may also reduce inflammation secondary to the raise in plasma HDL levels. Some studies suggest that niacin does not only increase HDL levels, but also modifies HDL composition, resulting in an improved anti-inflammatory and anti-oxidative capacity of HDL,[103] although results are not consistent.[104]

The reduction in plasma (V)LDL and increase in plasma HDL levels might contribute to the niacin-mediated reduction in systemic inflammation. However, studies in rabbits and apoE-deficient mice show that niacin reduces vascular inflammation also independent of changes in plasma lipid levels.[100, 105] In these studies, niacin reduced vascular VCAM-1, MCP-1 and TNFα expression and reduces macrophage and neutrophil infiltration into the plaque. This suggests that niacin has anti-inflammatory effects directly in the vasculature, which is supported by *in vitro* studies that reveal that niacin reduces TNFα-induced NF-κB activation, expression of VCAM-1 and MCP-1 in ECs as well as monocyte adhesion to ECs.[106] In addition to direct anti-inflammatory

effects in the vascular wall, niacin reduces secretion of proinflammatory cytokines by adipocytes *in vitro,*[94] that may contribute to the reduction in systemic inflammation upon niacin treatment *in vivo*.

Most of the anti-inflammatory effects of niacin are suggested to be mediated via the GPR109A that is not only expressed in adipocytes, but also in immune cells including macrophages.[107] In addition, some anti-inflammatory effects of niacin might be independent of the GPR109A, since *in vitro* studies revealed that in the absence of the GPR109A niacin reduces ROS production in ECs and inhibits EC-mediated LDL oxidation.[106] Whether these GPR109A independent effects occur *in vivo* remains uncertain, since LDLr-deficient mice receiving bone marrow cells derived from GPR109A-deficient mice did not benefit from the vascular anti-inflammatory effects of niacin.[101]

The fact that niacin reduces plasma lipid levels and inhibits inflammatory signaling through, at least partly, the GPR109A, emphasizes another close link between intracellular lipid and inflammatory pathways. Moreover, it shows that niacin, despite primarily being a lipid-lowering drug, has strong anti-inflammatory effects that contribute to the reduction in atherosclerosis.

5.4 Ezetimibe

Statins, fibrates and niacin are the current available drugs to treat patients with hyperlipidemia at risk for CVDs. Ezetimibe is approved as a lipid-lowering drug that may be used in combination with statins, but the outcomes of the first large clinical trials are still awaited.[108] Ezetimibe inhibits the activity of the Niemann-Pick C1 Like 1 (NPC1L1) transporter that is critical for intestinal cholesterol absorption.[109, 110] By doing so, ezetimibe reduces cholesterol absorption, thereby effectively reducing plasma cholesterol levels in hypercholesterolemic monkeys and mice.[111, 112] Human studies also show that ezetimibe effectively reduces LDL-cholesterol levels,[113] even on top of weight loss[114] or in combination with statins,[115] thereby thus further reducing the proatherogenic plasma lipoprotein profile. Ezetimibe indeed has shown to effectively reduce atherosclerosis in preclinical animal models, as recently reviewed by Davis *et al.*[112, 116]

Similar to the other lipid-lowering drugs, ezetimibe treatment attenuates systemic inflammation in human subjects (*i.e.* serum CRP levels)[114] as well as vascular inflammation in mice (*e.g.* aortic IL-6 and eNOS expression),[117, 118] which may contribute to the reduction in atherosclerosis upon ezetimibe treatment. The reduction in inflammation upon ezetimibe treatment may be secondary to the reduction in hepatic and plasma cholesterol levels, since this has been associated with reduced hepatic and vascular inflammation.[16, 29] Whether ezetimibe additionally possesses direct anti-inflammatory effects independent of the reduction in plasma and hepatic cholesterol levels, such as seen for statins, fibrates and niacin, is so far not known. One may hypothesize, since ezetimibe reduces not only intestinal cholesterol absorption, but to a minor extent also (saturated) FA absorption,[119] that ezetimibe additionally reduces intestinal LPS absorption. However, whether this really occurs and whether it is clinically relevant remains to be determined.

6. EFFECT OF INFLAMMATION-LOWERING DRUGS ON LIPID METABOLISM

Although inflammation clearly plays a role in the development of atherosclerosis, only few current human therapies primarily target inflammatory pathways in the treatment of atherosclerosis. The last decade, many papers have revealed that in animal studies, diminished activation of inflammatory pathways have marked effects on atherosclerosis.[120-123] Currently, anti-inflammatory therapies are under development for the treatment of atherosclerosis as recently reviewed by Charo *et al.*[124] Anti-inflammatory drugs may directly lower atherosclerosis, via inhibition of inflammatory mediators that contribute to atherosclerosis. In addition, anti-inflammatory drugs may reduce atherosclerosis by improving the atherogenic plasma lipid profile, since proinflammatory mediators induce a proatherogenic lipid profile as discussed in paragraph 4.2. However, since anti-inflammatory drugs are not the main drugs currently used for atherosclerosis treatment, less research has focused on the effects of anti-inflammatory drugs on lipid

Table 1: Therapeutic actions of lipid-lowering drugs and their effects on inflammation.

Drug	Therapeutic action	Effects on lipid metabolism	Effects on inflammation
Statin	**HMG-CoA red. inhibitor**	↓ **cholesterol synthesis**	↓ mevalonate pathway (isoprenoids)
		↓ plasma (V)LDL-cholesterol	↓ NF-κB and AP-1
			↓ inflammatory mediators
			(e.g. cytokines, adhesion molecules)
Fibrate	**PPARα activator**	↑ **plasma VLDL-TG clearance**	↓ NF-κB
		↓ **plasma TG**	↓ inflammatory mediators
		↓ plasma (V)LDL-cholesterol	(e.g. cytokines, adhesion molecules)
		↑ plasma HDL-cholesterol	
Niacin	**GPR109A agonist**	↓ **HSL activity**	↓ activity immune cells via GPR109A
		↓ FFA flux to liver	↓ NF-κB
		↓ VLDL-productie	↓ inflammatory mediators
		↓ plasma (V)LDL-cholesterol	(e.g. cytokines, chemokines)
		↑ plasma HDL-cholesterol	
Ezetimibe	**NPC1L1 inhibitor**	↓ **intestinal cholesterol absorption**	
		↓ plasma (V)LDL-cholesterol	↓ inflammatory mediators
			(e.g. cytokines, acute-phase proteins)

Primary actions are depicted in bold. AP-1, activator protein-1; GPR109A, G protein-coupled receptor 109A; HMG-CoA red., 3-hydroxy-3-methylglutaryl coenzyme A reductase; HSL, hormone-sensitive lipase; NF-κB, nuclear factor-kappaB; NPC1L1, Niemann-Pick C1 Like Protein 1; PPARα, peroxisome proliferator-activated receptor α.

metabolism. The studies that are available involve mainly pre-clinical studies (animal models) and are described below and summarized in table 2.

6.1 Salicylates (Salicylate/Aspirin/Salsalate)

Aspirin (acetyl salicylic acid) is a salicylate drug belonging to the NSAIDs (non-steroidal anti-inflammatory drugs) and is frequently used for prevention and treatment of CVD, due to its anti-platelet aggregating and anti-inflammatory activity. Aspirin is primarily known as a cyclooxygenase-1 (COX-1) inhibitor, thereby reducing synthesis of prostaglandins, which are involved among others in the regulation of hemostasis, platelet function, and macrophage differentiation.[125] Low-dose aspirin reduces vascular inflammation in mice[126] and plasma proinflammatory cytokine levels in patients at risk for CVD.[127] However, the anti-inflammatory effects of aspirin are most pronounced at high-dose aspirin treatment, when it also inhibits the activity of NF-κB.[128] As discussed above, NF-κB is a transcription factor crucially involved in many aspects of inflammation[10] and is activated in the atherosclerotic lesion.[129] Although the anti-platelet aggregating effects of aspirin contribute to the reduction in CVD, the fact that aspirin treatment reduces CVD mostly in patients with high inflammatory status indicates that the anti-inflammatory effects of aspirin strongly contribute to the reduction in CVD upon treatment.[130]

Although salicylate treatment reduces systemic inflammation, this is not the only way by which salicylate treatment prevents CVD. Treatment of hyperlipidemic APOE*3-Leiden mice with salicylate not only reduced hepatic and aortic NF-κB activity, but also markedly reduced plasma cholesterol levels,[29] demonstrating additional effects on lipid metabolism. Actually, since long it is known that high dose aspirin and salicylate treatment can reduce plasma FFA and TG levels,[131, 132] which was suggested to be caused by inhibition of lipogenesis[133] as well as inhibition of FA release from adipose tissue.[134] Recent mechanistic studies from our group in rodents showed that aspirin and salicylate treatment reduce plasma VLDL-TG and VLDL-cholesterol levels in atherosclerotic mouse models,[29, 135, 136] due to a reduction in VLDL-TG production.[135] The mechanism by which aspirin reduces hepatic VLDL-TG production could either be directly via its inhibitory effects on hepatic NF-κB, since hepatic NF-κB activity is positively associated with VLDL-TG production,[48] or indirectly via a drop in plasma FFA levels, thereby reducing the FA incorporation into VLDL-TG.[135]

These observations show that the anti-inflammatory salicylate drugs do not only reduce atherosclerosis by reducing inflammation, but additionally by inducing an anti-atherogenic lipid profile. However, although high-dose aspirin treatment has the most pronounced anti-inflammatory effects, caution should be taken regarding high-dose aspirin treatment, as this may increase the risk of gastrointestinal side effects.[125] Anti-inflammatory drugs that similarly reduce inflammation, plasma lipid levels and atherosclerosis, that do not have these side effects may need to be developed. In fact, salsalate is regarded as a safer alternative and is now used for clinical trials in type 2 diabetic patients. Recently, high-dose salsalate treatment has shown to reduce plasma TG levels in diabetic patients.[137]

6.2 Cytokine inhibition

The experience with salicylate treatment suggests that other anti-inflammatory agents may also be efficient in preventing CVDs, not only by reducing inflammatory processes, but also by reducing pro-atherogenic lipid profiles. Partly due to side effects of high-dose aspirin treatment, more attention is paid to develop new anti-inflammatory therapies.[124] We now discuss strategies to reduce signaling of the proinflammatory cytokines TNFα and IL-1ß, both of which have been shown to be involved in the development of atherosclerosis and affect lipid metabolism directly, as discussed in paragraph 4.2.

TNFα is a critical mediator of inflammation with an important role in the development of atherosclerosis. Anti-TNF therapy has been used for the treatment of chronic inflammatory rheumatoid arthritis (RA) and reduces cardiovascular events in these patients.[138] This reduction in cardiovascular events is most likely caused by attenuation of chronic inflammation, since it does not affect or even increase plasma total cholesterol, TG, LDL and HDL levels in RA patients.[139] Most experimental data are derived from genetic models showing that disrupted TNFα signaling is associated with reduced atherosclerosis development without affecting plasma lipid levels.[140] A single experimental report studied the effect of TNFα antagonism related to atherosclerosis in apoE-deficient mice and found no effect on plasma lipid levels and a tendency towards reduced atherosclerosis development.[122] Others evaluated the potency of anti-TNF treatment in metabolic diseases such as obesity and showed increased insulin sensitivity in insulin-resistant patients[141] and mice,[142] although results are not consistent.[143] Whether TNFα antagonism is able to reduce plasma lipid levels in hyperlipidemic patients needs to be evaluated in future studies.

IL-1β is a proinflammatory cytokine that mediates inflammation and is counterbalanced by IL-1 receptor antagonist (IL-1ra) via competitive binding to the IL-1 receptor (IL-1R). Genetic studies have revealed a link between IL-1β activity and atherosclerosis by showing that polymorphisms known to increase the IL-1β:IL-1ra ratio are associated with increased incidence of CVDs.[144, 145] In animal models, IL-1ß activity has causally been linked to the development of atherosclerosis.[146] As IL-1ß expression is elevated in the atherosclerotic plaque and secreted by ECs, SMCs and macrophages[147], IL-1ß may increase atherosclerosis by direct activation of inflammatory pathways in the plaque. Indeed, IL-1ra administration reduced atherosclerosis without changes in plasma lipid levels in apoE-deficient mice.[122] However, IL-1ß administration to rats causes hyperlipidemia,[148] suggesting that IL-1ra treatment may also be able to modulate plasma lipid levels. Absence of IL-1ra increases plasma cholesterol levels and reduces HDL levels in apoE-deficient mice,[149] which is amplified by a high-cholesterol diet.[150] In addition, a recent study showed that an IL-1ß polymorphism associated with increased IL-1ß activity, increases fasting and postprandial plasma lipids.[151] This raises the question whether IL-1ra administration may be able to reduce atherosclerosis by reducing plasma lipid levels, in addition to decreasing inflammation. The effects of IL-1ra administration on lipoprotein metabolism has not been studied in great detail. However, so far, subcutaneous

IL-1ra administration in mice,[122] in patients with RA[152] or in human subjects with the metabolic syndrome[153] did not influence plasma lipid levels.

Although data of anti-cytokine therapy in relation to lipid metabolism and atherosclerosis are scarce, the findings so far suggest that cytokine inhibition effectively reduces inflammation, but has no clear effect on plasma lipid levels. Possibly, inhibition of individual mediators of inflammation, like cytokines, simply lacks sufficient impact on pathways of lipid and lipoprotein metabolism. Cytokine inhibition reduces atherosclerosis in RA patients, and most likely also reduces atherosclerosis in other patients with a clearly enhanced chronic inflammatory state (*e.g.* inflammatory bowel disease), but the effect in for example hyperlipidemic or type 2 diabetic patients remains to be established and may be limited due to the lower inflammatory state of these patients.

Table 2: Therapeutic actions of inflammation-lowering drugs and their effects on lipid metabolism.

Drug	Therapeutic action	Effects on inflammation	Effects on lipid metabolism
Salicylates	**COX-1 inhibitor**	**↓ prostaglandin synthesis**	↓ VLDL production
		↓ platelet aggregation	↓ plasma cholesterol
		↓ NF-κB (high dose)	↓ plasma FFA
			↓ plasma TG
Anti-TNF	**TNFα inhibitor**	**↓ TNFα signaling**	No consistent effects
IL-1ra	**IL-1R antagonist**	**↓ IL-1ß signaling**	No effect on plasma lipids

Primary actions are depicted in bold. COX-1, cyclo-oxygenase-1; FFA, free fatty acid; IL-1β, interleukin-1 beta; IL-1ra, interleukin-1 receptor antagonist; NF-κB, nuclear factor-kappa B; TG, triglyceride; TNFα, Tumor necrosis factor -alpha.

7. CONCLUDING REMARKS

The last decade it has become clear that plasma lipid levels and plasma inflammatory mediators are not only independent risk factors for the development of atherosclerosis, but interaction exist between lipid and inflammatory pathways, thereby aggravating the development of atherosclerosis. *Vice versa*, the interaction between lipids and inflammatory mediators has shown to be beneficial in the treatment of atherosclerosis, since lipid-lowering drugs not only reduce plasma lipid levels, but also reduce levels of inflammatory mediators which further increase their efficiency in reducing atherosclerosis and CVDs.

It is interesting to note that various classes of lipid-lowering drugs all reveal anti-inflammatory properties. Part of the anti-inflammatory effects may be mediated by a similar mechanism, related to the reduction in (V)LDL and lipoprotein remnants.

In addition, however, they evoke (at least part of) their anti-inflammatory effects independent of lipid-lowering through different mechanisms: 1) statins interfere with multiple inflammatory pathways via inhibition of the mevalonate pathway, 2) fibrates inhibit the inflammatory NF-κB pathway through direct inhibitory actions of their target nuclear receptor PPARα on NF-κB activity, and 3) niacin may reduce inflammation directly via the GPR109A receptor on immune cells. Whether ezetimibe has direct anti-inflammatory effects is unknown, but it could possibly reduce intestinal endotoxin absorption. The fact these drugs all affect inflammatory pathways via different interactions in various cell types emphasizes that lipid- and inflammatory pathways are interrelated at multiple levels and in multiple tissues.

The effect of lipid-lowering therapies on inflammatory pathways has intensively been studied. In contrast, the effect of anti-inflammatory therapies on lipid metabolism has not been investigated to a great extent, although anti-inflammatory therapies are now under (pre-clinical) development for the treatment of atherosclerosis. So far, high-dose salicylate drugs such as aspirin have shown to reduce plasma cholesterol and TG levels, thereby contributing to the reduction in atherosclerosis in animal models. Cytokine inhibition effectively reduces inflammation, but the impact of this strategy appears too limited to also beneficially affect lipid pathways. Future studies may focus on the effects of new anti-inflammatory drugs with a broad spectrum on lipid metabolism. This will contribute to the understanding of the efficiency of these drugs in the treatment of atherosclerosis.

Further knowledge on the interaction between inflammation and lipid metabolism may improve treatment of patients at risk for CVD. Since CVD patients are very heterogeneous with respect to plasma lipid levels and inflammatory status, the exact contributions of lipids and inflammatory pathways to the development of atherosclerosis may vary between patients and need to be evaluated in subsets of clinical patients. Treatment strategies should subsequently be based on the type of patient at risk for CVD. Patients with an enhanced inflammatory status (*e.g.* RA, inflammatory bowel disease and chronic obstructive pulmonary disease patients) may benefit from lipid-lowering therapies with the strongest anti-inflammatory effects, or possibly anti-inflammatory therapy only. In fact, statins have been investigated for their efficacy in chronic inflammatory diseases such as RA.[154] Patients at risk for CVD with increased plasma lipid levels may benefit most from lipid-lowering strategies only, which will likely reduce (metabolic) inflammation as a consequence. Treatment with both a lipid-lowering drug in combination with an inflammation-lowering drug may also have an additional therapeutic value and should be focus of future investigation. Understanding the mechanisms by which lipid-lowering and anti-inflammatory therapies reduce atherosclerosis will optimize (personalized) therapy in the treatment of atherosclerosis.

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ASPIRIN REDUCES HYPERTRIGLYCERIDEMIA BY LOWERING VLDL-TRIGLYCERIDE PRODUCTION IN MICE FED A HIGH-FAT DIET

3

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Am J Physiol Endocrinol Metab 2011; 306: E1099-107

ABSTRACT

Systemic inflammation is strongly involved in the pathophysiology of the metabolic syndrome, a cluster of metabolic risk factors including hypertriglyceridemia. Aspirin treatment lowers inflammation via inhibition of NF-κB activity, but also reduces hypertriglyceridemia in humans. The aim of this study was to investigate the mechanism by which aspirin improves hypertriglyceridemia. Human apolipoprotein CI (apoCI)-expressing mice (*APOC1* mice), an animal model with elevated plasma triglyceride (TG) levels, as well as normolipidemic wild-type (WT) mice were fed a high-fat diet (HFD) and treated with aspirin. Aspirin treatment reduced hepatic NF-κB activity in HFD-fed *APOC1* and WT mice and in addition, aspirin decreased plasma TG levels (-32%; $p<0.05$) in hypertriglyceridemic *APOC1* mice. This TG-lowering effect could not be explained by enhanced VLDL-TG clearance, but aspirin selectively reduced hepatic production of VLDL-TG in both *APOC1* (-28%; $p<0.05$) and WT mice (-33%; $p<0.05$) without affecting VLDL-apoB production. Aspirin did not alter hepatic expression of genes involved in FA oxidation, lipogenesis and VLDL production, but decreased the incorporation of plasma-derived FA by the liver into VLDL-TG (-24%; $p<0.05$), which was independent of hepatic expression of genes involved in FA uptake and transport. We conclude that aspirin improves hypertriglyceridemia by decreasing VLDL-TG production without affecting VLDL particle production. Therefore, the inhibition of inflammatory pathways by aspirin could be an interesting target for the treatment of hypertriglyceridemia.

INTRODUCTION

The metabolic syndrome is a clustering of metabolic risk factors, including steatosis, insulin resistance and hyperlipidemia, predisposing to the early onset of atherosclerosis and cardiovascular morbidity and mortality. It is well established that the metabolic syndrome is associated with increased systemic inflammation.[1] Moreover, accumulating evidence suggests a strong involvement of systemic inflammation in the pathogenesis of components of the metabolic syndrome.[2] Hypertriglyceridemia, one of the components of the metabolic syndrome and an important risk factor for the development of cardiovascular disease, is strongly associated with increased inflammation.[3] Early studies show that sepsis, infection and inflammation are accompanied by hypertriglyceridemia.[4-6] More recent studies show that administration of LPS induces hypertriglyceridemia.[7,8] In addition, multiple cytokines, such as IL-6 and TNF-α, increase serum triglyceride (TG) levels.[9,10] Inhibition of inflammation might therefore be an attractive therapeutic target in patients with HFD-induced hypertriglyceridemia.

Non-steroidal anti-inflammatory drugs (NSAID) such as aspirin are known to inhibit the enzyme cyclooxygenase (COX). In addition, high doses of aspirin lower activation of inflammatory pathways by inhibition of the NF-κB pathway,[11,12] which plays a crucial role in the inflammation-mediated pathogenesis of the metabolic syndrome.[2] Interestingly, aspirin treatment diminishes hypertriglyceridemia in both obese rodents[13] and patients with type 2 diabetes mellitus.[14] However, the mechanism underlying this TG-lowering effect still has to be elucidated.

We previously found that human apolipoprotein CI (apoCI)-expressing (*APOC1*) mice have increased plasma TG, by a diminished clearance of VLDL particles through apoCI-mediated inhibition of lipoprotein lipase (LPL),[15] which is aggravated by high-fat diet (HFD) feeding (unpublished observation by I.O.C.M. Vroegrijk et al). Therefore, we reasoned that the HFD-fed *APOC1* transgenic mouse is an appropriate model to study the effectiveness of treatments targeting HFD-induced hypertriglyceridemia.

The aim of this study was to investigate the mechanism by which aspirin reverses HFD-induced hypertriglyceridemia. Therefore, we studied the effect of aspirin on VLDL-TG metabolism *in vivo* in HFD-fed hypertriglyceridemic *APOC1* mice as well as in C57Bl/6 wild-type (WT) mice, to extend any findings towards the mouse model that is most widely used for evaluation of treatments for the metabolic syndrome. Our results show that a high dose of aspirin improves hypertriglyceridemia as a consequence of a clear reduction of hepatic VLDL-TG production, mediated by a diminished hepatic incorporation of plasma-derived FA into VLDL-TG.

MATERIALS AND METHODS

Animals, diet and aspirin treatment

Transgenic *APOC1* mice with hemizygous expression of the human *APOC1* gene were generated as previously described and backcrossed at least 10 times to the C57Bl/6

background. The *APOC1* mouse model develops hypertriglyceridemia mainly due to a diminished clearance of VLDL particles through apoCI-mediated inhibition of lipoprotein lipase (LPL).[16,17] Male *APOC1* mice and WT mice (also on a C57Bl/6 background) were housed under standard conditions with a 12-hour light-dark cycle. At the age of 10–12 weeks, mice received a HFD (45 energy% derived from palm oil; D12451, Research Diet Services, Wijk bij Duurstede, The Netherlands) for a period of 6 weeks. Aspirin treatment (120 mg/kg/day in drinking water; pH 6.4) was given during the last 4 weeks on HFD and mice were subsequently used for experiments after an overnight fast at 9:00 am. Control mice received the same drinking water of pH 6.4 without the addition of aspirin. Mice were allowed free access to food and water. Animal experiments were approved by the institutional ethical committee on animal care and experimentation.

Liver NF-κB activation

Since the most common form of NF-κB is the p50/p65 heterodimer,[18] the activity of both the p50 and p65 subunits in liver tissue was determined using electrophoretic mobility shift assay (EMSA).[19] Shortly, tissues were homogenized in ice-cold Passive Lysis Buffer (Promega, Madison, WI) and centrifuged (14,000 rpm; 20 min; 4°C). Protein content of the supernatant was determined using the BCA protein assay kit (Pierce, Rockford, IL). For the EMSA, the gel shift assay system was purchased from Promega. The probe was end-labeled using T4 polynucleotide kinase and [^{32}P]ATP and purified on a Microspin G-25 column (GE Healthcare, Piscataway, NJ). For each sample, 50 µg protein was incubated with labelled probe and binding buffer (Promega) for 20 min at RT. Specific competition was done by adding unlabeled NF-kB binding probe to the reaction. The mixtures were run on 4.5% polyacrylamide gel electrophoresis in 0.5x Tris/Borate/EDTA (TBE) buffer. The gel was vacuum-dried and exposed to radiographic film.

Plasma parameters

Blood was collected from the tail vein into chilled paraoxon (Sigma, St Louis, MO)-coated capillaries to prevent ongoing lipolysis.[20] Capillaries were placed on ice, centrifuged and plasma was assayed for TG, total cholesterol (TC) and phospholipids (PL) using commercially available enzymatic kits from Roche Molecular Biochemicals (Indianapolis, IN) in 96-wells plates (Greiner Bio-One). Free fatty acids (FFA) were measured using NEFA-C kit from Wako Diagnostics (Instruchemie, Delfzijl, the Netherlands). ß-hydroxybutyrate (ß-HB) was determined using the enzymatic ß-HB Assay kit from BioVision (Mountain View, CA, USA)

Liver lipids

Lipids were extracted from livers according to a modified protocol from Bligh and Dyer.[21] Shortly, a small piece of liver was homogenized in ice-cold methanol. After centrifugation, lipids were extracted by addition of 1800 µL CH_3OH:$CHCl_3$ (3:1 v/v) to 45 µL homogenate. The $CHCl_3$ phase was dried and dissolved in 2% Triton X-100. Hepatic TG and TC concentrations were measured using commercial kits as described

earlier. Liver lipids were expressed per mg protein, which was determined using the BCA protein assay kit (Pierce).

Generation of VLDL-like emulsion particles

VLDL-like TG-rich emulsion particles were prepared and characterized as described previously.[22,23] Lipids (100 mg) at a weight ratio of triolein: egg yolk phosphatidylcholine: lysophosphatidylcholine: cholesteryl oleate: cholesterol of 70: 22.7: 2.3: 3.0: 2.0, supplemented with 200 µCi of glycerol tri[9,10(n)-^{3}H]oleate ([^{3}H]TO) were sonicated at 10 µm output using a Soniprep 150 (MSE Scientific Instruments, Crawley, UK). Density gradient ultracentrifugation was used to obtain 80 nm-sized emulsion particles, which were used for subsequent experiments. TG content of the emulsions was measured as described above. Emulsions were stored at 4°C under argon and used within 7 days.

In vivo clearance of VLDL-like emulsion particles

To study *in vivo* clearance of the VLDL-like emulsion particles, overnight fasted mice were anesthetized by intraperitoneal injection of acepromazine (6.25 mg/kg Neurotranq, Alfasan International BV, Weesp, The Netherlands), midazolam (6.25 mg/kg Dormicum, Roche Diagnostics, Mijdrecht, The Netherlands), and fentanyl (0.31 mg/kg Janssen Pharmaceuticals, Tilburg, The Netherlands). Mice were injected (t=0) via the tail vein with 200 µL of [^{3}H]TO-labeled emulsion particles at a dose of 100 µg of TG per mouse. Blood samples were taken from the tail vein at 1, 2, 5, 10 and 15 minutes after injection and plasma ^{3}H-activity was counted. Plasma volumes were calculated as 0.04706 x body weight (grams) as determined from ^{125}I-BSA clearance studies as described previously.[24] After taking the last blood sample, the liver, heart, spleen, muscle and white adipose tissue (*i.e.* gonadal, subcutaneous and visceral) were collected. Organs were dissolved overnight at 60°C in Tissue Solubilizer (Amersham Biosciences, Rosendaal, The Netherlands) and ^{3}H-activity was counted. Uptake of [^{3}H]TO-derived radioactivity by the organs was calculated from the ^{3}H activity in each organ divided by plasma-specific activity of [^{3}H]TG and expressed per mg wet tissue weight.

Hepatic VLDL-TG and VLDL-apoB production

To measure VLDL production *in vivo*, mice were fasted overnight as described above. Mice were injected intravenously with Tran^{35}S label (150 µCi/mouse; MP Biomedicals, Eindhoven, The Netherlands) to label newly produced apoB. After 30 minutes, at t=0 min, Triton WR-1339 (Sigma-Aldrich) was injected intravenously (0.5 mg/g body weight, 10% solution in PBS) to block serum VLDL clearance. Blood samples were drawn before (t=0) and 15, 30, 60 and 90 min after injection and used for determination of plasma TG concentration as described above. After 120 min, mice were exsanguinated via the retro-orbital plexus. VLDL was isolated from serum after density gradient ultracentrifugation at d<1.006 g/mL by aspiration[25] and counted for incorporated ^{35}S-activity.

Hepatic gene expression analysis

Total RNA was extracted from liver tissues using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. The quality of each mRNA sample was examined by lab-on-a-chip technology using Experion Stdsens analysis kit (Biorad, Hercules, CA). 1 µg of total RNA was reverse-transcribed with iScript cDNA synthesis kit (Bio-Rad) and obtained cDNA was purified with Nucleospin Extract II kit (Macherey-Nagel). Real-Time PCR was carried out on the IQ5 PCR machine (Biorad) using the Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). mRNA levels were normalized to mRNA levels of cyclophilin (*Cyclo*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Primer sequences are listed in table 1.

Table 1. Primers used for quantitative real-time PCR analysis.

Gene	Forward primer	Reverse primer
Acox1	TATGGGATCAGCCAGAAAGG	ACAGAGCCAAGGGTCACATC
Apob	GCCCATTGTGGACAAGTTGATC	CCAGGACTTGGAGGTCTTGGA
Cd36	GCAAAGAACAGCAGCAAAATC	CAGTGAAGGCTCAAAGATGG
Cpt1a	GAGACTTCCAACGCATGACA	ATGGGTTGGGGTGATGTAGA
Cyclo	CAAATGCTGGACCAAACACAA	GCCATCCAGCCATTCAGTCT
Dgat1	TCCGTCCAGGGTGGTAGTG	TGAACAAAGAATCTTGCAGACGA
Fasn	TCCTGGGAGGAATGTAAACAGC	CACAAATTCATTCACTGCAGCC
Fabp1	GAGGAGTGCGAACTGGAGAC	GTAGACAATGTCGCCCAATG
Gapdh	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
Mttp	CTCTTGGCAGTGCTTTTTCTCT	GAGCTTGTATAGCCGCTCATT
Ppara	ATGCCAGTACTGCCGTTTTC	GGCCTTGACCTTGTTCATGT
Slc27a2	ATCGCCTATGGTATGGGACA	ACTGGCTGGCTGAGAATTTG
Slc27a4	GCTTACTCCACGGCATGACT	GTGGCTGGTTCAGGAGGTAG
Slc27a5	ATGCAGAGCTGATGATGTGG	ATCACTGTTACGCCATGCTG
Srebf1	GGAGCCATGGATTGCACATT	CCTGTCTCACCCCCAGCATA

Acox1, acyl-Coenzyme A oxidase 1, palmitoyl; *Apob*, apolipoprotein B; *Cd36,* fatty acid translocase; *Cpt1a,* carnitine palmitoyltransferase 1a; *Dgat1*, diglyceride acyltransferase 1; *Fabp1*, fatty acid binding protein 1; *Fasn*, fatty acid synthase; *Mttp*, microsomal triglyceride transfer protein; *Ppara*, peroxisome proliferative activated receptor alpha; *Slc27a2*, fatty acid transport protein 2; *Slc27a4*, fatty acid transport protein 4; *Slc27a5*, fatty acid transport protein 5; *Srebf1*, sterol-regulatory element binding protein.

Contribution of plasma FA to VLDL-TG production

To measure the contribution of plasma derived FA to the VLDL-TG production *in vivo*, mice were fasted overnight as described above. Mice received a continuous i.v. infusion of ^{3}H-labeled FA ([9,10(n)-^{3}H] palmitic acid in PBS with 2% bovine serum albumin) at a rate of 100µL/h (1.6 µCi/h). After 2 hours of ^{3}H-labeled FA infusion a blood sample was taken (t=0 min), and Triton WR-1339 (Sigma-Aldrich) was injected

intravenously (0.5 mg/g body weight, 10% solution in PBS) to block serum VLDL clearance. Additional blood samples were drawn 15, 30, 60 and 90 min after injection and used for determination of ^{3}H activity in the TG fraction. Lipids were extracted by adding 10 µL plasma to 3.25 mL extraction fluid (heptane/methanol/chloroform; 100:128:137 (*v/v/v*)). ^{3}H-TG were subsequently separated from ^{3}H-FA; 1mL potassium carbonate (0.1M K_2CO_3, pH 10.5) was added followed by vortexing and centrifugation (3600 rpm; 15 min), leading to an upper alkaline-methanol-aqueous phase containing saponified ^{3}H-FA and a lower chloroform-organic phase containing ^{3}H-TG.[26] A fraction (0.5 mL) of the total aqueous phase (2.45 mL) was counted for ^{3}H in scintillation fluid. The amount of ^{3}H-TG in each sample was calculated by distracting total ^{3}H-FA activity from total ^{3}H activity.

Statistical analysis

Data are presented as means ± SD. Statistical differences were calculated using the Mann-Whitney test for two independent samples with SPSS 16.0 (SPSS Inc, Chicago, IL). P<0.05 was regarded statistically significant.

RESULTS

Aspirin reduces hepatic NF-κB activation

To verify that aspirin inhibits hepatic NF-κB activity, the activities of the NF-κB subunits p50 and p65 were measured in livers of *APOC1* and WT mice fed a HFD and treated with or without aspirin using a gel shift assay (**Fig. 1**). Aspirin indeed reduced the activity of both p50 (-69%; P<0.05) and p65 (-48%; P<0.05) in *APOC1* mice (Fig. 1A) and the activity of p50 in WT mice (-72%; P<0.05), while the reduction in the activity of p65 did not reach statistical significance (P=0.13) (Fig. 1B).

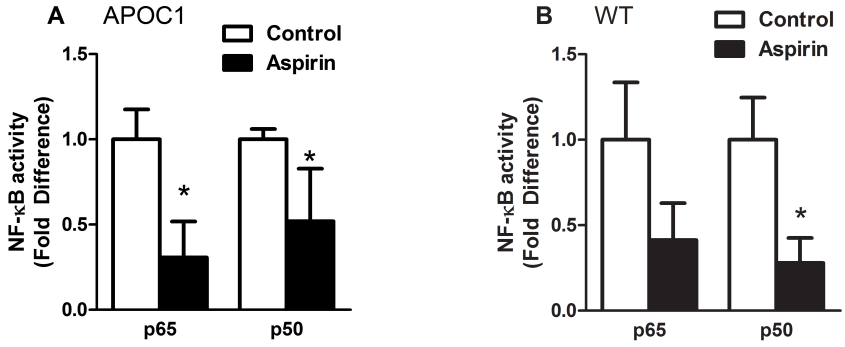

Figure 1. Aspirin reduces hepatic NF-κB activation. APOC1 and WT mice were fed a HFD for 6 weeks and treated without or with aspirin. Mice were sacrificed after an overnight fast and hepatic NF-κB activity was measured by electrophoretic mobility shift assay in liver tissue of APOC1 (A) and WT (B) mice treated without (open bars) or with (closed bars) aspirin. Activities of subunits p50 and p65 were measured. Values are means ± SD (n=3-4). *P<0.05

Aspirin lowers plasma triglyceride and cholesterol levels in HFD-fed *APOC1* mice

To examine whether aspirin could reduce hypertriglyceridemia in *APOC1* mice, hyperlipidemic *APOC1* mice were fed a HFD for 6 weeks and treated with or without aspirin and plasma lipids were determined (**Fig. 2**). Treatment of mice with aspirin reduced plasma TG levels by -32% (3.94 ± 0.15 to 2.67 ± 0.59 mmol/L; P<0.05; Fig. 2A) and plasma TC levels by -33% (4.09 ± 0.52 to 2.76 ± 0.90 mmol/L; P<0.05; Fig. 2B). Aspirin treatment did not affect plasma PL levels (Fig. 2C) and FFA levels (Fig. 2D). The reduction in plasma TG and TC levels was not caused by a reduction in bodyweight, since aspirin did not affect bodyweight in *APOC1* mice (control: 30.5 ± 2.1 g; aspirin: 28.9 ± 3.0 g). In WT mice fed a HFD for 6 weeks, aspirin did not affect plasma TG, TC, PL or FFA levels (Fig. 2E-H). In addition, aspirin did not affect bodyweight in WT mice (control: 30.3 ± 2.1 g; aspirin: 30.8 ± 1.9 g)

Aspirin attenuates VLDL-like emulsion particle-TG clearance in HFD-fed *APOC1*, but not WT mice

A reduction in fasted plasma TG levels can be explained by an increase in VLDL-TG clearance and/or a decrease in hepatic VLDL-TG production. To determine whether aspirin enhances the clearance of VLDL-TG, the plasma clearance and organ distribution of [^{3}H]-TO-labeled TG-rich VLDL-like emulsion particles was evaluated in aspirin and control treated hypertriglyceridemic *APOC1* mice (**Fig. 3**). Unexpectedly, aspirin inhibited, rather than enhanced, serum clearance of [^{3}H]TO (t ½ = 15.9 ± 6.6 *vs* 5.6 ± 2.6 min) (Fig. 3A) in *APOC1* mice. This reduction in [^{3}H]TO clearance upon aspirin was reflected by reduced uptake of [^{3}H]TO-derived radioactivity by the liver by -60% (123 ± 1 *vs* 308 ± 75 nmol/g; P<0.05), by skeletal muscle by -66% (11 ± 2 *vs* 31 ± 15 nmol/g; P<0.05) and by white adipose tissue (WAT), which reached statistical significance for gonadal WAT (12 ± 3 *vs* 44 ± 22 nmol/g; P<0.05) (Fig. 3B). Apparently, aspirin reduces rather than enhances TG clearance in *APOC1* mice and can, therefore, not explain the aspirin-induced reduction in VLDL-TG. In WT mice fed a HFD for 6 weeks, aspirin did not affect plasma clearance of [^{3}H]TO (Fig. 3C) or organ specific uptake of [^{3}H]TO-derived radioactivity (Fig. 3D) in WT mice. Apparently, the decreasing effect of aspirin on TG clearance may be specific for *APOC1* mice.

Aspirin lowers VLDL-TG production in HFD-fed *APOC1* and WT mice

Because the decrease in plasma TG levels in *APOC1* mice upon aspirin treatment was not caused by increased TG clearance, we investigated whether the decreased TG levels could be explained by diminished hepatic VLDL-TG production in *APOC1* mice. The rate of hepatic VLDL-TG production was measured by determining plasma TG levels after intravenous Triton WR1339 injection (**Fig 4**). We found a reduction in hepatic VLDL-TG secretion rate in *APOC1* mice treated with aspirin by -28% (3.42 ± 0.53 vs 4.95 ± 1.11 mM/h; P<0.05) (Fig. 4A), whereas aspirin did not affect the rate of VLDL-apoB production (Fig. 4B). Interestingly, similar to our observation in *APOC1* mice, aspirin did reduce the hepatic VLDL-TG secretion rate in HFD-fed WT mice by -33% (2.79 ± 0.47 mM/h vs 4.19 ± 0.48 mM/h; P<0.05; Fig. 4C), whereas VLDL-apoB

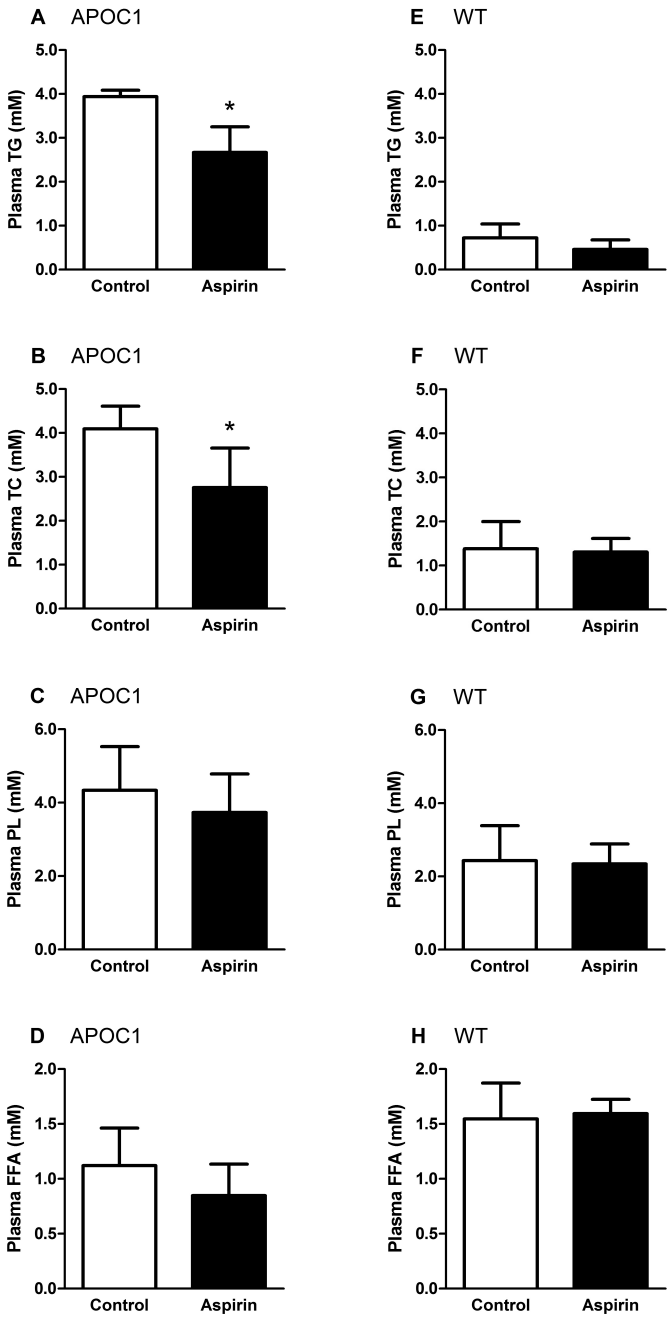

Figure 2. Aspirin lowers plasma triglyceride and cholesterol levels in HFD-fed APOC1 mice. Plasma triglycerides (TG) (A&E), total cholesterol (TC) (B&F), phospholipids (PL) (C&G) and free fatty acid (FFA) (D&H) levels were measured in plasma of overnight-fasted HFD-fed APOC1 and WT mice treated without or with aspirin. Values are means ± SD (n=4-5). *P<0.05.

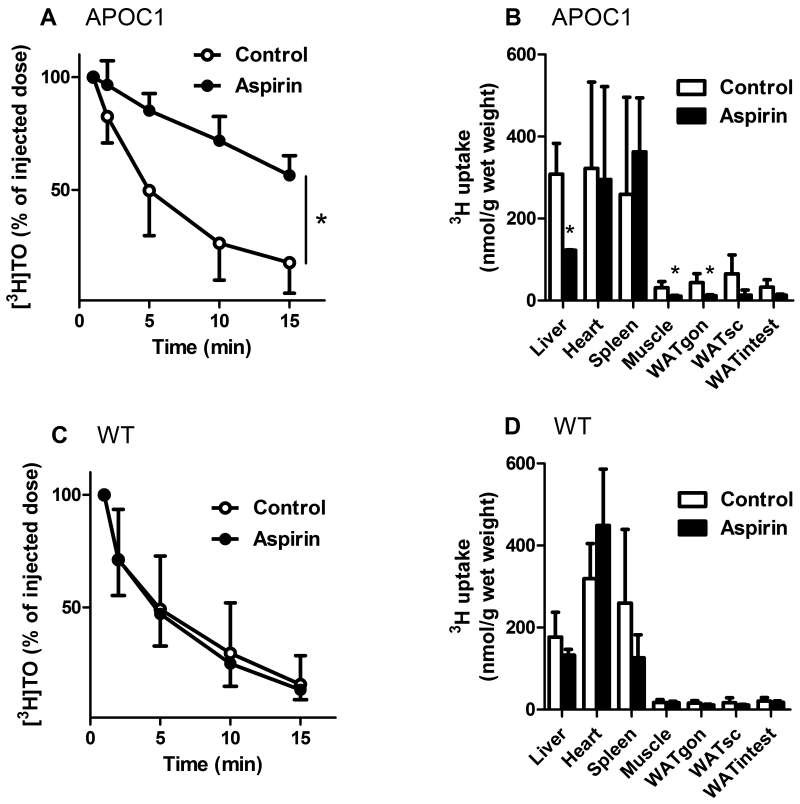

Figure 3. Aspirin attenuates TG clearance of VLDL-like emulsion particles in HFD-fed APOC1, but not WT mice. HFD-fed APOC1 and WT mice that were treated without or with aspirin were fasted overnight and injected with [3H]TO-labeled VLDL-like emulsion particles. Blood was collected at the indicated time points and radioactivity was measured in plasma of APOC1 (A) and WT (C) mice treated without (open circles) or with (closed circles) aspirin. Uptake of [3H]TO-derived activity by various organs was determined, and total FA uptake was calculated from the specific activity of TG in plasma, and expressed as nmol FA per mg wet tissue weight in APOC1 (B) and WT (D) mice. Values are means ± SD (n=4). *P<0.05. WAT, white adipose tissue; intest, intestinal; sc, subcutaneous; gon, gonadal.

production rate was also not affected (Fig. 4D). Apparently, aspirin generally reduces the VLDL-TG production in HFD-fed mice, independent of the genotype. Furthermore, since each VLDL particle contains a single apoB molecule, this observation shows that aspirin treatment inhibits hepatic VLDL-TG production, without affecting the rate of VLDL particle production.

