Novel pharmaeutical interventions in experimental atherosclerosis and myocardial infarction

Hoorn, Johanna Wijnanda Anthonia van der

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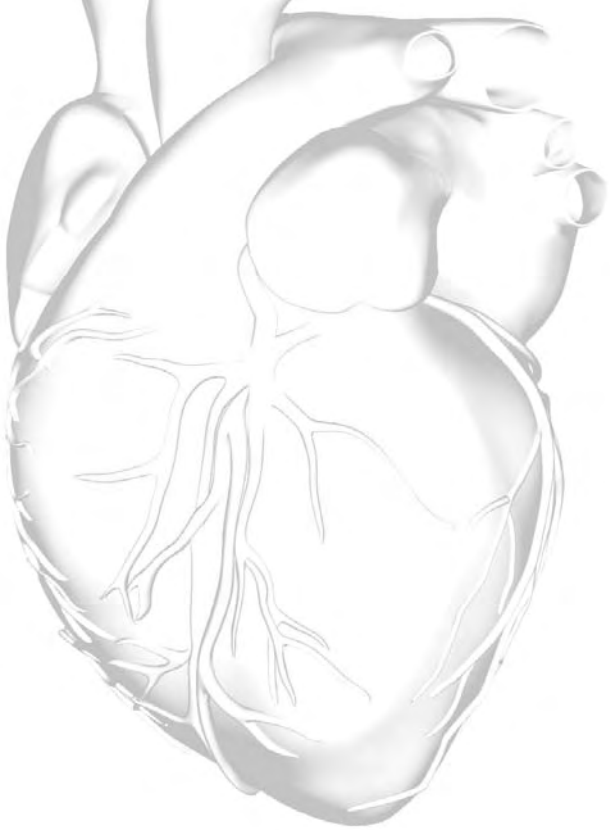

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ISBN
978-90-9023483-0

Cover illustration
© iStockphoto/Angelhell

Printed by
Drukkerij Haveka, Alblasserdam

The printing of this thesis was kindly supported by:

AB diets	Pfizer BV
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Merck Sharp and Dohme BV	Vakgarage Leo van der Hoorn

Novel Pharmaceutical Interventions in Experimental Atherosclerosis and Myocardial Infarction

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 30 oktober 2008

klokke 16.15 uur

door

Johanna Wijnanda Anthonia van der Hoorn

geboren te Woubrugge

in 1981

Promotiecommissie

Promotores:	Prof. Dr. J.W. Jukema Prof. Dr. Ir. L.M. Havekes
Co-Promotor:	Dr. H.M.G. Princen
Referent:	Prof. Dr. E.A.L. Biessen (Universiteit Maastricht)
Overige leden:	Prof. Dr. A. van der Laarse Prof. Dr. G. Pasterkamp (Universiteit Utrecht) Prof. Dr. P.H.A. Quax Dr. P.C.N. Rensen

The studies presented in this thesis were performed at the Gaubius Laboratory of TNO Quality of Life, BioSciences, Leiden, The Netherlands. The work was in part financially supported by AstraZeneca, Sankyo-Daiichi, Sanofi-Aventis and Servier.

Financial support by the Netherlands Heart Foundation and the J.E. Jurriaanse Stichting for the publication of this thesis is gratefully acknowledged.

"De moed erin houden en alles van de positieve kant bekijken"

Oma Van der Hoorn (1912-2004)

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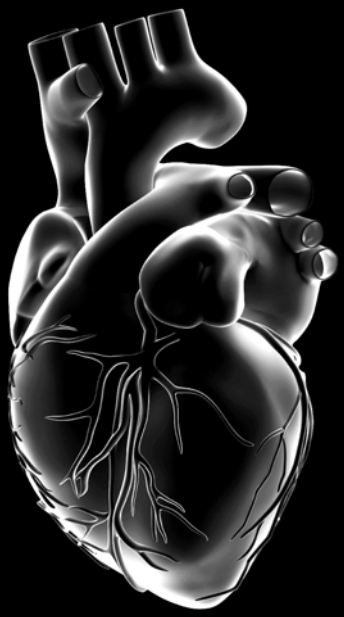

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General Introduction

Cardiovascular disease is the number one cause of death globally and is projected to remain the leading cause of death in the future. Estimates of the World Health Organization[1] show that 17.5 million people died from cardiovascular disease (CVD) in 2005, which represented 30% of all global deaths. It was estimated that if appropriate action is not taken, by 2015, 20 million people will die from CVD every year, mainly from myocardial infarction (MI) and stroke. The extensive frequency of CVD in the industrialized countries has been observed for decades. Therefore, in 1976 CVD risk equations were developed by the investigators of the Framingham Heart Study (FHS)[2], enabling clinicians to predict the development of coronary disease in individuals free of disease. The FHS is a longitudinal study, which started follow-up of healthy residents of Framingham (Massachusetts, USA) in 1948 and has included subsequent generations ever since. In a 12-year follow-up of a defined cohort of the FHS, the Framingham risk score was developed. It provides a 10-year hazard ratio for CVD based on sex, age, low density lipoprotein-cholesterol (LDL-C), high density lipoprotein (HDL)-C, blood pressure, diabetes and smoking habits[3,4].

The European Society of Cardiology initiated the development of a European risk score system (SCORE) and used data from 12 European cohort studies (n=205,178) covering a wide geographic spread of countries at different levels of cardiovascular risks[5]. The SCORE was even calibrated for the different countries; the one for The Netherlands is presented in **figure 1**. These new SCORE risk estimates of cardiovascular death are based on the same factors as the Framingham risk score with exception of diabetes. With a relative risk of approximately five in women and three in men, the impact of diabetes on CVD appeared to be much greater in these European studies. Therefore it was not included in the SCORE estimate but identified as an independent risk factor.

The single most important contributor to the growing burden of CVD is atherosclerosis, a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. The pathophysiology of this key problem has been studied extensively in the past century. Nowadays we consider atherosclerosis as a multifactorial disease