Aspirin does not affect liver lipid levels in HFD-fed *APOC1* and WT mice

To determine whether the attenuation in hepatic VLDL-TG production was the result of decreased lipid substrate availability in the liver, the effect of aspirin on hepatic lipid content was measured (**Fig. 5**). However, aspirin did not affect liver TG levels (Fig. 5A)

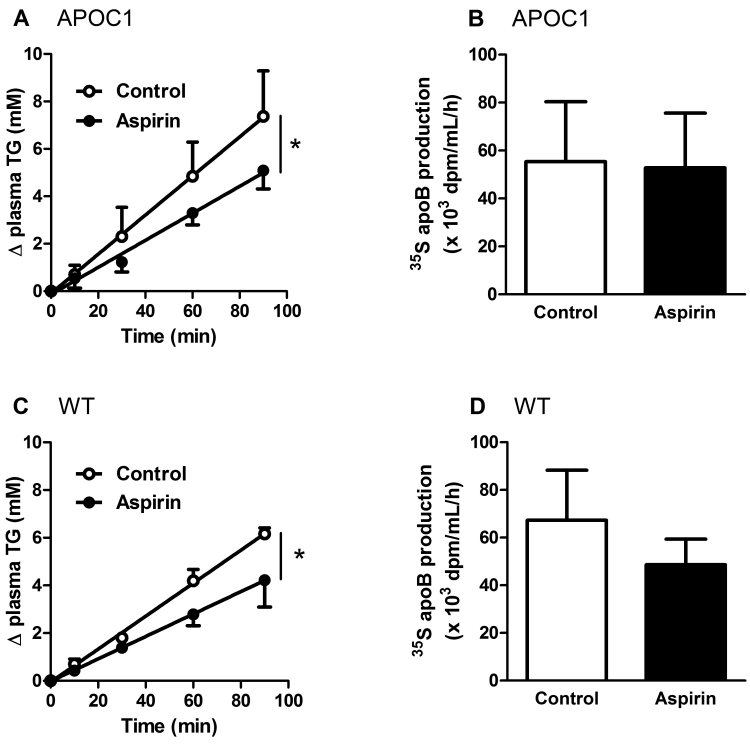

Figure 4. Aspirin decreases VLDL-TG production in HFD-fed APOC1 and WT mice. APOC1 and WT mice were fed a HFD and treated without or with aspirin. Overnight fasted mice were injected with Trans35S and TritonWR1339 and blood samples were drawn at the indicated time points. TG concentrations were determined in APOC1 (A) and WT (C) mice treated without (open circles) or with (closed circles) aspirin and plotted as the increase in plasma TG relative to t=0 (A). After 120 min, VLDL was isolated by ultracentrifugation, 35S-activity was counted and the production rate of newly synthesized VLDL-35S-apoB was determined for APOC1 (B) and WT (D). Values are means ± SD (n=5). *P<0.05.

and TC levels (Fig. 5B) in *APOC1* mice. Also, aspirin did not affect liver TG (Fig. 5C) or TC (Fig. 5D) levels in WT mice.

Aspirin treatment does not affect hepatic expression of genes involved in FA oxidation, lipogenesis or VLDL production

Since changes in hepatic gene expression could underlie the reduction in VLDL-TG production, we determined the effect of aspirin on expression of genes involved in FA oxidation, lipogenesis and VLDL production (**Table 2**). In both *APOC1* and WT mice, aspirin did not affect expression of peroxisome proliferative activated receptor alpha (*Ppara*), a transcription factor that regulates genes involved in FA oxidation and ketogenesis, nor did it affect its target genes acyl-Coenzyme A oxidase 1 (*Acox1*) and carnitine palmitoyltransferase 1a (*Cpt1a*). In line with these results, aspirin did not

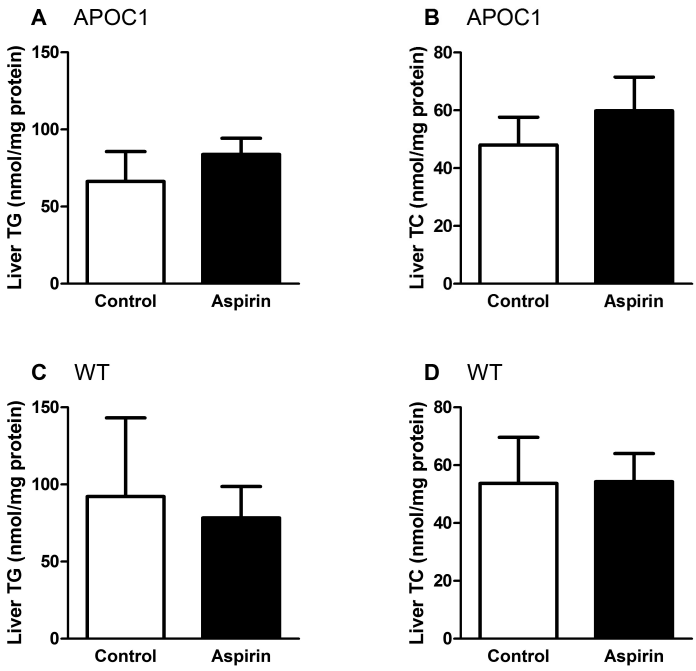

Figure 5. Aspirin does not affect liver lipids in HFD-fed APOC1 and WT mice. Livers were collected from overnight-fasted HFD-fed APOC1 and WT mice treated without or with aspirin. Lipids were extracted and triglyceride (TG, A&C) and total cholesterol (TC, B&D) concentrations were measured and expressed per mg protein. Values are means ± SD (n=6).

increase plasma ß-HB levels in WT mice (data not shown), which is a plasma marker for hepatic FA oxidation and ketogenesis. This implies that the reduced VLDL-TG production upon aspirin treatment is not caused by increased hepatic FA oxidation. We additionally determined the effect of aspirin on expression of genes involved in lipogenesis. In both APOC1 and WT mice, aspirin did not affect expression of sterol regulatory element binding protein 1c (*Srebp-1c*), which regulates genes required for de novo lipogenesis, nor did it affect acyl:diacylglycerol transferase 1 (*Dgat1*), which catalyzes the final and only committed step in TG synthesis, or FA synthase (*Fas*), which plays a key role in FA synthesis. These data suggests that aspirin does not affect genetic regulation of *de novo* lipogenesis. In addition, even though aspirin induced an increase of VLDL-TG secretion, aspirin did not affect hepatic gene expression of microsomal TG transfer protein (*Mttp*) which is involved in the assembly and secretion of VLDL. Furthermore, aspirin does not affect hepatic gene expression of apoB (*Apob*) in *APOC1* mice, which is in line with the observation that aspirin does not affect VLDL-apoB secretion *in vivo*. However, despite the fact that aspirin did not affect VLDL-apoB secretion in WT mice, gene expression of *Apob* was increased in WT mice.

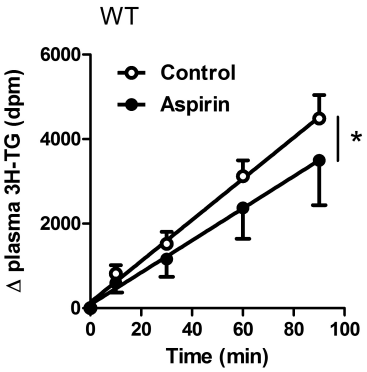

Figure 6. Aspirin reduces the contribution of plasma derived FA to the VLDL-TG production. WT mice were fed a HFD and treated without or with aspirin. Overnight fasted mice received a continuous i.v. infusion of 3H-labeled FA ([9,10(n)-3H] palmitic acid for 2 h, followed by an i.v. injection of TritonWR1339. Blood samples were drawn at the indicated time points and 3H activity in the TG fraction was determined in mice treated without (open circles) or with (closed circles) aspirin and plotted as the increase in plasma 3H-TG relative to t=0. Values are means ± SD (n=7). *P<0.05.

Aspirin treatment decreases the contribution of plasma-derived FA to the VLDL-TG production

Because the decrease in VLDL-TG production was not caused by a reduced hepatic lipid content or decreased expression of genes involved in *de novo* lipogenesis that could reduce lipid availability for VLDL-TG secretion, we investigated whether the decreased VLDL-TG production could be explained by a diminished contribution of plasma derived FA for VLDL-TG secretion in WT mice **(Fig. 6)**. The contribution of plasma derived FA was measured by determining plasma ^{3}H-TG levels after continuous ^{3}H-FA infusion and intravenous Triton WR1339 injection. We found that aspirin reduced hepatic ^{3}H-TG secretion rate in WT mice by -24% (3.1 ± 0.4 vs 2.4 ± 0.7 x 10^{3} dpm/h; P<0.05), which suggests that aspirin reduces VLDL-TG production by reducing the incorporation of plasma-derived FA into VLDL-TG. This reduction is not caused by a reduced hepatic expression of genes involved in hepatic FA uptake and transport (**Table 2**), since aspirin did not affect liver-type FA binding protein (*Fabp1*), FA transport proteins 2, 4 and 5 (*Slc27a2, Slc27a4, Slc27a5*) and even increased expression of FA translocase (*Cd36*) in *APOC1*, but not WT, mice. These data imply that aspirin reduced the VLDL-TG production independent of changes in hepatic expression of genes involved in FA uptake and transport.

DISCUSSION

Treatment of obese rodents and patients with type 2 diabetes with high dose aspirin reduces hypertriglyceridemia.[13,14] However, so far, the mechanistic basis for the relation between aspirin intake and reduced plasma TG levels has been poorly understood. In the present study we focused on the effects of aspirin on VLDL-TG metabolism in HFD-induced obese hyperlipidemic *APOC1* mice and additionally evaluated the effects of aspirin on VLDL-TG metabolism in HFD-fed normolipidemic WT mice. Our results document that aspirin treatment improves hypertriglyceridemia by reducing the hepatic production of VLDL-TG as a result of an attenuated hepatic incorporation of plasma derived FA into VLDL-TG, rather than from increased clearance of VLDL-TG from the circulation.

Table 2. Aspirin generally does not affect hepatic expression of genes involved in FA uptake and transport, FA oxidation, lipogenesis or VLDL secretion.

		APOC1			WT		
Gene	**Protein**	**Control**	**Aspirin**	**p-value**	**Control**	**Aspirin**	**p-value**
FA uptake and transport							
Fabp1	FABP1	1.00 ± 0.37	0.73 ± 0.35	0.22	1.00 ± 0.41	0.61 ± 0.25	0.14
Slc27a2	FATPa2	1.00 ± 0.45	1.23 ± 0.49	0.46	1.00 ± 0.33	0.74 ± 0.16	0.14
Slc27a4	FATPa4	1.00 ± 0.35	1.74 ± 0.51	0.13	1.00 ± 0.44	1.01 ± 0.47	0.77
Slc27a5	FATPa5	1.00 ± 0.17	1.20 ± 0.39	0.18	1.00 ± 0.40	0.96 ± 0.31	0.62
Cd36	CD36	1.00 ± 0.58	1.75 ± 0.40	0.05	1.00 ± 0.80	0.45 ± 0.22	0.23
FA oxidation							
Ppara	PPARα	1.00 ± 0.29	1.12 ± 0.46	0.62	1.00 ± 0.37	0.72 ± 0.18	0.18
Acox1	ACO	1.00 ± 0.42	1.55 ± 0.55	0.14	1.00 ± 0.36	0.59 ± 0.12	0.09
Cpt1a	CPT1a	1.00 ± 0.55	1.36 ± 0.43	0.22	1.00 ± 0.10	0.96 ± 0.11	0.46
Lipogenesis							
Dgat1	DGAT1	1.00 ± 0.37	1.20 ± 0.11	0.29	1.00 ± 0.42	1.06 ± 0.53	0.85
Fasn	FAS	1.00 ± 0.42	1.05 ± 1.09	0.81	1.00 ± 0.40	0.97 ± 0.27	0.90
Srebf1	SREBP-1c	1.00 ± 0.40	1.22 ± 0.53	0.41	1.00 ± 0.53	0.85 ± 0.68	0.72
VLDL secretion							
Apob	ApoB	1.00 ± 0.46	1.20 ± 0.26	0.73	1.00 ± 0.32	1.59 ± 0.31*	0.03
Mttp	MTP	1.00 ± 0.37	0.87 ± 0.21	0.56	1.00 ± 0.39	1.21 ± 0.12	0.34

Livers were isolated from overnight fasted *APOC1* and WT mice fed a HFD and treated without or with aspirin. mRNA was isolated and mRNA expression of the indicated genes was quantified by RT-PCR. Genes are grouped as genes involved in FA uptake and transport, FA oxidation, lipogenesis and VLDL production. Data are calculated as fold difference as compared to the control group. Values are means ± SD (n=4-5). *P<0.05 compared to control group. *Acox1,* acyl-Coenzyme A oxidase 1, palmitoyl; *Apob*, apolipoprotein B; *Cd36,* fatty acid translocase; *Cpt1a,* carnitine palmitoyltransferase 1a, liver; *Dgat1*, diglyceride acyltransferase 1; *Fabp1*, fatty acid binding protein 1, liver; *Fasn*, fatty acid synthase; *Mttp*, microsomal triglyceride transfer protein; *Ppara*, peroxisome proliferative activated receptor alpha; *Slc27a2*, fatty acid transport protein 2; *Slc27a4*, fatty acid transport protein 4; *Slc27a5*, fatty acid transport protein 5; *Srebpf1*, sterol-regulatory element binding protein.

In the present study, aspirin treatment decreased plasma TG and TC levels in HFD fed *APOC1* mice that display hypertriglyceridemia. This improvement in hyperlipidemia is in accordance with earlier studies, showing reduced serum TG concentrations upon aspirin or salicylate treatment in patients with type 2 diabetes mellitus[14] and in diabetic rats.[13]

Our data show that aspirin treatment attenuated the clearance of VLDL-like TG-rich particles in *APOC1* mice. Therefore, the decrease in plasma TG levels by aspirin can not be explained by increased TG clearance. Earlier studies report that high dose LPS injections reduce the clearance of TG-rich lipoproteins by inhibition of the LPL activity, mediated by cytokines.[6,27] If indeed inflammation inhibits clearance of TG, inhibition

of inflammation by aspirin is expected to increase TG-rich lipoprotein clearance, which is in contrast to our observation in *APOC1* mice. It should be noted that aspirin, in addition to inhibition of inflammation via NF-κB, also inhibits prostaglandin synthesis, which has been demonstrated to restore the LPS-induced inhibition of LPL.[28] Moreover, an early report has shown that aspirin treatment inhibits post-heparin LPL activity in humans.[29] It would be interesting to elucidate the mechanism by which aspirin reduces the VLDL-TG clearance, however this is beyond the scope of the current manuscript as it does not explain the reduction in hypertriglyceridemia that we observe. Moreover, the observation may be a specific feature of the *APOC1* transgenic mouse model, since we did not observe such an effect in WT mice.

Aspirin very effectively reduced hepatic secretion of VLDL-TG in *APOC1* mice, explaining the reduction in hypertriglyceridemia upon aspirin treatment. In addition, aspirin equally reduced hepatic secretion of VLDL-TG in WT mice, indicating that the effects of aspirin on the VLDL-TG production do not exclusively occur in hypertriglyceridemic mouse models such as the *APOC1* mouse. To our knowledge, we show for the first time that a decrease in inflammation corresponds with a drop in VLDL-TG production. The reduction of VLDL-TG secretion in our study is not paralleled by a reduction in apoB secretion in both *APOC1* and WT mice, suggesting that aspirin reduces the lipidation of VLDL particles rather than reducing the number of particles that are secreted by the liver. In contrast to our data on apoB secretion, a recent study by Tsai et al[30] observed that suppression of IKK with BMS345541 decreased apoB secretion *in vitro* in primary hamster hepatocytes and HepG2 cells. Even though differences between species might explain these conflicting findings, both these published *in vitro* studies and our present *in vivo* study point towards a link between the IKK/NF-κB pathway and the regulation of VLDL production. Moreover, we have recently shown that *activation* of the hepatic IKK/NF-κB pathway increases VLDL-TG production,[31] supporting the hypothesis that the effects of aspirin on the VLDL-TG production are mediated via a reduction in hepatic NF-κB activity. Nevertheless, activation of hepatic IKK/NF-κB increases hepatic *Fas* expression,[31] while aspirin in the current study did not change hepatic expression of *Fas*, neither did it change expression of other genes involved in TG synthesis, such as *Dgat1* and *Srepb-1c,* suggesting that aspirin more likely lowers VLDL-TG production by other mechanisms than via its effects on hepatic NF-κB activity.

A reduction in hepatic lipid availability by increased lipid oxidation could underlie the mechanism by which aspirin reduces hepatic VLDL-TG production. However, aspirin did not affect expression of genes involved in FA oxidation nor plasma levels of ß-HB, a marker of hepatic FA oxidation and ketogenesis. Similarly, aspirin did not affect expression of genes involved in de novo lipogenesis or VLDL production, suggesting that aspirin does not reduce VLDL-TG production by changing expression of genes involved in hepatic lipid metabolism.

It has been suggested that the decrease in plasma TG concentration that occurs upon aspirin treatment might be secondary to the fall in plasma FFA levels.[32] A reduction in

FFA delivery to the liver could result in a reduced availability of FA for the release of VLDL-TG by the liver.[33] Indeed, although aspirin did not change plasma FFA levels, it changed the turnover of FA as reflected by a -24% reduction in the incorporation of plasma derived FA into VLDL-TG, showing that aspirin in fact lowers the availability of plasma derived FA for VLDL-TG production. This reduction of FA incorporation into VLDL-TG upon aspirin treatment was not caused by a reduced hepatic expression of FA transporter proteins, suggesting that aspirin reduces the FA incorporation via another mechanism. It is possible that aspirin reduces posttranscriptional processing of FA transporters independent of mRNA expression, since expression of FA transporters does not always correlate with changes in protein content or the rate of FA transport.[34] Alternatively, aspirin might increase FA uptake and transport via simple diffusion, since FA uptake has been described independent of any FA transporter.[34]

The decrease of FA turnover that we observed could be secondary to an increased insulin sensitivity of adipose tissue, thereby decreasing FA mobilization to plasma. Indeed, high dose salicylates, such as aspirin have shown to increase insulin sensitivity[13] and the reduction in VLDL-TG production in our study is similarly accompanied by an increased insulin sensitivity (unpublished observation). However, the aspirin-induced reduction in FA utilization and subsequent VLDL-TG secretion in our study were determined under fasting conditions, when the role of insulin is marginal. In fasting conditions, the lipolytic activity of adipocytes is stimulated by catecholamines. Interestingly, aspirin has been reported to reduce catecholamine-stimulated lipolysis, which is therefore a more likely explanation for our findings.[35,36] In addition, it has been shown that aspirin reduces release of FA from adipose tissue directly via inhibition of TNF-α induced lipolysis.[37] We therefore propose that the fact that aspirin reduces plasma derived FA utilization by the liver, is likely caused via direct inhibition of intracellular lipolysis in adipose tissue, which reduces plasma FA availability. Adipose tissue lipolysis might be further inhibited in the fed state by an increased sensitivity for insulin.

In conclusion, our data show that aspirin inhibits NF-κB and decreases HFD-induced hypertriglyceridemia by reducing hepatic VLDL-TG secretion rather than by accelerating the tissue distribution of VLDL-TG. The reduction in VLDL-TG is not caused by a decreased steatosis, increased FA oxidation or changes in *de novo* lipogenesis, but by an attenuation of hepatic incorporation of plasma derived FA into VLDL-TG. In scope of our findings, aspirin could potentially be a new therapeutic drug in the treatment of hypertriglyceridemia. However, chronic high-dose aspirin is associated with risk for bleeding. Salsalate on the other hand is a non-steroidal anti-inflammatory drug with similar structure that is regarded as a safer alternative. High-dose salsalate treatment has recently shown to reduce TG levels in diabetic patients similar to high-dose aspirin treatment,[38] and could therefore potentially be a new drug for the treatment of hypertriglyceridemia.

ACKNOWLEDGEMENTS

The authors are grateful to A. Logiantara for excellent technical assistance. This work was supported by grants from the Netherlands Organization for Scientific Research (NWO Zon-MW; 917.76.301 to P.J. Voshol) and the Dutch Diabetes Research Foundation (2005.01.003 to P.J. Voshol). I.O.C.M. Vroegrijk is supported by the seventh framework program of the EU-funded "LipidomicNet" (202272). P.C.N. Rensen is an Established Investigator of the Netherlands Heart Foundation (2009T038).

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HEPATOCYTE-SPECIFIC IKK-β ACTIVATION ENHANCES VLDL-TRIGLYCERIDE PRODUCTION IN APOE*3-LEIDEN MICE

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J Lipid Res 2011; 52: 942-50

ABSTRACT

Low-grade inflammation in different tissues, including activation of the nuclear factor κB (NF-κB) pathway in liver, is involved in metabolic disorders such as type 2 diabetes and cardiovascular diseases (CVD). In this study we investigated the relation between chronic hepatocyte-specific overexpression of IKK-β and hypertriglyceridemia, an important risk factor for CVD, by evaluating whether activation of IKK-β only in the hepatocyte affects VLDL-triglyceride (TG) metabolism directly. Transgenic overexpression of constitutively active human IkB kinase (IKK-β) specifically in hepatocytes of hyperlipidemic APOE*3-Leiden mice clearly induced hypertriglyceridemia. Mechanistic *in vivo* studies revealed that the hypertriglyceridemia was caused by increased hepatic VLDL-TG production, rather than a change in plasma VLDL-TG clearance. Studies in primary hepatocytes showed that IKK-β overexpression also enhances TG secretion *in vitro*, indicating a direct relation between IKK-β activation and TG production within the hepatocyte. Hepatic lipid analysis and hepatic gene expression analysis of pathways involved in lipid metabolism suggested that hepatocyte specific IKK-β overexpression increases VLDL production not by increased steatosis or decreased FA oxidation, but most likely by ChREBP-mediated upregulation of *Fas* expression. These findings implicate that specific activation of inflammatory pathways exclusively within hepatocytes induces hypertriglyceridemia. Furthermore, we identify the hepatocytic IKK-β pathway as a possible target to treat hypertriglyceridemia.

INTRODUCTION

Obesity is associated with diseases such as dyslipidemia, type 2 diabetes and cardiovascular disease (CVD). The accumulation of lipids in numerous tissues is accompanied by increased inflammatory processes, such as macrophage infiltration and production of inflammatory mediators in white adipose tissue. In liver, fat accumulation increases the activity of the pro-inflammatory nuclear factor κB (NF-κB), and liver-specific activation of NF-κB induces metabolic disturbances.[1,2]

Hypertriglyceridemia is caused by accumulation of VLDL particles in the plasma as a consequence of changes in lipid metabolism that are associated with obesity. Proinflammatory cytokines can cause hypertriglyceridemia[3] and, conversely, suppression of inflammation may reduce hypertriglyceridemia[4] suggesting a direct causal role for inflammatory pathways in the development of hypertriglyceridemia. In fact, administration of lipopolysaccharide (LPS), an inflammatory component of the outer membrane of Gram-negative bacteria, increases plasma triglyceride (TG) levels.[5] However, many inflammatory mediators affect multiple tissues, such as muscle, adipose tissue and liver and, moreover, they can act on multiple cell types including macrophages. The specific contribution of hepatocytes in the relation between inflammation and TG metabolism has never been studied.

In the current study we, therefore, aimed to investigate whether activation of the inflammatory NF-κB pathway exclusively in hepatocytes affects VLDL-TG metabolism and, as a consequence, causes hypertriglyceridemia. To this end, we used hepatocyte-specific transgenic IKK-β (LIKK) mice, which have been described before.[1] LIKK mice have an albumin promoter to drive expression of constitutively active human IκB kinase β (IKK-β), which activates the NF-κB pathway selectively in hepatocytes. To study the effects of the hepatocyte-specific inflammation on VLDL-TG metabolism, we crossbred the LIKK mouse with the transgenic APOE*3-Leiden (E3L) mouse that expresses human APOE*3-Leiden (a mutant form of APOE3) and human APOC1,[6] both of which attenuate the clearance of apoE-containing TG-rich lipoproteins. Therefore, the E3L mouse shows increased plasma TG and cholesterol levels and is a well-established model of human-like lipoprotein metabolism.[7] By using the E3L. LIKK mouse, we were able to study the effects of the inflammatory NF-κB pathway in the hepatocyte on TG-rich lipoprotein metabolism directly. Our results show that activation of NF-κB in hepatocytes of E3L mice induces hypertriglyceridemia by enhancing VLDL-TG production directly within hepatocytes.

MATERIALS AND METHODS

Animals

LIKK mice, which express constitutively active human IKK-β selectively in hepatocytes under control of the albumin promoter[1] were crossbred with E3L mice,[6] expressing both human APOE*3-Leiden and human APOC1, in our animal facility to obtain heterozygous E3L.LIKK mice on a C57Bl/6J background. Male E3L.LIKK and E3L

littermates were housed under standard conditions with a 12-hour light-dark cycle and were fed a standard mouse chow diet with free access to water. Experiments were performed in 14-week old animals after an overnight fast. All experiments were approved by the institutional ethical committee on animal care and experimentation.

Western blot analysis

Tissues were homogenized by Ultraturrax (22,000 rpm; 2x5 sec) in an ice-cold buffer (pH 7.4) containing 30 mM Tris.HCl, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 0.5% (v/v) Triton X-100, 1% (v/v) SDS and protease inhibitors (Complete, Roche, Mijdrecht, The Netherlands) at a 1:6 (w/v) ratio. Homogenates were centrifuged (16,000 rpm; 15 min, 4°C) and the protein content of the supernatant was determined using the BCA protein assay kit (Pierce, Rockford, IL). Proteins (20-50 µg) were separated by 7-10% SDS-PAGE followed by transfer to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked for 1 h at room temperature in Tris-buffered saline with Tween-20 (TBST) with 5% non-fat dry milk followed by an overnight incubation with the following antibodies: p-Ser536 NF-κB p65 (#3031), NF-κB p65 (#3034), p-Ser32/36 IκBα (#9246), IκBα (#9242) (all from Cell Signalling), MTP (#612022) (BD Biosciences, Erembodegem, Belgium) and DGAT1 (#54037) (Abcam, Cambridge, UK). Blots were then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence (ECL) and quantified using Image J (NIH).

Plasma lipids and lipoprotein profiles

Blood was collected from the tail vein into chilled paraoxon (Sigma, St Louis, MO)-coated capillaries to prevent ongoing lipolysis.[8] Capillaries were placed on ice, centrifuged and plasma was assayed for TG, total cholesterol (TC), and phospholipids (PL) using commercially available enzymatic kits from Roche Molecular Biochemicals (Indianapolis, IN). Free fatty acids (FFA) were measured using NEFA-C kit from Wako Diagnostics (Instruchemie, Delfzijl, the Netherlands). For the determination of lipid distribution over plasma lipoproteins, 50 µL of pooled plasma was used for fast performance liquid chromatography (FPLC). Plasma was injected onto a Superose 6 column (Äkta System; Amersham Pharmacia Biotech, Piscataway, NJ), and eluted at a constant flow rate of 50 µL/min with PBS pH 7.4. TG and TC were measured as described above in collected fractions of 50 µL.

Liver lipids

Lipids were extracted from livers according to a modified protocol from Bligh and Dyer.[9] Briefly, a small piece of liver was homogenized in ice-cold methanol. After centrifugation, lipids were extracted by addition of 1800 µL CH_3OH:$CHCl_3$ (3:1 v/v) to 45 µL homogenate. The $CHCl_3$ phase was dried and dissolved in 2% Triton X-100. Hepatic TG and TC concentrations were measured using commercial kits as described earlier. Liver lipids were expressed per mg protein, which was determined using the BCA protein assay kit.

FA composition of liver TG

Liver samples (100 mg) were homogenized with 0.5 mL saline. Subsequently, 5 mL of chloroform: methanol (2: 1 by volume) was added containing butylated hydroxytoluene (BHT). In addition, an internal TG standard was added before extraction. Liver lipids were extracted according to the method of Folch et al.[10] Total TG were separated by spotting lipid extracts onto silica gel 60 (Merk) thin-layer chromatography plates and running in hexane: diethyl ether: acetic acid (85: 15: 1, v/v/v). Lipid bands were visualized under UV light after spraying with 0.1% ANS (8-anilino-1-naphthalene sulfonic acid), and identified using commercial standards. TG bands were scraped into glass tubes and methylated at 80°C with 1.5% H_2SO_4 in methanol for 2 h. TG-derived FA were eluted into hexane. Separation and quantification of the FA methyl esters (FAMEs) from liver TG was achieved using gas chromatography, on an Agilent 6890 GC (Agilent Technologies, UK) fitted with a 30 m x 0.53 mm (film thickness 1 μm) capillary column (RTX-Wax). Individual FA peaks were identified by a reference containing known FAMEs. FA compositions (mol%) were then determined.

Generation of VLDL-like emulsion particles

VLDL-like TG-rich emulsion particles were prepared and characterized as described previously.[11,12] Lipids (100 mg) at a weight ratio of triolein: egg yolk phosphatidylcholine: lysophosphatidylcholine: cholesteryl oleate: cholesterol of 70: 22.7: 2.3: 3.0: 2.0, supplemented with 200 μCi of glycerol tri[9,10(n)-^{3}H]oleate ([^{3}H]TO) were sonicated at 10 μm output using a Soniprep 150 (MSE Scientific Instruments, Crawley, UK). Density gradient ultracentrifugation was used to obtain 80 nm-sized emulsion particles, which were used for subsequent experiments. TG content of the emulsions was measured as described above. Emulsions were stored at 4°C under argon and used within 7 days.

In vivo clearance of VLDL-like emulsion particles

To study the *in vivo* clearance of the VLDL-like emulsion particles, overnight fasted mice were anesthetized by intraperitoneal injection of acepromazine (6.25 mg/kg Neurotranq, Alfasan International BV, Weesp, The Netherlands), midazolam (6.25 mg/kg Dormicum, Roche Diagnostics, Mijdrecht, The Netherlands), and fentanyl (0.31 mg/kg Janssen Pharmaceuticals, Tilburg, The Netherlands). Mice were injected (t=0) via the tail vein with 200 μL of [^{3}H]TO-labeled emulsion particles at a dose of 100 μg of TG per mouse. Blood samples were taken from the tail vein at 1, 2, 5, 10 and 15 minutes after injection and plasma ^{3}H-activity was counted. Plasma volumes were calculated as 0.04706 x body weight (g) as determined from ^{125}I-BSA clearance studies as described previously.[13] After taking the last blood sample, the liver, heart, spleen, muscle and white adipose tissue (*i.e.* gonadal, subcutaneous and visceral) were collected. Organs were dissolved overnight at 60°C in Tissue Solubilizer (Amersham Biosciences, Rosendaal, The Netherlands) and ^{3}H-activity was counted. Uptake of [^{3}H]TO-derived radioactivity by the organs was calculated from the ^{3}H activity in each organ divided by plasma-specific activity of [^{3}H]TG and expressed per mg wet tissue weight.

In vivo hepatic VLDL-TG and VLDL-apoB production

To measure VLDL production *in vivo*, mice were fasted overnight and anesthetized as described above. Mice were injected intravenously with Tran^{35}S label (150 µCi/mouse; MP Biomedicals, Eindhoven, The Netherlands) to label newly produced apolipoprotein B (apoB). After 30 minutes, at t=0 min, Triton WR-1339 (Sigma-Aldrich) was injected intravenously (0.5 mg/g body weight, 10% solution in PBS) to block serum VLDL clearance. Blood samples were drawn before (t=0) and 15, 30, 60 and 90 min after injection and used for determination of plasma TG concentration as described above. After 120 min, mice were exsanguinated via the retro-orbital plexus and euthanized by cervical dislocation. VLDL was isolated from serum after density gradient ultracentrifugation at d<1.006 g/mL by aspiration[14] and counted for incorporated ^{35}S-activity.

Isolation of primary mouse hepatocytes.

Primary hepatocytes were isolated from mouse livers according to the method of Berry and Friend[15] modified by Groen et al.[16] Briefly, the portal vein was cannulated and liver was first perfused with a calcium-free Krebs/bicarbonate buffer, saturated with 95% O_2 and 5% CO_2 at a flow rate of 5 mL/min. Subsequently, perfusion of the liver was continued with calcium-containing Krebs/bicarbonate buffer with 0.0125% collagenase (Roche, Penzberg, Germany) during 10-15 min until cellular dissociation was observed. Cells were gently released and centrifuged four times at 50g for 1 min at 4°C to remove non-parenchymal cells from pelleted hepatocytes. Isolated hepatocytes were washed and suspended in complete Williams' E medium containing insulin (Actrapid), fetal calf serum, dexamethasone and penicillin/streptomycin (P/S). Hepatocytes were isolated with similar yields from livers of E3L.LIKK and E3L mice, 70-80% viable, as assessed by trypan blue dye exclusion, and 99% free of non-parenchymal cells by visual inspection. No differences with respect to viability were observed between cells isolated from E3L.LIKK and E3L mice. Cells were seeded into 12-well dishes (Greiner Bio-One), pre-coated with collagen at a density of $1.0x10^6$ viable cells/well in 2 mL complete Williams' E medium. After a 2 h adherence period, non-attached cells were removed from the cultures by careful washing.

In vitro measurement of TG secretion by hepatocytes

TG secretion *in vitro* was measured as described previously.[17] After an overnight incubation, cells were washed 2 times and incubated 4 h in fetal calf serum-free and hormone-free (SF-HF) Williams' E medium. To measure rates of secretion of TG, cells were subsequently incubated in SF-HF medium containing 4.4 µCi of [^{3}H]glycerol (Amersham; UK) with or without 0.75 mM oleate (C18:1) complexed with BSA to stimulate lipogenesis. After 1, 2, 4 or 20 h incubation, medium was collected and cells were washed three times and harvested in 2 mL PBS. Lipids were extracted from medium according to a modified protocol from Bligh and Dyer.[9,17] The lipids were dried under nitrogen, dissolved into chloroform with 2 mM tripalmitin added as a carrier

and subjected to TLC (Silica gel 60, Merck, Belgium) using hexane: diethylether: acetic acid (80/20/1; v/v/v) as mobile phase. Lipid spots were visualized using iodine vapor, and tripalmitin-positive spots were scraped off, dissolved in 0.5 M acetic acid, and assayed for radioactivity by scintillation counting. Protein content of the cells was determined using the BCA protein assay kit as described earlier. Data are expressed as dpm/mg protein.

Hepatic gene expression analysis

Total RNA was extracted from liver tissues using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. RNA quality of each sample was examined by lab-on-a-chip technology using Experion Std Sens analysis kit (Biorad, Hercules, CA, USA). One µg of total RNA was reverse-transcribed with iScript cDNA synthesis kit (Bio-Rad) and the obtained cDNA was purified with Nucleospin Extract II kit (Macherey-Nagel). Real-Time PCR was carried out on the IQ5 PCR machine (Biorad) using the Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). mRNA levels were normalized to mRNA levels of cyclophilin (*Cyclo*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Primer sequences are listed in **Supplemental Table S1**.

Statistical analysis

Data are presented as means ± SD. Statistical differences were calculated using the Mann-Whitney test for two independent samples with SPSS 16.0 (SPSS Inc, Chicago, IL). $P<0.05$ was regarded statistically significant.

RESULTS

LIKK increases liver NF-kB signalling in E3L mice

To verify that LIKK expression in E3L mice increases hepatic NF-κB signalling, livers from E3L and E3L.LIKK mice were assayed for the presence of phosphorylated over total NF-κB and IκBα using Western blot (**Fig. 1**). Indeed, expression of LIKK increased the ratio of pNF-κB Ser^{536} over NF-κB (1.6 ± 0.4 fold; $P<0.05$) (Fig. 1A, B) as well as that of pIκBα $Ser^{32/36}$ over IκBα (1.9±0.6 fold; $P<0.05$) (Fig. 1C, D). The increased ratio of pIκBα $Ser^{32/36}$ over IκBα was mainly caused by a decrease of total IκBα (0.8±0.1 fold; $P<0.05$), indicating increased IκBα ubiquitination and degradation by the proteasome, which reflects activation of the NF-κB pathway. These data are in line with the increased NF-κB signalling previously observed in LIKK mice as compared to wild-type (WT) mice.[1]

LIKK induces hypertriglyceridemia in E3L mice

To determine whether the hepatocyte-specific inflammation affects plasma lipid levels, TG, TC, PL and FFA levels were measured in plasma of E3L and E3L.LIKK mice (**Fig. 2**). LIKK expression in E3L mice increased TG by +39% (2.90±0.52 vs 2.09±0.28 mmol/L; $P<0.05$; Fig. 2A), TC by +18% (2.24±0.25 vs 1.90±0.28 mmol/L; $P<0.05$;

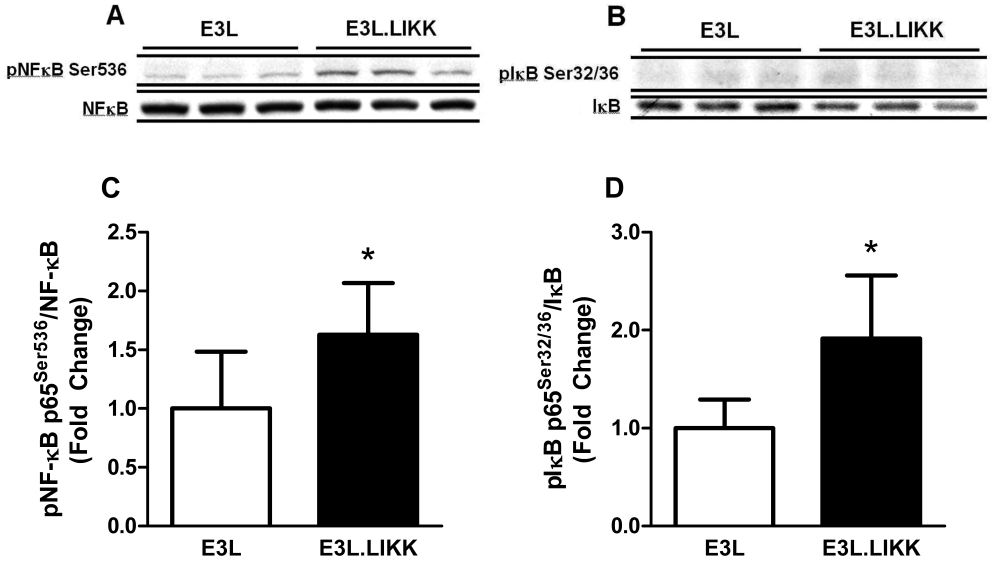

Figure 1. LIKK increases hepatic NF-κB signaling in E3L mice. E3L and E3L.LIKK mice were fed a chow diet and sacrificed at the age of 14 weeks after an overnight fast. NF-κB signaling was measured in liver tissue by phosphorylation of NF-κB (A,C) and IκB (B,D). Representative Western blots of phosphorylated NF-κB (NF-κB Ser536) and total NF-κB (A) and phosphorylated IκBα (pIκBα Ser32/36) and total IκBα (B) are shown for 3 mice per group. Ratios of phosphorylated proteins over total proteins were quantified (B,D). Values are means ± SD (n=5-7). *P<0.05.

Fig. 2B), and PL by +22% (2.12±0.18 vs 1.74±0.27 mmol/L; P<0.05; Fig. 2C). LIKK did not affect plasma FFA levels (Fig. 2D). Lipoprotein profiling showed that the LIKK-induced increase in plasma TG could be explained by a rise in VLDL-TG (+42%) (Fig. 2E). Likewise, the increase in TC was mainly reflected by an increase in VLDL-C (+54%), LDL-C (+34%) and HDL-C (+25%) (Fig. 2F).

LIKK does not affect clearance of VLDL-like emulsion particle-TG in E3L mice

Hypertriglyceridemia is caused by a decrease in VLDL-TG clearance and/or an increase in hepatic VLDL-TG production. To investigate whether LIKK inhibits the clearance of VLDL-TG, the plasma clearance and organ distribution of [^{3}H]TO-labeled TG-rich VLDL-like emulsion particles was evaluated in E3L.LIKK versus E3L mice (**Fig. 3**). LIKK did not affect the plasma half-life of [^{3}H]TO (Fig. 3A), nor the uptake of [^{3}H]TO-derived fatty acids (FA) by the various organs (Fig. 3B), indicating that LIKK does not increase plasma TG levels by decreasing TG clearance.

LIKK increases VLDL-TG production in E3L mice

As no difference was observed in TG clearance between E3L.LIKK and E3L mice, it is likely that the LIKK-induced increase in plasma TG levels can be explained by

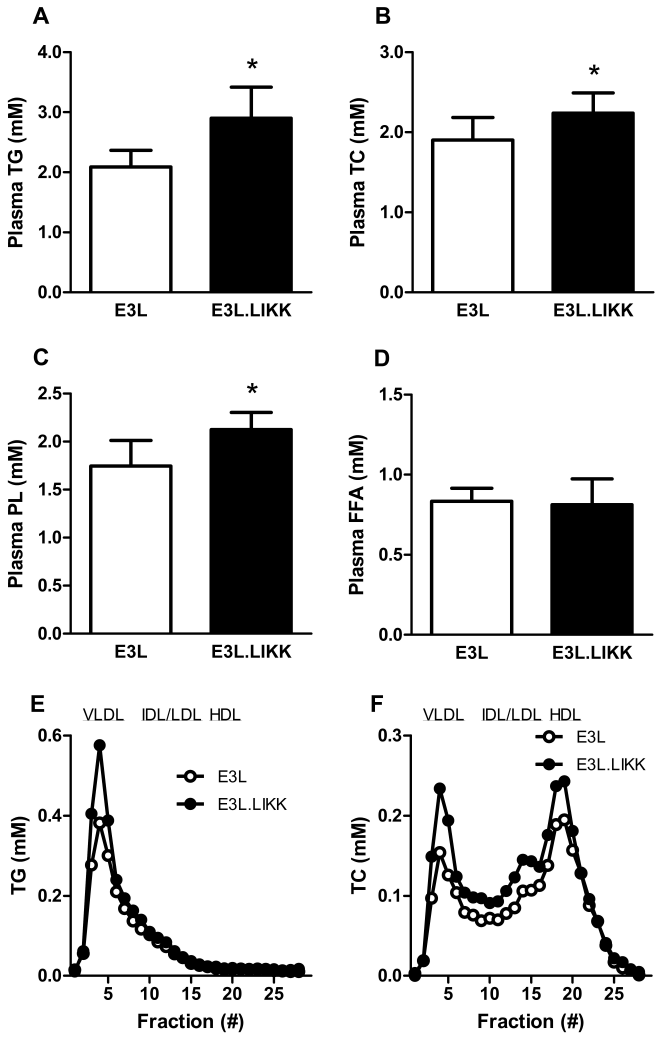

Figure 2. LIKK induces hyperlipidemia in E3L mice. Plasma triglycerides (TG) (A), total cholesterol (TC) (B), phospholipids (PL) (C) and free fatty acid (FFA) (D) levels were measured in plasma of overnight fasted E3L and E3L.LIKK mice. Values are means ± SD (n=5-7). *P<0.05. Plasma was collected, pooled per group, and subjected to FPLC to separate lipoproteins. Distribution of TG (E) and TC (F) over lipoproteins was determined.

an increase of VLDL-TG production. The rate of hepatic VLDL-TG production was measured by determining plasma TG levels after intravenous Triton WR1339 injection (**Fig. 4**). Indeed, LIKK strongly increased the accumulation of plasma TG at all time points (Fig. 4A). The VLDL-TG production rate, as determined from the slope of the curve from all individual mice, was increased by +48% (3.90 ± 1.01 vs 2.64 ± 0.82 mM/h, P<0.05) (Fig. 4B), whereas the rate of VLDL-apoB production did not

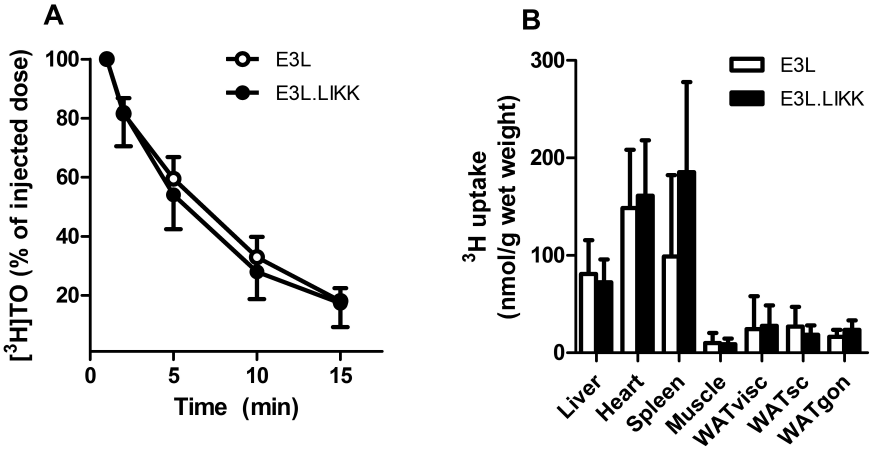

Figure 3. LIKK does not affect clearance of VLDL-like emulsion particle-TG in E3L mice. E3L and E3L.LIKK mice that were fasted overnight were injected with [3H]TO-labeled VLDL-like emulsion particles. Blood was collected at the indicated time points and radioactivity was measured in plasma (A) of E3L mice (open circles) and E3L.LIKK mice (closed circles). Uptake of [3H]TO-derived activity by various organs was determined, and total FA uptake was calculated from the specific activity of TG in plasma, and expressed as nmol FA per mg wet tissue weight (B). Values are means ± SD (n=8). WAT, white adipose tissue; visc, visceral; sc, subcutaneous; gon, gonadal.

change significantly (P=0.52) (Fig. 4C). Since each VLDL particle contains a single apoB molecule, LIKK apparently increases plasma TG levels by enhancing VLDL-TG production without affecting VLDL particle production.

LIKK does not affect liver lipid levels

To investigate whether the increase in hepatic VLDL-TG production was the result of increased lipid substrate availability in the liver, the effect of LIKK on the hepatic lipid content was investigated (**Fig. 5**). However, E3L and E3L.LIKK mice did not differ with respect to liver TG levels (Fig. 5A) and TC levels (Fig. 5B). LIKK did not influence the FA composition of hepatic TG, apart from a mild increase in the relative abundance of linoleic acid (18:2 n-6) by +19% (P<0.05) (**Suppl. Fig. S1**).

LIKK directly increases TG secretion in hepatocytes from E3L mice

To evaluate whether IKK-ß overexpression in hepatocytes directly increases VLDL-TG production, we next studied TG secretion from isolated hepatocytes of E3L and E3L.LIKK mice *in vitro*. We used [^{3}H]glycerol as precursor for TG synthesis, by measuring the accumulation of [^{3}H]TG in the medium (**Fig. 6**). In the absence of oleate, the [^{3}H]TG secretion was low, but LIKK significantly increased the [^{3}H]TG secretion after 20 h of incubation as compared to the [^{3}H]TG secretion from control E3L hepatocytes (2.3-fold; P<0.05) (Fig. 6A). In the presence of oleate, as a substrate for TG synthesis, [^{3}H]

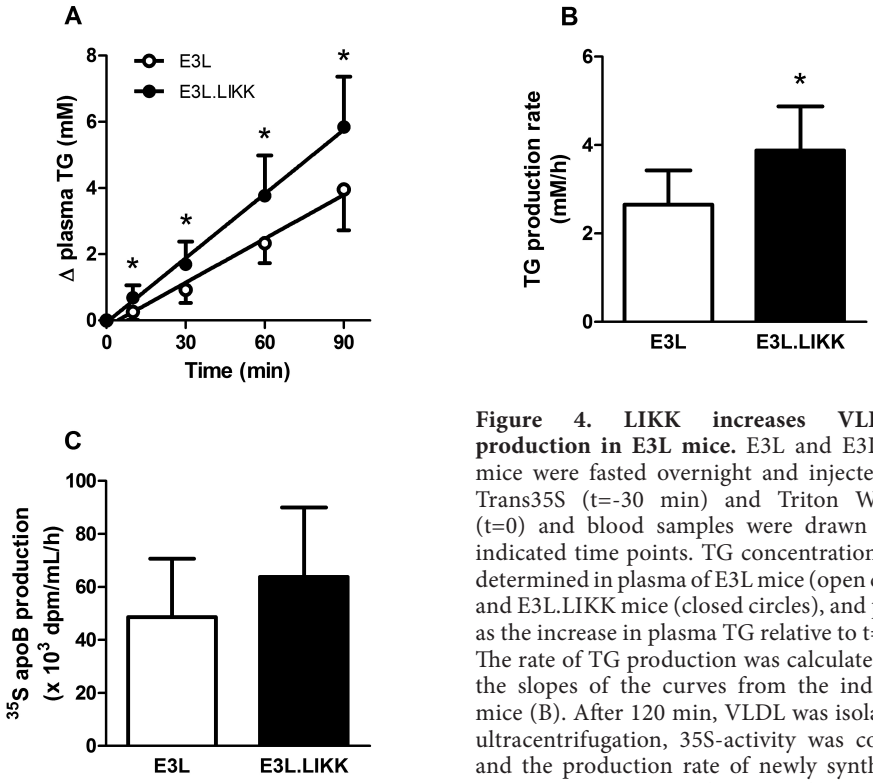

Figure 4. LIKK increases VLDL-TG production in E3L mice. E3L and E3L.LIKK mice were fasted overnight and injected with Trans35S (t=-30 min) and Triton WR1339 (t=0) and blood samples were drawn at the indicated time points. TG concentrations were determined in plasma of E3L mice (open circles) and E3L.LIKK mice (closed circles), and plotted as the increase in plasma TG relative to t=0 (A). The rate of TG production was calculated from the slopes of the curves from the individual mice (B). After 120 min, VLDL was isolated by ultracentrifugation, 35S-activity was counted, and the production rate of newly synthesized VLDL-35S-apoB was determined (C). Values are means ± SD (n=5-8). *P<0.05.

TG secretion was markedly increased, and LIKK caused an additional increase in [^{3}H] TG secretion, reaching significance after 20 h of incubation (1.9-fold at 20 h; P<0.05) (Fig. 6B).

LIKK increases hepatic expression of fatty acid synthase, but does not affect protein levels or expression of genes involved in VLDL production

To obtain further insight into the mechanism underlying the effects of IKK-ß overexpression on VLDL-TG production, we evaluated the hepatic expression of genes involved in VLDL secretion, lipogenesis, FA oxidation, cholesterol metabolism, bile acid metabolism, lipid droplets and nuclear receptors in livers of E3L and E3L.LIKK mice (Table 1). Even though LIKK induced an increase of VLDL-TG production *in vivo* and *in vitro*, LIKK did not affect hepatic gene expression or protein level (**Suppl. Fig. S2A,B**) of microsomal TG transfer protein (*Mttp*), which is involved in the assembly and secretion of VLDL. In addition, LIKK did not affect apoB (*Apob*) expression, in line with the observation that LIKK did not increase VLDL-apoB secretion *in vivo*. Also, LIKK did not affect expression of sterol regulatory element binding protein 1c

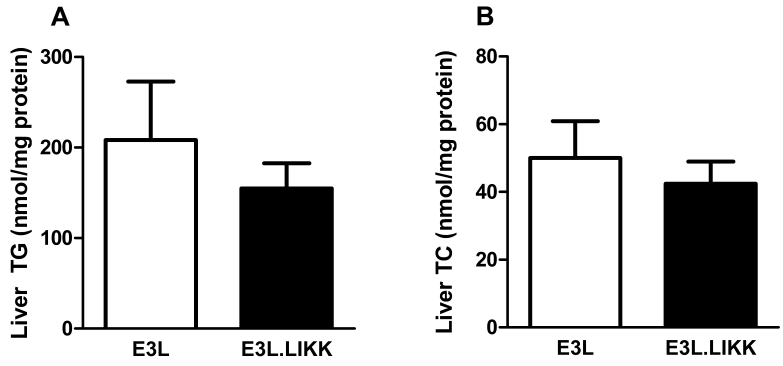

Figure 5. LIKK does not affect liver lipid content in E3L mice. Livers were obtained from overnight fasted E3L and E3L.LIKK mice and lipids were extracted. Triglycerides (TG, A) and total cholesterol (TC, B) concentrations were measured and expressed per mg protein. Values are means ± SD (n=5-7). *P<0.05.

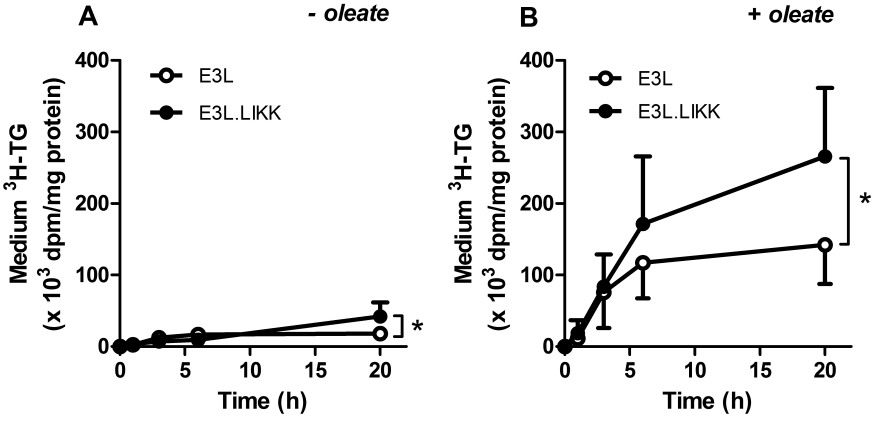

Figure 6. LIKK increases TG secretion in hepatocytes from E3L mice. Hepatocytes were isolated from E3L (open circles) and E3L.LIKK mice (closed circles), cultured overnight, and incubated without or with oleate complexed with bovine serum albumin. [3H]Glycerol was added to quantify newly synthesized triacylglycerols. Medium was collected at the indicated time points, [3H]TG was measured and expressed as dpm per mg cell protein. Values are means ± SD of 3-6 mice per group, in vitro experiments were performed in triplicate (n=3-6). *P<0.05.

(*Srebp-1c*), which regulates genes required for *de novo* lipogenesis, nor did it affect expression or protein levels (Suppl. Fig. S2A,C) of acyl:diacylglycerol transferase 1 (*Dgat1*), which catalyzes the final and only committed step in TG synthesis.

In addition, LIKK did not largely affect clusters of genes involved in FA oxidation (acyl-Coenzyme A oxidase 1 (*Acox1)* and carnitine palmitoyltransferase 1a *(Cpt1a*), cholesterol metabolism (ATP-binding cassette sub-family G member 5 (*Abcg5),*

ATP-binding cassette sub-family G member 8 (*Abcg8) and* HMG-CoA reductase *(Hmgcr*) or bile acid metabolism (cholesterol 7 alpha hydroxylase *(Cyp7a1) and* sterol 12 alpha-hydrolase *(Cyp8b1*)), apart from a 1.9-fold increase in cholesterol 27 hydroxylase *(Cyp27a1)* expression. Additionally, LIKK did not affect clusters of genes involved in lipid droplet formation (perilipin 2 (*Plin2),* fat-specific protein FSP27 (*Cidec) and* cell death activator CIDE-A *(Cidea*)),or expression of nuclear receptors (peroxisome proliferator activated receptor alpha *(Ppara),* PPAR-gamma coactivator 1-beta *(Ppargc1) and* liver X receptor alpha *(Nr1h3)*), apart from a 1.6-fold increase in expression of perilipin 5 (*Plin5)* and farnesoid X activated receptor *(Nr1h4)* respectively.

However, LIKK did increase expression of FA synthase (*Fas*), which plays a key role in FA synthesis, by 2.4-fold, and of liver-type pyruvate kinase (Pklr) by 1.7-fold, both of which are target genes of ChREBP. Taken together, these data suggest that LIKK increases VLDL-TG production by ChREBP-mediated upregulation of Fas expression, suggesting an increase in *de novo* lipogenesis.

DISCUSSION

Obesity leads to an increase in inflammatory processes in numerous organs including the liver.[18] In the current study, we questioned whether increased activation of inflammatory pathways in the liver, specifically in hepatocytes, induces hypertriglyceridemia. Indeed, we show that chronic activation of the inflammatory NF-κB pathway specifically in hepatocytes increases plasma TG, which was caused by an increased VLDL-TG production rather than a decreased clearance of VLDL-TG. Furthermore, we provide evidence that the increased TG production induced by hepatocyte-specific IKKβ overexpression is a direct effect of the transgene expression in the hepatocyte.

The strong relation between inflammation and hypertriglyceridemia has largely been derived from the observed increase in plasma TG during acute infection, which is believed to contribute to the host defense.[19] However, although similar inflammatory pathways are involved, metabolic inflammation is clearly different from acute inflammation with respect to its cause, intensity and duration. The inflammation that is observed in obesity, is a chronic and low-grade inflammation that is caused by a metabolic overload, rather than a pathogen.[20] The NF-κB activity in the liver of E3L. LIKK mice in this study is about 1.5-fold higher compared to control E3L mice, which is similar to hepatic NF-κB activation levels seen after HFD feeding and in obesity.[1] The present study shows that this low-grade activation of hepatocyte-specific IKK-β induces an increase in plasma TG levels in E3L mice, a model for human-like lipoprotein metabolism, which was due to an increase in plasma VLDL-TG levels. Additional investigation of VLDL-TG metabolism revealed that the increased VLDL-TG levels were not caused by decreased clearance of TG from VLDL-like particles, but rather by increased hepatic production of VLDL-TG. These findings are in line with a study showing that injection of a low dose of LPS increases secretion of VLDL-TG, without

Table 1. Effect of LIKK on hepatic expression of genes involved in lipid metabolism in E3L mice.

Gene	Protein	E3L	E3L.LIKK	Change
VLDL secretion				
Apob	ApoB	1.00 ± 0.35	1.01 ± 0.34	n.s.
Mttp	MTP	1.00 ± 0.46	1.34 ± 0.36	n.s.
Lipogenesis				
Srebp-1c	SREBP1c	1.00 ± 0.39	1.10 ± 0.74	n.s.
Dgat1	DGAT1	1.00 ± 0.32	1.21 ± 0.41	n.s.
Fas	FAS	1.00 ± 0.52	2.43 ± 1.07**	+143%
FA oxidation				
Acox1	ACO	1.00 ± 0.70	1.13 ± 0.20	n.s.
Cpt1a	CPT1a	1.00 ± 0.58	0.95 ± 023	n.s.
Glucose metabolism				
Pklr	L-PK	1.00 ± 0.48	1.72 ± 0.80*	+72%
Cholesterol metabolism				
Abcg5	ABCG5	1.00 ± 0.22	1.05 ± 0.31	n.s.
Abcg8	ABCG6	1.00 ± 0.16	0.93 ± 0.16	n.s.
Hmgcr	HMG-CoA	1.00 ± 0.19	0.98 ± 0.08	n.s.
Bile acid metabolism				
Cyp7a1	CYP7A1	1.00 ± 0.59	1.19 ± 0.55	n.s.
Cyp8b1	CYP8B1	1.00 ± 0.60	1.21 ± 0.27	n.s.
Cyp27a1	CYP27A1	1.00 ± 0.41	1.85 ± 0.51**	+85%
Lipid droplets				
Plin2	PLIN2/ADRP	1.00 ± 0.98	1.04 ± 0.33	n.s.
Plin5	PLIN5/PAT-1	1.00 ± 0.72	1.64 ± 0.55*	+64%
Cidec	CIDE-3/FSP27	1.00 ± 0.98	1.05 ± 0.42	n.s.
Cidea	CIDEA	1.00 ± 0.65	0.85 ± 0.59	n.s.
Transcription factors				
Ppara	PPARα	1.00 ± 0.28	0.89 ± 0.16	n.s.
Ppargc1b	PGC-1β	1.00 ± 0.63	0.77 ± 0.26	n.s.
Nr1h3	LXRα	1.00 ± 0.35	1.09 ± 0.09	n.s.
Nr1h4	FXR	1.00 ± 0.50	1.58 ± 0.43*	+58%

Livers were isolated from overnight fasted E3L and E3L.LIKK mice. mRNA was isolated and mRNA expression of the indicated genes was quantified by RT-PCR. Data are calculated as fold difference as compared to the control group. Values are means ± SD (n=8). *P<0.05 and **P<0.01 compared to the control group. n.s., not significant. *Abcg5*, ATP-binding cassette sub-family G member 5 ; *Abcg8*, ATP-binding cassette sub-family G member 8; *Acox1*, acyl-Coenzyme A oxidase 1; *Apob*, apolipoprotein B; *Cidea*, cell death activator CIDE-A; *Cidec*, fat-specific protein FSP27; *Cpt1a*, carnitine palmitoyltransferase 1a; *Cyp27a1*, cholesterol 27 hydroxylase; *Cyp7a1*, cholesterol 7 alpha hydroxylase; *Cyp8b1*, sterol 12 alpha-hydrolase; *Dgat1*, diglyceride acyltransferase 1; *Fas*, fatty acid synthase; *Hmgcr*, HMG-CoA reductase; *Mttp*, microsomal triglyceride transfer protein; *Nr1h3*, liver X receptor alpha; *Nr1h4*, farnesoid X activated receptor; *Pklr*, liver-type pyruvate kinase; *Plin2*, perilipin 2; *Plin5*, perilipin 5; *Ppara*, peroxisome proliferator activated receptor alpha; *Ppargc1b*, PPAR-gamma coactivator 1-beta; *Srebp-1c*, sterol-regulatory element binding protein.

affecting its clearance.[5] However, LPS associates with macrophages rather than with hepatocytes,[21] which hampers interpretation which cell type is primarily responsible for the increase in VLDL-TG secretion. In addition to LPS, individual cytokines, that activate various cell types, increase VLDL-TG production.[3,22] Since both LPS and cytokines can activate NF-κB signalling, our findings could suggest that the increase in VLDL secretion caused by LPS and cytokines in these earlier studies has been mediated, at least in part, via direct or indirect activation of NF-κB in the hepatocytes. In the present study, even though LIKK clearly increased VLDL-TG secretion, there were no significant effects of LIKK on apoB production or hepatic *Apob* gene expression. This suggests that NF-κB activation increases the intracellular lipidation of apoB, but not the number of VLDL-particles secreted. This is in contrast with a study of Tsai et al,[23] showing that adenoviral-mediated overexpression of IKK did increase apoB secretion in HepG2 cells. This discrepancy could possibly be explained by the level of IKK overexpression, which was higher with adenoviral-mediated IKK overexpression in their *in vitro* HepG2 model than with transgenic overexpression in our *in vivo* study. It is thus reasonable to postulate that low grade NF-κB activity mainly increases lipidation of the VLDL particles, whereas a higher degree of NF-κB activation could in addition increase the number of secreted VLDL-particles.

It is interesting to speculate about the mechanism why hepatocyte-specific NF-κB activation increases VLDL-TG secretion, as many different factors could theoretically be involved. For example, IKK-β overexpression can cause insulin resistance,[1] which could result in an inability of insulin to suppress VLDL-TG production.[24] Furthermore, Kupffer cells have been suggested to play an important role in hepatic lipid metabolism.[25] Additionally, Kupffer cell products could possibly suppress lipid oxidation in hepatocytes via NF-κB mediated suppression of PPARα activity.[26] Furthermore, although plasma FFA levels were unaltered by LIKK, liver-directed FA flux may have been influenced, resulting in altered substrate availability for VLDL-TG production. Therefore, to evaluate the effect of IKK-β overexpression in hepatocytes on VLDL-TG production in absence of these potentially confounding factors, we studied the effect of IKK-β in hepatocytes on TG production *in vitro*. In fact, IKK-β expression in hepatocytes *per se* appeared to directly increase VLDL-TG production. Although additional factors may contribute to the effect of LIKK on VLDL-TG production *in vivo*, a direct effect of IKK-β overexpression in hepatocytes thus at least contributes to this phenomenon.

PPAR-α, LXR and FXR have shown to be activated during inflammation and interact with inflammatory processes[27,28] and could possibly underlie the mechanism by which hepatocyte-specific NF-κB activation increases VLDL-TG secretion directly within the hepatocyte. However, no change was observed in expression of hepatic PPAR-α and LXR or expression of their target genes. NF-κB activation did increase FXR expression, but FXR activation has been linked to a lower VLDL-TG secretion,[29] making a causal relationship between FXR activation and the increase in VLDL-TG secretion unlikely. Apparently, chronic hepatocyte-specific activation of NF-κB by

IKK-β overexpression does not induce identical changes in lipogenic pathways that are seen in acute inflammation, however, it clearly increases VLDL-TG production and induces hypertriglyceridemia. Increased hepatic lipid availability, by increased lipogenesis and/or decreased lipid oxidation, could also underlie the mechanism by which hepatocyte-specific NF-κB increases VLDL-TG secretion. Acute inflammation has been shown to increase hepatic lipogenesis as measured by incorporation of $^{3}H_{2}O$ into FA *in vivo.*[5,30,31] In our study we measured expression of genes involved in hepatic FA oxidation and *de novo* lipogenesis. Despite the fact that LIKK did not decrease the expression of genes involved in FA oxidation, LIKK clearly increased expression of *Fas*, which is a key enzyme in the regulation of FA synthesis. Although upregulation of *Fas* could be mediated by the transcription factors LXR, Srebp-1c and ChREBP,[32,33] the observed upregulation of *Pklr* as a main ChREBP target gene suggests that LIKK most likely activates ChREBP, thereby increasing *Fas* expression. The fact that activation of NF-κB has been linked to local disturbances in glucose metabolism that could activate ChREBP would underscore this observation.[1,34,35] It is thus conceivable that increased ChREBP mediated *Fas* expression increases hepatic lipogenesis and thereby increases lipid availability for VLDL-TG production.[36] In fact, activation of lipogenesis results in large, but not more, VLDL particles, which is consistent with our findings[37]. The fact that we did not observe an increase in the hepatic TG content or FA oxidation that could have been expected by increased *Fas* expression,[38] can be explained by efficient incorporation of newly synthesized TG into nascent VLDL resulting in the increased hepatic VLDL-TG secretion.

In conclusion, we show that activation of hepatocyte-specific NF-κB through overexpression of IKK-ß increases TG levels in E3L mice by stimulation of VLDL-TG secretion, directly within the hepatocyte, without effects on VLDL-TG clearance. The stimulation of VLDL-TG secretion is not driven by increased steatosis or decreased FA oxidation, but most likely by ChREBP mediated upregulation of *Fas* expression.

ACKNOWLEDGEMENTS

The authors are grateful to A. Logiantara for excellent technical assistance. This work was supported by grants from the Netherlands Organization for Scientific Research (NWO Zon-MW; 917.76.301 to P.J.V.) and the Dutch Diabetes Research Foundation (2005.01.003 to P.J.V.). M.C.W. is supported by a Mosaic grant of the Dutch Organization for Scientific Research (NWO 017.003.83). P.C.N.R. is an Established Investigator of the Netherlands Heart Foundation (2009T038).

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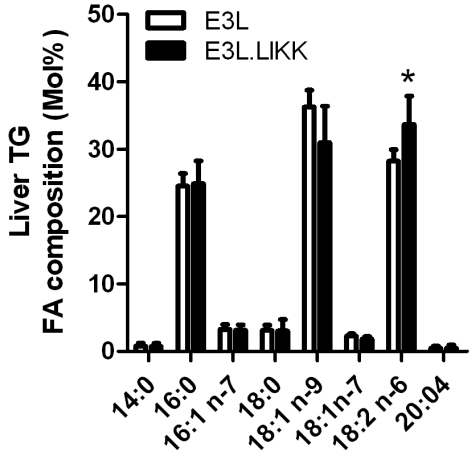
SUPPLEMENTARY DATA

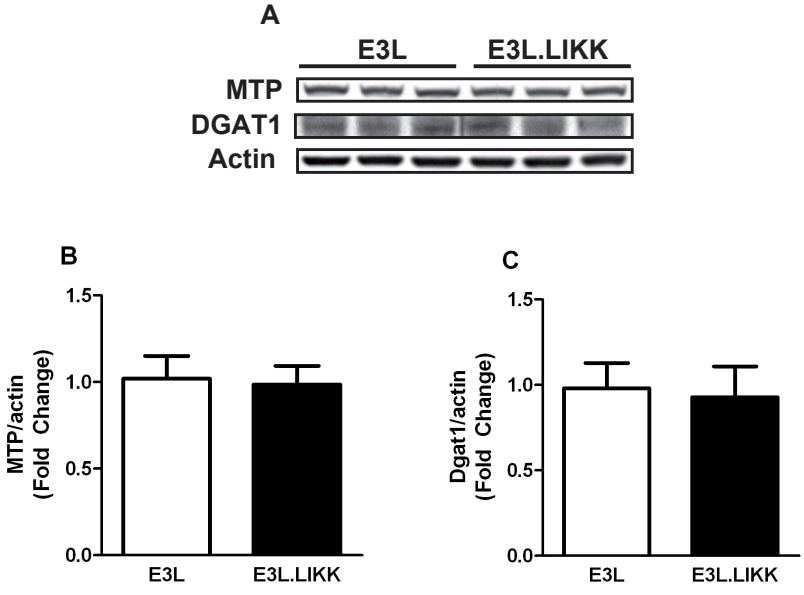
Supplemental Table S1. Primers used for quantatitive real-time PCR analysis.

Gene	Forward primer	Reverse primer
Abcg5	TGTCCTACAGCGTCAGCAACC	GGCCACTCTCGATGTACAAGG
Abcg8	GACAGTTCACAGCCCACAA	GCCTGAAGATGTCAGAGCGA
Acox1	TATGGGATCAGCCAGAAAGG	ACAGAGCCAAGGGTCACATC
Apob	GCCCATTGTGGACAAGTTGATC	CCAGGACTTGGAGGTCTTGGA
Cidea	CTCGGCTGTCTCAATGTCAA	CCGCATAGACCAGGAACTGT
Cidec	CTGGAGGAAGATGGCACAAT	GGGCCACATCGATCTTCTTA
Cpt1a	GAGACTTCCAACGCATGACA	ATGGGTTGGGGTGATGTAGA
Cyclo	CAAATGCTGGACCAAACACAA	GCCATCCAGCCATTCAGTCT
Cyp27a1	TCTGGCTACCTGCACTTCCT	CTGGATCTCTGGGCTCTTTG
Cyp7a1	CAGGGAGATGCTCTGTGTTCA	AGGCATACATCCCTTCCGTGA
Cyp8b1	GGACAGCCTATCCTTGGTGA	CGGAACTTCCTGAACAGCTC
Dgat1	TCCGTCCAGGGTGGTAGTG	TGAACAAAGAATCTTGCAGACGA
Fasn	TCCTGGGAGGAATGTAAACAGC	CACAAATTCATTCACTGCAGCC
Gapdh	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
Hmgcr	CCGGCAACAACAAGATCTGTG	ATGTACAGGATGGCGATGCA
Mttp	CTCTTGGCAGTGCTTTTTCTCT	GAGCTTGTATAGCCGCTCATT
Nr1h3	CTGCACGCCTACGTCTCCAT	AAGTACGGAGGCTCACCAGCT
Nr1h4	GGCCTCTGGGTACCACTACA	ACATCCCCATCTCTTTGCAC
Pklr	GCAGAACGAGTCACAGCAAT	GTGGAGGCTTCCTTCAAGTG
Plin2	CAGGATGGAGGAAAGACTGC	CTTATCCACCACCCCTGAGA
Plin5	TGTCCAGTGCTTACAACTCGG	CAGGGCACAGGTAGTCACAC
Ppara	ATGCCAGTACTGCCGTTTTC	GGCCTTGACCTTGTTCATGT
Ppargc1b	TTGTAGAGTGCCAGGTGCTG	CCTCCATAGCTCAGGTGGAA
Srebp-1c	GGAGCCATGGATTGCACATT	CCTGTCTCACCCCCAGCATA

Abcg5, ATP-binding cassette sub-family G member 5 ; Abcg8, ATP-binding cassette sub-family G member 8; Acox1, acyl-Coenzyme A oxidase 1; Apob, apolipoprotein B; Cidea, cell death activator CIDE-A; Cidec, fat-specific protein FSP27; Cpt1a, carnitine palmitoyltransferase 1a; Cyp27a1, cholesterol 27 hydroxylase; Cyp7a1, cholesterol 7 alpha hydroxylase; Cyp8b1, sterol 12 alpha-hydrolase; Dgat1, diglyceride acyltransferase 1; Fasn, fatty acid synthase; Hmgcr, HMG-CoA reductase; Mttp, microsomal triglyceride transfer protein; Nr1h3, liver X receptor alpha; Nr1h4, farnesoid X activated receptor; Pklr, liver-type pyruvate kinase; Plin2, perilipin 2; Plin5, perilipin 5; Ppara, peroxisome proliferator activated receptor alpha; Ppargc1b, PPAR-gamma coactivator 1-beta; Srebp-1c, sterol-regulatory element binding protein.



Supplemental Figure S1. Effect of LIKK on fatty acid composition of hepatic triglycerides in E3L mice.. Livers were obtained from overnight fasted E3L and E3L.LIKK mice and lipids were extracted. TG were isolated by thin-layer chromatography followed by fatty acid separation and quantification using gas chromatography. Fatty acid composition was then determined (in Mol%). Values are means ± SD (n=5-7).



Supplemental Figure S2. LIKK does not affect hepatic MTP and DGAT1 protein levels. E3L and E3L.LIKK mice were fed a chow diet and sacrificed at the age of 14 weeks after an overnight fast. MTP and DGAT1 levels were measured in liver tissue by Western blots and Actin was used as an internal control. Representative Western blots are shown for 3 mice per group (A). Ratios of MTP (B) and DGAT1 (C) proteins over Actin levels were quantified. Values are means ± SD (n=5-7).

HEPATOCYTE-SPECIFIC IKK-β EXPRESSION AGGRAVATES ATHEROSCLEROSIS DEVELOPMENT IN APOE*3-LEIDEN MICE

5

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Atherosclerosis 2012; 220: 362-8

ABSTRACT

The liver is the key organ involved in systemic inflammation, but the relation between hepatic inflammation and atherogenesis is poorly understood. Since nuclear factor-κB (NF-κB) is a central regulator of inflammatory processes, we hypothesized that chronically enhanced hepatic NF-κB activation, through hepatocyte-specific expression of IκB kinase-β (IKKβ) (*LIKK*), will aggravate atherosclerosis development in *APOE*3-Leiden* (*E3L*) mice. *E3L.LIKK* and *E3L* control littermates were fed a Western-type diet for 24 weeks. *E3L.LIKK* mice showed a 2.3-fold increased atherosclerotic lesion area and more advanced atherosclerosis in the aortic root with less segments without atherosclerotic lesions (11 *vs.* 42%), and more segments with mild (63% *vs.* 44%) and severe (26% *vs.* 14 %) lesions. Expression of *LIKK* did not affect basal levels of inflammatory parameters, but plasma cytokine levels tended to be higher in *E3L.LIKK* mice after lipopolysaccharide (LPS) administration. *E3L.LIKK* mice showed transiently increased plasma cholesterol levels, confined to (V)LDL. This transient character resulted in a mild (+17%) increased cumulative plasma cholesterol exposure. We conclude that selective activation of NF-κB in hepatocytes considerably promotes atherosclerosis development which is (at least partly) explained by an increased sensitivity to proinflammatory triggers and transiently increased plasma cholesterol levels.

INTRODUCTION

Increased inflammation, in addition to disturbances in lipid metabolism, is the other main contributor to the development of atherosclerosis [1]. Nuclear factor-κB (NF-κB) has been identified as the most important transcription factor in the regulation of inflammatory processes during atherosclerosis development [2]. In unstimulated cells, NF-κB p65/p50 dimer is kept inactive by its inhibitory protein: inhibitor of κB (IκB). A wide range of extracellular stimuli, including cytokines, microbial components, and also free fatty acids, induce activation of the IκB kinase complex, which consists of two kinases (IKKα and -β) and a regulatory subunit, NEMO/IKKγ. This complex mediates the phosphorylation of IκB, resulting in its ubiquitination and degradation, leaving the NF-κB dimer free to translocate to the nucleus and activate its target genes [2].

While general inhibition of the NF-κB pathway by pharmacological agents reduces atherosclerosis development in mice [3,4], the relative contribution of NF-κB may differ at cellular- or tissue-specific level. Suppression of the NF-κB pathway in endothelial cells by ablation of NEMO/IKKγ has been shown to decrease atherosclerosis development [5]. In murine bone marrow transplantation models, inhibition of the NF-κB pathway at distinct levels in hematopoietic cells can have different outcomes, *i.e.* deficiency of the NF-κB p50 subunit resulted in smaller atherosclerotic lesions [6], whereas deletion of IKKβ increased atherosclerosis development [7]. Surprisingly, the role of the NF-κB pathway in hepatocytes on atherosclerosis development has not been investigated thus far.

The liver plays a central role in both lipid metabolism [8] and inflammation [9]. Disturbances in lipid metabolism and increased inflammation are the two main risk factors for atherogenesis [1]. Hepatocytes form the largest population of cells in the liver and execute most of its important functions. During inflammation, acute phase proteins are mainly synthesized by the hepatocytes [10]. Interestingly, hepatocyte-specific deficiency of gp130, a receptor component of IL-6 signaling which signals independent of the NF-κB pathway, decreases atherosclerosis in *apoe*$^{-/-}$ mice [11], suggesting that reduced hepatic inflammation is associated with less atherosclerosis development.

Despite ample evidence implicating the involvement of NF-κB in atherogenesis, the hepatocyte-specific role of NF-κB in atherosclerosis has not been investigated directly. Therefore, in this study we aimed to investigate whether chronic activation of hepatocyte-specific NF-κB aggravates atherosclerosis development. We used transgenic mice with hepatocyte-specific expression of human IKKβ (Liver-specific IKKβ or *LIKK* mice), resulting in an increase of active NF-κB [12], crossbred with atherosclerosis-prone *APOE*3-Leiden* (*E3L*) mice. *E3L* mice exhibit a human-like lipoprotein distribution on a cholesterol-rich diet due to transgenic expression of a human mutant of the *APOE3* gene, and are therefore susceptible to atherosclerosis development [13]. Collectively, our results show that hepatocyte-specific NF-κB activation markedly aggravates atherosclerosis development in *E3L* mice.

METHODS

Animals and study design

Transgenic *LIKK* mice expressing constitutively active human IKKβ in the hepatocytes by an albumin promoter [12] were crossbred with *E3L* mice to generate heterozygous *E3L.LIKK* and control *E3L* littermates, as described before [14]. Mice were housed under standard conditions with a 12-hour light/dark cycle and had free access to food and water. Female mice of 10-12 weeks of age were fed a Western-type diet containing 15% (w/w) cacao butter supplemented with 0.25% (w/w) cholesterol (Hope Farms, Woerden, The Netherlands). Food intake and body weight were measured weekly. Unless indicated otherwise, blood was drawn every 4 weeks after 4 hours of fasting in EDTA-containing tubes by tail bleeding, and plasma was isolated by centrifugation. All animal experiments were approved by the Institutional Ethical Committee on Animal Care and Experimentation of the Leiden University Medical Center (Leiden, The Netherlands).

mRNA expression analysis

Total RNA from livers of *E3L* and *E3L.LIKK* mice was isolated using an RNA isolation kit according to manufacturer's specifications, including a 15 min. DNAse I treatment (Macherey-Nagel, Düren, Germany). Quality control of the isolated RNA was checked with the lab-on-a-chip technology using Experion Stdsens analysis kit (Bio-Rad, Hercules, CA). One μg of total RNA was converted to cDNA with iScript cDNA Synthesis kit (Bio-Rad) and purified with Nucleospin Extract II kit (Macherey-Nagel, Düren, Germany). Real time PCR (RT-PCR) was carried out on an iQ5 Single-Color real-time PCR detection system (Bio-Rad) using the Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). Expression levels were normalized using hypoxanthine-guanine phosphoribosyl transferase (*Hprt*), cyclophilin (*Cyclo*) and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). Primer sequences are listed in Supplemental Table 1.

Western blot analysis

Liver tissue was homogenized by Ultraturrax (22,000 rpm; 2x5 sec) in an ice-cold buffer (pH 7.4) containing 30 mM Tris.HCl, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 0.5% (v/v) Triton X-100, 1% (v/v) SDS and protease inhibitors (Complete, Roche, Mijdrecht, The Netherlands) at a 1:6 (w/v) ratio. Homogenates were centrifuged (16,000 rpm; 15 min., 4°C) and the protein content of the supernatant was determined using the BCA protein assay kit (Pierce, Rockford, IL). Proteins (20-50 μg) were separated by 7-10% SDS-PAGE followed by transfer to a polyvinylidene fluoride membrane. Membranes were blocked for 1 hour at room temperature in Tris-buffered saline with Tween-20 with 5% non-fat dry milk followed by an overnight incubation with either IKKβ (ab32135, Abcam, Cambridge, UK), tubulin, pSer536 NF-κB p65 or NF-κB p65 antibodies (#2148, #3031, #3034, resp., Cell Signaling, Danvers, MA). Blots were then incubated with horseradish peroxidase-conjugated secondary antibodies for

1 hour at room temperature. Bands were visualized by enhanced chemiluminescence and quantified using Image J software (NIH, USA).

Plasma inflammatory markers

Plasma levels of serum amyloid A (SAA) were determined using the murine Phase Serum Amyloid A Assay kit (Tridelta, County Kildare, Ireland) according to manufacturer's instructions. Plasma levels of inflammatory cytokines and chemokines (IFNγ, IL-12 p70, IL-1β, IL-6, TNFα and IL-10) were measured using a multiplex murine inflammatory cytokine profile immunoassay from Meso Scale Discovery (MSD) on a MSD 2400 plate reader according to the manufacturer's protocol (MSD, Gaithersburg, MD).

Lipopolysaccharide stimulation

Mice were injected i.v. with *Salmonella minnesota* Re595 lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO) (50 mg/kg body weight). Blood was collected 90 min. after injection in heparin-coated capillaries. Plasma was assayed for cytokines as described above.

FACS staining of leukocyte subpopulations in peripheral blood

Nonfasted, whole blood was drawn in EDTA-containing tubes by tail bleeding and incubated with antibodies against Ly6C-FITC (kindly provided by Dr P.J. Leenen, Erasmus University, Rotterdam, The Netherlands), Ly6G-PE, CD115-biotin and CD11b-APC (all from Pharmingen, Alphen a/d Rijn, The Netherlands). In between all steps, cells were washed with PBS containing 1% BSA and 0.05% Na-azide.

Binding of anti-CD115-biotin was detected with streptavidin conjugated with PerCP-Cy5.5 (Pharmingen, Alphen a/d Rijn, The Netherlands). Red blood cells were lysed with shock-buffer, cells were fixed with 1% paraformaldehyde and measured with an LSRII (Becton Dickinson, Erembodegem, Belgium).

To obtain leukocyte profiles, the following gating strategy was applied: debris was gated out in a forward (FSC)/side scatter (SSC) plot and leukocytes minus debris were divided according to their CD11b expression (myeloid lineage: CD11b-pos; lymphoid lineage: CD11b-neg). CD11b-pos cells were then selected and using Ly6G expression and SSC, cells were divided in neutrophilic granulocytes (Ly6G-hi/SSC-hi), eosinophilic granulocytes (Ly6G-neg/SSC-hi) and a non-granulocyte population (Ly6G-neg/SSC-low). The non-granulocyte population was then selected and using their CD11b and CD115 expression, monocytes (CD11b-pos/CD115-pos) and NK cells (CD11b-med/CD115-neg) were identified. Next, the monocyte population was selected and using Ly6C expression and FSC, classical monocytes (Ly6C-hi/FSC-low), non-classical monocytes (Ly6C-low/FSC-low) and intermediate monocytes (Ly6C-med/FSC-low) were identified. Going back to the lymphoid lineage (CD11b-neg cell population), using Ly6C-expression, lymphocytes (Ly6C-low/FSC-low) and lymphoblasts (Ly6-ly6C-med/FSC-hi) were identified.

Plasma lipids, lipoprotein profile, hepatic VLDL-TG production and liver lipids analysis

Plasma total cholesterol (TC), triglycerides (TG) and phospholipids (PL) levels were determined using enzymatic kits from Roche Molecular Biochemicals (Woerden, The Netherlands) according to the manufacturer's protocols. We used the following formula to calculate the cumulative plasma TC levels over 24 weeks of Western-type diet feeding which equals the area under the curve for the plasma TC in time: $4*TC_{t=0} + 0.5*4*(TC_{t=4} - TC_{t=0}) + 4*TC_{t=4} + 0.5*4*(TC_{t=8} - TC_{t=4}) + 4*TC_{t=8} + 0.5*4*(TC_{t=12} - TC_{t=8}) + 4*TC_{t=12} + 0.5*4*(TC_{t=16} - TC_{t=12}) + 4*TC_{t=16} + 0.5*4*(TC_{t=20} - TC_{t=16}) + 4*TC_{t=20} + 0.5*4*(TC_{t=24} - TC_{t=20})$. The correlation between cumulative plasma TC level and atherosclerotic lesion area was assessed for both *E3L* and *E3L.LIKK* mice. For the determination of lipid distribution over plasma lipoproteins, pooled plasma per group was size-fractionated using an ÄKTA fast performance liquid chromatography (FPLC) system (Pharmacia, Roosendaal, The Netherlands). Each sample was injected onto a Superose 6 HR3.2/30 column and eluted at a constant flow rate of 50 µL/min in PBS, pH 7.4. Fractions of 50 µL were collected and assayed for TC as described above.

Mice were fasted for 4 hours prior to the start of the hepatic VLDL-TG production experiment, and, subsequently, sedated with 6.25 mg/kg acepromazine (Alfasan), 6.25 mg/kg midazolam (Roche), and 0.3125 mg/kg fentanyl (Janssen-Cilag). At timepoint zero, blood was taken via tail bleeding and mice were i.v. injected with 100 µL PBS containing 100 µCi Trans^{35}S label to measure *de novo* total apolipoprotein B (apoB) synthesis. After 30 min., the animals received 500 mg of tyloxapol (Triton WR-1339, Sigma-Aldrich) per kg body weight as a 10% (w/w) solution in sterile saline, to prevent systemic lipolysis of newly secreted hepatic VLDL-TG. Additional blood samples were taken at regular timepoints after tyloxapol injection and used for determination of plasma TG concentration. At timepoint 120 min., the animals were sacrificed and blood was collected by orbital puncture for isolation of VLDL by density gradient ultracentrifugation. ^{35}S-labeled total apoB content was measured in the VLDL fraction after precipitation with isopropanol.

Lipids from liver tissue were extracted according to a protocol adapted from Bligh and Dyer. Hepatic TG, PL concentrations were determined using the enzymatic kits as described previously. TC, free cholesterol (FC) and cholesteryl ester (CE) content were assessed with a cholesterol/cholesteryl ester quantitation enzymatic kit according to manufacturer's specifications (Biovision Research Products, Mountain View, CA). Liver lipids were expressed per mg protein, which was measured using the BCA protein assay kit (Pierce, Rockford, IL).

Atherosclerosis quantification

Mice were euthanized by carbon dioxide inhalation after 24 weeks of diet. Hearts were isolated and fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin, and were cross-sectioned (5 µm) throughout the entire aortic root area. Of each mouse, 4 sections with 50-µm intervals were used for quantification of atherosclerotic lesion

area. Characterization of lesion severity was performed separately in each of the 3 segments between the aortic valves in the 4 sections.

Sections were stained with hematoxylin-phloxine-saffron (HPS). Atherosclerotic lesions were categorized for severity by one blinded observer, according to the guidelines of the American Heart Association, adapted for mice. Various types of lesions were distinguished: type 0 (no lesions) (Supplemental Fig. 8A), type I to III (early fatty streak-like lesions containing foam cells) (Supplemental Fig. 8B-D), and type IV to V (advanced lesions containing foam cells in the media, presence of fibrosis, cholesterol clefts, mineralization, and/or necrosis) (Supplemental Fig. 8E-F).

Immunohistochemistry for determination of adhering monocytes and macrophage-, and smooth muscle cell content in the lesions was performed as described previously. The sections were incubated overnight with antibody M18 (1:100, Santa Cruz Biotechnology, Santa Cruz, Calif) and, subsequently, with biotinylated rabbit anti-goat conjugate(1:400, Brunschwig chemie, Amsterdam, The Netherlands) for quantification of MCP-1. AIA 31240 rabbit antiserum (1:1000, Accurate Chemical and Scientific, Westbury, NY) in combination with biotinylated donkey anti-rabbit conjugate (1:3000, Amersham Pharmacia Biotech) were used for quantification of both the number of monocytes adhering to the endothelium as well as the macrophage area. Antibody M0851 (1:800, Dako, Carpinteria, CA) and biotinylated horse anti-mouse conjugate (1:400, Vector Laboratories, Burlingame, CA) were used to quantify smooth muscle actin. Immunostaining was amplified using Vector Laboratories Elite ABC kit (Vector Laboratories, Burlingame, CA) and the immunoperoxidase complex was visualized with Nova Red (Vector Laboratories, Burlingame, CA). Counterstaining was performed with Mayer's haematoxylin. Sirius red was used to stain for collagen in the lesions (Chroma, Stuttgart, Germany).

Total lesion size, MCP-1-, macrophage-, smooth muscle cell-, and collagen content were quantified using Cell^D image analysis software (Olympus Soft Imaging Solutions, Münster, Germany).

Statistical analysis

Data are presented as means ±SEM. SPSS 17.0 for Windows (SPSS, Chicago, Ill) was used for statistical analysis. Statistical differences were assessed with the Mann-Whitney *U* test. For lesion typing, differences were determined by the χ^2 test. To assess the correlation between cumulative cholesterol exposure and atherosclerotic lesion area, the Pearson correlation test was performed after log transformation of the atherosclerotic lesion area. Differences at $P<0.05$ were regarded as statistically significant.

RESULTS

LIKK causes low-grade inflammation

The overall appearance of *E3L* and *E3L.LIKK* mice was similar. To assess whether expression of *LIKK* affects body weight gain, we measured food intake and body weight

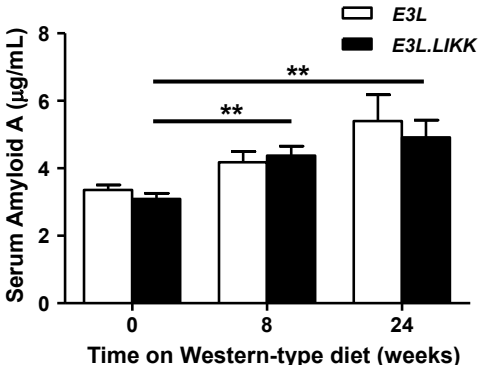

Figure 1. LIKK does not increase plasma SAA levels. SAA levels were determined in plasma from E3L.LIKK (black bars) and E3L (white bars) mice fed a Western-type diet for 0, 8 and 24 weeks. Values are means ±SEM; n=15/group; **P<0.01.

weekly. Both were not different between *E3L.LIKK* and *E3L* control mice (Supplemental Fig. 1A-B). The liver- and spleen weight and histological morphology of the liver were also comparable between *E3L.LIKK* and *E3L* mice (data not shown). To gain more insight in the effects of *LIKK* on inflammation, we determined whether *LIKK* expression increased the inflammatory state of the liver and systemic inflammatory markers in *E3L.LIKK* mice on a Western-type diet. We confirmed previous findings [14] showing that the enhanced expression of hepatocyte-specific human IKKβ (Supplemental Fig. 2A) resulted in a 1.4-fold increased hepatic NF-κB activation, displayed by an enhanced expression the phosphorylated p65 subunit (pNF-κB^{Ser536}) (Supplemental Fig. 2B). IKKβ kinase phosphorylates subunit p65 of NF-κB at the position Ser536, which activates the transcriptional activity of NF-κB [15]. The transgenic expression of human IKKß mRNA was present only in *E3L.LIKK* mice and did not alter murine IKKß mRNA expression (Supplemental Fig. 2C-D). The enhanced hepatic NF-κB activation in *E3L.LIKK* mice did not result in increased IL-6 expression in whole liver, but did result in a tendency towards increased IL-1β expression (*P*=0.085) and a significant increase in MCP-1 expression (Supplemental Table 2).

To evaluate whether the increased hepatocyte-specific NF-κB activation in *E3L.LIKK* mice enhanced the systemic inflammatory state, we determined the plasma inflammation marker SAA and plasma cytokines under basal conditions. *LIKK* expression did not affect SAA before (3.1 ±0.17 *vs.* 3.4 ±0.15 µg/mL) and after 8 weeks (4.4 ±0.28 *vs.* 4.2 ±0.31 µg/mL) and 24 weeks (4.9 ±0.51 *vs.* 5.4 ±0.78 µg/mL) of Western-type diet feeding (Fig. 1), and neither the determined plasma cytokine levels (Supplemental Fig. 3A-F). SAA levels increased significantly with Western-type diet feeding in *E3L.LIKK* mice (Fig. 1).

Since we did not observe a clear increased systemic proinflammatory state under basal conditions, we challenged the mice with LPS to boost the inflammatory response. Interestingly, after injection of LPS, proinflammatory cytokines (*e.g.* IL-1β, IFNγ) showed a tendency towards increased plasma levels in *E3L.LIKK* mice as compared to *E3L* mice (Fig. 2A-F). The anti-inflammatory IL-10:IL-1β ratio was significantly lower in *E3L.LIKK* mice (Fig. 2G). Overall, these data indicate that *E3L.LIKK* mice are more sensitive to proinflammatory triggers compared to their *E3L* littermates.

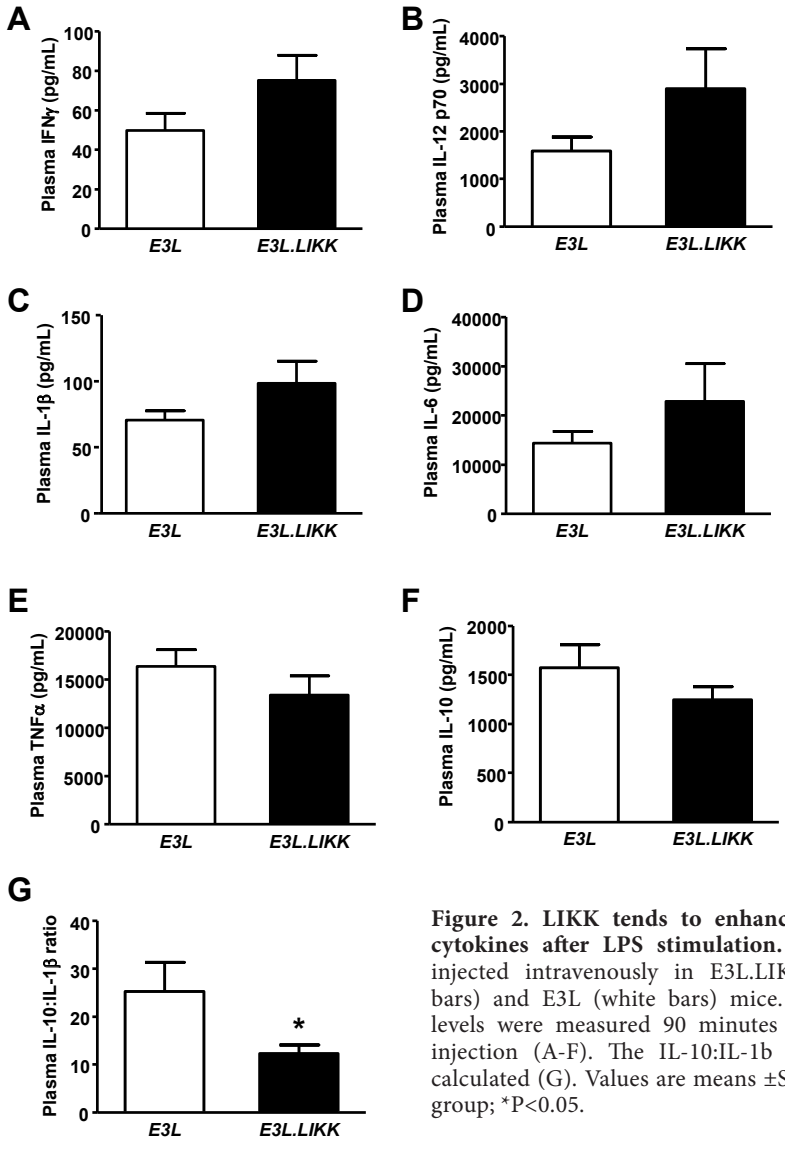

Figure 2. LIKK tends to enhance plasma cytokines after LPS stimulation. LPS was injected intravenously in E3L.LIKK (black bars) and E3L (white bars) mice. Cytokine levels were measured 90 minutes after LPS injection (A-F). The IL-10:IL-1b ratio was calculated (G). Values are means ±SEM; n=7/group; *P<0.05.

To study whether this chronic low-grade inflammation in *E3L.LIKK* mice also resulted in increased inflammatory cell counts in liver and plasma, we determined the hepatic mRNA expression of various cell-type markers of inflammatory cells present in the liver, which are likely to influence atherogenesis [16], and the number of circulating monocytes. Hepatic mRNA expression of CD68 (Kupffer cells), CD3 ((NK)T cells), and Vα14 (NKT cells) were not different between the genotypes (Supplemental Table 2), neither were the total number of circulating monocytes, the proinflammatory Ly6C-hi monocyte subset, the intermediate Ly6C-med monocyte subset and the less

inflammatory Ly6C-lo monocyte subset (Supplemental Fig. 4A-D). Together, the above findings indicate that the enhanced hepatocyte-specific NF-κB activation in *E3L.LIKK* mice results in a tendency towards a mildly enhanced hepatic proinflammatory state and an elevated sensitivity to proinflammatory stimuli as compared to *E3L* littermates.

LIKK transiently enhances VLDL cholesterol levels

To assess the effect of hepatocyte-specific NF-κB activation on plasma lipid levels, TC, TG and PL concentrations were determined every 4 weeks in *E3L.LIKK* and *E3L* mice. *LIKK* expression caused a transient increase of plasma TC levels only at 8 weeks (+50%; $P<0.0001$) and 12 weeks (+28%; $P<0.05$) of Western-type diet feeding (Fig. 3A). Accordingly, the cumulative total cholesterol exposure was higher in *E3L.LIKK* than in *E3L* mice (+17%; $P<0.05$; Fig. 3B). A similar transient increase was found for plasma TG and PL levels (Supplemental Fig. 5A-B).

To determine which lipoproteins contribute to the transient elevated plasma TC levels, lipoproteins were size-fractionated by FPLC, and cholesterol was measured in the individual fractions. The transient increase in plasma TC levels at 8 weeks of Western-type diet feeding in *E3L.LIKK* mice was confined to (V)LDL, whereas at 16 weeks the lipoprotein distribution in the *E3L.LIKK* mice was similar to that of the *E3L* mice, in line with the plasma lipid levels (Fig. 3C). In line with our previous finding that expression of *LIKK* increased the VLDL production in male mice on chow diet [14], we found that expression of *LIKK* increased, albeit not significantly, the VLDL-TG production rate (+24%) (Supplemental Fig. 6A), and tended to increase the VLDL-apolipoprotein B (apoB) production rate (+33%) (Supplemental Fig. 6B). No differences were observed in the liver lipid content between *E3L.LIKK* and *E3L* mice (Supplemental Fig. 7A-E). Taken together, these findings indicate that hepatocyte-specific NF-κB activation results in a modest and transient increase in plasma lipid levels in *E3L* mice.

LIKK enhances atherosclerosis development

To investigate the effect of *LIKK* expression on atherosclerosis development, *E3L.LIKK* and *E3L* mice were sacrificed after 24 weeks of Western-type diet feeding, and lesion size and severity were measured in the aortic root. Representative pictures of both groups are shown in Fig. 4A. *E3L.LIKK* mice developed more than 2-fold larger atherosclerotic lesions (+131%; $P<0.05$; Fig. 4B) as compared to their *E3L* littermates. This increased lesion area coincided with more advanced lesion progression, since we found markedly fewer segments without atherosclerotic lesions (11% *vs.* 42%; $P<0.001$) and more segments with mild (63% *vs.* 44%; $P<0.001$) and severe lesions (26% *vs.* 14%; $P<0.001$) as compared to *E3L* mice (Fig. 4C). Examples of mild and severe lesions are shown in Supplemental Figure 8. These data indicate that chronic hepatocyte-specific NF-κB activation severely augments atherosclerosis development in *E3L* mice.

LIKK aggravates atherosclerotic lesion composition

We next evaluated whether *LIKK* expression would affect monocyte adherence and recruitment to the vascular wall, as well as the composition of the atherosclerotic

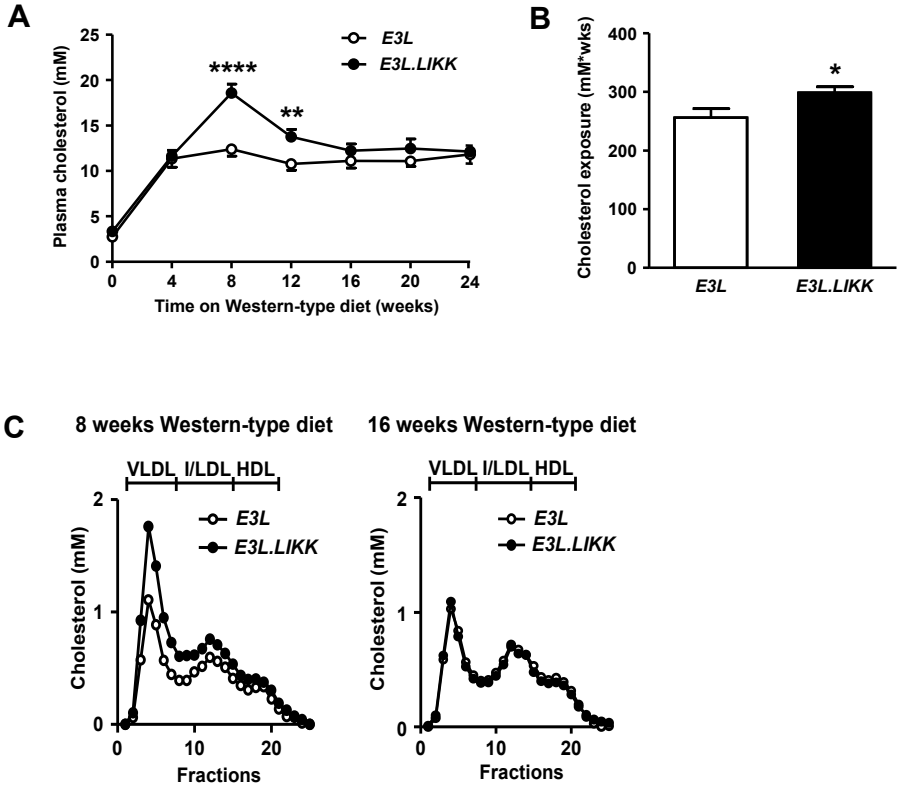

Figure 3. LIKK transiently increases (V)LDL. Plasma cholesterol levels of E3L.LIKK (black symbols) and E3L (white symbols) mice fed a Western-type diet were assessed (A), and cumulative total cholesterol exposure was calculated (B). Lipoprotein profiles were determined at 8 (left) and 16 (right) weeks (C). Values are means ±SEM; n=15/group; *P<0.05, **P<0.01, ****P<0.0001.

lesions with respect to the macrophage, smooth muscle cell, and collagen content of the lesions. Adherence of monocytes to the vessel wall and the content of the chemokine monocyte chemoattractant protein-1 (MCP-1) of the atherosclerotic lesions were not significantly enhanced in *E3L.LIKK* mice as compared to *E3L* mice (Fig. 5A-B). *LIKK* expression did not affect the relative macrophage and collagen content of the lesions (Fig. 5C+E), but did result in an increased smooth muscle cell content of the lesions (+79%, *P*<0.05; Fig. 5D).

Aggravated atherosclerosis development in E3L.LIKK mice does not solely depend on the transient increase in plasma cholesterol levels

In *E3L* mice on Western-type diet, the cumulative plasma cholesterol exposure is highly predictive for the atherosclerotic lesion area (unpublished data, J.F.P. Berbée, P.C.N. Rensen). To verify if the transient increase in plasma TC levels (Fig. 3) alone could

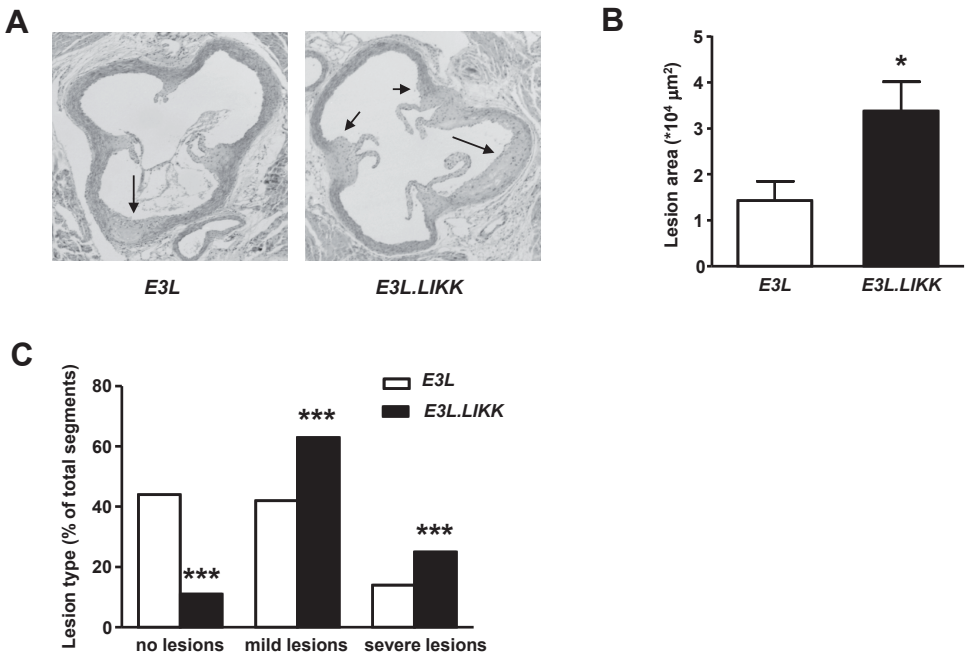

Figure 4. LIKK aggravates atherosclerotic lesion area and severity. After 24 weeks of Western-type diet feeding, E3L.LIKK (black bars) and E3L (white bars) mice were sacrificed and cross-sections of aortic roots were stained with HPS. Representative pictures are shown. Arrows indicate lesions (A). Total lesion area was assessed in 4 sections of the aortic root (B) and lesion severity was determined separately in each of the 3 segments between the aortic valves of the 4 sections (C). Statistical analysis for lesion area was performed by Mann-Whitney U test, for lesion severity was determined by the x^2 test. Values are means ±SEM; n=15/group; *P<0.05, ***P<0.001.

account for the aggravation in atherosclerosis development observed in *E3L.LIKK* mice, or whether additional mechanism(s) could contribute, including the low-grade systemic inflammation, we assessed the correlation between the cumulative plasma total cholesterol exposure and the atherosclerotic lesion area of the *E3L.LIKK* and *E3L* mice. As expected, there was a significant positive logarithmic correlation between the atherosclerotic lesion area and the cumulative plasma cholesterol exposure in the control *E3L* mice (Supplemental Fig. 9A; r^2=0.757, *P*=0.002). However, we did not observe such a correlation in the *E3L.LIKK* mice (Supplemental Fig. 9B; r^2=-0.250, *P*=0.369), indicating that in addition to the transient increase in plasma TC levels in *E3L.LIKK* mice, additional mechanism(s), most likely the augmented sensitivity to proinflammatory stimuli, contributed to the aggravated atherosclerosis development in these mice.

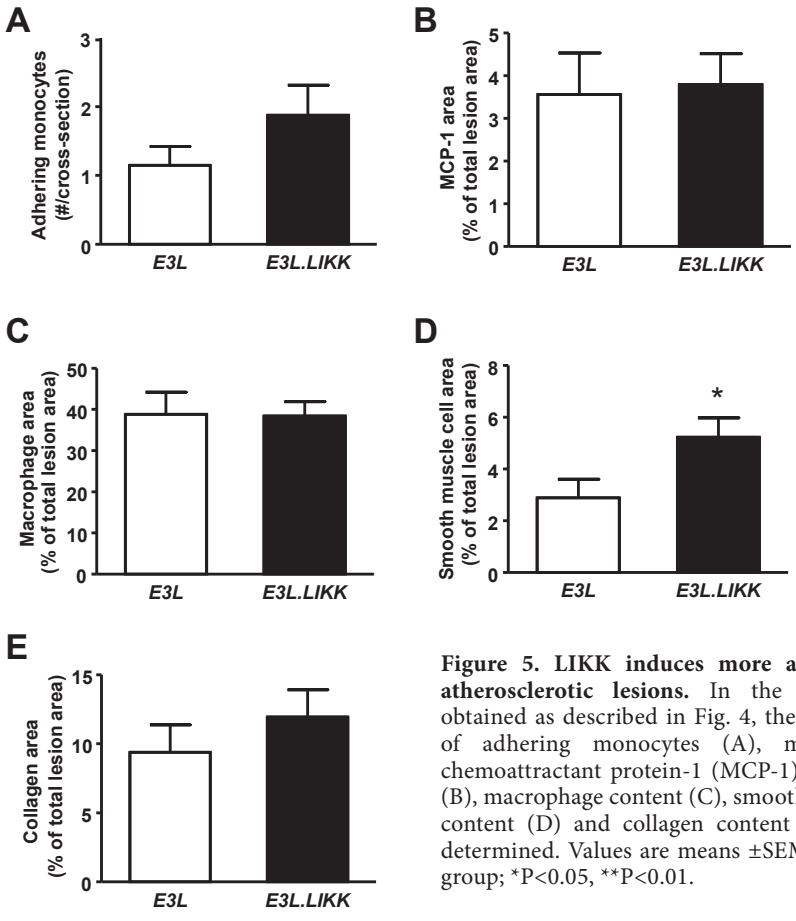

Figure 5. LIKK induces more advanced atherosclerotic lesions. In the sections obtained as described in Fig. 4, the number of adhering monocytes (A), monocyte chemoattractant protein-1 (MCP-1) content (B), macrophage content (C), smooth muscle content (D) and collagen content (E) was determined. Values are means ±SEM; n=15/group; *P<0.05, **P<0.01.

DISCUSSION

NF-κB is regarded as a potential therapeutic target in atherosclerosis [3, 4] and studying tissue- and cell-specific effects of NF-κB in atherogenesis will expand our knowledge in the comprehensive actions of NF-κB on atherosclerosis development. The present study demonstrates for the first time that chronic, hepatocyte-specific expression of IKKß (*LIKK*) and subsequent activation of NF-κB aggravates atherosclerosis development in *E3L* mice. In addition, the atherosclerotic lesion composition with respect to the macrophage and collagen content was not affected by *LIKK*, but in accordance with the presence of more advanced lesions, the smooth muscle cell content was increased. Expression of *LIKK* resulted in transiently increased plasma cholesterol levels and an enhanced sensitivity to proinflammatory triggers, which both are likely to have contributed to the increased atherosclerotic lesion size and severity. Since lesion size and severity are often correlated in atherosclerosis studies with different murine models

[13, 17], the increased lesion severity in *E3L.LIKK* mice is likely to be mainly attributed to the larger size of the lesions.

Expression of *LIKK* in *E3L* mice increased the activation of the NF-κB pathway in the liver, in line with our previous report [14]. In addition, hepatic mRNA expression of inflammatory parameters was increased or tended to be increased in *E3L.LIKK* mice, indicating that inflammatory mediators at local tissue level were enhanced in *E3L.LIKK* mice. This enhanced activation of hepatocyte-specific NF-κB in *E3L.LIKK* mice, however, did not result in a significant increased systemic proinflammatory state under basal conditions as compared to their *E3L* littermates. Importantly, Cai *et al.* [12] demonstrated that in *LIKK* mice on a wild-type background, systemic levels of IL-6 were only mildly elevated, while IL-1β and TNFα levels were similar as in wild-type mice. Our results show that *LIKK* expression on an *E3L* background resulted in a less pronounced hepatic inflammatory state as compared to *LIKK* expression on a wild-type background as described by Cai *et al.* [12], as reflected in a smaller increase in active NF-κB (1.4- *vs.* 2.2-fold) and mRNA levels of proinflammatory cytokines levels in the liver. Furthermore, under basal conditions *E3L* mice have lower levels of active NF-κB present in the liver as compared to wild-type mice (unpublished data, J.A. van Diepen, M.C. Wong, P.J. Voshol). This implies that *E3L* mice have a lower chronic inflammatory state than wild-type mice, which could interfere with the proinflammatory effects caused by expression of *LIKK* in the present study. Also, in comparison with other murine atherosclerosis models, *e.g.* the *apoe*$^{-/-}$ and *ldlr*$^{-/-}$ mice, *E3L* mice display a milder phenotype with respect to hyperlipidemia and increased inflammation [18, 19]. In the current study, basal circulating levels of some cytokines were at borderline of the detection limit of current assays (Supplemental Fig. 3) and as expected, the levels increased 5-3700x after LPS injection (Fig. 2). Furthermore, after stimulation with LPS, *E3L.LIKK* mice showed a tendency towards a higher systemic inflammatory state than *E3L* mice.

There is a strong interaction between inflammation and lipid metabolism [20]. For example, lowering inflammation using salicylate did not only reduced NF-κB activation, but concomitantly also reduced circulating cholesterol levels in *E3L* mice [21]. In line with this observation, in the present study we found higher plasma lipid levels at 8 weeks of Western-type diet feeding in female *E3L.LIKK* compared to *E3L* mice, which were confined to (V)LDL. We hypothesize that the increased lipid levels at this time point is accompanied by a maximal enhanced systemic inflammatory state. As mentioned above, lipid metabolism and inflammation strongly influence each other [20]. A possible cause for the increased plasma lipid levels at 8 weeks of diet is therefore a more enhanced inflammation in the liver, possibly due to an increased activation of the NF-κB pathway in the liver.

We recently reported that male *E3L.LIKK* mice on chow diet also showed enhanced (V)LDL levels as a result of an increased hepatic VLDL-TG production rate [14], and found in the current study a trend towards an enhanced VLDL-apoB production in female *E3L.LIKK* mice on Western-type diet, with a similar effect-size. Possible reasons for the less apparent increase of VLDL-TG production in females compared to males

are differences in gender and/or diet. Although the increase in VLDL-TG production is more apparent in male *E3L.LIKK* mice, we used female mice in the present study. The main reason for this is that female *E3L* mice are more susceptible to develop atherosclerosis. In order for male *E3L* mice to become similarly atherosclerosis-prone they need to be fed Western-type diets not only with higher percentages of cholesterol, but also containing cholate. In addition, fructose was added in the drinking water to further raise their (V)LDL-cholesterol levels [22]. The increase in (V)LDL levels in females in the current study was only transient at 8 weeks of Western-type diet feeding and disappeared at 16 weeks. Since no differences in plasma lipid levels and hepatic mRNA expression of genes involved in lipid metabolism were detected between both groups at 24 weeks of diet, the increased VLDL-TG production at 8 weeks of diet is likely to be transient. At present, we cannot explain the transient nature of this increase in (V)LDL levels, but it may be the result of a progressive negative feedback mechanism to reduce the hepatic VLDL production which takes place during long-term Western-type diet feeding.

Dyslipidemia is regarded as the classical risk factor for atherosclerosis development. The transiently enhanced total cholesterol levels, resulting in a modest increase (+17%) in cumulative total cholesterol exposure upon *LIKK* expression, thus likely contributed to the enhanced atherosclerosis development. Previous diet-induced atherosclerosis studies in *E3L* mice have consistently demonstrated that there is a positive logarithmic relation between the cumulative cholesterol exposure during the study and the atherosclerotic lesion area (J.F.P. Berbée, P.C.N. Rensen, unpublished data). In agreement with these previous observations, we did observe such a significant logarithmic relation in *E3L* mice but not in *E3L.LIKK* mice. This suggests that the increase in atherosclerotic lesion area in *E3L.LIKK* mice can only partly be attributed to the transiently enhanced plasma cholesterol levels and that additional mechanisms are involved.

Inflammation is the second main risk factor for atherosclerosis. Enhanced extravascular or systemic inflammation, by the periodontal pathogen *Porphyromonas gingivalis* [23] or by repeated administration of LPS [24], respectively, promotes atherosclerosis development. In addition, in humans, low-grade systemic inflammation is associated with enhanced risk of coronary artery disease [25, 26]. It is thus likely that, as discussed above, the increased sensitivity for proinflammatory triggers, such as LPS, in *E3L.LIKK* mice also directly contributed to the enhanced atherosclerotic lesion formation.

We excluded higher circulating levels of proinflammatory Ly6C-hi monocytes as being another possible contributor to the aggravated atherosclerosis development in *E3L.LIKK* mice. Adhesion of monocytes to endothelial cells and subsequent migration into the vessel wall is one of the crucial steps in atherosclerotic lesion formation. Ly6C-hi monocytes are more prone to adhere to activated endothelium than Ly6C-lo monocytes and are, therefore, associated with enhanced atherosclerosis development [27]. We found that *E3L.LIKK* mice had similar levels of circulating subsets of monocytes as compared to their *E3L* controls, which is consistent with the observed similar number of adhering monocytes to the vascular wall.

In line with the enhanced atherosclerosis development that we observed in *E3L.LIKK* mice, Luchtefeld *et al*. [11] have reported that gp130-deficient mice with defective IL-6 signaling specifically in hepatocytes, develop less atherosclerosis, indicating that modulation of hepatic inflammation can have profound effects on atherogenesis. These studies also underscore that enhanced inflammation in the liver, *e.g.* due to viral hepatitis or steatohepatitis, may augment atherosclerosis development. Indeed, in several clinical studies, such hepatic pathological conditions are associated with an elevated occurrence of CVD [28, 29, 30]. Even after adjustment for classical risk factors for CVD, such as LDL cholesterol levels, chronic hepatitis C infection was still significantly associated with increased atherosclerosis in a cross-sectional study [30]. Together, these findings suggest that there is a direct effect of hepatic inflammation on atherosclerosis development, independent of systemic lipid levels. Moreover, they suggest that in addition to the currently used lipid-targeted drugs such as statins, reducing NF-κB activity in the liver may be a promising additive therapeutic strategy against atherosclerosis development.

In conclusion, we have shown that hepatocyte-specific activation of NF-κB leads to larger and more advanced atherosclerotic lesions. Our studies furthermore suggest that both the transient elevated (V)LDL cholesterol levels as well as the increased sensitivity to proinflammatory stimuli are most likely responsible for this aggravating effect on atherosclerosis. These findings contribute to the present understanding of the role of the liver, and more specifically the role of hepatic NF-κB, in atherosclerosis development and may help to develop new innovative anti-atherosclerotic strategies.

ACKNOWLEDGEMENTS

This work was supported by grants from the Netherlands Organization for Scientific Research (NWO Mosaic; 017.003.83 [to L.H.]) and F.R. Nieuwenkamp stichting. P.C.N. Rensen is an Established Investigator of the Netherlands Heart Foundation (Grant 2009T038).

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SUPPLEMENTARY DATA

Supplemental Table 1. Primers used for RT-PCR.

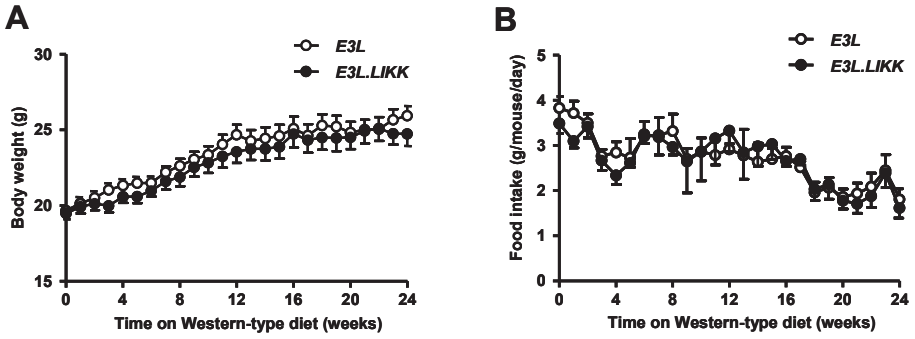
Gene	Forward primer	Reverse primer
Human target		
IKKβ	GGAGCTCTGTGGGCGGGAGA	GGCCCATGGGGCTCCTCTGT
Murine target		
Apob	GCCCATTGTGGACAAGTTGATC	CCAGGACTTGGAGGTCTTGGA
CD3	CTGTCTAGAGGGCACGTCAA	GATGCGGTGGAACACTTTCT
CD68	CCTCCACCCTCGCCTAGTC	TTGGGTATAGGATTCGGATTTGA
Cpt1a	GAGACTTCCAACGCATGACA	ATGGGTTGGGGTGATGTAGA
Cyclo	CAAATGCTGGACCAAACACAA	GCCATCCAGCCATTCAGTCT
Fas	TCCTGGGAGGAATGTAAACAGC	CACAAATTCATTCACTGCAGCC
Gapdh	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
Hmgcr	CCGGCAACAACAAGATCTGTG	ATGTACAGGATGGCGATGCA
Hprt	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
IKKβ	GCCCTCTGCTCCCGGCTAGA	CCAGTCTAGAGTCGTGAAGCTTCTGT
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTGGGTCCGTCAACT
IL-6	CCGGAGAGGAGACTTCACAG	TTCTGCAAGTGCATCATCGT
MCP-1	GCATCTGCCCTAAGGTCTTCA	TTCACTGTCACACTGGTCACTCCTA
Mttp	CTCTTGGCAGTGCTTTTTCTCT	GAGCTTGTATAGCCGCTCATT
Vα14	GTGGGTGGCTGGCAAGAC	TCTCCCTGACGCACAACCA
Srebp-1c	GGAGCCATGGATTGCACATT	CCTGTCTCACCCCCAGCATA

IKKβ, IκB kinase-β; *Apob*, apolipoprotein B; *Cd3*, marker for (NK)T cells; *CD68*, marker for macrophages (Kupffer cells); *Cpt1a*, carnitine palmitoyltransferase 1a; *Cyclo*, cyclophilin; *Fas*, fatty acid synthase; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Hmgcr*, HMG-CoA reductase; *Hprt*, hypoxanthine-guanine phosphoribosyl transferase; *IL-1β*, interleukin-1b; *IL-6*, interleukin-6; *MCP-1*, monocyte chemoattractant protein-1; *Mttp*, microsomal triglyceride transfer protein; *Srebp-1c*, sterol-regulatory element binding protein; *Vα14*, marker for NKT cells.

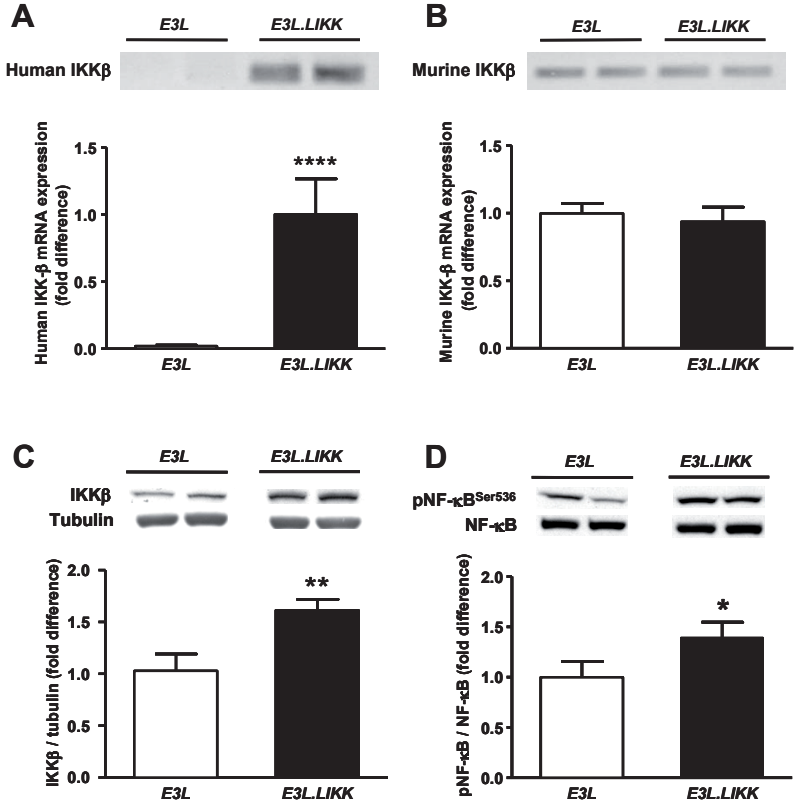
Supplemental Table 2. Expression of *LIKK* increases hepatic MCP-1 expression.

Gene	E3L	E3L.LIKK	Significance
Cytokines and chemokines			
IL-1β	1.00 ± 0.05	1.24 ± 0.14	P = 0.085
Il-6	1.00 ± 0.13	1.24 ± 0.20	n.s.
MCP-1	1.00 ± 0.27	1.39 ± 0.32*	P = 0.049
Inflammatory cells			
CD68	1.00 ± 0.29	1.15 ± 0.14	n.s.
CD3	1.00 ± 0.14	0.69 ± 0.30	n.s.
Vα14	1.00 ± 0.08	0.94 ± 0.21	n.s.
VLDL secretion			
Apob	1.00 ± 0.24	1.00 ± 0.33	n.s.
Mttp	1.00 ± 0.13	1.15 ± 0.28	n.s.
Lipogenesis			
Srebp-1c	1.00 ± 0.19	1.46 ± 0.49	n.s.
Fas	1.00 ± 0.21	0.83 ± 0.19	n.s.

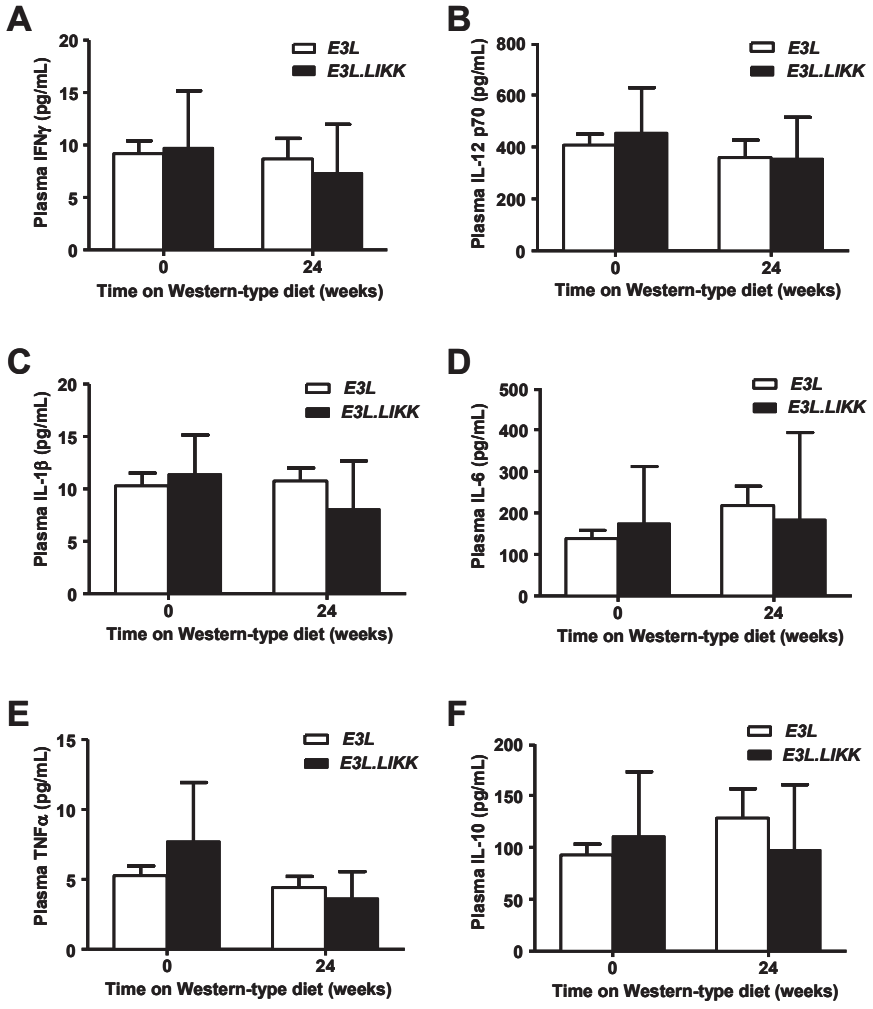
After 24 weeks of Western-type diet, *E3L.LIKK* and *E3L* mice were sacrificed, livers were isolated and mRNA expression of indicated targets were quantified by RT-PCR. Data are calculated as fold difference as compared to the control group. Values are means ± SEM (n=6-15). *$P<0.05$ compared to the control group. n.s., not significant. *IKKβ*, IκB kinase-β; *Apob*, apolipoprotein B; *Cd3*, marker for (NK)T cells; *CD68*, marker for macrophages (Kupffer cells); *Cpt1a*, carnitine palmitoyltransferase 1a; *Cyclo*, cyclophilin; *Fas*, fatty acid synthase; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Hmgcr*, HMG-CoA reductase; *Hprt*, hypoxanthine-guanine phosphoribosyl transferase; *IL-1β*, interleukin-1b; *IL-6*, interleukin-6; *MCP-1*, monocyte chemoattractant protein-1; *Mttp*, microsomal triglyceride transfer protein; *Srebp-1c*, sterol-regulatory element binding protein; *Vα14*, marker for NKT cells.



Supplemental Fig. 1. Expression of LIKK does not affect body weight and food intake. E3L.LIKK (black symbols) and E3L mice (white symbols) were fed a Western-type diet for 24 weeks. Body weight (A) and food intake (B) were measured weekly. Values are means ±SEM; n=15/group.

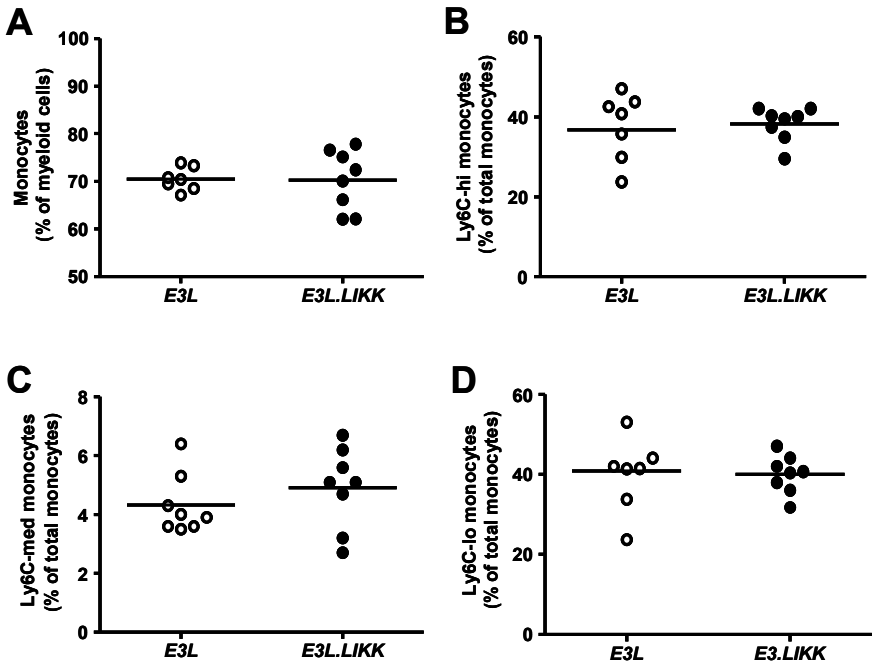


Supplemental Fig. 2. Expression of LIKK increases hepatic IKKb mRNA and protein expression, and NF-kB protein activity. After 24 weeks of Western-type diet, E3L.LIKK (black bars) and E3L mice (white bars) were sacrificed, livers were isolated and hepatic mRNA ▸

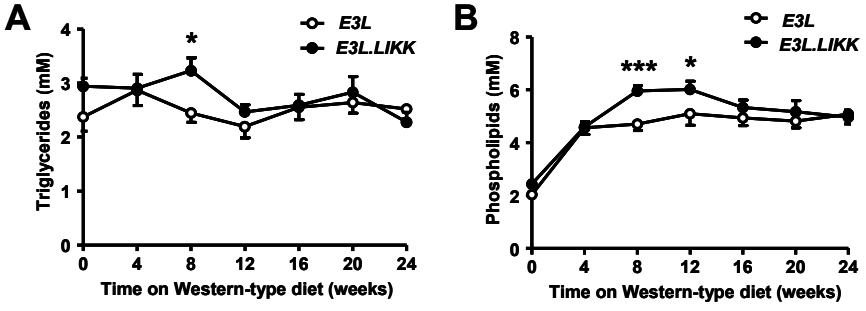


Supplemental Fig. 3. LIKK does not affect plasma cytokines after 0 and 24 weeks of diet. E3L.LIKK (black bars) and E3L (white bars) were fed 24 weeks of Western-type diet. Plasma levels of the indicated cytokines were measured at t=0 and 24 weeks of diet. Values are means ±SEM; n=15/group.

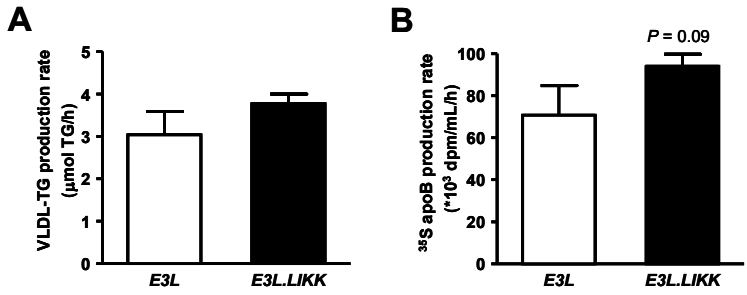
- expression of human IKKß (A) and murine IKKb (B) was quantified by RT-PCR. Human IKKb is expressed as fold difference compared to E3L.LIKK mice and murine IKKb to E3L mice. The inserts show representative bands of two mice per group of the qPCR product run on a gel. Values are mean ±SEM; n=14-15/group; nd: not detectable, ****P<0.0001 versus E3L. Hepatic protein expression of IKKb normalized to tubulin (C) and phosphorylated NF-κB p65Ser536 normalized to total NF-kB (D) was assessed in E3L.LIKK (black bars) and E3L mice (white bars). Values are mean ±SEM; n=6-7/group; *P<0.05, **P<0.01 versus E3L controls.



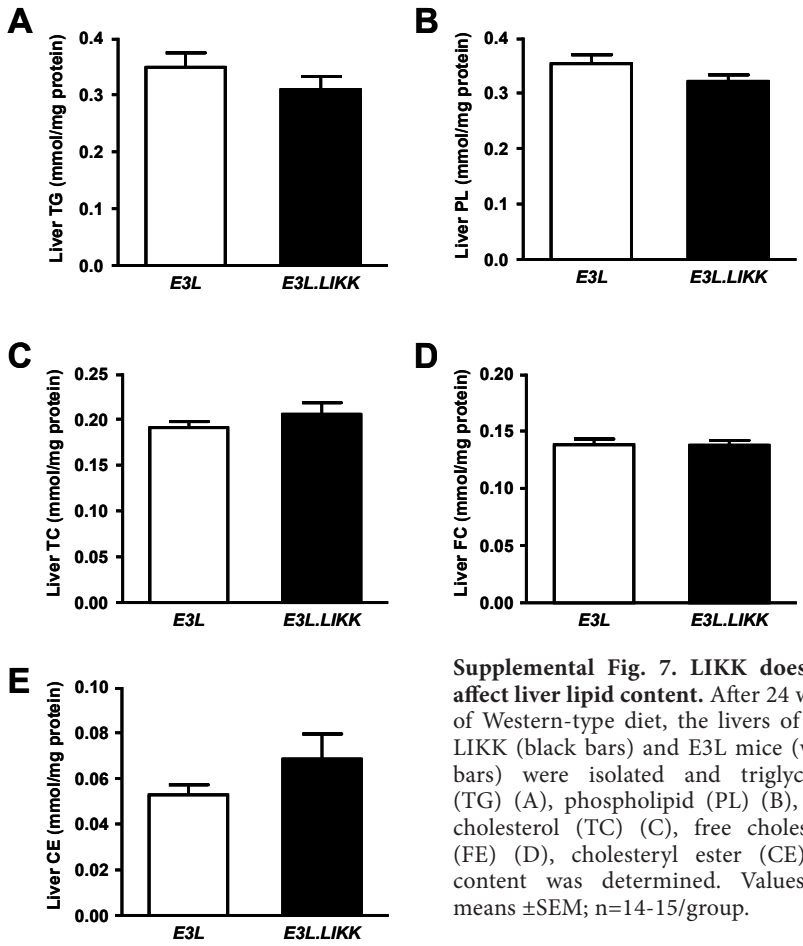
Supplemental Fig. 4. LIKK does not affect circulating subsets of monocytes. Blood was drawn from E3L.LIKK (black circles) and E3L (open circles) mice fed a Western-type diet for 8 weeks. The number of monocytes (A) and Ly6C-hi (B), Ly6C-med (C) and Ly6C-lo (D) expressing subsets were determined by FACS analysis. n=7-8/group.



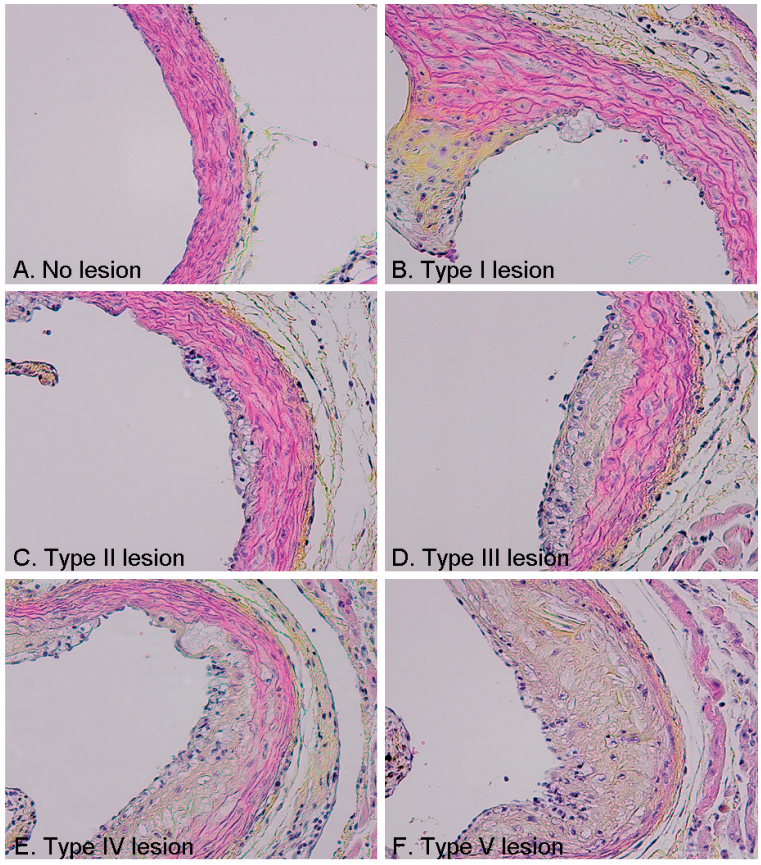
Supplemental Fig. 5. LIKK transiently increases plasma triglycerides and phospholipids. E3L.LIKK (black symbols) and E3L mice (white symbols) were fed a Western-type diet for 24 weeks. Plasma was obtained every 4 weeks to determine triglycerides (TG) (A) and phospholipids (PL) (B) concentration over time. Values are means ±SEM; n=15/group; *P<0.05, ***P<0.001.



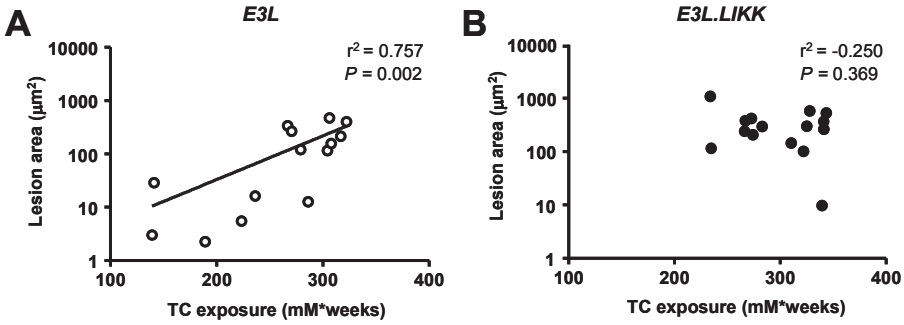
Supplemental Fig. 6. Expression of LIKK tends to increase VLDL-apoB production. After 8 weeks of Western-type diet, E3L.LIKK (black bars) and E3L mice (white bars) were fasted 4 hours and injected with Trans35S and tyloxapol, and blood samples were drawn after tyloxapol injection. The rate of TG production was calculated from the slopes of the curves from the individual mice (A). After 120 min., the total VLDL-fraction was isolated by ultracentrifugation, 35S-activity was counted, and the production rate of newly synthesized VLDL-35S-apoB was determined (B). Values are means ±SEM; n=5-11/group.



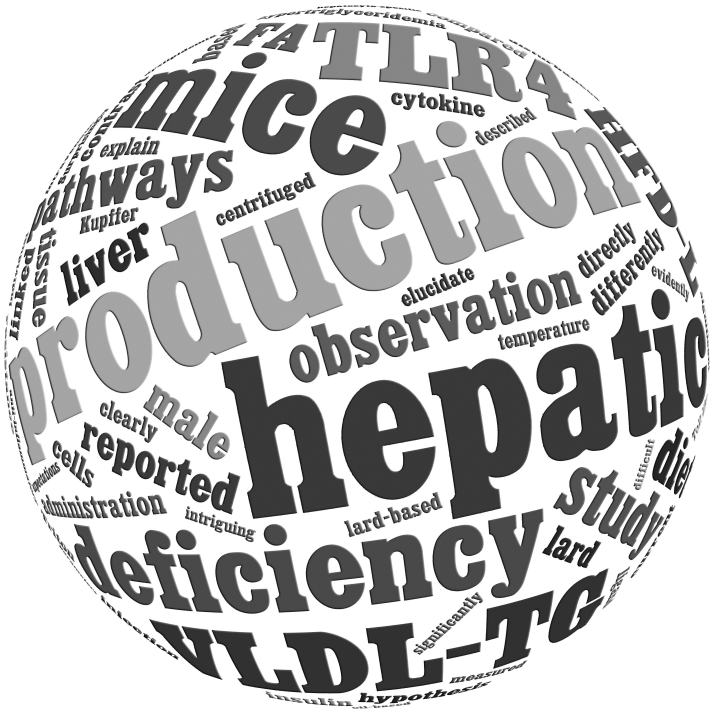
Supplemental Fig. 7. LIKK does not affect liver lipid content. After 24 weeks of Western-type diet, the livers of E3L.LIKK (black bars) and E3L mice (white bars) were isolated and triglyceride (TG) (A), phospholipid (PL) (B), total cholesterol (TC) (C), free cholesterol (FE) (D), cholesteryl ester (CE) (E) content was determined. Values are means ±SEM; n=14-15/group.



Supplemental Fig. 8. Representative pictures of HPS-stained segments classified in different severities. Atherosclerotic lesions are categorized into mild (type I-III) and severe (type IV-V) phenotypes. Magnification 200x. (A) No lesion. (B) Type I, early fatty streak: per section up to 10 foam cells present in the intima. (C) Type II, regular fatty streak: more than 10 foam cells present in the intima. (D) Type III, mild plaque: extension of foam cells into the media and covered by a fibrotic cap. (E) Type IV, moderate plaque: a more progressive lesion infiltrating into the media, fibrosis in the media, without loss of architecture. (F) Type V, severe plaque: the media is severely damaged, elastic lamina are broken, presence of cholesterol clefts, mineralization and/or necrosis.



Supplemental Fig. 9. Correlation between cumulative plasma TC exposure and atherosclerotic lesion area. The correlation between the cumulative plasma TC exposure and atherosclerotic lesion area, after log transformation, in E3L (open circles) (A) and the E3L.LIKK mice (closed circles) (B) was determined using the Pearson correlation test. n=15/group.



TOLL-LIKE RECEPTOR 4 DEFICIENCY DOES NOT REDUCE VLDL-TRIGLYCERIDE PRODUCTION IN MICE FED A HIGH-FAT DIET

6

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In preparation

ABSTRACT

High-fat diet (HFD) feeding increases hepatic inflammation, as evidenced by increased Toll-like receptor 4 (TLR4)/nuclear factor κB (NF-κB) signaling in livers of HFD-fed mice. In lean animal models, administration of lipopolysaccharide (LPS), a well-known ligand for TLR4, as well as activation of hepatic NF-κB both directly increase VLDL-TG production, pointing towards an important link between TLR4/NF-κB signaling and hepatic VLDL-TG production. Furthermore, TLR4 deficiency has recently been shown to protect against HFD-induced hepatic inflammation. The aims of this study were to investigate 1) whether FA composition of HFD based on lard (HFD-L) and palm oil (HFD-P) differentially affect hepatic NF-κB signaling and VLDL-TG production and 2) whether TLR4 deficiency reduces the hepatic VLDL-TG production in HFD-fed mice. We demonstrate that FA composition of the HFD strongly affects hepatic inflammation, whereby HFD-P, but not HFD-L, markedly increased hepatic NF-κB signaling. However, the increase in hepatic NF-κB signaling was not accompanied by an increased VLDL-TG production. Furthermore, in contrast to our hypothesis, TLR4 deficiency did not affect hepatic VLDL-TG production in mice fed HFD-L and even increased VLDL-TG production in mice fed HFD-P. We therefore conclude that 1) FA composition determines the ability of HFD to induce hepatic inflammation, 2) HFD-P-induced hepatic inflammation does not increase VLDL-TG production, and 3) TLR4 deficiency does not reduce VLDL-TG production in HFD-fed mice.

INTRODUCTION

Obesity and high-fat diet (HFD) feeding are associated with a chronic low-grade inflammation as characterized by activation of inflammatory pathways in numerous organs including the liver, which have been linked to metabolic disturbances such as insulin resistance and dyslipidemia.[1,2] Activation of inflammatory pathways has shown to causally interact with metabolic pathways.[1,3] We recently reported that hepatic activation of the inflammatory IKK-ß/NF-κB pathway induced hypertriglyceridemia by increasing hepatic VLDL-TG production [2]. In addition, administration of bacterial lipopolysaccharide (LPS) in rodents directly increases VLDL-TG production.[4] LPS is a major component of the cell wall of Gram-negative bacteria and is a potent inducer of NF-κB activity via activation of Toll-like receptor (TLR) 4,[5] proposing the TLR4/NF-κB pathway as a candidate linking inflammation and dyslipidemia.

Interestingly, accumulating evidence exists that beside LPS, also saturated fatty acids (SFAs) may serve as ligands for TLR4.[6, 7] Moreover, SFAs have been reported to increase NF-κB activity and proinflammatory cytokine expression in a TLR4 dependent manner in various tissues,[7,8] while unsaturated fatty acids (UFAs) can have anti-inflammatory effects.[9] It has been suggested that HFD feeding, by increasing plasma and tissue SFA concentrations, could induce local tissue inflammation via activation of TLR4. Accordingly, TLR4 deficient mice are protected against hepatic inflammation induced by both SFA infusion or HFD feeding.[7,10]

The aims of the current study were to investigate 1) whether FA composition of the HFD based on lard (HFD-L) and palm oil (HFD-P) differently affects NF-κB signaling and VLDL-TG production, and 2) whether TLR4 deficiency reduces the hepatic VLDL-TG production in HFD-fed mice. We show that HFD-P, but not HFD-L, markedly induced NF-κB signaling, which however did not increase hepatic VLDL-TG production. In addition, in contrast to our hypothesis, TLR4 deficiency did not affect hepatic VLDL-TG production in mice fed a HFD-L and even increased VLDL-TG production in mice fed a HFD-P.

MATERIALS AND METHODS

Animals and diets

TLR4 deficient (TLR4-/-) mice on a C57Bl/6J background that have been described before[11] were kindly provided by Dr. S. Akira and Dr. T. van der Poll, and bred in our animal facility. Male TLR4-/- mice and wild-type (WT) littermates were housed under standard conditions with a 12-hour light-dark cycle and had free access to food and water. At the age of 12 weeks, animals were assigned to a high fat diet (HFD), which provided 45% energy from lipids (D12451, Research Diet Services, Wijk bij Duurstede, The Netherlands). The HFDs that were used were identical, except for the source of fat in the diet, that was either lard (lard-based high fat diet; HFD-L) or palm oil (palm oil-based high fat diet; HFD-P), which have been described before.[12] Macronutrient composition of both diets is listed in table 1. In addition, details of FA composition

Table 1. Macronutrient composition of the high fat lard diet (HFD-L) and high fat palm oil diet (HFD-P)

Macronutrients	HFD-L (g/kg)	HFD-P (g/kg)
Casein	200	200
L-cystine	3	3
Corn starch	72.8	72.8
Maltodextrin	100	100
Sucrose	172.8	172.8
Cellulose	50	50
Soybean oil	25	25
Palm oil	0	177.5
Lard	177.5	0
Mineral Mix	10	10
Dicalcium phosphate	13	13
Calcium carbonate	5.5	5.5
Potassium citrate	16.5	16.5
Vitamin mix	10	10
Choline bitartrate	2	2
Other	141.85	141.85
FD&C red dye	0.05	0.05
Total	858.15	858.15

of both diets are shown in table 2. All experiments were approved by the institutional ethical committee on animal care and experimentation of the Leiden University Medical Center.

Western blot analysis

Liver tissue was homogenized by Ultraturrax (22,000 rpm; 2x5 sec) in an ice-cold buffer (pH 7.4) containing 30 mM Tris.HCl, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 0.5% (v/v) Triton X-100, 1% (v/v) SDS and protease inhibitors (Complete, Roche, Mijdrecht, The Netherlands) at a 1:10 (w/v) ratio. Homogenates were centrifuged (16,000 rpm; 15 min, 4°C) and the protein content of the supernatant was determined using the BCA protein assay kit (Pierce, Rockford, IL). Proteins (20-50 µg) were separated by 7-10% SDS-PAGE followed by transfer to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked for 1 h at room temperature in Tris-buffered saline with Tween-20 (TBST) with 5% non-fat dry milk followed by an overnight incubation with the following antibodies: p-Ser536 NF-κB p65 (#3031), NF-κB p65 (#3034), IκBα (#9242) (all from Cell Signalling) or ß-actin (#A5441; Sigma). Blots were then incubated with a horseradish peroxidase (HRP)-conjugated

Table 2. Fatty acid composition of the high fat lard diet (HFD-L) and high fat palm oil diet (HFD-P)

Fatty acid	HFD-L (% of total)	HFD-P (% of total)
C12:0	0	0.3
C14:0	0.8	0.9
C16:0	29.2	35.6
C16:1	2.8	0.2
C18:0	15.0	4.4
C18:1	43.3	40.6
C18:2	8.9	16.5
C18:3	0	0.7
C20:0	0	0.5
C20:1	0	0.1
C22:0	0	0.2
Total	100	100

secondary antibodies for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence (ECL) and quantified using Image J (NIH, USA).

Hepatic VLDL-TG production

To measure VLDL production *in vivo*, mice were fasted for 4 hours and anesthetized by intraperitoneal injection of acepromazine (6.25 mg/kg Neurotranq, Alfasan International BV, Weesp, The Netherlands), midazolam (6.25 mg/kg Dormicum, Roche Diagnostics, Mijdrecht, The Netherlands), and fentanyl (0.31 mg/kg Janssen Pharmaceuticals, Tilburg, The Netherlands). At t=0 min, Triton WR-1339 (Sigma-Aldrich, St Louis, MO) was injected intravenously (0.5 mg/g body weight, 10% solution in PBS) to block serum VLDL clearance. Blood samples were drawn before (t=0) and 15, 30, 60 and 90 min after injection into chilled capillaries, centrifuged and used for determination of plasma TG concentration using a commercially available enzymatic kit from Roche Molecular Biochemicals (Indianapolis, IN).

Statistical analysis

Data are presented as means ± SD. Statistical significant differences were calculated using a Student's T-test (SPSS Inc, Chicago, IL). $P<0.05$ was regarded statistically significant.

RESULTS

A palm oil-based, but not a lard-based HFD, increases hepatic NF-κB signaling in WT mice

We first determined whether FA composition of a HFD would affect hepatic NF-κB signaling. Therefore, WT mice were fed a HFD based on either lard (HFD-L) or palm oil

(HFD-P) or a control chow diet for 5 weeks. Hepatic NF-κB signaling was determined by western blot on homogenized liver tissue. Surprisingly, the HFD-L did not increase hepatic pNF-κB signaling compared to a chow diet (**Fig 1**). In contrast, the HFD-P strongly increased hepatic phosphorylation of NF-κB over total NF-κB compared to the chow diet (+175%; P<0.05; Fig 1C) and also tended to decrease total IκBα over ß-actin (Fig 1D). This indicates increased IκBα ubiquitination and degradation by the proteasome, which reflects activation of the NF-κB pathway, albeit that the reduction in total IκBα did not reach statistical significance (P=0.09). Taken together, these data show that a HFD-P, but not a HFD-L, increases hepatic NF-κB signaling in WT mice.

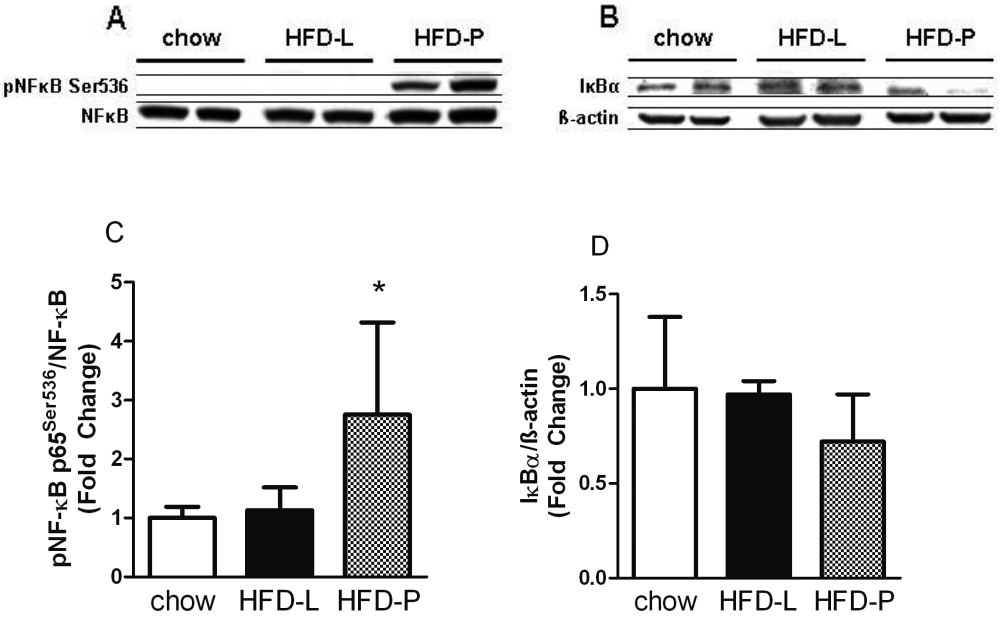

Figure 1. A high fat palm oil diet (HFD-P), but not a high fat lard diet (HFD-L), increases hepatic NF-κB signaling in WT mice. WT mice were either fed a HFD-L, HFD-P or a control chow diet (chow) for 5 weeks. NF-κB signaling was measured in liver tissue by phosphorylation of NF-κB (A,C) and total IκBα protein (B,D). Representative Western blots are shown for 2 mice per group (A,B). Ratios of phosphorylated NF-κB over total NF-κB and IκBα over ß-actin were quantified (B,D). Values are means ± SD (n=7-8). *P<0.05.

The increased hepatic NF-κB signaling in HFD-P-fed mice does not translate into an increased VLDL-TG production

Since activation of hepatic NF-κB signaling has been shown to increase VLDL-TG production,[2] we investigated whether a HFD-P increases the VLDL-TG production compared to a HFD-L. Therefore, WT mice were fed a HFD-P or HFD-L and the rate of VLDL-TG production was measured by determining plasma TG levels after intravenous

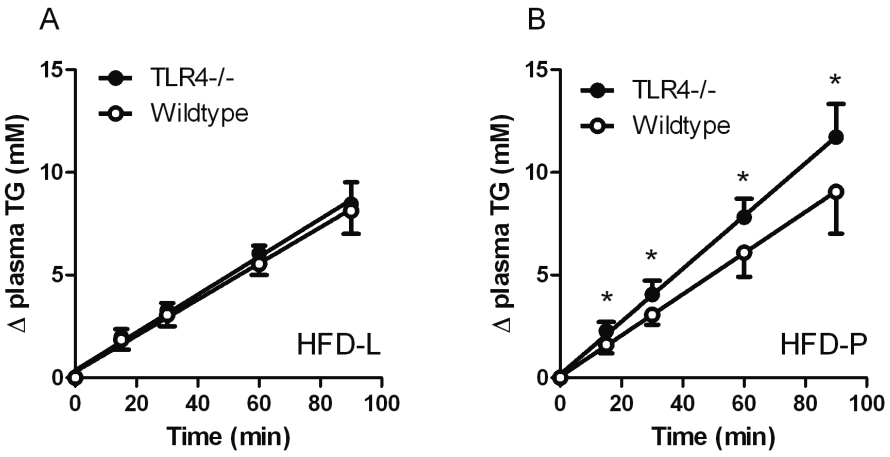

Figure 2. TLR4 deficiency does not affect VLDL-TG production in mice fed a high fat lard diet (HFD-L) and increases VLDL-TG production in mice fed a high fat palm oil diet (HFD-P). TLR4-/- and WT mice were fed a HFD weeks and VLDL-TG production was measured after a 4 hour fasting period. Mice were intravenously injected with Triton WR1339 (t=0) and blood samples were drawn at the indicated time points. TG concentrations were determined in plasma of WT mice (open circles) and TLR4-/- mice (closed circles) fed a HFD-L (A) or HFD-P (B) and plotted as the increase in plasma TG relative to t=0 (A). Values are means ± SD (n=7-8).

Triton WR1339 injection. Surprisingly, the increased hepatic NF-κB signaling in HFD-P-fed mice that we had observed did not translate into an increased hepatic VLDL-TG production (Fig. 2A and B). The VLDL-TG production, as determined from the slope of the curve from all individual mice, was similar between mice fed a HFD-P and HFD-L (6.01 ± 1.32 vs 5.28 ± 0.95 mM/h; N.S.)

TLR4 deficiency does not affect VLDL-TG production in mice fed a HFD-L and increases VLDL-TG production in mice fed a HFD-P

Previous studies reported that TLR4 deficiency reduces hepatic NF-κB signaling in HFD-fed mice.[13] Therefore, we investigated whether TLR4 deficiency would also decrease VLDL-TG production in HFD-fed mice. TLR4-/- and WT mice were fed a HFD-L or HFD-P and the rate of VLDL-TG production was measured by determining plasma TG levels after intravenous Triton WR1339 injection. In HFD-L fed mice, TLR4 deficiency did not affect VLDL-TG production (Fig 2A). The slope of the curve from all individual mice, was similar between TLR4-/- and WT mice (5.53 ± 0.97 vs 5.28 ± 0.95 mM/h; N.S.). In accordance, basal plasma TG levels were also not affected (0.47 ± 0.12 vs 0.48 ± 0.18 mM; N.S.).

In mice fed a HFD-P, TLR4 deficiency did not affect basal plasma TG levels (0.56 ± 0.19 vs 0.55 ± 0.17 mM; N.S.) but in contrast to our hypothesis, TLR4 deficiency clearly increased VLDL-TG production (Fig 2B). The rate of VLDL-TG production, as determined from the slope of the curve from all individual mice, was significantly higher in TLR4-/- compared to WT mice (7.71 ± 1.01 vs 6.01 ± 1.32 mM/h; $P<0.05$).

DISCUSSION

High-fat diet (HFD) feeding induces activation of inflammatory pathways in numerous organs including liver.[1,14] These inflammatory pathways have shown to cause metabolic disturbances such as insulin resistance and hypertriglyceridemia.[1,2] Both administration of LPS, the natural ligand for TLR4, as well as direct hepatic activation of NF-κB, have been reported to increase hepatic VLDL-TG production,[2,4] thereby contributing to hypertriglyceridemia. Deficiency of TLR4 protects against HFD induced hepatic inflammation[7,13] but so far it is unknown whether TLR4 deficiency is able to reduce VLDL-TG production after HFD-feeding. Surprisingly, our results document that TLR4 deficiency did not affect hepatic VLDL-TG production in animals fed a HFD-L, and even increased hepatic VLDL-TG production in animals fed a HFD-P. Therefore, we conclude that, in opposite to expectations, TLR4 deficiency did not reduce hepatic VLDL-TG production in HFD-fed mice.

Although it has previously been shown that HFD feeding induces hepatic inflammation [1], in the current study we reveal that the type of HFD that is used can highly affect the activation of hepatic NF-κB signaling; a HFD based on palm oil clearly induced hepatic NF-κB activity, while a HFD based on lard did not affect hepatic NF-κB activity. It will be interesting to elucidate which specific FAs in the HFD-P are responsible for the activation of hepatic inflammation, which is difficult to obtain from this study since the HFD-P and HFD-L differ with respect to more than one FA. In general, it has been shown that SFAs activate inflammatory pathways, while unsaturated fatty acids (UFAs) conduct anti-inflammatory effects.[6,9] Therefore, it is most likely that the increase in one or more of the SFAs (C12:0, C16:0, C20:0 or C22:0) or the reduction in one of the UFAs (C16:1 or C18:1) in the HFD-P are responsible for the increased hepatic inflammation in mice fed this HFD.

Another intriguing observation of the current study is that the HFD-P induced 2.8-fold increase in hepatic NF-κB activity did not translate into an increased VLDL-TG production. We recently showed that hepatocyte-specific overexpression of IKK-ß induced a 1.6-fold increase in hepatic NF-κB activity, which significantly increased VLDL-TG production.[2] In the liver, hepatocytes are responsible for the production of VLDL-TG, while Kupffer cells are the resident macrophages that are responsible for a first line of defense against inflammatory agents.[15, 16] Although *in vitro*, SFAs have shown to directly activate inflammatory pathways in hepatocytes,[9,17] HFD-derived SFAs *in vivo* might primarily act on Kupffer cells rather than on hepatocytes. It is conceivable that Kupffer cells require a certain level of activation of inflammatory signaling to secondarily activate inflammatory signaling in hepatocytes via local cytokine production. This would explain why administration of LPS, that strongly increases inflammatory pathways, but not HFD-derived SFAs, that only moderately increases inflammatory pathways, is able to increase hepatic VLDL-TG production.[4]

The second hypothesis of the current study was that TLR4 deficiency decreases the VLDL-TG production HFD-fed mice. From our data we conclude that, opposed to expectations, TLR4 deficiency did not reduce hepatic VLDL-TG production in

HFD-fed mice. One mechanism that could explain this unexpected observation is the fact we used only male mice for our experiments. A sexual dimorphism has been reported for TLR deficient mice, showing that TLR2 and TLR4 deficient female mice fed a HFD are more evidently protected against metabolic disturbances compared with males.[7,18] In the study of Shi et al,[7] although male TLR4 deficient male mice were clearly protected against HFD-induced hepatic inflammation, they were not protected against the development of insulin resistance. Since insulin resistance has been linked to an increased VLDL-TG production,[19] this could possibly explain why TLR4 deficiency in male mice did not reduce the VLDL-TG production in the current study.

It is remarkable that the type of HFD strongly influences the rate of VLDL-TG production in TLR4 deficient mice. A previous study from our group already revealed that the HFD-L and HFD-P differently affect other metabolic parameters such as tissue-specific insulin resistance [12]. One of the most intriguing findings of this study however is the observation that TLR4 deficiency actually *increased* the hepatic VLDL-TG production in mice fed a HFD-P, an observation that is in contrast to our hypothesis and rather difficult to explain. Earlier studies have reported that TLR4 deficiency decreases hepatic NF-κB activity and cytokine expression in HFD-fed mice.[7,13] In our study we did not yet verify whether TLR4 deficiency indeed decreased hepatic inflammation. It is possible that absence of TLR4 causes compensatory upregulation of other TLRs such as TLR2, which has also been linked to HFD-induced hepatic inflammation.[18] Actually, both C12:0 and C16:0 that are more abundant in the HFD-P have shown to be able to activate TLR2 *in vitro*.[20,21] Theoretically, upregulation of TLR2 in TLR4 deficient mice fed a HFD-P could, therefore, increase rather than decrease hepatic inflammation and consequently increase the VLDL-TG production. Future studies should elucidate whether FA composition of the HFD affect metabolic parameters by activation of specific TLRs. In addition, whether counteracting upregulation of TLR2 occurs in livers of TLR4 deficient mice remains to be determined; no change in TLR2 expression has been observed in adipose tissue of TLR4 deficient mice.[22]

In general, we can conclude that TLR4 deficiency does not reduce VLDL-TG production in HFD-fed mice. In addition, it appears that the FA composition of the HFD strongly affects the VLDL-TG production in TLR4 deficient mice. Our observations imply that the FA composition of the HFD in experimental studies on metabolism and inflammation can strongly affect the conclusions to be drawn.

ACKNOWLEDGEMENTS

We thank Dr. S. Akira (Department of Biochemistry, Hyogo College of Medicine, Hyogo, Japan) and Dr. T. van der Poll (Center for Experimental and Molecular Medicine, Academic Medical Center, Amsterdam, The Netherlands), who kindly provided the TLR4 deficient mice. This work was supported by the Netherlands Organization for Scientific Research (grant 917.76.301; J.v.D.), the seventh framework program of the EU-funded "LipidomicNet" (proposal number 2202272; J.v.D and I.O.C.M.V.) and the Center for Translational Molecular Medicine (www.ctmm.nl) project PREDICCt

(grant 01C-104; M.C.L.). P.C.N.R. is an Established Investigator of the Netherlands Heart Foundation (2009T038).

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THE INFLAMMASOME IS A CENTRAL PLAYER IN THE INDUCTION OF OBESITY AND INSULIN RESISTANCE

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Proc Natl Acad Sci U S A 2011; 108: 15324-9

ABSTRACT

Inflammation plays a key role in the pathogenesis of obesity. Chronic overfeeding leads to macrophage infiltration in the adipose tissue, resulting in pro-inflammatory cytokine production. Both microbial and endogenous danger signals trigger assembly of the intracellular innate immune sensor Nlrp3 resulting in caspase-1 activation and production of pro-inflammatory cytokines IL-1β and IL-18. Here, we showed that mice deficient in Nlrp3, ASC and caspase-1 were resistant to the development of high fat diet-induced obesity, which correlated with protection from obesity-induced insulin resistance. Further, hepatic triglyceride content, adipocyte size and macrophage infiltration in adipose tissue were all reduced in mice deficient in inflammasome components. Monocyte chemoattractant protein (MCP)-1 is a key molecule that mediates macrophage infiltration. Indeed, defective inflammasome activation was associated with reduced MCP-1 production in adipose tissue. Furthermore, plasma leptin and resistin that affect energy use and insulin sensitivity were also changed by inflammasome-deficiency. Detailed metabolic and molecular phenotyping demonstrated that the inflammasome controls energy expenditure and adipogenic gene expression during chronic overfeeding. These findings reveal a critical function of the inflammasome in obesity and insulin resistance and suggest inhibition of the inflammasome as a potential therapeutic strategy.

INTRODUCTION

The discovery of NOD-like receptors (NLRs) as essential components of the immune system triggered significant interest in the study of their contribution to the pathogenesis of inflammatory and autoimmune diseases. NLRs comprise a large family of intracellular proteins that are believed to be primarily involved in the innate immune response to microbial pathogens through the recognition of conserved pathogen-associated molecular patterns.[1-3] However, they also contribute to inflammation by sensing 'danger signals', i.e. endogenous molecules that are produced during tissue damage or inflammation.[3-5] A prominent example of an NLR protein implicated in auto-inflammatory disease is Nlrp3 (also called Cryopyrin)[6]. Nlrp3 activation induces the recruitment and autocatalytic activation of the cystein protease caspase-1 in a large cytosolic protein complex named the 'inflammasome.[2-7] The bipartite adaptor protein ASC bridges the interaction between Nlrp3 and the caspase-1 by means of homotypic interactions involving its pyrin and CARD motifs, making it essential for activation of the inflammasome.[7] Activated caspase-1 processes the cytosolic precursors of the related cytokines interleukin (IL)-1β and IL-18, thus allowing secretion of the biologically active cytokines. Hence, mice lacking caspase-1 are defective in the maturation and secretion of IL-1β and IL-18.[8-10] IL-1β participates in the generation of systemic and local responses to infection, injury and immunological challenges by inducing the "acute phase response" characterized by fever, synthesis of acute phase proteins and leukocytosis.[11] Although IL-18 lacks the pyrogenic activity of IL-1β, it is involved in the induction of several secondary pro-inflammatory cytokines, chemokines and cell adhesion molecules.[12,13]

Obesity is accompanied by the development of a chronic low grade inflammation that is promoted by expanding adipose tissue.[14,15] Expansion of fat mass characterized by adipocyte enlargement fuels the infiltration of macrophages into the adipose tissue.[16,17] Altogether, the enhanced inflammatory trait of the adipose tissue instigates the production of cytokines that contribute to the development of insulin resistance.[18-19] IL-1β and IL-18 have also been linked to the development of obesity-induced insulin resistance. IL-1β has been reported to inhibit adipocyte differentiation,[20] while the absence of IL-18 induced obesity and insulin resistance.[21,22] Moreover, high fat diet feeding resulted in the activation of caspase-1 in adipose tissue in mice.[23] Interestingly, absence of NLRP3 has recently been shown to prevent the development of obesity-induced insulin resitance.[24] However, the role of caspase-1 and the inflammasome member ASC in the development of high fat diet-induced obesity has not been characterized. Chronic activation of inflammasome-mediated caspase-1 activity may underlie the development of obesity-induced insulin resistance. To understand the role of the inflammasome in obesity and insulin resistance, we studied the response of *Nlrp3-/-*, *ASC-/-* and C*asp1-/-* mice to high fat diet (HFD) feeding. Our results indicate a major role for the inflammasome in modulating obesity and reveal its critical function in obesity-induced inflammation and insulin resistance.

MATERIAL AND METHODS

Animals

Nlrp3-/-, *ASC-/-* and *Casp1-/-* mice backcrossed to C57BL/6 background for at least 10 generations have been described before.[33,34] Mice were housed in a pathogen-free facility and the animal studies were conducted under protocols approved by St. Jude Children's Research Hospital Committee on Use and Care of Animals. All mice were male, 8-10 weeks old at the start of the diet intervention and maintained in a SPF facility. All experiments were conducted under protocols approved by the St. Jude Children's research Hospital Committee on Use and Care of Animals and the animal experimentation committee of Leiden University Medical Center.

Diet intervention

Male mice received a low fat diet (LFD) or high fat diet (HFD) for 16 weeks, providing 10 or 45% energy percent in the form of fat (D12450B or D12451, Research Diets). At the end of the feeding experiment, blood was collected in EDTA-coated tubes and centrifuged to collect plasma. Liver and epididymal white adipose tissue were dissected, weighed, and immediately frozen in liquid nitrogen. All studies described below were performed in animals fed the HFD.

Hyperinsulinemic euglycemic clamp

The hyperinsulinemic euglycemic clamp study was performed as published previously.[21,35,36] Plasma glucose, insulin and free fatty acid levels were determined using commercially available kits (Instruchemie, crystal chem. INC. and Wako Pure Chemical Industries).

Calculations

Turnover rates of glucose (μmol/min/kg) were calculated during the basal period and in steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of 3H-glucose (dpm/μmol). All metabolic parameters were expressed per kilogram of bodyweight. The hepatic glucose production (EGP) is calculated from the rate of disappearance (Rd) and glucose infusion rate (GIR) by the following equation: Rd = EGP + GIR. The Rd is measured from Steele's equation in steady state using the tracer infusion rate (Vin) and plasma specific activity (SA) of 3Hglucose (dpm/μmol) by the following formula: Rd = Vin/SA.

Indirect calorimetry

Groups of 8 mice per genotype were subjected to individual indirect calorimetry and metabolic cage measurements for a period of 84 hours (Comprehensive Laboratory Animal Monitoring System, Columbus Instruments). A period of 24 hours prior to the start of the experiment allowed the acclimatization of the animals to the cages and single housing. Experimental analysis started at 07:00h and continued for 60 hours. *Statistical analysis indirect calorimetry:* Averages were tested for significant differences

between groups using student T-Test after D'Agostino and Pearson test for normality. The statistical significance threshold was set at 0.05.

Histology/immunohistochemistry

Morphometry of individual fat cells was assessed using digital image analysis. Microscopic images were digitized in 24 bit RGB (specimen level pixel size 1.28x1.28 um2). Recognition of fat cells was initially performed by applying a region growing algorithm on manually indicated seed points, and minimum Feret diameter were calculated. For detection of macrophages/monocytes, an F4/80+ antibody (Serotec) was used. Visualization of the complex was done using 3,3'-diaminobenzidene for 5 minutes. Negative controls were used by omitting the primary antibody. Haematoxylin and Eosin staining of sections was done using standard protocols.

Liver triglycerides

Liver triglycerides were determined in 10% liver homogenates prepared in buffer containing 250 mM sucrose, 1mM EDTA and 10 mM Tris-HCl at pH 7.5.

Cell culture

Human SGBS cells were differentiated towards adipocytes using a standard protocol. In short, differentiation of cells was induced by treatment with transferrin, insulin, cortisol, T3, dexamethasone and IBMX. IL-1β (5 ng/ml), IL-18 (25 ng/ml) or IL-1ra (5 µg/ml) were added during differentiation. After 10 days of differentiation, RNA was isolated, cDNA was prepared and qPCR analysis was performed as described below.

RNA isolation and qPCR analysis

RNA from animal tissues or cultured cells was isolated using Trizol Reagent (Invitrogen) following manufacturer's instructions. RNA was reverse transcribed (iScript cDNA Synthesis Kit, Bio-Rad Laboratories) and real-Time PCR was done with a Power Sybr Green PCR master mix (Applied Biosystems) using a 7300 Real-Time PCR System (Applied Biosystems). Melt curve analysis was included to assure a single PCR product was formed. Values were corrected using the housekeeping gene 36B4 or beta2-microglobulin (B2M).

Plasma adipokines/adipose tissue-chemokines

Plasma concentrations of insulin, leptin and resistin are determined using Luminex techniques following manufacturer's instructions. Concentration of different chemokines in adipose tissue was determined by ELISA following manufacturer's instructions (R&D systems) or radio immune assays. Values were expressed as total amount per milligram WAT.

Microarray gene expression analysis

RNA quality was determined by analysis on the Agilent 2100 Bioanalyzer, and all samples had a RIN>8. Total RNA (100ng) was labeled, and processed automatically

on an HT MG-430 PM array plate using the Affymetrix GeneTitan system in the St. Jude microarray core according to the manufacturer's instructions. The probes on the HT MG-430 PM array were redefined according to Dai *et al*.[37] Normalized expression estimates were generated from the raw intensity values using the RMA algorithm in the Bioconductor library AffyPLM.[38] Differentially expressed probesets were identified using linear models, applying moderated t-statistics that implement empirical Bayes regularization of standard errors.[39] To adjust for both the degree of independence of variances relative to the degree of identity and the relationship between variance and signal intensity, the moderated t-statistic was extended by a Bayesian hierarchical model to define a intensity-based moderated T-statistic (IBMT).[40] P-values were corrected for multiple testing using a false discovery rate method.[41] Probesets with a FDR < 10% (q-value < 0.1) were considered to be significantly regulated. Changes in gene expression were related to functional changes using gene set enrichment analysis (GSEA).[42] Gene sets were derived from Gene Ontology, KEGG, NCI, PFAM and Biocarta pathway databases. Enrichment Map was used for interpretation of the GSEA results.[43] Only gene sets consisting of more than 10 and less than 500 genes were taken into account. The enrichment map was generated with gene sets that passed the significance threshold of p-value < 0.005 and similarity cut-off value of 0.6, resulting in a network of 226 nodes (gene sets) and 670 edges (interactions). All singletons were removed to create the final gene set interaction network.

Statistical analysis

Statistical significant differences were calculated using a Student's T-test. The cut-off for statistical significance was set at a P-value of 0.05 or below.

RESULTS

Absence of the inflammasome protects from high fat diet-induced obesity

To investigate the role of the inflammasome in the adipose tissue, we first analyzed the expression and activation of inflammasome downstream molecule caspase-1 in adipose tissue. In line with our previous work,[23] caspase-1 gene expression and activation in white adipose tissue (WAT) of HFD fed wild-type mice were increased (Fig.1a,b). We next assessed the functional roles of Nlrp3, ASC and Caspase-1 in the development and progression of HFD-induced obesity using mice lacking Nlrp3, ASC or Caspase-1. During 16 weeks of HFD-feeding, food intake and bodyweight development were examined weekly. HFD-feeding of wild-type, *Nlrp3-/-* (Fig. 1c), *ASC-/-* (Fig. 1d) and C*asp1-/-* (Fig. 1e) animals demonstrated that the absence of the inflammasome components protects against the development of high fat diet-induced obesity. The observed lean phenotype in *Nlrp3-/-*, *ASC-/-*, and *Casp1-/-* mice was due to defective inflammasome activation confirmed by significantly reduced IL-1β production, but not IL-6, in WAT of HFD-fed mice as compared to wild-type mice (Supplementary Fig. 1). The daily caloric food intake of all genotypes fed the LFD or HFD was similar (Fig. 1f). Concurrent with the development of HFD-induced obesity, HFD-fed

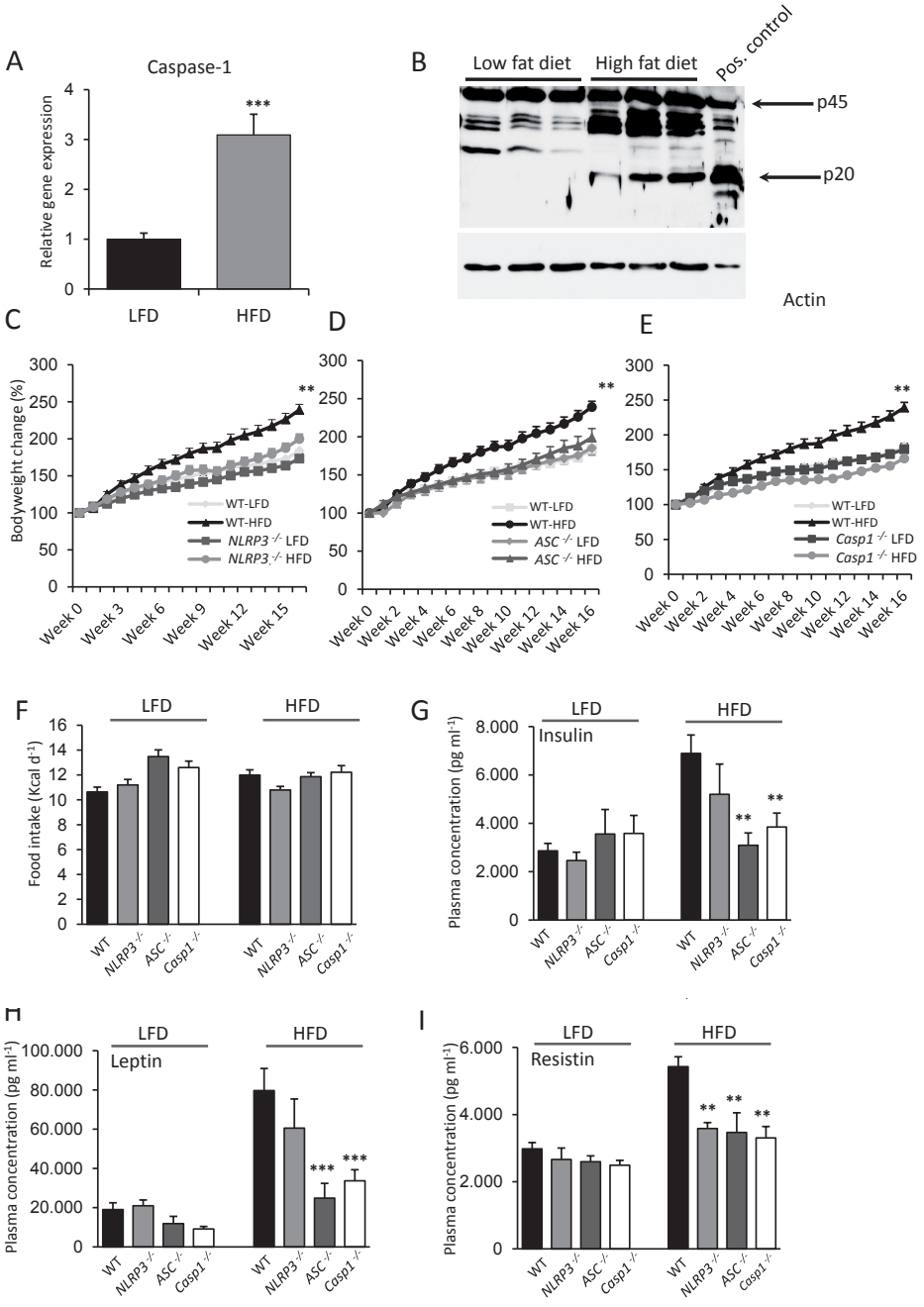

Figure 1. Absence of the Nlrp3-inflammasome protects against the development of HFD-induced obesity. qPCR analysis of caspase-1 gene expression levels in epididymal WAT of LFD and HFD fed wild-type C57/Bl6 animals after 16 weeks of diet-intervention (A). Caspase-1 protein levels in WAT of LFD vs. HFD-fed wild-type animals (B). Comparison of bodyweight gain in wild-type, Nlrp3-/-(C), ASC-/- (D) and Casp1-/- (E) animals on LFD or HFD during 16 weeks. Daily food intake of wild-type, Nlrp3-/-, ASC-/- and Casp1-/- animals fed a LFD or HFD (F). Plasma concentrations of insulin (G), leptin (H) and resistin (I) in LFD and HFD-fed wild-type, Nlrp3-/-, ASC-/- and Casp1-/- mice. **P < 0.01; **P < 0.001; n= 6-8 mice per group. Error bars represent s.e.m.

wild-type animals displayed hyperinsulinemia (Fig. 1g). In contrast, HFD-fed *ASC*-/- and *Casp1*-/- animals had significantly lower plasma insulin and leptin levels (Fig. 1g,h). Moreover, plasma concentrations of resistin, which is known to impair glucose tolerance and insulin action,[25] were significantly reduced in HFD-fed *Nlrp3*-/-, *ASC*-/- and *Casp1*-/- mice (Fig. 1i). However, plasma triglycerides and cholesterol levels were not significantly different between all genotypes (**Table 1**).

Table 1. Total plasma cholesterol en triglyceride concentrations were measured after 16 weeks of low fat or high fat diet feeding. Values are means ± SEM (n=5-10).

	Triglycerides (mg/dL)	**Cholesterol (mg/dL)**
Wt-LFD	129 ± 11	163 ± 8
Wt-HFD	143 ± 6	185 ± 7
$Casp1^{-/-}$ LFD	146 ± 23	125 ± 5
$Casp1^{-/-}$ HFD	116 ± 14	161 ± 9
$ASC^{-/-}$ LFD	131 ± 15	132 ± 10
$ASC^{-/-}$ HFD	132 ± 11	174 ± 14
$NLRP3^{-/-}$ LFD	104 ± 10	178 ± 7
$NLRP3^{-/-}$ HFD	144 ± 7	157 ± 7

ASC deficient mice are protected from HFD-induced insulin resistance, liver steatosis and adipocyte hypertrophy

To explore the effects of the key inflammasome adaptor ASC on HFD-induced insulin resistance, we performed insulin and glucose tolerance tests. As shown in Fig. 2a, both insulin sensitivity and glucose tolerance were improved in *ASC*-/- mice fed the HFD compared to wild-type mice. We next analyzed liver and adipose tissue morphology after 16 week of HFD intervention in wild-type and *ASC*-/- mice. The development of liver steatosis was blunted in *ASC*-/- mice (Fig. 2b,c). However, such striking effect of reduced liver Triglyceride (Tg) was not observed in *Nlrp3*-/- mice (Supplementary Fig. 2a). In line with the lower body weight and plasma leptin levels, epididymal adipose tissue mass was reduced in HFD-fed *ASC*-/- mice (Fig. 2d). Further analysis of the adipose tissue morphology in HFD-fed *ASC*-/- mice (Fig. 2e) revealed a significant reduction in adipocyte size (Fig. 2f,g) suggestive of an improvement in adipose tissue dynamics.[26] In contrast, obesity-induced macrophage infiltration into the adipose tissue was not prevented by the absence of ASC as determined by an immunohistochemical localization of macrophages (Fig. 2h). These results were further confirmed by qPCR analysis of the macrophage marker CD68 in total adipose tissue (wild-type LFD: 1 ± 0.29, wild-type HFD: 6.86 ± 1.87, *ASC*-/- LFD: 1.91 ± 0.31, *ASC*-/- HFD: 7.17 ± 2.17). Adipose tissue morphology was also examined in *Nlrp3*-/- mice which showed a marginal reduction in adipocyte size compared to the wild-type (Supplementary Fig. 2b).

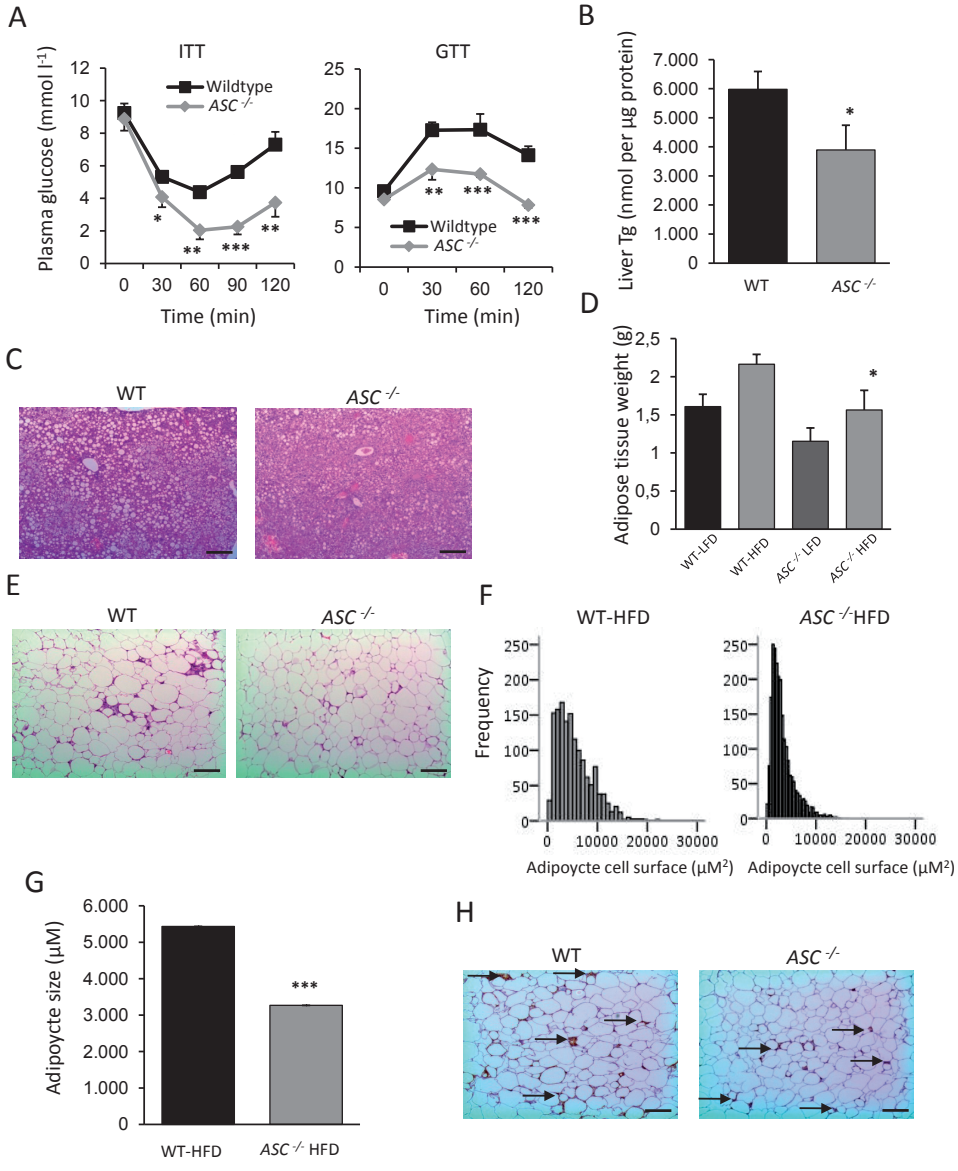

Figure 2. ASC-/- animals are protected against HFD-induced insulin resistance, steatosis and adipocyte hypertrophy. Insulin tolerance test (ITT) and glucose tolerance tests (GTT) of HFD-fed wild-type and ASC-/- animals (A). Liver triglyceride (Tg) levels in HFD-fed wild-type and ASC-/- mice (B). Liver histology as determined by hematoxylin and eosin (H&E) staining (C). Scale bars: 100 µm. Epididymal adipose tissue weight of LFD and HFD-fed wild-type or ASC-/- mice (D). Adipose tissue morphology after H&E staining of HFD-fed wild-type and ASC-/- mice. Scale bars: 100 µm (E) Quantification of adipocyte size using software analysis (F,G) Localization of macrophages in WAT of HFD-fed wild-type and ASC-/- at week 16 (H). Arrow indicates macrophage specific immunoreaction. Scale bars: 100 µm.

Hyperinsulinemic euglycemic clamp studies reveal a critical role for inflammasome in HFD induced insulin resistance

We next investigated the role of inflammasome in HFD-induced insulin resistance. Hyperinsulinemic euglycemic clamp studies revealed that glucose infusion rates were higher 5 in HFD-fed *Casp1-/-* mice (Fig. 3a,b) in line with an improvement in insulin sensitivity due to the absence of caspase-1. Blood glucose concentrations during the clamp as well as basal and hyperinsulinemic plasma, insulin and FFA levels are shown in Supplementary Fig. 3 and Supplemental Table 1. The elevated glucose infusion rate during hyperinsulinemia was caused by an increase of peripheral glucose uptake (Fig. 3c) while hepatic glucose production was unchanged (Fig. 3d) compared to HFD-fed wildtype animals. Similarly, inhibition of caspase-1 using pralnacasan in Ob/ob animals improved insulin sensitivity[23] paralleled by an increase in circulating plasma

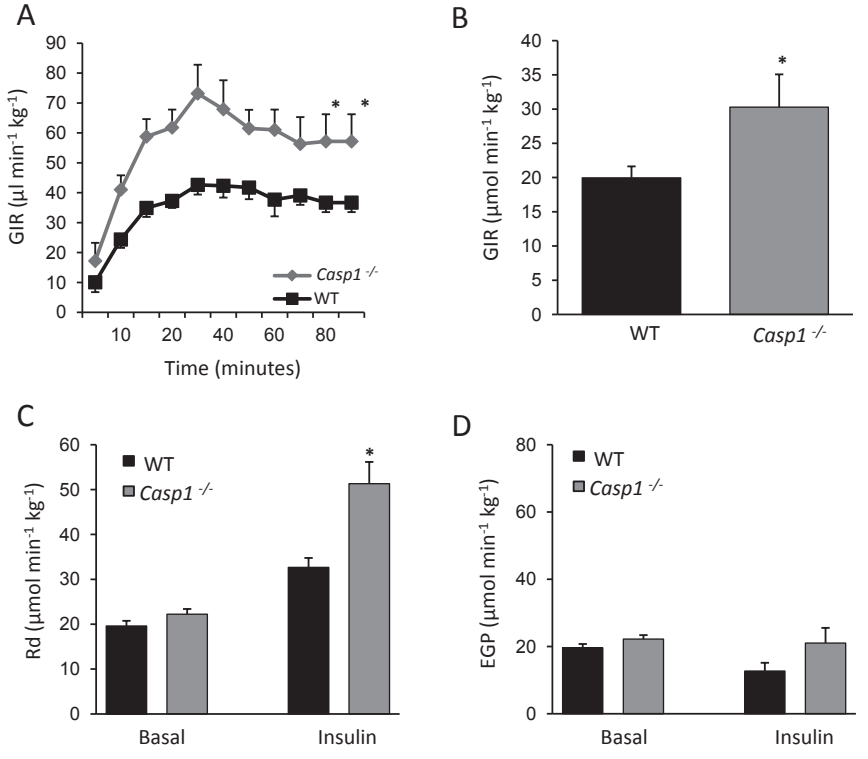

Figure 3. Absence of caspase-1 protects against the development of HFD-induced insulin resistance as determined by hyperinsulinemic euglycemic clamp analysis. Glucose infusion rates (GIR) during the euglycemic hyperinsulinemc clamp in HFD-fed wild-type and Casp1-/- animals (A). Average glucose infusion rate of wild-type and Casp1-/- animals fed a HFD for 16 weeks (B). Rate of disappearance (Rd), a measure of peripheral glucose uptake in wild-type and Casp1-/- animals (C). Endogenous (hepatic) glucose production (EGP) during the clamp (D). Mice were maintained on a HFD during 16 weeks prior to the clamp experiment. **P < 0.01; **P < 0.001; n= 6-8 mice per group. Error bars represent s.e.m.

adiponectin levels (Ob/ob + vehicle vs. Ob/ob + pralnacasan: 697.5 ± 40.1 vs. 797.5 ± 24.7, p-value< 0.05).

Caspase-1 mediates macrophage influx into adipose tissue

Since caspase-1 is the core molecule of inflammasome complex, we focused on *Casp1*-/- mice to understand the role of the inflammasome in adipose tissue function after HFD-feeding. The resistance to HFD-induced body weight-gain in *Casp1*-/- animals (Fig. 1e) was accompanied by a dramatic reduction in WAT mass as determined by DEXA-scan analysis (Fig. 4a). Notably, liver triglyceride storage upon HFD feeding was unchanged in *Casp1*-/- animals as compared to wild-type mice (Supplemental Fig. 4). We next analyzed morphological changes of the WAT induced by HFD-feeding. As shown in Fig. 4b, histological analysis revealed the presence of smaller adipocytes in HFD-fed *Casp1*-/- animals (Fig. 4b-d). Immunohistochemical localization of macrophages in WAT of HFD-fed animals showed a reduction in the number of macrophages in *Casp1*-/- mice (Fig. 4e). These results were confirmed by qPCR analysis of the macrophage marker CD68 in WAT (Fig. 4f). Additionally, as MCP-1 is responsible for macrophage influx,[27] we analyzed protein levels of MCP-1 in adipose tissue of HFD-fed animals. As shown in Fig. 4g, MCP-1 protein levels were significantly reduced ($P < 0.01$) in adipose tissue of *Casp1*-/- animals compared to wild-type mice. To test the relative contribution of IL-1β and IL-18 to this phenomenon, human adipocytes (SGBS cell line) were differentiated towards adipocytes in the presence of IL-1β or IL-18. As shown in Supplemental Figure 5, MCP-1 gene expression levels were enhanced by treatment of the cells with IL-1β (fold change vs. control: 3.6, p-value < 0.001) whereas IL-18 (fold change vs. control: 0.57, p-value: NS) had no effect. Moreover, blockade of endogenous IL-1 bioactivity by treatment with IL-1 receptor antagonist (IL-1ra) during differentiation of the cells, led to a significant reduction in MCP-1 gene expression levels (fold change vs. control: 0.25, p-value < 0.05) suggesting that the lower levels of MCP-1 in adipose tissue of HFD-fed *Casp-1*-/- animals (Fig. 4g), can mainly be attributed to the absence of IL-1β. To acquire more information regarding the molecular pathways controlled by caspase-1 in adipose tissue during HFDfeeding, we performed a microarray analysis comparing HFD-fed wild-type and *Casp1*-/- animals (Fig. 4h) that revealed a substantial overlap of pathways regulated in both *Casp1*-/- and *ASC*-/- animals including cell cycle and immunometabolism that encompasses functional gene sets both involved in immunity and metabolism. However, several pathways were regulated specifically in one genotype. For example, sphingolipid metabolism that may generate ligands including ceramide, is specifically regulated in the absence of caspase-1. A complete overview of differentially expressed genes in adipose tissue of HFD-fed animals is available online (http://humannutrition2.wur.nl/stienstra2011).

Defects in inflammasome increase energy expenditure in HFD-fed mice

We further analyzed the role of the inflammasome in obesity by examining energy expenditure, fecal output and caloric content in HFD-fed wild-type and *Casp1*-/- animals. Detailed analysis of the food intake revealed that, although the absence of

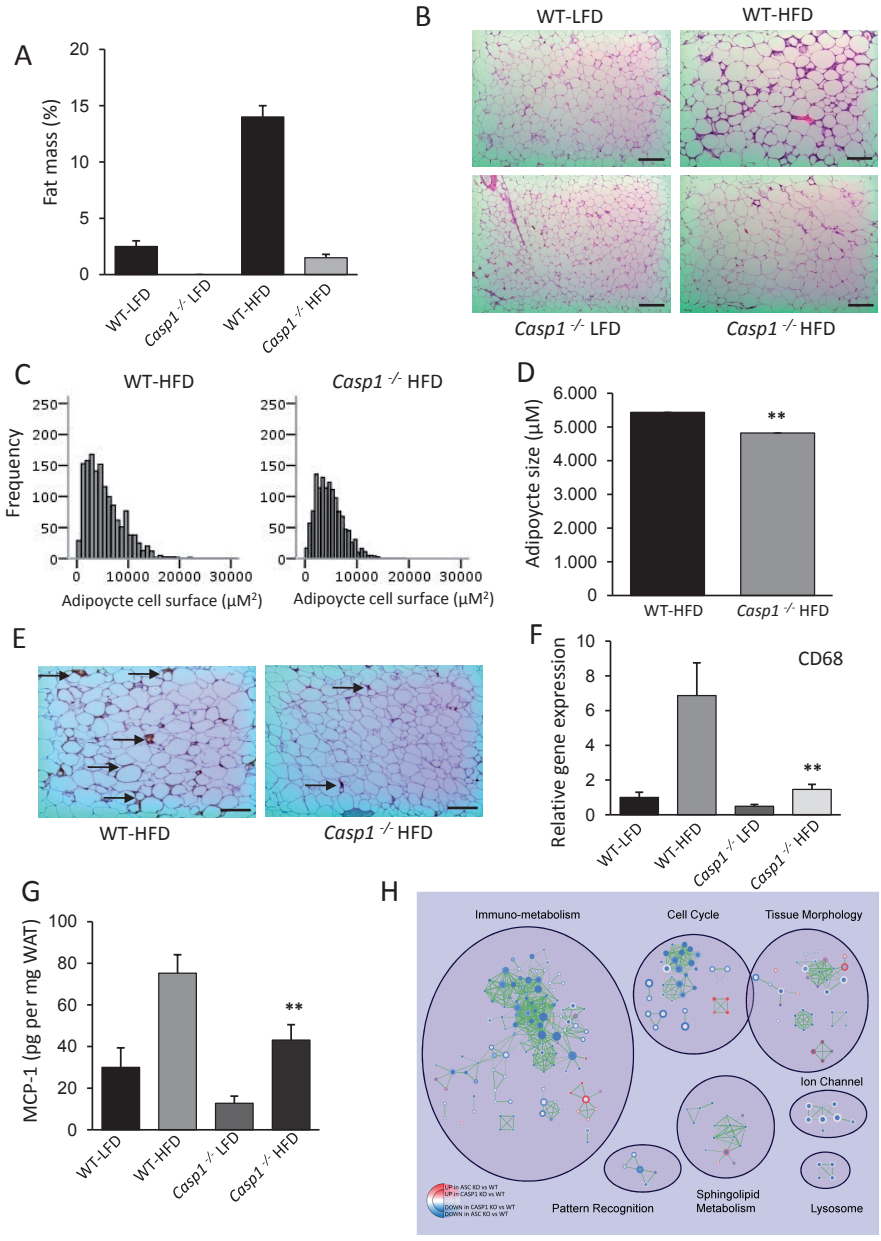

Figure 4. HFD-fed Casp1-/- animals are protected against obesity-induced adipocyte hypertrophy and macrophage influx into the adipose. Total % WAT as determined by DEXA scan analysis of wild-type and Casp1-/- on LFD or HFD for 16 weeks (A). Histology as determined by hematoxylin and eosin staining of WAT. Scale bars: 100 µm (B). Software analysis of adipocyte size (C) and quantification (D). Macrophage influx into the WAT as determined by immunohistochemistry (E). Arrow indicates macrophage specific immunoreaction. Scale bars: 100 µm. qPCR analysis of the macrophage marker CD68 in white adipose tissue (F). Concentration of MCP-1 in adipose tissue (G). **P < 0.01; n= 6-8 mice per group. Error bars represent s.e.m. ▸

caspase-1 protects against the development of obesity, *Casp1-/-* animals eat more as compared to wild-type mice (Fig. 5a). However, as shown in Fig. 5b, fecal output was significantly enhanced in *Casp1-/-* animals. Analysis of the fat content of the feces as determined by the percent of steatocrit, revealed no significant difference between both genotypes (Fig. 5c). Finally, caloric content of the feces did not differ between both genotypes on the HFD (Fig. 5d) suggesting that the net energy intake is similar in HFD-fed wild-type and *Casp1-/-* mice. However, although total daily caloric intake was highly similar, feeding behavior was strikingly different between wild-type and *Casp1-/-* mice (Supplemental Fig. 6a). Whereas wild-type animals displayed a constant intake, the *Casp1-/-* mice were characterized by the consumption of relatively large amounts of food followed by periods in which the animals did not eat at all. Inasmuch gut hormones are known to control food intake, we measured circulating concentrations of GIP, PYY and ghrelin before and two hours after the administration of a lipid bolus. Circulating postprandial levels of the satiety signals GIP and PYY were not different between both phenotypes (Supplemental Fig.9b).In contrast, whereas circulating levels of ghrelin were reduced in wildtype animals after receiving the lipid bolus (Supplemental Fig. 6b), this postprandial reduction was not observed in *Casp1-/-* mice suggesting that an orexigenic action of ghrelin may contribute to modulation of the feeding pattern. In contrast, analysis of the energy expenditure in HFD-fed caspase-1-deficient mice unveiled significantly higher energy expenditure (Fig. 5e,f). These results indicate that although total energy intake does not differ, energy expenditure is enhanced in *Casp1-/-* animals.

DISCUSSION

The prevalence of obesity has reached epidemic proportions worldwide. During the development of obesity, the morphology and functional properties of adipose tissue change dramatically. In addition to adipocyte hypertrophy, adipose tissue turns into an inflamed tissue characterized by macrophage infiltration and altered secretion of adipokines.[14,17] Whereas the secretion of proinflammatory cytokines is enhanced, the production of insulin-sensitizing adipokines such as adiponectin is reduced. To date, several pro-inflammatory cytokines have been linked to the development of insulin resistance including IL-1β and IL-18.[14] These cytokines are both produced as inactive precursors in the cytosol, and are released following their maturation by the cysteine protease caspase-1. The cytosolic zymogen form of caspase-1 consists of an N-terminal

▸ Enrichment map for gene expression in WAT of Casp-1-/- (n=3) (inner node area) and ASC-/- (n=3) (node borders) compared to WT (n=4) at 16 weeks after HFD intervention (H). Nodes represent functional gene sets, and edges between nodes their similarity. Color intensity of node area or border is proportional to enrichment significance in Casp-1-/- or ASC-/- mice compared to WT; red indicates increased and blue suppressed gene sets in null mice compared to WT. Node size represents the gene set size, and edge thickness represent degree of overlap between two connected gene sets. Clusters were manually circled and labeled to highlight the prevalent biological functions among related gene sets.

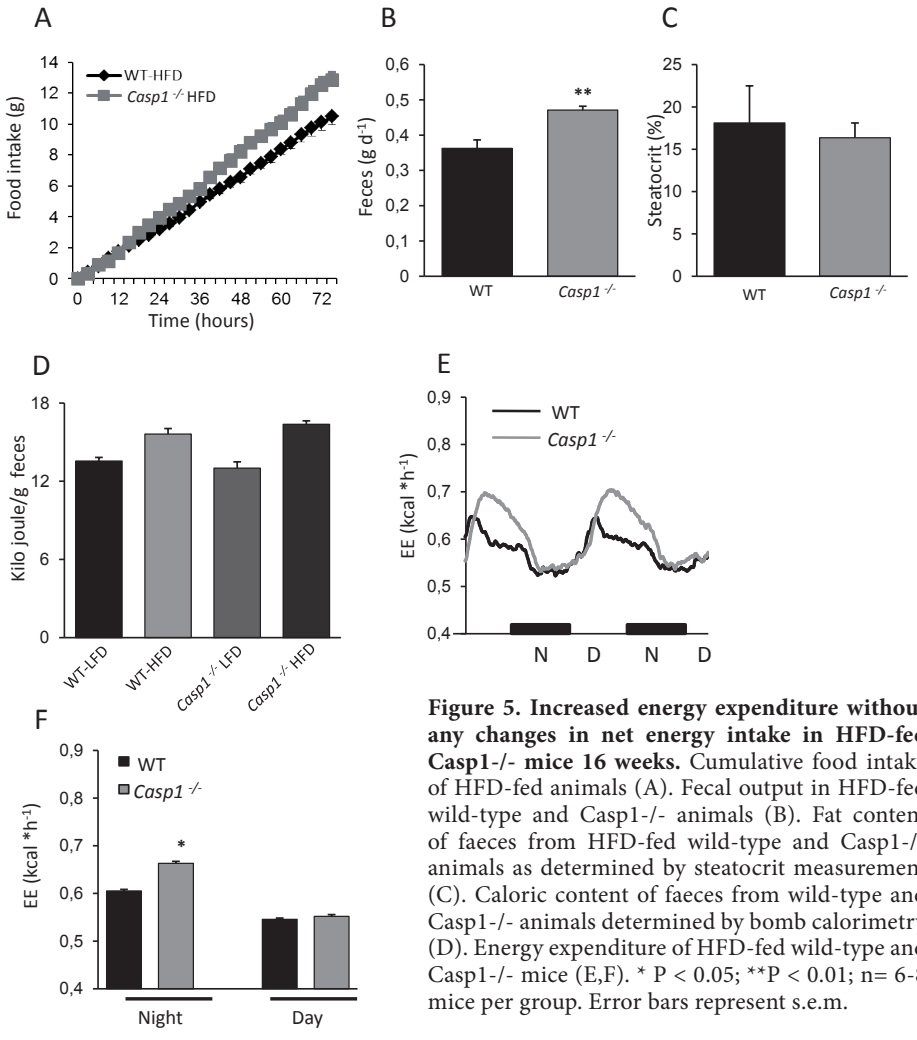

Figure 5. Increased energy expenditure without any changes in net energy intake in HFD-fed Casp1-/- mice 16 weeks. Cumulative food intake of HFD-fed animals (A). Fecal output in HFD-fed wild-type and Casp1-/- animals (B). Fat content of faeces from HFD-fed wild-type and Casp1-/- animals as determined by steatocrit measurement (C). Caloric content of faeces from wild-type and Casp1-/- animals determined by bomb calorimetry (D). Energy expenditure of HFD-fed wild-type and Casp1-/- mice (E,F). * $P < 0.05$; **$P < 0.01$; n= 6-8 mice per group. Error bars represent s.e.m.

prodomain that is processed and activated within the inflammasomes, which are assembled on NLR protein scaffolds. Because of the metabolic effects described for IL-1β and IL-18, and due to the crucial role of the inflammasome in the activation of these cytokines, we set out to test the role of the inflammasome in obesity. Our studies demonstrate that the mice deficient in inflammasome components are protected from HFD-associated body weight gain, adipocyte hypertrophy, hyperinsulinemia and hyperresistinemia. Importantly, HFD-induced production of the protein resistin was significantly reduced in inflammasome-deficient animals implying its critical role in driving the inflammatory potential of adipocytes. Further, chronic overfeeding resulted in pheno- and genotypical differences in WAT of inflammasome-deficient animals illustrated by a divergent macrophage influx and gene expression profile of the adipose tissue.

Deficiency in caspase-1 or ASC results in the same phenotype, which strongly suggests a role for the inflammasome in obesity. However, there are slight differences between ASC- and caspase-1-deficient mice. Although changes in adipogenic gene expression in caspase-1 and ASC-deficient animals show a considerable overlap, subtle differences exist. Whereas ASC may mediate effects on adipocyte hypertrophy as observed in *Casp-1-/-* animals, macrophage influx in the *ASC-/-* animals may partly be regulated independently of caspase-1. In this regard, recent studies reported that ASC directly mediates gene expression[28] and has a critical role in the development of experimental autoimmune encephalomyelitis and arthritis independently of the inflammasome.[29,30] However, in obesity, several of the parameters tested suggest that ASC and caspase-1 work in the same pathway. Indeed, caspase-1 appears to have a central role in adipose tissue functioning during chronic overfeeding by regulating macrophage influx and systemic insulin sensitivity. Although part of the protective effects in HFD-fed *Casp1-/-* animals are explained by the absence or lower levels of IL-1β, caspase-1 may also control alternative pathways. The wide range of genes differentially regulated between HFD-fed wild-type and *Casp1-/-* animals support the hypothesis of novel functions of caspase-1 in adipose tissue. In addition, the increase in energy expenditure observed in *Casp1-/-* animals identifies caspase-1 as a metabolic regulator. Finally, caspase-1 appears to drive the production of different chemokines in adipose tissue that regulated inflammatory cell influx. We postulate that caspase-1 downstream signaling contributes to the enhanced energy expenditure observed in HFD-fed *Casp1-/-* animals. Indeed, using the diagonal gel proteomic approach, several proteins that are involved in energy metabolism were identified as caspase-1 substrates.[31] Additional substrates of caspase-1 in adipose tissue may be identified by *in silico* analysis and may help to explain its contribution to the development of adipose tissue dysfunction during HFD-induced obesity.

One of the future challenges is to delineate the exact role of adipose-tissue specific caspase-1 to the development of HFD-induced obesity and insulin resistance. Moreover, future studies in animals that have adipocyte-specific overexpression of caspase-1 will be helpful to identify the differential contribution of macrophage- or adipocyte-derived caspase-1 to adipose tissue dysfunction and obesity. It will also be important to identify metabolic signals activating the inflammasome during the development of obesity. Interestingly, ceramide, a lipid molecule, has been described as a potent danger signal for inflammasome activation.[24] However, these results were generated in vitro and do not rule out alternative pathways that mediate activation of caspase-1. Indeed, high levels of glucose have also been shown to activate caspase-1 within adipose tissue.[32] Possibly, multiple danger signals exist that may use specificinflammasome pathways all leading to caspase-1 activation. Although we have clearly established the importance of the inflammasome during the development of obesity in animal models, additional studies focused on unraveling the importance of the inflammasome activation in human adipose tissue are needed. In conclusion, our study shows that the inflammasome, in addition to its role in the innate immune response, contributes to the development

of obesity-induced insulin resistance. Therefore, the inflammasome represent a useful therapeutic target in the treatment of obesity and insulin resistance.

ACKNOWLEDGEMENTS

We thank Anthony Coyle, John Bertin, Ethan Grant (Millennium Pharmaceuticals), Gabriel Nunez (University of Michigan), Richard Flavell (Yale University) and Shizuo Akira (Osaka university) for generous supply of mutant mice and Tim Koenen (Radboud University Nijmegen Medical Centre) for technical assistance. This work was supported by grants from the National Institutes of Health grant number AR056296 and AI088177, the American Lebanese and Syrian Associated Charities to T-D.K, the Dutch Diabetes Research Foundation (RS, MGN, CJT) and the European Association for the Study of Diabetes. M.G.N. was supported by a Vici grant of the Netherlands Organization for Scientific Research. J.v.D. is supported by the Netherlands Organization for Scientific Research (grant 917.76.301). P.C.N.R. is an Established Investigator of the Netherlands Heart Foundation (grant 2009T038). I.O.C.M. Vroegrijk is supported by the seventh framework program of the EU-funded "LipidomicNet" (202272).

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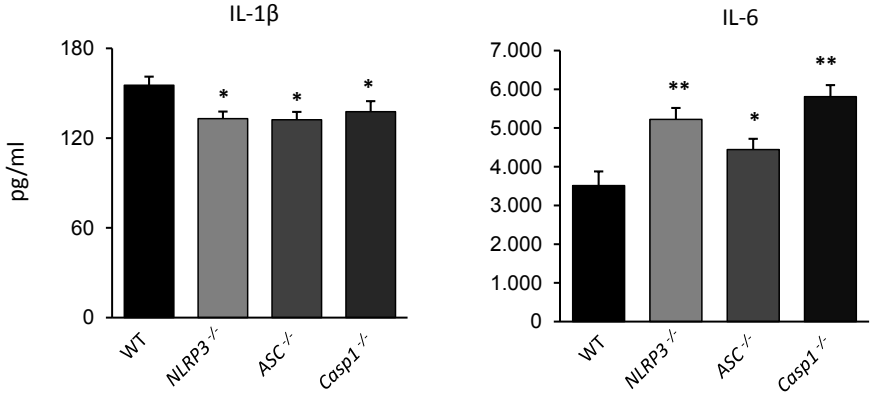
SUPPLEMENTARY DATA

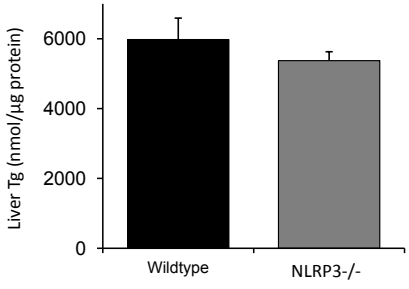
Suppl. Table S1. Plasma FFA and insulin levels during the euglycemic hyperinsulinemic clamp analysis.

	FFA (mM)		Insuline (ng/mL)	
	Basal	**Hyperinsulinemic**	**Basal**	**Hyperinsulinemic**
Wildtype	0.74 ± 0.07	0.58 ± 0.04	1.38 ± 0.40	4.74 ± 0.40
Casp1$^{-/-}$	0.71 ± 0.04	0.42 ± 0.06*	0.64 ± 0.15	3.30 ± 0.30*

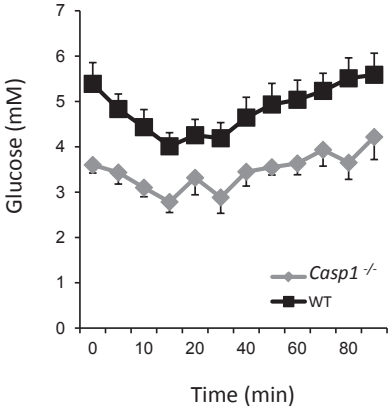
Euglycemic hyperinsuliemic clamp was performed after an overnight fast in caspase1 deficient and wild-type mice fed a HFD. Values are means ± SEM (n=6-9). *P<0.05



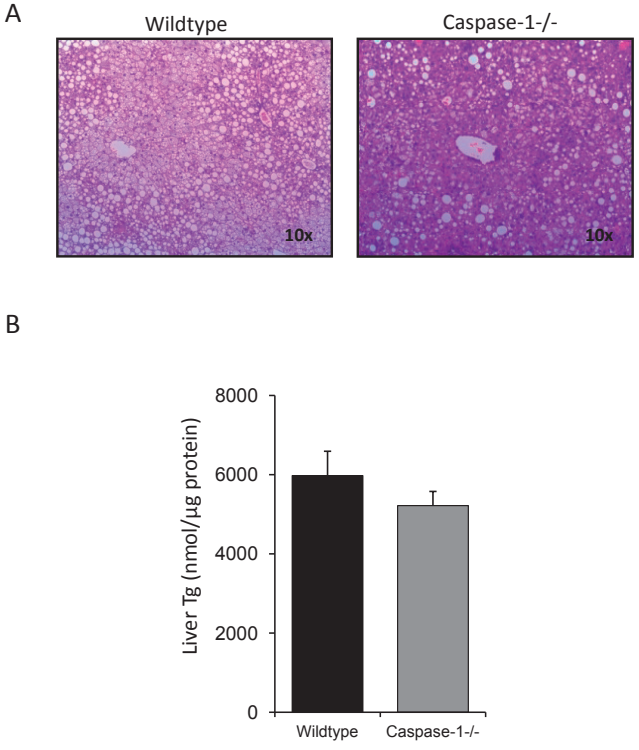
Suppl. Fig. S1: Cytokine analysis in WAT from HFD-fed wild-type, Nlrp3-/-, ASC-/-, and Casp1-/- mice. Total adipose of HFD-fed animals (16 weeks of diet intervention) was used to analyze concentrations of IL-1β and IL-6. * p-value < 0.05 vs. WT, ** p-value <0.01 vs. WT



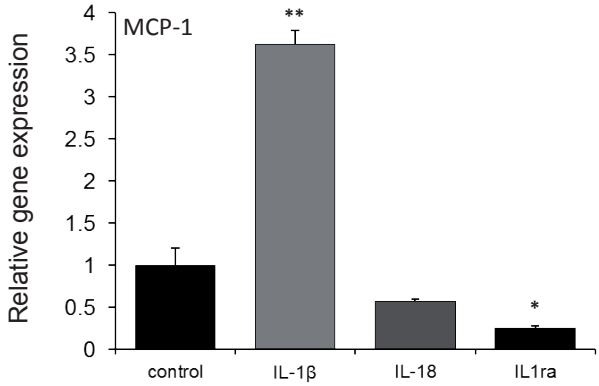
Suppl. Fig. S2: Liver triglyceride content (A) and H&E staining of white adipose tissue (B) in HFD-fed wild-type and Nlrp3-/- animals.



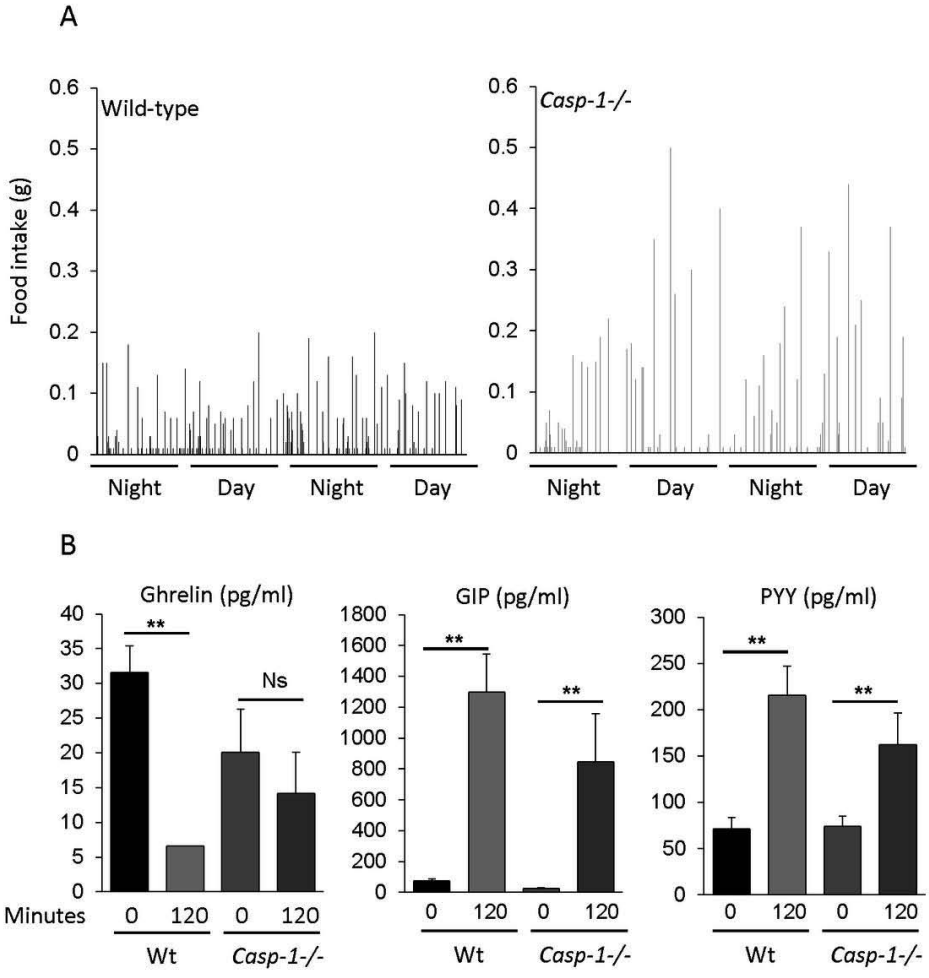
Suppl. Fig. S3: Glucose levels during euglycemic hyperinsulinemic clamp analysis of HFD-fed wildtype and Casp1-/- animals.



Suppl. Fig. S4: Liver histology as determined by H&E staining and liver triglyceride content in HFD-fed wild-type and Casp1-/- animals.



Suppl. Fig. S5: Monocyte chemotactic protein 1 (MCP-1) gene expression results in human SGBS adipocytes treated with IL-1β, IL-18 or IL-1 receptor antagonist during differentiation. MCP-1 gene expression results were analyzed in SGBS cells after 10 days of differentiation in the absence or presence of recombinant IL-1β (5 ng/ml), IL-18 (25 ng/ml) or IL-1ra (5 µg/ml). ** p-value < 0.001 * p-value < 0.05 vs. control



Suppl. Fig. S6: Feeding behaviour (a) and gut hormones levels after a postprandial response (b) in wild-type and Casp1-/- animals.

CASPASE-1 DEFICIENCY REDUCES INTESTINAL AND HEPATIC TRIGLYCERIDE-RICH LIPOPROTEIN SECRETION

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Submitted

ABSTRACT

Inflammasome-mediated caspase-1 activates the pro-inflammatory cytokines interleukin (IL)-1ß and IL-18. Recently, we showed that caspase-1 deficiency strongly reduces high fat diet (HFD)-induced weight gain, but the mechanism is still unclear. We now aimed to elucidate the mechanism by which caspase-1 deficiency modulates resistance to HFD-feeding by focusing on the role of caspase-1 in the regulation of triglyceride (TG)-rich lipoprotein metabolism. Postprandial TG kinetics, intestinal TG absorption, very low-density lipoprotein (VLDL)-TG production as well as TG clearance, all of which strongly contribute to the supply of TG for storage in adipose tissue, were measured in caspase-1 deficient and compared to results obtained in wild-type mice (both C57Bl/6 background). Microarray and qPCR analysis were used to identify intestinal and hepatic metabolic pathways involved. Caspase-1 deficiency reduced the postprandial response to an oral lipid load. The tissue-specific clearance of TG-rich lipoproteins was not changed, evidenced by unaltered kinetics of i.v. administered VLDL-like emulsion particles. An oral gavage of [^{3}H]TG-containing olive oil revealed that caspase-1 deficient mice had decreased intestinal chylomicron-TG production and reduced uptake of [^{3}H]TG-derived fatty acids (FA) in liver, muscle, and adipose tissue. Similarly, despite an elevated hepatic TG content, caspase-1 deficiency reduced the hepatic VLDL-TG production without reducing VLDL-apoB production. Pathway analysis of microarray data revealed that caspase-1 deficiency reduces intestinal and hepatic expression of genes involved in lipogenesis. The reduced adiposity of caspase-1 deficient mice is caused by a hampered assembly and secretion of TG-rich lipoproteins, which decreases the availability of TG-derived FA for uptake by peripheral organs including adipose tissue. We anticipate that caspase-1 represents a novel link between innate immunity and the regulation of TG-rich lipoprotein metabolism.

INTRODUCTION

Obesity is accompanied by low-grade chronic systemic inflammation, characterized by increased circulating levels of proinflammatory cytokines including interleukin (IL)-1β, tumor necrosis factor-α (TNF-α) and IL-6.[1] Activation of inflammatory pathways induces metabolic disturbances, leading to insulin resistance, dyslipidemia and cardiovascular diseases. Caspase-1 is a cysteine protease that is crucial for the activation of the pro-inflammatory cytokines IL-18 and IL-1β. Caspase-1 activation is in turn controlled by the inflammasome, a multiprotein complex consisting of a member of the Nod-like receptor family (e.g. NLRP3), and the inflammasome adaptor molecule ASC.[2] A close interaction has been described between the innate immune system and lipoprotein metabolism in general, and triglyceride (TG) metabolism in particular.[3] This interaction has largely been derived from studies that evaluated the effects of lipopolysaccharide (LPS), a major component of the cell wall of Gram-negative bacteria, and individual cytokines on lipoprotein metabolism.[4-6]

Although inflammasome-mediated caspase-1 activity has recently been linked to metabolic disturbances such as steatohepatitis[7] and cardiovascular diseases,[8] the role of caspase-1 in lipoprotein metabolism has never been elucidated. We recently showed that absence of caspase-1 in mice is accompanied by a profound decrease in both adipocyte size and total adipose tissue mass upon high-fat diet (HFD)-induced obesity[9]. Remarkably, inhibition of caspase-1 in adipocytes *in vitro* actually increases adipocyte differentiation,[9] suggesting that the decrease in adipose tissue mass *in vivo* is not due to a primary defect in adipogenesis, yet may be the result of a lack of sufficient lipid supply.

Dietary intake and subsequent intestinal absorption of fat strongly contribute to the supply of lipids for storage in adipose tissue. Dietary fat is extremely efficiently absorbed in the small intestine and incorporated into chylomicrons for distribution to other tissues. Interestingly, caspase-1 deficient (caspase-$1^{-/-}$) mice fed a HFD display an increased feces weight,[10] suggesting a decreased intestinal capacity for the absorption of dietary fat. We therefore hypothesized that caspase-1 might play a direct role in the regulation of (postprandial) TG-rich lipoprotein metabolism that could underlie the reduction in adipose tissue mass in caspase-$1^{-/-}$ mice.

In the current study, we investigate the role of caspase-1 in TG metabolism and show that caspase-1 deficiency markedly reduces intestinal TG absorption as well as hepatic very low-density lipoprotein (VLDL)-TG production, thereby limiting the availability of lipids for peripheral storage.

MATERIALS AND METHODS

Animals

Caspase-$1^{-/-}$ mice[9] were backcrossed ten generations to C57Bl/6 mice and age-matched wild-type C57Bl/6J mice were used as control mice. Mice were housed under standard conditions with a 12-hour light-dark cycle and were fed a standard mouse chow diet

with free access to water. Experiments were performed in 14 to 16-week old animals. When indicated, mice were fasted overnight (from 18.00-8.00 h) or for 4 h (from 8.00-12.00 h). All experiments were approved by the institutional ethical committee on animal care and experimentation of the Leiden University Medical Center.

Fatty acid composition of feces

Mice were individually housed for 4 days to collect feces quantitatively. Feces were weighed, freeze-dried, grounded and fecal fatty acids (FA) were subsequently derivatized by methyl esterification. Therefore, 2 mL methanol/hexane (4:1 v/v) containing 80 µg pentadecanoic acid (C15:0) as an internal standard (Fluka) was added to 15 mg feces. Then, 200 µL acetyl chloride (Merck) was added and samples were incubated at 95°C. After subsequent cooling to 4°C, 5 mL 6% K_2CO_3 (Sigma) was added and samples were centrifuged (10 min, 4000 rpm, 4°C). The upper hexane layer was isolated and used for GC analysis of FA methyl esters (FAME). FAME were separated on a 50 m x 0.25 mm capillary GC column (CP Sil 88, Agilent technologies) in a 3800 GC gas chromatograph (Varian) equipped with a flame ionization detector. The injector and flame ionization detector were kept at 270°C. The column temperature was programmed from 170°C to 210°C. FAME were introduced by split injection (split ratio 20:1). Quantification was based on the area ratio of the individual FA to the internal standard.

Postprandial TG response

To measure the postprandial response, overnight fasted mice received an intragastric load of 200 µL olive oil (Carbonell, Cordoba, Spain). Blood samples were drawn before (t=0) and 1, 2, 4 and 8 h after the bolus into chilled capillaries coated with paraoxon (Sigma, St Louis, MO) to prevent ongoing lipolysis.[11] Plasma was assayed for TG with the commercially available enzymatic kit from Roche Molecular Biochemicals (Indianapolis, IN) and for free FA (FFA) using NEFA-C kit from Wako Diagnostics (Instruchemie, Delfzijl, The Netherlands).

In vivo clearance of TG-rich emulsion particles

VLDL-like TG-rich emulsion particles (80 nm) labeled with glycerol tri[^{3}H]oleate [triolein (TO)] and [^{14}C]cholesteryl oleate (CO) were prepared and characterized as described previously.[12] To study the *in vivo* clearance of the VLDL-like TG-rich particles, mice were fasted for 4 h and injected (t=0) via the tail vein with 200 µL of emulsion particles (1.0 mg TG per mouse). Blood samples were taken from the tail vein at 2, 5, 10 and 15 min after injection to determine the serum decay of [^{3}H]TO and [^{14}C]CO. Plasma volumes were calculated as 0.04706 x body weight (g) as determined from ^{125}I-BSA clearance studies as described previously.[13] After taking the last blood sample, the liver, hindlimb muscle, brown adipose tissue (BAT) and gonadal (gWAT), subcutaneous (sWAT) and visceral (vWAT) white adipose tissues were collected. Organs were dissolved overnight at 60°C in Tissue Solubilizer (Amersham Biosciences, Rosendaal, The Netherlands) and ^{3}H- and ^{14}C-activity was counted. Uptake of [^{3}H]

TO- and [^{14}C]CO-derived radioactivity by the organs was corrected for plasma radioactivity present in the respective tissues and expressed per mg wet tissue weight.

Intestinal TG absorption

To measure intestinal lipid absorption, overnight fasted mice received an intragastric load of [^{3}H]triolein ([^{3}H]TO) (5 μCi; GE Healthcare, Little Chalfont, UK) in 200 μL olive oil (Carbonell, Cordoba, Spain). Blood samples were drawn before gavage (t=0) and at 1 and 2 h after gavage and serum ^{3}H-activity was counted. Plasma volumes (mL) were calculated as 0.04706 x body weight (g).[13] Two hours after the oral lipid load, liver, muscle, BAT and gWAT, sWAT and vWAT were collected. Organs were dissolved overnight at 60°C in Tissue Solubilizer (Amersham Biosciences, Rosendaal, The Netherlands) and counted to determine ^{3}H uptake. In addition, the intestinal tract (*i.e.* duodenum, proximal jejunum, distal jenunum and ileum) were isolated and washed 2 times in 10 mL phosphate-buffered saline (PBS). Both the intestinal tissue and the non-absorbed luminal content (in PBS) were counted for ^{3}H-activity to determine the amount of absorbed versus non-absorbed olive oil present in the intestinal tract.

In vivo hepatic VLDL-TG and VLDL-apoB production

To measure VLDL production *in vivo*, mice were fasted for 4 h and anesthetized by intraperitoneal injection of acepromazine (6.25 mg/kg Neurotranq, Alfasan International BV, Weesp, The Netherlands), midazolam (6.25 mg/kg Dormicum, Roche Diagnostics, Mijdrecht, The Netherlands), and fentanyl (0.31 mg/kg Janssen Pharmaceuticals, Tilburg, The Netherlands). Mice were injected intravenously with Tran[^{35}S] label (150 μCi/mouse; MP Biomedicals, Eindhoven, The Netherlands) to label newly produced apolipoprotein B (apoB). After 30 min at t=0 min, Triton WR-1339 (Sigma-Aldrich) was injected intravenously (0.5 mg/g body weight, 10% solution in PBS) to block serum VLDL clearance. Blood samples were drawn before (t=0) and at 15, 30, 60 and 90 min after injection and used for determination of plasma TG concentration as described above. After 120 min, mice were exsanguinated via the retro-orbital plexus. VLDL was isolated from serum after density gradient ultracentrifugation at d<1.006 g/mL by aspiration[14] and counted for incorporated ^{35}S-activity.

Liver lipids

Lipids were extracted from livers of mice that were in a fed state and after 4h-fasting according to a modified protocol from Bligh and Dyer.[15] Briefly, a small piece of liver was homogenized in ice-cold methanol. After centrifugation, lipids were extracted by addition of 1800 μL CH_3OH:$CHCl_3$ (3:1 v/v) to 45 μL homogenate. The $CHCl_3$ phase was dried and dissolved in 2% Triton X-100. Hepatic TG, total cholesterol (TC) and phospholipid (PL) concentrations were measured using commercial kits from Roche Molecular Biochemicals (Indianapolis, IN). Liver lipids were expressed per mg protein, which was determined using the BCA protein assay kit.

RNA isolation and qPCR analysis.

Total RNA was obtained from the epithelial layer of the duodenum, jejunum and ileum 2 h after an oral lipid bolus as well as from 4 h-fasted livers. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), followed by DNAse treatment and column purification using the RNeasy mini kit (Qiagen, Hilden, Germany). The RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer and subsequent cDNA analysis was done using iScript reagent (Biorad). qPCR analysis was carried out on a Bio-Rad MyIQ. 36B4 was used as housekeeping genes and PCR primer sequences were taken from the PrimerBank and ordered from Eurogentec (Seraing, Belgium). Sequences of the primers used are available upon request.

Microarray analysis

RNA quality was assessed with 6000 Nano chips (Bioanalyzer 2100; Agilent, Amstelveen, The Netherlands). RNA was judged as being suitable when RIN (RNA integrity number) was above 8.0. Total RNA (100 ng) was labelled and processed automatically on Affymetrix GeneChip Mouse Gene 1.1 ST arrays using an Affymetrix GeneTitan Instrument, according to the manufacturer's recommendations. Quality control of the datasets was performed using Bioconductor[16] packages integrated in an on-line pipeline.[17] Various advanced quality metrics, diagnostic plots, pseudo-images and classification methods were applied to ascertain only excellent quality arrays were used in the subsequent analyses.[18] The probes on the array were redefined according to Dai et al.[19] utilizing current genome information. In this study probes were reorganized based on the Entrez Gene database, build 37, version 2 (remapped CDF v14). Normalized expression estimates were obtained from the raw intensity values using the robust multiarray analysis (RMA) preprocessing algorithm.[20] To determine the intestinal expression level for each gene, expression data of all three segments (duodenum, jejunum, ileum) were combined and averaged per mouse. Changes in gene expression were related to functional changes using gene set enrichment analysis (GSEA). Gene sets were derived from Gene Ontology, KEGG, National Cancer Institute, PFAM, Biocarta, Reactome, and WikiPathway curated pathway databases. Enrichment Map was used for interpretation of the GSEA results. Only gene sets consisting of more than 15 and fewer than 500 genes were taken into account. The enrichment map was generated with gene sets that passed the combined significance threshold of P value < 0.01 and FDR q-value < 0.5, and similarity (Jaccard index) cut-off value of 0.375. All singletons were removed to create the final gene set interaction network.

Statistical analysis

Data are presented as means ± SD. Statistical significant differences were calculated using a Student's T-test (SPSS Inc, Chicago, IL). $P<0.05$ was regarded statistically significant.

RESULTS

Absence of caspase-1 reduces white adipose tissue mass

Caspase-1$^{-/-}$ and wild-type (WT) mice were evaluated for body weight and adipose tissue mass. In line with results from our previous study,[9] caspase-1 deficiency markedly reduced white adipose tissue (WAT) mass, while body weight was similar between caspase-1$^{-/-}$ vs WT mice (30.0 ± 3.2 vs 30.8 ± 2.5). The reduction in WAT was reflected by a reduction in the amount of gWAT (-69%; P<0.001) and sWAT (-34%; P<0.01), and a trend toward reduction in the amount of vWAT (-17%; P=0.16).

Absence of caspase-1 reduces the postprandial response to an oral lipid load

As intestinal absorption of fat strongly contributes to the supply of lipids for storage in adipose tissue, we investigated whether the reduction in adipose tissue mass and the resistance to obesity upon high fat feeding in caspase-1$^{-/-}$ mice[9, 10] reflects impaired postprandial lipid handling. Hereto, overnight fasted WT and caspase-1$^{-/-}$ mice received an intragastric olive oil bolus (200 µL) and the appearance of TG and FFA in plasma were determined (**Fig 1**). In caspase-1$^{-/-}$ mice, the postprandial increase in TG levels was completely abolished (peak TG level -59% at t=2 h; P<0.01; Fig 1A), which was paralleled by a postprandial drop in serum FFA (peak FFA level -38% at t=4 h; P<0.05; Fig 1B) levels.

Caspase-1 deficiency does not affect TG-rich particle clearance

The postprandial TG response is determined by the balance between intestinal TG production and the rate of plasma TG clearance. To determine whether the decreased postprandial response in caspase-1$^{-/-}$ mice was caused by an increased clearance of

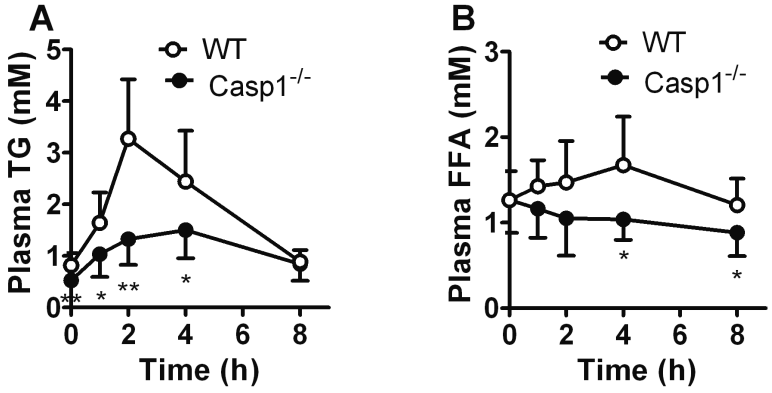

Figure 1. Caspase-1 deficiency reduces the postprandial lipid response. Overnight fasted WT and caspase-1$^{-/-}$ mice received an intragastric olive oil gavage and blood samples were drawn at the indicated time points. Plasma TG **(A)** and FFA **(B)** concentrations were determined in WT mice (open circles) and caspase-1$^{-/-}$ mice (closed circles). Values are means ± SD (n=8). *P<0.05 and **P<0.01.

TG-rich particles, we evaluated plasma clearance and organ distribution of [^{3}H]TO and [^{14}C]CO-double-labeled TG-rich emulsion particles in WT and caspase-1$^{-/-}$ mice (**Fig 2**). Caspase-1 deficiency slightly delayed clearance of [^{3}H]TG from plasma (Fig 2A). However, at 15 min after the injection, both WT and caspase-1$^{-/-}$ mice had equally cleared the [^{3}H]TG from the circulation. Caspase-1 deficiency did not affect ^{3}H uptake per g of tissue by the liver, muscle, BAT and vWAT, while ^{3}H uptake in gWAT and sWAT was even increased in caspase-1$^{-/-}$ mice (Fig 2B). This observation implies that the reduction in adiposity observed in caspase-1$^{-/-}$ mice can not be explained by a defect in clearance and/or uptake of TG-derived [^{3}H]FA by WAT. Similarly, caspase-1 deficiency did not affect [^{14}C]CO clearance (Fig 2C) or [^{14}C]CO uptake by the various organs (Fig 2D), indicating that TG-rich particle turnover was similar in caspase-1$^{-/-}$ and WT mice.

Absence of caspase-1 reduces intestinal TG absorption and increases fecal FA content

Since the markedly decreased postprandial TG response in caspase-1$^{-/-}$ mice was not explained by increased plasma TG clearance, we then evaluated intestinal lipid absorption

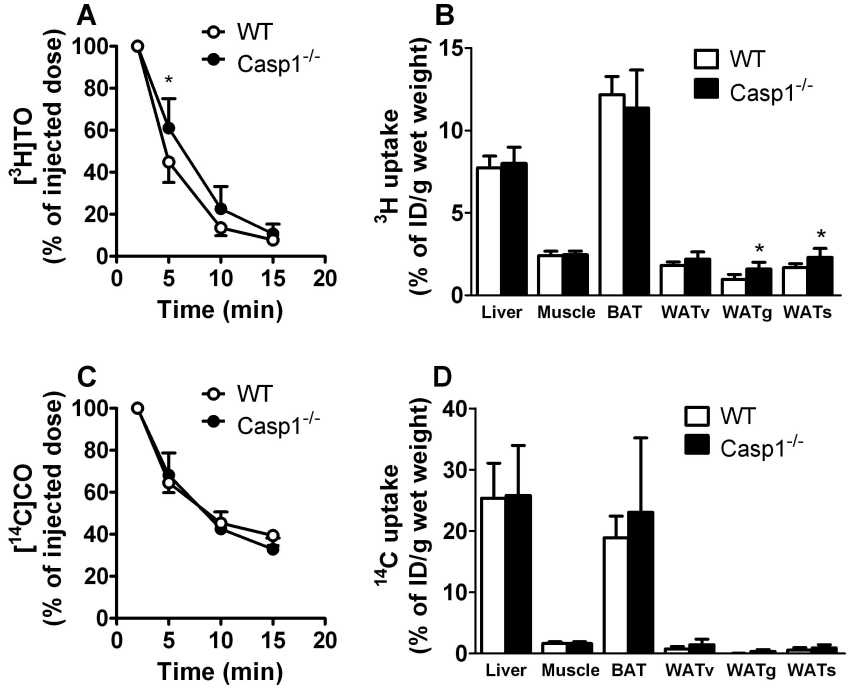

Figure 2. Caspase-1 deficiency does not affect clearance of VLDL-like TG-rich particles. WT and caspase-1$^{-/-}$ mice were fasted for 4 h and received an i.v. bolus of [^{3}H]TO and [^{14}C]CO-double labeled TG-rich emulsion particles (1 mg TG per mouse) (n=7-8). Blood was collected at the indicated time points and ^{3}H **(A)** and ^{14}C **(C)** activities were counted in plasma of WT mice (open circles) and caspase-1$^{-/-}$ mice (closed circles). At 15 min after injection, organs were isolated and uptake of ^{3}H **(B)** and ^{14}C **(D)** activity by the organs was measured. Values are means ± SD. *P<0.05.

in caspase-1$^{-/-}$ and WT mice after an overnight fast. Mice were challenged with an oral lipid load containing 5 μCi of glycerol tri[^{3}H]oleate in 200 μL olive oil, followed by assessment of the appearance of ^{3}H-activity in plasma and uptake of ^{3}H-activity by tissues (**Fig 3**). Two hours after the gavage, the appearance of ^{3}H activity in plasma of caspase-1$^{-/-}$ mice was significantly reduced (-37%; P<0.001; Fig 3A), suggesting a reduction in chylomicron-TG production. In line with this finding, a decreased amount of ^{3}H-activity was retrieved in the liver (-44%), muscle (-45%), BAT (-72%) and gWAT (-65%) of caspase-1$^{-/-}$ mice (Fig 3B). Since we observed that caspase-1 deficiency does not affect clearance of TG-rich particles *per se*, these data confirm that caspase-1 deficiency reduces the intestinal TG production after an oral lipid load, and as a consequence reduces uptake and incorporation of intestinally derived TG into peripheral lipid stores.

To determine whether the reduced intestinal TG production was secondary to a decreased FA retention by the enterocytes from the luminal tract, we isolated the intestinal tract 2 h after the gavage and determined both the amount of ^{3}H that was present in the intestinal tissue and the non-absorbed amount of ^{3}H that was still present in the lumen of the intestinal tract. Caspase-1 deficiency did not affect the presence of [^{3}H]TG-derived tissue ^{3}H-activity in the different parts of the intestinal tract (Fig 3C), suggesting that the reduction in intestinal TG production in absence of caspase-1 was not caused by a reduced postprandial intracellular TG-derived FA availability, which was confirmed by Oil red O staining (not shown). Instead, caspase-1 deficiency resulted in the accumulation of non-absorbed [^{3}H]TG in the lumen of the distal part of the intestinal tract (Fig 3D), indicating reduced TG absorption.

To investigate whether the decrease in postprandial intestinal TG production results in long-term reduction of intestinal lipid absorption, feces was collected from individually housed mice over a 4-day period and the fecal FA content was evaluated. Indeed, the feces of caspase-1$^{-/-}$ mice displayed an increased amount of C14:0, C16:0, C18:1, C18:2 and C18:3 compared to wild-type mice (Fig 3E), resulting in an increase of the total fecal FA content (85.7 ± 9.4 vs 69.4 ± 7.3 μmol/g feces; P<0.01; Fig 3F).

Caspase-1 deficiency decreases VLDL-TG production, without affecting VLDL-apoB production, despite increased hepatic lipid content.

In fasting conditions, the availability of lipids for peripheral organs is determined by the production of VLDL-TG by the liver through mechanisms similar to intestinal chylomicron synthesis. Since caspase-1 deficiency reduces postprandial chylomicron-TG production, we hypothesized that caspase-1 deficiency could similarly affect hepatic VLDL-TG production. VLDL-TG production was measured in 4 h fasted mice by determining plasma TG levels after Triton WR1339 injection (**Fig 4**). Caspase-1$^{-/-}$ mice indeed showed a strong reduction of TG accumulation in plasma compared to WT mice (Fig 4A). The VLDL-TG production rate, as determined from the slope of the curve from all individual mice, was decreased by -42% (3.6 ± 0.5 vs 6.3 ± 0.7 mM/h; P<0.001), whereas the rate of VLDL-apoB production did not change significantly (Fig 4B). As the reduced rate of VLDL-TG production could be

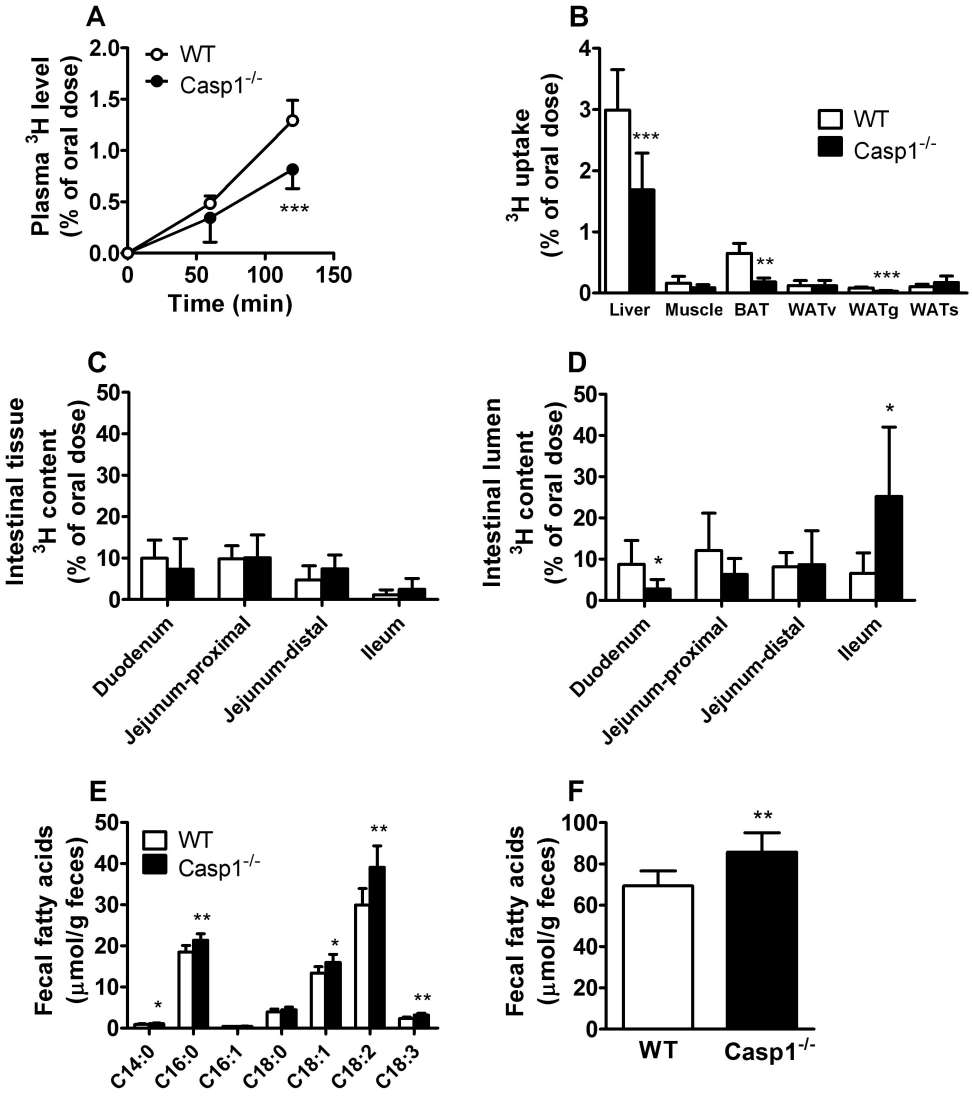

Figure 3. Caspase-1 deficiency reduces intestinal TG production. Overnight fasted WT and caspase-1$^{-/-}$ mice received an intragastric load of 200 µL olive oil containing [^{3}H]triolein ([^{3}H]TO) (n=6-7). Blood samples were drawn at the indicated timepoints and ^{3}H activity was counted in plasma of WT mice (open circles) and caspase-1$^{-/-}$ mice (closed circles) **(A)**. At 2 h after the oral lipid load, organs were collected and uptake of ^{3}H activity was measured **(B)**. Intestine was isolated and washed twice to remove intestinal luminal non-absorbed olive oil. Both the ^{3}H activity in the intestinal tissue **(C)** and the non-absorbed ^{3}H activity in the lumen **(D)** were determined. Feces was collected from individual housed WT and caspase-1$^{-/-}$ mice (n=8) and fecal fatty acids were derivatized and extracted followed by fatty acid separaton and quantification using gas chromatography. Individual fatty acids were determined **(E)** and total fatty acid content (µmol/g feces) was calculated from the sum of all individual fatty acids **(F)**. Values are means ± SD. *P<0.05 and ***P<0.001.

caused by a decreased hepatic lipid substrate availability in the liver, we evaluated the hepatic TG (Fig 4C) and TC (Fig 4D) content in livers from both fed and 4 h-fasted WT and caspase-1$^{-/-}$ mice. In the fed state, hepatic lipid content was not affected by caspase-1 deficiency. Surprisingly, 4 h of fasting strongly increased hepatic TG content in caspase-1$^{-/-}$ compared to WT mice (+64%; $P<0.05$), which was confirmed by Oil Red O staining (data not shown). These data show that caspase-1 deficiency reduces VLDL-TG production despite an increased availability of lipids. This suggests that caspase-1 deficiency reduces secretion of TG from the liver thereby causing the increased hepatic TG content.

Caspase-1 deficiency is thus associated with a reduction of both intestinal chylomicron-TG- and hepatic VLDL-TG production, suggesting a general role of caspase-1 in the assembly and/or secretion of TG-rich lipoprotein particles.

Reduced hepatic and intestinal expression of genes involved in FA metabolism and lipogenesis in caspase-1 deficient mice.

To investigate whether caspase-1 deficiency influences intestinal molecular pathways involved in postprandial lipid handling, we performed microarray analysis on postprandial intestinal samples of WT and caspase-1$^{-/-}$ mice. Comparison of expression of function gene sets revealed that caspase-1 deficiency downregulated metabolic clusters of genes involved in FA metabolism and oxidation (**Fig 5**).

The secretion of intestinal chylomicron-TG in the postprandial state and hepatic VLDL-TG in the fasted state are regulated by similar pathways.[21] We, therefore, further analyzed a selection of genes that are directly involved in the hepatic and intestinal secretion of TG (FA uptake, lipogenesis and VLDL/chylomicron secretion) in caspase-1$^{-/-}$ and WT mice by qPCR (**Table 1**). Mice deficient for caspase-1 had normal expression of hepatic- and intestinal apoB (*Apob*) and microsomal TG transfer protein (*Mttp*), both involved in the assembly and secretion of chylomicrons and VLDL. Likewise, genes involved in FA uptake and transport, apart from increasing expression of *Cd36*, were largely unaffected in these mice. Rather, caspase-1 deficiency reduced expression of the main regulator of lipogenesis, *Srebf1*, in liver (-80%; $P<0.05$) and intestine (-50%; $P<0.05$). Furthermore, caspase-1$^{-/-}$ mice showed reduced expression of other genes involved in lipogenesis in liver (*Scd1*) and intestine (*Srebf2* and *Fasn*).

DISCUSSION

Inflammasome-mediated caspase-1 activity has recently been established as an important mediator of obesity and obesity-related insulin resistance, as caspase-1$^{-/-}$ mice are protected against HFD-induced obesity and associated insulin resistance [9, 10]. We have now established a role for caspase-1, either direct or indirect, in the regulation of the production of chylomicron-TG by the intestine and VLDL-TG by the liver. Animals lacking caspase-1 showed reduced postprandial chylomicron-TG secretion as well as fasting VLDL-TG secretion, leading to a reduced availability of TG-derived

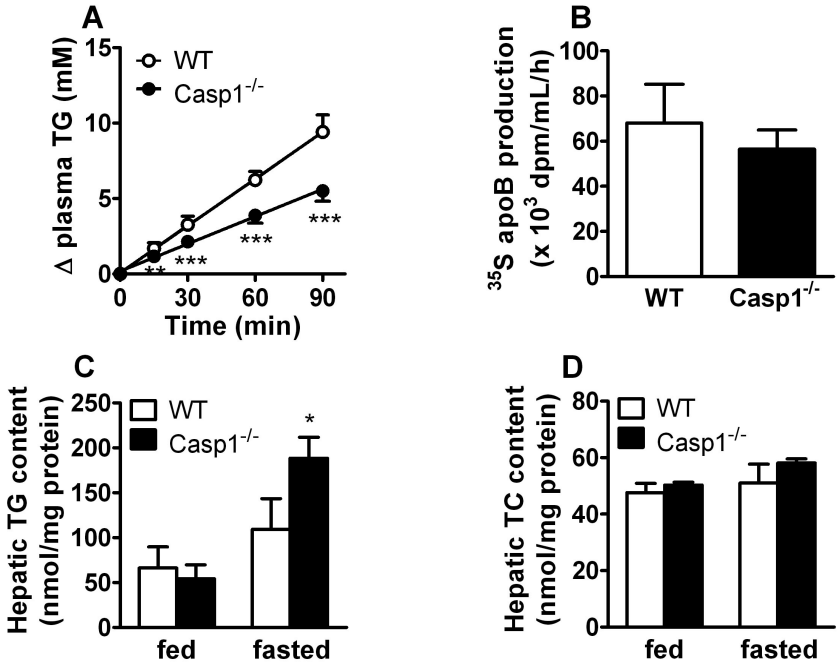

Figure 4. Caspase-1 deficiency decreases VLDL-TG production without affecting VLDL-apoB production, and increases hepatic steatosis. WT and caspase-1$^{-/-}$ mice were fasted for 4 hours and received injections of Tran^{35}S to label newly synthesized protein and Triton WR1339 to block lipolysis (n=8). Blood samples were drawn at the indicated timepoints and TG concentrations were determined in plasma of WT mice (open circles) and caspase-1-/- mice (closed circles) and plotted as the increase in plasma TG relative to t=0 **(A)**. TG production rates were determined by linear regression analysis. At 120 min, mice were exsanguinated and VLDL was isolated and assayed for ^{35}S-apoB **(B)**. Livers were obtained from fed and 4 h-fasted WT and caspase-1$^{-/-}$ mice and lipids were extracted (n=3). Triglycerides (TG) (**C**) and total cholesterol (TC) (**D**) were measured and expressed per mg protein. Values are means ± SD. *P<0.05 and ***P<0.001.

FA for uptake by peripheral organs and increased fat excretion in the feces. These data provide a mechanistic explanation for the reduced adipose tissue mass observed in caspase-1$^{-/-}$ mice upon HFD-feeding and suggest that caspase-1 represents a novel link between innate immunity and the regulation of TG-rich lipoprotein production.

Our observations demonstrate that caspase-1 deficiency does not affect the uptake of TG-derived FA by adipose tissue *per se*, by showing that adipose tissue is fully capable of taking up [^{3}H]FA derived from TG-rich particles that are administered directly into the circulation. This, together with our observation that *in vitro* adipogenesis is enhanced rather than reduced in adipocytes in the absence of caspase-1,[9] suggests that adipose tissue mass in caspase-1$^{-/-}$ mice is not reduced as a result of an impaired adipose tissue functioning by means of FA uptake and adipogenesis. Data from the current study show that caspase-1 deficiency rather reduces TG-derived FA delivery to the adipose tissue secondary to a reduction in TG-rich lipoprotein secretion.

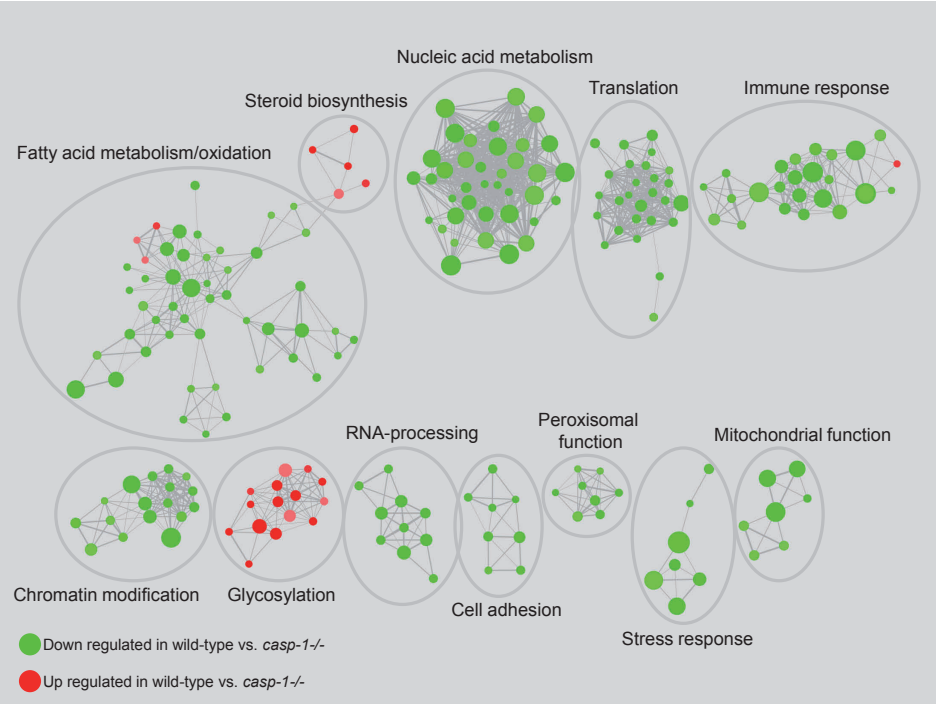

Figure 5. Caspase-1 deficiency reduces intestinal signaling pathways involved in FA metabolism. Overnight fasted WT and caspase-1$^{-/-}$ mice received an intragastric olive oil gavage. After 2 h, duodenum, jejunum and ileum were isolated and mRNA from the epithelial layer was used for micro-array analysis (n=3). Expression data of all three segments (duodenum, jejunum and ileum) were combined and averaged for each gene. Nodes represent functional gene sets, for which red indicates increased and green suppressed gene sets in caspase-1$^{-/-}$ mice. Node size represents the size of the gene set. Clusters were manually circled and labeled to highlight the prevalent biological functions among related gene sets.

It is interesting to speculate how caspase-1 deficiency reduces hepatic and intestinal TG secretion. The secretion of TG-rich lipoproteins is dependent on TG availability, which can be derived from FA supply from the diet (intestine), plasma (liver) or from *de novo* lipogenesis. The reduction in intestinal TG secretion in caspase-1$^{-/-}$ mice can probably not be explained by a reduction of FA uptake *per se* by enterocytes from the diet, since both tracer studies and Oil Red O staining of intestinal sections revealed that enterocytes of both caspase-1$^{-/-}$ and WT mice were capable of FA uptake. Also, caspase-1 deficiency did not reduce intestinal or hepatic expression of *Cd36, Slc27a4* and *Fabp*, proteins involved in FA transport and activation, implying that the intestine and liver of caspase-1$^{-/-}$ mice were able to take up FA. Actually, intestinal expression of *Cd36* was increased in caspase-1$^{-/-}$ mice, which may be a compensatory reaction to the reduced chylomicron secretion, since *Cd36* has shown to be essential for the production of chylomicrons.[22]

Table 1. qPCR analysis of caspase-1 and wild-type fasted liver and postprandial intestinal samples reveals a global reduction in lipogenesis.

		Liver		Intestine					
				Duodenum		Jejunum		Ileum	
		Relative to WT				Relative to WT			
Gene	Protein	WT	Casp-1$^{-/-}$	WT	Casp-1$^{-/-}$	WT	Casp-1$^{-/-}$	WT	Casp-1$^{-/-}$
Fatty acid uptake and transport									
Slc27a4	FATP4	1.00 ± 0.71	0.97 ± 0.76	1.00 ± 0.62	0.42 ± 0.19	1.05 ± 0.65	0.71 ± 0.31	0.31 ± 0.16	0.16 ± 0.09
Fabp1	L-FABP	1.00 ± 0.79	0.61 ± 0.54	-	-	-	-	-	-
Fabp2	I-FABP	-	-	1.00 ± 0.26	0.66 ± 0.20	0.75 ± 0.31	0.60 ± 0.43	0.01 ± 0.00	0.01 ± 0.01
Cd36	CD36	1.00 ± 0.74	0.32 ±0.28	1.00 ± 0.41	2.07 ± 1.00*	1.55 ± 0.90	3.37 ± 2.07	0.03 ± 0.06	0.06 ± 0.06
Lipogenesis									
Srebf1	SREBP1a/c	1.00 ± 0.65	0.20 ± 0.16*	1.00 ± 0.48	0.50 ± 0.16*	1.11 ± 0.74	1.46 ± 0.79	0.07 ± 0.01	0.05 ± 0.02
Srebf2	SREBP2	1.00 ± 0.98	1.13 ± 1.03	1.00 ± 0.36	0.52 ± 0.11*	0.91 ± 0.30	0.57 ± 0.24	0.92 ± 0.36	0.45 ± 0.16*
Dgat1	DGAT1	1.00 ± 0.84	1.00 ±0.73	1.00 ± 0.43	0.67 ± 0.26	1.18 ± 0.76	0.77 ± 0.33	0.25 ± 0.12	0.13 ± 0.06
Dgat2	DGAT2	1.00 ± 0.98	0.63 ± 0.46	1.00 ± 0.35	0.72 ± 0.27	0.67 ± 0.36	0.50 ± 0.53	0.06 ± 0.04	0.03 ± 0.02
Mogat1	MGAT1	N.D.	N.D.	1.00 ± 0.62	0.83 ± 0.30	1.24 ± 0.85	0.68 ± 0.28	0.28 ± 0.16	0.13 ± 0.09
Scd1	SCD1	1.00 ± 0.73	6.27 ± 4.56*	1.00 ± 0.41	1.44 ± 2.48	2.12 ± 2.31	0.71 ± 0.55	4.33 ± 7.23	12.27 ± 14.76
Fasn	FAS	1.00 ± 1.18	1.54 ± 0.76	1.00 ± 0.58	0.69 ± 0.11	0.85 ± 0.27	0.49 ± 0.20*	0.71 ± 0.17	0.75 ± 0.40
Lipoprotein assembly									
Mttp	MTP	1.00 ± 0.78	0.82 ± 0.63	1.00 ± 0.28	0.71 ± 0.14	0.92 ± 0.39	0.55 ± 0.35	0.09 ± 0.06	0.04 ± 0.02
Apob	ApoB	1.00 ± 0.42	1.12 ± 0.84	1.00 ± 0.33	1.02 ± 0.36	1.60 ± 1.00	1.81 ± 1.13	0.44 ± 0.20	0.28 ± 0.13

Livers (4 h fasted) and intestines (2 h postprandial) were isolated from WT and caspase-1 deficient mice. mRNA was isolated and mRNA expression of the indicated genes was quantified by qPCR. Data are calculated as fold change as compared with wild-type (WT) livers or WT duodenum (for intestines). Values are means ± SD (n=5-6). *P<0.05 compared with WT. *Apob,* apolipoprotein B; *Cd36*, fatty acid translocase; *Dgat,* diglyceride acyltransferase; *Fabp1,* liver type fatty acid binding protein; *Fabp2,* intestinal fatty acid binding protein; *Fasn,* fatty acid synthase; *Mogat,* monoacylglycerol O-acyltransferase 1; *Mttp,* microsomal triglyceride transfer protein; *Ppara,* peroxisome proliferator activated receptor alpha; *Ppargc1b,* PPAR-gamma coactivator 1-beta; *Scd1,* stearoyl-Coenzyme A desaturase 1; *Slc27a4,* fatty acid transport protein 4; *Srebf,* sterol-regulatory element binding protein.

It appears that caspase-1 deficiency is associated with reduced expression of the intestinal- and hepatic lipogenic genes such as *Srebf1, Srebf2* and *Fasn*, which are involved in FA synthesis and intracellular resynthesis of TG for the secretion of lipid-rich lipoproteins. However, the reduced expression of lipogenic genes in liver and intestine was not paralleled by reduced lipid content. Actually, hepatic TG secretion is lower in caspase-$1^{-/-}$ animals despite an increased TG availability in the liver, suggesting that the reduced expression of lipogenic genes that we observe is a consequence rather than cause of the reduced TG secretion. Other studies indeed observed a reduction in expression of *Srebf1* as a response to steatosis.[23] These data implicate that a reduction in lipogenesis is not the predominant mediator of the reduced TG secretion, as caspase-1 deficiency is associated with reduced intestinal TG secretion despite adequate availability of lipids. The fact that TG secretion is reduced without changes in hepatic apoB secretion or intestinal and hepatic expression of *Apob,* suggests that caspase-1 deficiency mainly affects apoB lipidation and secretion of the lipoprotein particle.

As caspase-1 processes a number of cytokines, including pro-IL-1ß and pro-IL-18 into the active form, absence of caspase-1 is associated with reduced levels of bioactive IL-1ß and IL-18. To what extent these differences are involved in the reduced production of TG-rich lipoprotein secretion in caspase-$1^{-/-}$ animals remains to be determined. Injection of pro-inflammatory IL-1ß in rats has since long been known to increase lipogenesis and hepatic secretion of TG-containing lipoproteins,[6] suggesting that absence of bioactive IL-1ß in caspase-$1^{-/-}$ mice could well be responsible for the reduction in secretion of TG-containing lipoproteins. Accordingly, a recent report revealed that IL-1ß gene polymorphisms affect the postprandial TG response in humans.[24] Subjects with the polymorphism that conveys higher IL-1ß activity displayed an increased postprandial TG response. Our data suggest that these observations may thus be explained by an increased intestinal and/or hepatic TG production, rather than by reduced LPL activity, which has been suggested as a possible mechanism for their observation.[25] An IL-1ß-mediated increase in intestinal TG secretion may also contribute to the tendency towards higher abdominal obesity previously observed in these subjects.[26] Similar to IL-1ß, genetic variation in IL-18 has been associated with postprandial TG levels,[27] suggesting that absence of caspase-1 may reduce TG-rich lipoprotein production indirectly via these cytokines. However, in addition to IL-1ß and IL-18, caspase-1 has recently been shown to be able to directly cleave additional substrates.[28, 29] Future studies will need to identify new potential cleavage sites in proteins involved in lipoprotein assembly that may contribute to the reduction in TG secretion in caspase-$1^{-/-}$ mice.

In conclusion, we show that mice deficient for caspase-1 show an attenuated intestinal chylomicron-TG production and hepatic VLDL-TG production, both of which limit the availability of FA for uptake by adipose tissue. The current study reveals a novel function for caspase-1, or caspase-1 derived substrates, in controlling intestinal and hepatic TG-rich lipoprotein assembly and secretion and thus reveals a new link between innate immunity and lipoprotein metabolism. We anticipate that

caspase-1 may be a novel therapeutic target for the treatment of obesity and obesity-related disorders such as cardiovascular disease.

ACKNOWLEDGEMENTS

The authors are grateful to A.C.M. Pronk, E.J. Pieterman, D.C.F. Salvatori and C.J.A. van der Wee-Pals for excellent technical assistance. This work was supported by an Amylin Paul Langerhans grant from the European Foundation for the Study of Diabetes, the seventh framework program of the EU-funded "LipidomicNet" (202272), and the Center of Medical Systems Biology (CMSB) and the Netherlands Consortium for Systems Biology (NCSB) established by The Netherlands Genomics Initiative/ Netherlands Organization for Scientific Research (NGI/NWO). R.S. was supported by a grant from the Dutch Diabetes Research Foundation. M.G.N. was supported by a Vici grant from the Netherlands Organization for Scientific Research. P.C.N.R. is an Established Investigator of the Netherlands Heart Foundation (grant 2009T038).

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9

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

An overload of nutrients in the diet is known to cause so called diet-induced inflammation or "metabolic inflammation", a low-grade systemic inflammation characterized by elevated levels of inflammatory cytokines and other markers of inflammation in the circulation. The development of metabolic inflammation may depend on the composition of nutrients in the diet, the overall exposure to the nutrients and the type of tissue involved. White adipose tissue (WAT) and liver appear to be largely involved in the onset and development of metabolic inflammation, although the exact contribution of these tissues and the inflammatory pathways involved are still under investigation. The studies described in this thesis evaluated the role of inflammation in WAT and liver in the development of insulin resistance, hypertriglyceridemia and atherosclerosis.

WHITE ADIPOSE TISSUE INFLAMMATION AND INSULIN RESISTANCE

Obesity induces expansion of adipose tissue that coincides with the influx of macrophages and secretion of pro-inflammatory cytokines.[1,2] The cytokines IL-1β and IL-18 are secreted by the adipose tissue upon expansion and have been linked to the development of obesity and insulin resistance, although both cytokines appear to have opposite effects. Whereas IL-1β induces insulin resistance, IL-18 protects against obesity and insulin resistance.[3-7] In **chapter 7**, we revealed that absence of their common activator, caspase-1, strongly reduces high fat diet (HFD)-induced obesity and insulin resistance. Caspase-1 becomes active as part of a multiprotein complex called 'the inflammasome'. Interestingly, absence of the other components of the inflammasome (*i.e.* NLRP3 and ASC) similarly protected against HFD-induced obesity, and absence of ASC additionally protected against HFD-induced insulin sensitivity. This suggests that the inflammasome is an important mediator in the development of obesity and insulin resistance. Our findings were confirmed by others, showing that mice deficient for NLRP3 or ASC are protected from both palmitate- and HFD-induced insulin resistance[8,9]

The NLRP3 inflammasome is the most well known inflammasome, although other inflammasomes exist that can activate ASC and caspase-1 (*i.e.* NLRP1, NLRC4 or AIM2).[10] Processes that are known to induce insulin resistance (i.e. mitochondrial dysfunction, endoplasmatic reticulum stress and ceramide production) have recently been shown to activate the NLRP3 inflammasome,[9,11,12] suggesting that the NLRP3 inflammasome may be an important mediator in the induction of insulin resistance by these processes. However, we show that NLRP3 deficiency reduced plasma markers of obesity and insulin resistance to a more mild extent compared to deficiency of the other inflammasome components ASC and caspase-1 (**chapter 7**). Additionally, another study showed that the loss of NLRP3 reduced, but not eliminated, caspase-1 function in obesity,[9] suggesting that other inflammasomes such as NLRP1, NLRC4 or AIM2 may contribute to the pathophysiology of obesity and insulin resistance, which needs to be evaluated by future research.

We and others showed that obesity in mice increases expression of components of the inflammasome in adipose tissue (**chapter 7**),[9] which correlates with body weight.[9]

In line with these data, weight loss clearly reduces expression of components of the inflammasome in adipose tissue of obese subjects.[9] The exact contribution of adipocytes and macrophages to the expression and function of components of the inflammasome is so far not fully understood. A recent study showed that, in adipose tissue of obese subjects, caspase-1 was highly expressed in adipocytes, while ASC expression mainly originated from the stromavascular fraction.[13] This dissociation suggests that caspase-1 and ASC may function separately in both cell types. In line with this, we show that deficiency of caspase-1 and ASC in mice differently affects metabolic pathways in adipose tissue (**chapter 7**). Caspase-1 deficiency reduces macrophage infiltration and ASC deficiency reduces adipocyte size; both components influence another cell type than where they are highest expressed in human adipose tissue. The underlying mechanism for this is so far unknown and may be caused by species differences. Future studies need to unravel how components of the inflammasome become activated in adipocytes and macrophages, and how they exert their metabolic effects in these, or other, cell types.

ROLE OF METABOLIC INFLAMMATION IN TG-RICH LIPOPROTEIN METABOLISM

A close interaction has been described between the immune system and lipoprotein metabolism in general and triglyceride (TG) metabolism in particular. Observations in patients with acute inflammation reveal that bacterial and viral infection is accompanied by hypertriglyceridemia.[14, 15] Most of the current knowledge on the mechanistic relation between inflammation and TG metabolism has largely been derived from studies that evaluated the effects of lipopolysaccharide (LPS), a major component of the cell wall of Gram-negative bacteria, and individual cytokines on lipoprotein metabolism.[16] In this thesis we investigated the effects of various inflammatory pathways on TG-rich lipoprotein metabolism (*i.e.* VLDL and chylomicron), in order to gain more insight into the effects of metabolic inflammation on the development of hypertriglyceridemia.

Hepatic inflammation and VLDL-TG secretion

HFD feeding and obesity not only induce inflammation in WAT, but also in liver, as reflected by increased markers of hepatic inflammation in obese subjects (SAA and CRP)[17,18] and increased hepatic NF-κB activity in mice fed a HFD.[19,20] Hepatic lipid accumulation has long been thought to induce inflammation. However, not all steatotic livers display increased inflammation,[21] suggesting that other factors may be involved. We show that the type of HFD that is consumed can strongly affect the activation of hepatic inflammation (**chapter 6**). A HFD based on palm oil (HFD-P) clearly induced hepatic NF-κB activity, while a HFD based on lard (HFD-L) did not affect hepatic NF-κB activity. In addition to FA composition of the diet, other studies show that addition of cholesterol to a HFD strongly increases hepatic inflammation[22,23] and seems more important for the induction of inflammatory pathways than hepatic TG accumulation.[22]

The liver consists of multiple cell types including Kupffer cells (the resident macrophages) and hepatocytes. It is known that inflammatory stimuli such as cytokines

or LPS induce hypertriglyceridemia by increasing hepatic VLDL-TG secretion,[24] although the role of Kupffer cells and hepatocytes in this process is not fully clear. This is because LPS associates with macrophages rather than with hepatocytes,[25] but the secretion of VLDL-TG is a metabolic process that is executed by hepatocytes in the liver, suggesting cross-talk between both cell types. In **chapter 4**, we revealed that chronic activation of the inflammatory transcription factor NF-κB specifically in the hepatocyte increased hepatic VLDL-TG production, both *in vivo* and *in vitro*, thereby inducing hypertriglyceridemia. These findings demonstrate a direct intracellular effect of NF-κB activity on the secretion VLDL-TG. In addition, this suggests that hepatocytes are the cells ultimately responsible for the metabolic disturbances that are associated with hepatic inflammation.

The relative contribution of Kupffer cells and hepatocytes to the development of HFD-induced hepatic inflammation is still under investigation. Dietary cholesterol can induce hepatic inflammation by Kupffer cell-mediated internalization of modified lipoproteins, suggesting that Kupffer cells play a major role in the cholesterol-induced hepatic inflammation[26] FAs on the other hand have been suggested to induce hepatic inflammation by acting as endogeneous ligands for Toll-like receptors (TLR) 2 and TLR4, two receptors that can activate NF-κB signaling. TLR2 and TLR4 are expressed on both Kupffer cells as well as hepatocytes,[45] suggesting that FA may activate both cell types. Indeed, addition of palmitic acid to hepatocytes and macrophages *in vitro* at equal concentrations similarly induces inflammatory signaling in both cell types.[27] It remained to be established, however, whether FA similarly induces inflammation in both cell types in a more physiological situation such as HFD feeding *in vivo*. In **chapter 6** we show that a HFD- increased total hepatic NF-κB activity, which however did not translate towards an increased VLDL-TG production. Since we showed that a similar activation of NF-κB within the hepatocyte directly increased VLDL-TG production (**chapter 4**), this implies that the increased hepatic NF-κB activity upon HFD-P feeding reflects an increased NF-κB activity in Kupffer cells rather than hepatocytes.

As summarized in Figure 1, we hypothesize that not only cholesterol derived from (ox)LDL[26] and LPS,[25] but also FA (**chapter 6**), activate inflammation, primarily in Kupffer cells. Activated Kupffer cells can subsequently secrete pro-inflammatory mediators including the cytokines TNFα and IL-1ß that increase NF-κB activity in adjacent hepatocytes,[28] which increases the secretion of VLDL-TG (**chapter 4**). Following this hypothesis, data from **chapter 6** suggest that activation of NF-κB activity in hepatocytes occurs only when inflammation within Kupffer cells reaches a certain threshold to secrete a sufficient amount of cytokines. This threshold would be consistent with the exponential rather than linear interrelationship between dietary cholesterol and hepatic inflammation.[23]

Inhibition of hepatic inflammation and VLDL-TG secretion

The causal link between hepatocyte-specific NF-κB activity and VLDL-TG production suggests that inhibition of NF-κB activity in the hepatocyte may reduce hypertriglyceridemia by reducing VLDL-TG production. Although it is not known

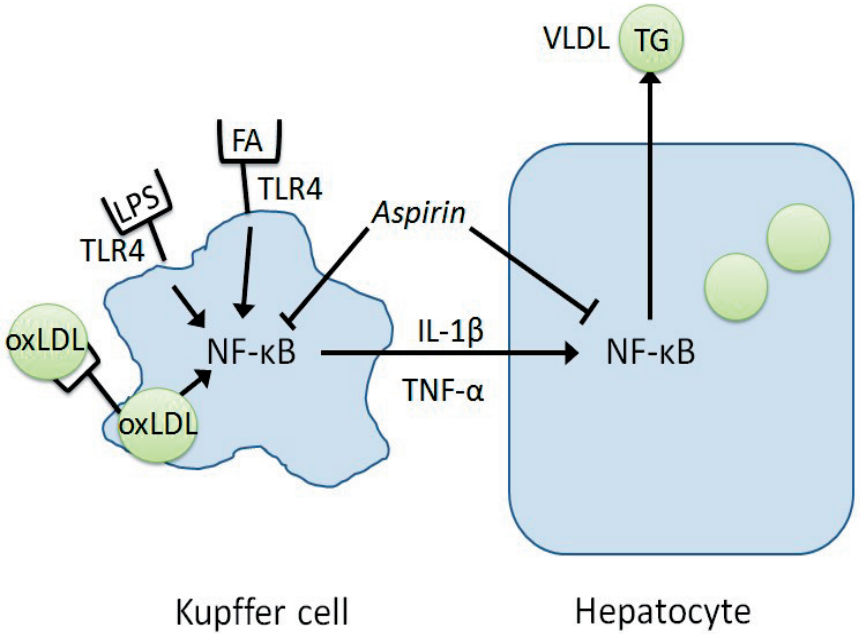

Figure 1. Schematic model showing how hepatic inflammation, originating in macrophages, may affect VLDL-TG secretion in hepatocytes. See text for explanation.

whether aspirin primary acts on Kupffer cells or hepatocytes, we show that high-dose aspirin that decreases total hepatic NF-κB activity,[19,29] also decreases hepatic VLDL-TG production in HFD-fed mice, thereby reducing hypertriglyceridemia in a hypertriglyceridemic mouse model (**chapter 3**).

High-dose aspirin may therefore be an interesting anti-inflammatory therapy to reduce hypertriglyceridemia, but aspirin dose is associated with side effects,[30] urging the need to search for additional therapies that may reduce NF-κB activity within the hepatocyte. TLR4 deficiency reduces HFD-induced hepatic NF-κB activity,[31] which would suggest that TLR4 deficiency also reduces hepatic VLDL-TG production. To our surprise, TLR4 deficiency did not reduce hepatic VLDL-TG production in HFD-fed mice (**chapter 6**). According to our hypothesis presented in Figure 1, these data would suggest that TLR4 deficiency reduced NF-κB activity in the macrophage rather than hepatocyte, therefore not affecting VLDL-TG secretion. TLR4 may indeed be predominantly present on Kupffer cells, based on the fact that LPS, the primary ligand for TLR4, associates with Kupffer cells rather than hepatocytes.[25] Even more surprising was that TLR4 deficiency increased VLDL-TG in mice fed a HFD-P, by a mechanism that is still unknown. We did not measure hepatic NF-κB activity in our study, but it may be speculated that deficiency of TLR4 induces upregulation of other TLRs involved in inflammatory signaling that may counteract any effects of TLR4 deficiency on NF-κB activity.

It must be noted that in humans, general inhibition of inflammation (*e.g.* with aspirin) is associated with a reduction in hypertriglyceridemia. On the other hand, inhibition of specific cytokines, such as TNFα or IL-1ß, does not reduce or even increases hypertriglyceridemia (discussed in **chapter 2**), implying that counter regulatory mechanisms may be involved. This suggests that general inhibition of inflammation may be more useful to reduce hypertriglyceridemia, rather than targeting a single receptor or cytokine involved in inflammation. Future research should thus further delineate the exact contribution of (combinations of) specific components of inflammatory pathways on VLDL-TG production and hypertriglyceridemia.

In **chapter 8** we identified caspase-1 as a potential new therapeutic target to reduce hypertriglyceridemia. Deficiency of caspase-1 highly reduced both VLDL-TG production as well as chylomicron-TG production, suggesting that it generally reduces assembly and secretion of TG-rich lipoproteins. Theoretically, this may reduce hypertriglyceridemia and atherosclerosis, which needs to be further evaluated in hypertriglyceridemic mouse models. Interestingly, a recent report showed that absence of the activator of caspase-1, the NLRP3 inflammasome, in bone marrow-derived cells reduces atherosclerosis development in hyperlipidemic LDLr-deficient mice.[32] This, however, occurred independently of plasma lipid levels that were unchanged. In scope of our findings, we could hypothesize that (additional) caspase-1 deficiency in hepatocytes would further reduce atherosclerosis by lowering circulation of TG-rich lipoproteins. The effect of total caspase-1 deficiency on atherosclerosis has so far only been evaluated in hyperlipidemic apoE-deficient mice, where it did not reduce atherosclerosis.[33] However, it should be realized that the apoE-deficient mouse model already has a markedly impaired VLDL-TG secretion[34] which may not be further reduced by caspase-1 deficiency. This makes the apoE-deficient mouse a less suitable model to verify whether caspase-1 deficiency reduces hypertriglyceridemia and subsequent atherosclerosis. It would be rather interesting to investigate the contribution of caspase-1 deficiency to hyperlipidemia and atherosclerosis in other hyperlipidemic mouse models such as the LDLr deficient mouse or the APOE*3 Leiden mouse model with unaltered VLDL production. Bone marrow transplantation studies using these models may reveal the exact contribution of caspase-1 in hepatocytes versus macrophages in the development and/or regression of atherosclerosis.

Hepatic inflammation and atherosclerosis

Inflammatory processes in one organ may activate and aggravate inflammatory processes in other organs via secretion of pro-inflammatory cytokines. In line with this idea, we showed that specific activation of NF-κB signaling in hepatocytes increases the macrophage content in plaques in the vasculature (**chapter 5**), implying crosstalk between inflammatory processes in liver and vasculature. It remains to be elucidated whether pro-inflammatory cytokines play a prominent role in this crosstalk. We showed that specific activation of NF-κB in hepatocytes did not increase fasted serum cytokines, although this may not reflect the actual flux of cytokines from the liver to the vasculature.

We rather observed an elevated plasma cytokine response to LPS stimulation. LPS levels are known to increase after consumption of a fat-rich meal.[35,36] Therefore, the hepatocyte-specific activation of NF-κB signaling may aggravate the postprandial circulation of cytokines that could activate vascular inflammatory processes such as macrophage infiltration. Alternatively, hepatocyte-specific activation of NF-κB may induce vascular inflammation by (temporarily) increased secretion of VLDL-TG (**chapter 4 and 5**), thereby increasing circulating levels of lipoproteins and lipoprotein remnants that are prone to modification and can activate macrophages elsewhere.

Metabolic inflammation and intestinal chylomicron production

By assembly and secretion of TG-rich chylomicrons via the lymph into the circulation, the intestine regulates transport of dietary lipids to peripheral tissues. We showed that deficiency of caspase-1, an important regulator of the innate immune response, markedly reduces intestinal chylomicron-TG secretion (**chapter 8**), suggesting an important role for inflammatory pathways in the regulation of intestinal lipid metabolism. The role of inflammation in intestinal lipid metabolism is strengthened by a recent report revealing that IL-1β gene polymorphisms affect the postprandial response in humans. Subjects with higher IL-1β activity displayed an increased postprandial TG response,[37] sustaining our observation that caspase-1 deficiency reduces intestinal TG secretion.

It remains to be established why components of the immune system such as caspase-1 would affect the secretion of intestinal chylomicron-TG. It has been shown that increased circulation of TG-rich lipoproteins can protect against bacterial infections.[38] The involvement of caspase-1 in the regulation of TG-rich lipoprotein production may therefore be beneficial for protection or even survival during infections. It is interesting to note that the intestinal tract contains an immense amount of bacteria, called the intestinal microbiota, that play an important role in the defense against invading pathogens. Intestinal epithelial cells, including enterocytes, sense microbial products that are able to activate inflammasome-mediated caspase-1[39-41] that may further initiate activation of immune cells. Our data suggest that caspase-1 activation may simultaneously increase secretion of chylomicrons to increase defense mechanisms. Interestingly, gut microbiota have lately received a lot of attention as they have been suggested to regulate energy homeostasis and lipid metabolism and modulate obesity.[42] Germ-free mice are resistant to obesity[43] and supplementation of probiotics that modulate microbiota, reduce intestinal lipid absorption in rats.[44] The mechanisms explaining the effects of (changes in) microbiota on lipid metabolism are so far unknown. Future studies may evaluate whether caspase-1 is a mediator in the relation between gut microbiota and postprandial lipid metabolism.

CONCLUDING REMARKS

Obesity and high-fat diets (HFD) that trigger obesity coincide with the presence of chronic inflammation that is causally involved in the pathology of metabolic disturbances such as hypertriglyceridemia, atherosclerosis and insulin resistance.

Therefore, reducing inflammation may provide new therapeutic strategies in the treatment of related diseases such as cardiovascular disease (CVD) and type 2 diabetes mellitus (T2D). For the development of new drugs that reduce inflammation, it is however essential to evaluate the contribution of individual inflammatory pathways to specific metabolic disturbances. In this thesis we showed that augmenting hepatocyte-specific NF-κB activity directly increases the VLDL-TG production, thereby inducing hypertriglyceridemia. This, in addition to an increased sensitivity for inflammatory stimuli, increases atherosclerosis. Inhibition of NF-κB activity in the hepatocyte (e.g. with aspirin) may therefore represent an interesting therapy to treat hypertriglyceridemia and atherosclerosis. Furthermore, we identified caspase-1 a new link between innate immunity and triglyceride metabolism. Inflammasome-mediated caspase-1 appeared involved in the development of obesity, adipose tissue inflammation and insulin resistance. Our data suggest that caspase-1 represents a novel target to treat obesity and insulin resistance.

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SUMMARY

Obesity is frequently accompanied by the development of metabolic disturbances such as insulin resistance that ultimately results in the onset of type 2 diabetes mellitus, and hyperlipidemia, an important risk factor for the development of atherosclerosis. Obesity is accompanied by low-grade systemic inflammation that is secondary to the onset and development of metabolic inflammation in peripheral tissues such as adipose tissue and liver. In fact, metabolic inflammation appears to be causally involved in the onset and development of metabolic disturbances such as insulin resistance and atherosclerosis.

Both hyperlipidemia and inflammation are well known risk factors for the development of atherosclerosis and have been shown to play a causal role in the progression of atherosclerotic plaques. In **chapter 2,** we discussed the interplay between lipid metabolism and inflammatory pathways, which may explain why lipid-lowering drugs and anti-inflammatory drugs reduce atherosclerosis beyond expectations as solely based on their primary mode of action. We therefore discussed the direct and indirect effects of lipid-lowering drugs (*i.e.* statin, fibrates, niacin, ezetimibe) on inflammatory processes, as well as the effect of inflammation-lowering drugs (*i.e.* salicylate, anti-TNF, IL-1ra) on lipid metabolism, both of which may be of additive value in the treatment of atherosclerosis.

Systemic inflammation induces an increase in plasma triglyceride (TG) levels, also called hypertriglyceridemia. Aspirin treatment lowers inflammation via inhibition of nuclear factor κB (NF-κB) activity, but also reduces hypertriglyceridemia in humans. The aim of **chapter 3** was to investigate the mechanism by which aspirin improves hypertriglyceridemia. Therefore, human *APOC1* mice, an animal model with elevated plasma TG levels, as well as normolipidemic wild-type (WT) mice were fed a high-fat diet (HFD) and treated with aspirin. Aspirin treatment reduced hepatic NF-κB activity in HFD-fed *APOC1* and WT mice and in addition, aspirin decreased plasma TG levels in hypertriglyceridemic *APOC1* mice. This TG-lowering effect could not be explained by enhanced VLDL-TG clearance, but aspirin selectively reduced hepatic production of VLDL-TG in both *APOC1* and WT mice without affecting VLDL-apoB production. Aspirin did not alter hepatic expression of genes involved in fatty acid (FA) oxidation, lipogenesis and VLDL production, but decreased the incorporation of plasma-derived FA by the liver into VLDL-TG, which was independent of hepatic expression of genes involved in FA uptake and transport. These data led us to conclude that aspirin improves hypertriglyceridemia by decreasing VLDL-TG production without affecting VLDL particle production. Our findings suggest that the inhibition of inflammatory pathways by aspirin could be an interesting target for the treatment of hypertriglyceridemia.

The liver is an organ that is involved in both inflammation and lipid metabolism. However, it is not known whether the liver plays a direct role in the interaction between inflammation and hypertriglyceridemia. Since NF-κB is a central regulator of inflammatory processes, **in chapter 4** we investigated the relation between chronically enhanced hepatic NF-κB activation and hypertriglyceridemia. To this end, we evaluated whether hepatocyte-specific overexpression of IκB kinase (IKK-β) would affect

VLDL-TG metabolism directly in hyperlipidemic APOE*3-Leiden (E3L) mice, a well-established model for human-like lipoprotein metabolism. We found that hepatocyte-specific IKK-β overexpression induced hypertriglyceridemia. With mechanistic *in vivo* studies we revealed that this that was caused by increased hepatic VLDL-TG production, rather than by reduced VLDL-TG clearance from plasma. Studies in primary hepatocytes showed that IKK-β overexpression also enhances TG secretion *in vitro*, indicating a direct relation between IKK-β activation and TG production within the hepatocyte. Hepatic lipid analysis and hepatic gene expression analysis of pathways involved in lipid metabolism suggested that the increased VLDL production was not caused by increased steatosis or decreased FA oxidation, but most likely by ChREBP-mediated upregulation of *Fas* expression. These findings implicate that specific activation of inflammatory pathways exclusively within hepatocytes induces hypertriglyceridemia. Furthermore, we identified the hepatocytic IKK-β pathway as another possible target to treat hypertriglyceridemia.

Inflammation is a causal factor in the development of atherosclerosis and, as mentioned above, the liver is a key organ involved in inflammation. The relation between hepatic inflammation and atherogenesis is however poorly understood. In **chapter 5** we investigated whether hepatocyte-specific IKK-β overexpression aggravates atherosclerosis development in E3L mice fed a Western-type diet for 24 weeks. We showed that hepatocyte-specific IKK-β overexpression increased the atherosclerotic lesion area in the aortic root and increased lesion severity. Hepatocyte-specific IKK-β overexpression did not affect basal levels of inflammatory parameters, but tended to increase plasma cytokine levels after administration of an inflammatory stimulus (lipopolysaccharide, LPS). In addition, hepatocyte-specific IKK-β overexpression transiently increased plasma cholesterol levels, confined to (V)LDL, which resulted in a mild increased cumulative plasma cholesterol exposure. Taken together, we showed that selective activation of IKK-β in hepatocytes considerably promotes atherosclerosis development which is (at least partly) explained by an increased sensitivity to proinflammatory triggers as well as transiently increased plasma cholesterol levels.

HFD feeding increases hepatic inflammation, as evidenced by increased hepatic Toll-like receptor 4 (TLR4)/NF-κB signaling. In lean animal models, administration of LPS, a well-known ligand for TLR4, as well as activation of hepatic NF-κB both directly increase VLDL-TG production, pointing towards an important link between TLR4/NF-κB signaling and hepatic VLDL-TG production. Furthermore, TLR4 deficiency has been shown to protect against HFD-induced hepatic inflammation. In **chapter 6**, we therefore aimed to investigate 1) whether FA composition of HFD based on lard (HFD-L) and palm oil (HFD-P) differentially affect hepatic NF-κB signaling and VLDL-TG production and 2) whether TLR4 deficiency reduces the hepatic VLDL-TG production in HFD-fed mice. We demonstrated that FA composition of the HFD strongly affects hepatic inflammation, whereby HFD-P, but not HFD-L, markedly increased hepatic NF-κB signaling. However, the increase in hepatic NF-κB signaling was not accompanied by an increased VLDL-TG production. Furthermore, in contrast

to our hypothesis, TLR4 deficiency did not reduce hepatic VLDL-TG production in mice fed HFD-L and even increased VLDL-TG production in mice fed HFD-P. Based on these data, we therefore concluded that 1) FA composition determines the ability of HFD to induce hepatic inflammation, 2) HFD-P-induced hepatic inflammation does not increase VLDL-TG production, and 3) TLR4 deficiency does not reduce VLDL-TG production in HFD-fed mice.

Besides hepatic inflammation, HFD feeding induces adipose tissue inflammation, characterized by macrophage infiltration and production of proinflammatory cytokines that play a causal role in the development of insulin resistance and type 2 diabetes mellitus. The proinflammatory cytokines interleukin (IL)-1β and IL-18 require cleavage by caspase-1 to become activated. This enzyme is part of an intracellular multi-protein complex called "the inflammasome" that besides caspase-1 consists of NLRP3 and adaptor protein ASC. In **chapter 7**, we aimed to investigated the role of these components of the inflammasome in HFD-induced obesity, adipose tissue inflammation and insulin resistance. We showed that mice deficient for NLRP3, ASC or caspase-1 were resistant to the development of HFD-induced obesity and had lower plasma leptin and resistin levels. Furthermore, absence of components of the inflammasome reduced hepatic TG content, adipocyte size, and macrophage infiltration in adipose tissue, as well as adipose tissue expression of monocyte chemoattractant protein (MCP)-1, a key molecule that mediates macrophage infiltration. The reduction in HFD-induced obesity and adipose tissue inflammation in mice deficient for ASC and caspase-1 was paralleled by increased insulin sensitivity. Detailed metabolic and molecular phenotyping demonstrated that the inflammasome is involved in energy expenditure and adipogenic gene expression during chronic overfeeding, although the exact mechanisms are still unclear. These findings revealed a critical function of the inflammasome in HFD-induced obesity and insulin resistance.

Since the mechanism underlying the observation that absence of caspase-1 strongly reduced HFD-induced weight gain remained unclear, we aimed in **chapter 8** to elucidate the mechanism by which caspase-1 deficiency modulates resistance to HFD-feeding by focusing on the role of caspase-1 in the regulation of TG-rich lipoprotein metabolism. To this end, we used caspase-1 deficient and wild-type control mice to determine postprandial TG kinetics, intestinal TG absorption, VLDL-TG production as well as TG clearance, all of which strongly contribute to the supply of TG for storage in adipose tissue. We showed that caspase-1 deficiency reduced the postprandial response to an oral lipid load. The tissue-specific clearance of TG-rich lipoproteins was not changed, evidenced by unaltered kinetics of i.v. administered VLDL-like emulsion particles. An oral gavage of radiolabeled TG-containing olive oil revealed that caspase-1 deficient mice had decreased intestinal chylomicron-TG production and reduced uptake of TG-derived FA in liver, muscle, and adipose tissue. Similarly, despite an elevated hepatic TG content, caspase-1 deficiency reduced the hepatic VLDL-TG production without reducing VLDL-apoB production. Pathway analysis

of microarray data revealed that caspase-1 deficiency reduces intestinal and hepatic expression of genes involved in lipogenesis. From these data we concluded that the resistance to HFD-induced obesity in caspase-1 deficient mice is caused by a hampered assembly and secretion of TG-rich lipoproteins, which decreases the availability of TG-derived FA for uptake by peripheral organs including adipose tissue. These studies revealed that that caspase-1 represents a novel link between innate immunity and the regulation of TG-rich lipoprotein metabolism. Based on **chapter 7** and **chapter 8**, we thus anticipated that inhibition of the inflammasome could be a potential therapeutic strategy in the treatment of obesity and insulin resistance.

Taken together, the studies described in this thesis show that inflammation plays an important role in TG-rich lipoprotein metabolism both in the intestine, liver and adipose tissue. We show that inhibition of inflammation by aspirin reduces the hepatic VLDL-TG production, while hepatocyte-specific NF-κB activation enhances the hepatic VLDL-TG production. Our studies reveal a central role of the liver in the inflammation-induced hypertriglyceridemia and atherosclerosis. In addition, we identified caspase-1 as a novel link between the innate immune system and TG-rich lipoprotein assembly and secretion and as a new therapeutic target in the treatment of obesity and insulin resistance.

NEDERLANDSE SAMENVATTING VOOR NIET-INGEWIJDEN

De belangrijkste vetten in ons dieet zijn **triglyceriden** (TG) en cholesterol, beide essentieel voor het menselijk lichaam. Omdat vetten slecht oplosbaar zijn in bloed, worden ze in het lichaam getransporteerd in zogenaamde **lipoproteïnen.** Deze deeltjes lossen wel op in bloed en brengen de vetten van en naar verschillende organen. Chylomicronen zijn lipoproteïnen die vetten na een maaltijd vanuit de darm naar overige organen zoals vet, spier, hart en lever transporteren. Als men een tijdje niet heeft gegeten zorgt de lever voor voldoende beschikbaarheid van vetten door productie van zogenaamde zeer lage dichtheids lipoproteïnen (**VLDL**). Zowel chylomicronen als VLDL bevatten voornamelijk TG en daarom noemen we deze deeltjes ook wel **TG-rijke lipoproteïnen**. In het bloed worden de TG vanuit het VLDL en chylomicronen na lipolyse opgenomen in diverse organen zoals wit vetweefsel (voor opslag), spier en hart (voor leveren van energie) of bruin vetweefsel (voor warmteproductie). Als de energie-inname het energieverbruik overstijgt (d.w.z. een positieve energiebalans), worden de TG opgeslagen in het vetweefsel. Op lange termijn zal een postitieve energiebalans daarom leiden tot overgewicht en obesitas.

Obesitas gaat vaak gepaard met de ontwikkeling van metabole stoornissen. Een voorbeeld hiervan is **insulineresistentie** dat uiteindelijk leidt tot het ontstaan van type 2 diabetes mellitus. Een ander voorbeeld is **hyperlipidemie**, een belangrijke risicofactor voor de ontwikkeling van **atherosclerose** (aderverkalking). Obesitas gaat ook gepaard met een milde systemische ontsteking in het lichaam. Deze ontsteking wordt veroorzaakt door de ontwikkeling van **metabole ontsteking** in perifere organen zoals vetweefsel en de lever. Metabole ontsteking lijkt bovendien causaal gerelateerd te zijn aan de ontwikkeling van insulineresistentie en atherosclerose.

Zowel hyperlipidemie als ontsteking zijn bekende risicofactoren voor de ontwikkeling van atherosclerose en beide spelen een causale rol bij de initiatie en progressie van atherosclerotische lesies. In **hoofdstuk 2** beschrijven we de interactie tussen vetmetabolisme en ontstekingsprocessen. Deze interactie kan mogelijkerwijs verklaren waarom zowel lipidenverlagende als anti-inflammatoire geneesmiddelen de mate van atherosclerose meer verminderen dan verwacht op basis van hun primaire werkingsmechanisme. We bespreken daarom de directe en indirecte effecten van lipidenverlagende middelen waaronder statines, fibraten, niacine en ezetimibe op ontstekingsprocessen, alsook de effecten van ontstekingsverlagende middelen waaronder salicylaat, anti-TNF en IL-1ra op het lipidenmetabolisme.

Systemische ontsteking veroorzaakt een stijging van het plasma TG niveau, wat ook wel **hypertriglyceridemie** wordt genoemd. Behandeling met **aspirine** verlaagt ontsteking via remming van de nucleaire factor kB (**NF-κB**) activiteit, en vermindert ook hypertriglyceridemie bij mensen. In **hoofdstuk 3** wilden we het mechanisme onderzoeken waardoor aspirine hypertriglyceridemie verlaagt. Hiervoor gebruikten we ***APOC1* muizen**, een diermodel met verhoogde plasma TG concentraties, evenals controle muizen met normale plasma TG concentraties. Deze muismodellen voerden we een vetrijk dieet, met of zonder toegevoegd aspirine in het drinkwater. Behandeling met aspirine verminderde zoals verwacht de NF-κB activiteit in de lever. Bovendien

zorgde aspirine voor een daling in het plasma TG niveau in de hypertriglyceridemische *APOC1* muizen. Een daling in plasma TG kan worden veroorzaakt door een verhoogde **klaring** en/of verlaagde **productie** van VLDL-TG. Mechanistische studies toonden aan dat aspirine selectief de productie van VLDL-TG door de lever verlaagde in zowel *APOC1* als controle muizen, terwijl de productie van VLDL-apoB, het belangrijkste eiwit van VLDL, niet veranderde. Dit geeft aan dat de productie van het aantal VLDL deeltjes hetzelfde was, maar dat elk VLDL deeltje minder TG bevatte. Vervolgens hebben we geanalyseerd op welke manier aspirine de productie van VLDL-TG verlaagde. Onze bevindingen lieten zien dat aspirine geen invloed had op expressie van genen in de lever die betrokken zijn bij vetzuuroxidatie, lipogenese en VLDL productie, maar zorgde voor een daling in de omzetting van vetzuren vanuit het plasma in VLDL-TG door de lever. Dit was onafhankelijk van hepatische expressie van genen betrokken bij FA opname en transport. Samengevat concludeerden wij dat aspirine hypertriglyceridemie verbetert door het verlagen van de productie van VLDL-TG. Onze bevindingen suggereren dat de remming van ontsteking door aspirine een interessante therapie kan zijn voor de behandeling van hypertriglyceridemie.

De lever is een orgaan dat betrokken is bij zowel ontsteking als vetmetabolisme. Het is echter niet bekend of de lever een directe rol speelt in de interactie tussen ontsteking en hypertriglyceridemie. Aangezien NF-κB een centrale rol speelt in inflammatoire processen, hebben we in **hoofdstuk 4** gekeken naar het verband tussen chronisch verhoogde NF-κB activatie in the lever en hypertriglyceridemie. Daarvoor bestudeerden we of levercel (**hepatocyt**)-specifieke overexpressie van IκB kinase (**IKK-β**), dat leidt tot verhoogde NF-κB activatie, het metabolisme van VLDL-TG rechtstreeks beïnvloedt in hyperlipidemische APOE*3-Leiden (**E3L**) **muizen**. De E3L muis is een muismodel dat bekend staat om zijn menselijke lipoproteïnenmetabolisme. We observeerden dat de hepatocyt-specifieke IKK-β overexpressie leidde tot hypertriglyceridemie. Met *in vivo* studies hebben we aangetoond dat dit werd veroorzaakt door een toegenomen VLDL-TG productie door de lever, en niet door een verminderde VLDL-TG klaring uit het plasma. Studies in geïsoleerde primaire hepatocyten toonden aan dat IKK-β overexpressie ook *in vitro* de secretie van TG verhoogde, wat wijst op een directe relatie tussen de IKK-β activatie en TG productie binnen de hepatocyt. Analyse van leverlipiden en expressie van genen die betrokken zijn bij het vetmetabolisme in de lever suggereerden dat de toegenomen VLDL productie niet werd veroorzaakt door een verhoogde vetstapeling of afgenomen FA oxidatie. We vonden echter een verhoging van de ChREBP-gemedieerde inductie van *Fas* expressie. Aangezien *Fas* codeert voor het eiwit vetzuursynthase dat een belangrijke rol speelt bij de aanmaak van nieuwe vetzuren, zou een verhoging van *Fas* expressie het aanbod van vetten in de hepatocyt voor de VLDL-TG productie mogelijk kunnen verhogen. Onze bevindingen impliceren dat specifieke activatie van inflammatoire pathways in de hepatocyt leidt tot hypertriglyceridemie. Verder zou IKK-β in de hepatocyt een mogelijk doelwit kunnen zijn voor de behandeling van hypertriglyceridemie.

Ontsteking is een causale factor in de ontwikkeling van atherosclerose (**atherogenese**) en de lever is dus een belangrijk orgaan betrokken bij ontstekingsprocessen. De relatie tussen leverontsteking en atherogenese is echter nog onduidelijk. In **hoofdstuk 5** hebben we onderzocht of hepatocytspecifieke IKK-β overexpressie de ontwikkeling van atherosclerose verergert in E3L muizen die een vetrijk westers dieet kregen gedurende 24 weken. We toonden aan dat hepatocytspecifieke IKK-β overexpressie leidde tot grotere en ernstigere **atherosclerotische lesies** in de aorta. We onderzochten vervolgens waarom de leverontsteking leidde tot meer atherosclerose ontwikkeling. Hepatocytspecifieke IKK-β overexpressie had geen invloed op basale plasmaniveaus van inflammatoire parameters, maar vertoonde een tendens tot verhoogde plasma **cytokine** concentraties na een inflammatoire stimulus (lipopolysaccharide, LPS). Daarnaast verhoogde hepatocytspecifieke IKK-β overexpressie tijdelijk het plasma **cholesterol** niveau, beperkt tot (V)LDL, wat resulteerde in een licht toegenomen cumulatieve blootstelling aan plasma cholesterol. Samenvattend hebben we hebben aangetoond dat selectieve activatie van IKK-β in hepatocyten atherosclerose ontwikkeling aanzienlijk bevordert, wat tenminste deels verklaard kan worden door zowel een verhoogde gevoeligheid voor pro-inflammatoire stimuli als een tijdelijk verhoogd plasma cholesterolniveau.

Een vetrijke voeding (hoog-vet dieet; **HFD**) verhoogt leverontsteking, zoals blijkt uit toegenomen hepatische Toll-like receptor 4 (**TLR4**)/NF-κB signalering. Het is bekend dat activatie van TLR4 door toediening van LPS, een bekend ligand voor TLR4, evenals activatie van NF-κB in de lever, beide direct de VLDL-TG productie verhogen. Dit wijst op een belangrijke schakel tussen TLR4/NF-κB signalering en VLDL-TG productie door de lever. Verder is aangetoond dat deficiëntie van TLR4 beschermt tegen HFD-geïnduceerde lever ontsteking. In **hoofdstuk 6** hebben we ons daarom gericht op het onderzoeken of 1) de FA samenstelling van HFD gebaseerd op **reuzel** (HFD-L) en **palmolie** (HFD-P) verschillend van invloed zijn op de lever NF-κB activatie en VLDL-TG productie en 2) TLR4 deficiëntie leidt tot een verlaging van de VLDL-TG productie in HFD-gevoede muizen. Onze resultaten lieten zien dat de samenstelling van het HFD sterk van invloed is op de leverontsteking, waarbij HFD-P, maar niet HFD-L, de NF-κB activiteit in de lever sterk verhoogde. Echter, de toename van de NF-κB signalering in de lever door HFD-L ging niet gepaard met een verhoogde VLDL-TG productie. In tegenstelling tot onze hypothese, verlaagde TLR4 deficiëntie bovendien de VLDL-TG productie in HFD-L gevoede muizen niet en verhoogde TLR4 deficiëntie zelfs de VLDL-TG productie in HFD-P gevoede muizen. Op basis van deze gegevens hebben we dan ook geconcludeerd dat 1) de samenstelling van een HFD van invloed is op het ontstaan van leverontsteking, 2) HFD-P-geïnduceerde lever ontsteking niet leidt tot een verhoogde VLDL-TG productie, en 3) TLR4 deficientie niet leidt tot een verlaging van de VLDL-TG productie in HFD-gevoede muizen.

Naast leverontsteking induceert vetrijke voeding ook ontsteking in het **vetweefsel**. Deze ontsteking wordt gekenmerkt door infiltratie van macrofagen in het vetweefsel

en de productie van pro-inflammatoire cytokinen, waarvan is aangetoond dat deze een causale rol spelen in de ontwikkeling van insuline resistentie en type 2 diabetes mellitus. De pro-inflammatoire cytokinen interleukine **(IL)-1β** en **IL-18** hebben worden geactiveerd door **caspase-1**. Dit enzym maakt deel uit van een groot eiwitcomplex, het "**inflammasoom**", dat naast caspase-1 bestaat uit **NLRP3** en **ASC**. In **hoofdstuk 7** hebben we onderzoek gedaan naar de rol van deze componenten van het inflammasoom bij HFD-geïnduceerde obesitas, vetweefselontsteking en insulineresistentie. We zagen dat muizen met een deficiëntie voor NLRP3, ASC of caspase-1 alle beschermd waren tegen de ontwikkeling van HFD-geïnduceerde obesitas. Bovendien bleek dat de afwezigheid van componenten van het inflammasoom leidde tot minder vetstapeling in de lever, kleinere vetcellen en minder macrofaaginfiltratie in het vetweefsel. Daarnaast zagen we een lagere expressie van het monocyte chemoattractant proteïne (MCP)-1 in het vetweefsel, een eiwit dat een belangrijke rol speelt bij de macrofaaginfiltratie. De verminderde obesitas en ontsteking van het vetweefsel in muizen met een deficiëntie voor ASC en caspase-1 ging gepaard met verhoogde gevoeligheid voor insuline. Een gedetailleerde analyse van metabole en moleculaire processen toonde aan dat het inflammasoom betrokken is bij het energieverbruik evenals expressie van genen betrokken bij adipogenese tijdens chronische overvoeding, hoewel de exacte mechanismen nog onduidelijk zijn. Deze bevindingen onthulden een cruciale rol van het inflammasoom in HFD-geïnduceerde obesitas en insulineresistentie.

Omdat onduidelijk was op welke manier caspase-1 deficiëntie beschermde tegen HFD-geïnduceerde obesitas, hebben we in **hoofdstuk 8** uitgezocht wat het mechanisme is dat hieraan ten grondslag ligt, door de rol van caspase-1 in de regulatie van het TG-rijke lipoproteïnenmetabolisme te bestuderen. Hiervoor gebruikten we caspase-1 deficiënte muizen en controle muizen en bestudeerden we processen die allemaal sterk bijdragen aan de levering van TG voor opslag in het vetweefsel, te weten postprandiale TG kinetiek, intestinale TG absorptie, VLDL-TG productie en TG klaring. Onze resultaten lieten zien dat caspase-1-deficiëntie leidde tot een sterk verminderde **postprandiale TG respons** na een orale bolus van olijfolie. Een verminderde postprandiale respons kan veroorzaakt worden door verminderde **TG absorptie** vanuit de darm of door een toename van TG klaring uit het plasma. We lieten echter zien dat de weefselspecifieke klaring van TG-rijke lipoproteïnen niet veranderd was, zoals bleek uit de ongewijzigde kinetiek van VLDL-achtige emulsiedeeltjes die direct in het bloed waren toegediend. Een oraal toegediende bolus van olijfolie met radioactief gemerkt TG toonde aan dat caspase-1 deficiënte muizen een verminderde productie hadden van chylomicron-TG vanuit de darm wat leidde tot een verminderde opname van vetzuren afkomstig van TG in de lever, de spier en vetweefsel. Op dezelfde manier bleek ook dat, ondanks een verhoogde TG voorraad in de lever, caspase-1-deficiëntie leidde tot een verminderde productie van VLDL-TG. We lieten verder zien dat caspase-1-deficiëntie de expressie van genen die betrokken zijn bij de vetsynthese vermindert in de darm en in de lever. Op basis van onze studies concludeerden we dat de bescherming tegen HFD-geïnduceerde obesitas in caspase-1 deficiënte muizen

wordt veroorzaakt door een verminderde secretie van TG-rijke lipoproteïnen (zowel chylomicronen als VLDL), wat leidt tot een verminderde beschikbaarheid van TG voor opname door perifere organen, inclusief het vetweefsel. Deze studies laten zien dat caspase-1 een nieuwe link is tussen ontstekingsprocessen en de regulatie van het TG-rijke lipoproteïnenmetabolisme. We voorzien dat op basis van hoofdstuk 7 en hoofdstuk 8 de remming van het inflammasoom, waaronder caspase-1, een nieuwe therapeutische mogelijkheid zou kunnen zijn voor de behandeling van obesitas en insulineresistentie.

Samengevat laten de studies in dit proefschrift zien dat ontsteking een belangrijke rol speelt in het metabolisme van TG-rijke lipoproteïnen, in zowel de darm, de lever en het vetweefsel. We tonen aan dat remming van ontsteking door aspirine de productie van VLDL-TG in de lever vermindert, terwijl hepatocytspecifieke NF-κB activatie de productie van VLDL-TG in de lever verhoogt. Onze studies onthullen een centrale rol van de lever in ontstekingsafhankelijke hypertriglyceridemie en atherosclerose. Daarnaast identificeerden we caspase-1 als een nieuwe schakel tussen ontstekingsprocessen en de productie van TG-rijke lipoproteïnen, en als een nieuw doelwit voor de behandeling van obesitas en insulineresistentie.

DANKWOORD

Het doen van onderzoek en schrijven van een proefschrift is in veel opzichten te vergelijken met de beklimming van een flinke Alpen-col op de fiets; er is enige training vereist, maar daarna begin je met veel enthousiasme. Als snel blijken sommige stukken steiler dan je verwacht en moet je extra aanzetten, op andere momenten kun je even herstellen in een ruimte bocht en lijkt het zelfs bijna vanzelf te gaan. Maar het aller belangrijkste; samen is het veel makkelijker en leuker dan alleen. Na ruim vier jaar wil ik mijn proefschrift daarom graag afsluiten met een woord van dank aan kopmannen, ploeggenoten en supporters, zonder wie dit proefschrift niet op deze manier tot stand had kunnen komen.

Dr. Voshol, beste Peter, je hebt me aan het begin van mijn promotietraject een vlotte start gegeven en me veel vaardigheden bijgebracht op het (proefdier)lab. Bedankt voor alle leuke discussies, gesprekken en het vertrouwen dat je me gaf om mijn onderzoek vorm te geven en samenwerkingen aan te gaan. Prof. Rensen, beste Patrick, wat heb ik ontzettend veel van je mogen leren tijdens de laatste twee jaar van mijn promotieonderzoek! Ik bewonder je enorme efficiëntie en precisie en heb genoten van je aanstekelijke enthousiasme voor het onderzoek. Prof. Havekes, beste Louis, het faciliteren van een jaarlijks weekend naar Frankrijk en het belang dat je hecht aan koffiepauzes zijn typerend voor je aandacht voor teambuilding; het heeft de beklimming van mijn col een stuk aangenamer gemaakt, veel dank daarvoor. Prof. Romijn, beste Hans, mijn dank gaat uit naar je positieve stimulerende input en alle constructieve commentaren op mijn manuscripten.

Mijn directe collega's wil ik bedanken voor de samenwerking, maar vooral voor de ontzettend leuke tijd die ik heb gehad tijdens mijn promotieonderzoek. Irene, wat hebben we veel uren doorgebracht op het LGP. Ik denk ook met veel plezier terug aan onze clamp-activiteiten en werkbezoeken in Dundee, Graz, Leuven en Seattle. We kunnen samen ontzettend hard werken en lachen, ik ben blij dat je mijn paranimf wilt zijn! Verder bedank ik Man-Chi, voor de prettige samenwerking aan ons LIKK-project. Jimmy, voor je hulp tijdens mijn opstartfase en je waardevolle bijdrage aan hoofdstuk 2. Sjoerd en Amanda, voor onmisbare hulp bij veel van mijn experimenten op het LGP. Mieke, Janine, Hanna, Nancy en Edwin voor het verstoren van de rust op C5, jullie waren geweldige 'kantoortuin-genoten'. Ook alle andere collega's van de lipidengroep wil ik in het bijzonder bedanken: Claudia, Mariëtte, Illiana, Jitske, Willeke, Silvia, Marieke, Lihui, Ko, Vanessa, Mattijs, Emma, Lianne, Jan, Sam, alsmede TNO-collega's José, Anita, Susan en Wen, voor alle input tijdens werkbesprekingen en koffiepauzes. Alle andere collega's van het Endo-lab: Christel, Eric, Geertje, Guido, Henny, Henry, Hermine, Hetty, Ineke, Isabel, Ivo, Janny, Jeroen, Judith, Karien, Laura, Lianne, Maggy, Maj, Martiene, Rasvan, Thomas, Trea en Vicky voor alle hulp en gezelligheid op het lab. In het bijzonder Chris, voor je rust en je hulp, niet alleen bij de eerste 10 km die ik ooit heb gerend. Ik bewonder de manier waarop jij met een diverse groep mensen om kan gaan.

Ook buiten de Endocrinologie zijn er een aantal mensen die een bijdrage hebben geleverd aan dit proefschrift. Bruno, thank you for the western blots that you performed for my studies and the critical revision of my manuscripts. Jasper en Adriaan hebben als stagiaires een bijdrage geleverd aan dit proefschrift. Ik hoop dat jullie hier veel van hebben opgestoken, ik heb in ieder geval veel van jullie geleerd. Ben en Fred wil ik bedanken voor de liefdevolle verzorging van alle kleine deelnemers aan mijn experimenten, de goede sfeer op het LGP en later op de E0.

Rinke, de laatste twee hoofdstukken in dit proefschrift zijn het resultaat van een open en constructieve samenwerking met jou. Nieuwe resultaten leidden steevast tot meer vragen dan antwoorden. Het is leuk en interessant om met je te brainstormen over nieuwe hypotheses en experimenten. Ik hoop dat het in de nabije toekomst tot nog meer mooie projecten en artikelen zal leiden, samen met onze 'vette' groep in Nijmegen.

Niet alleen op het werk, maar zeker ook daarbuiten zijn verschillende mensen een grote energiebron voor me geweest tijdens mijn promotie-traject. Het is jammer dat we niet meer op een kluitje in Maastricht wonen, maar ik ben blij dat ik de meeste van jullie ondanks dat nog vaak kan zien. Lauw, Mijn, Simone, Annie, Merel, Annet en Monique, jullie zijn nu eenmaal ontzettend gaaf! En Sanne, Inge, Anne, Elze, Anouk, Margje, Dor, Emile, Yvonne en iedereen die ik hier niet kan noemen, dankjewel voor alle mooie en leuke momenten van de afgelopen jaren.

Mijn schoonfamilie, Piet, Carla, Angela, Christian en Patrick, bedankt voor alle gezelligheid. Ik voel me heel erg thuis bij jullie.

Lieve Maartje, Hester, Arjen en ook Jasper en Erik, bedankt voor de vele ontspannende uren beachvolleyballen en fietsen, de beste manier om mijn hoofd leeg te maken. Bovenal zijn jullie degenen die me het langste en beste kennen en met wie ik nooit raak uitgepraat, een bijzonder vertrouwd gevoel. Hes, het is ontzettend leuk om mijn zusje als (bijna) collega te hebben, ik ga het missen om je zo vaak te kunnen zien! Fijn dat je me als paranimf helpt met de laatste loodjes.

Lieve mems, peps, wat is het toch altijd fijn om in Wageningen te zijn! Jullie staan altijd voor ons klaar en zijn ontzettend betrokken. Bedankt voor de onvoorwaardelijke steun bij al mijn keuzes.

Lieve Ed, van potentieel proefpersoon uitgegroeid tot mijn allergrootste maat! Met jou kan ik praten, lachen, genieten en me verwonderen zoals ik dat met niemand anders kan. Ver weg of dichtbij, je maakt me blij, gewoon door te zijn wie je bent.

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CURRICULUM VITAE

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Janna Alida van Diepen werd geboren op 31 januari 1983 te Wageningen. Na het behalen van haar Gymnasium diploma aan Regionale Scholengemeenschap Pantarijn te Wageningen in 2001, startte zij datzelfde jaar met de studie Gezondheidswetenschappen aan de Universiteit van Maastricht. In 2002 behaalde zij haar propedeuse, waarna zij zich voor haar doctoraal specialiseerde in de richting Biologische Gezondheidskunde. Hiervoor heeft zij zes maanden gestudeerd aan de Faculty of Sport Sciences aan de University of Aberdeen (Schotland, UK). Haar afstudeeronderzoek voerde zij uit bij de vakgroep Humane Biologie van de Universiteit Maastricht, onder begeleiding van Dr. M. Mensink en Dr. P. Schrauwen. Hiervoor bestudeerde ze de relatie tussen oxidatieve capaciteit en insuline gevoeligheid in patiënten met type 2 diabetes. In augustus 2005 behaalde zij haar Master diploma, waarna ze een periode heeft gewerkt als vrijwilliger bij Safe Passage (Guatemala) en een aantal maanden als onderzoeksassistent bij de Nutrition, Metabolism en Genomics group van Wageningen University onder leiding van Dr. S. Kersten. In juni 2007 startte zij als promovenda met haar promotieonderzoek bij de afdeling Endocrinologie van het LUMC, onder supervisie van Prof. Dr. L.M. Havekes, Prof. Dr. J.A. Romijn, Prof. Dr. P.C.N. Rensen en Dr. P.J. Voshol. Voor presentaties over haar onderzoek won zij de Amsterdam Young Scientist Award 2009 van de European Conference on Obesity, en de Presentation Award 2010 van de 2nd International Symposium on Chylomicrons in Disease. Het promotieonderzoek, waarvan de resultaten zijn beschreven in dit proefschrift, werd afgerond in september 2011. Janna werkt momenteel als postdoc onderzoeker op de afdeling Algemene Interne Geneeskunde van het Radboud Universitair Medisch Centrum Nijmegen.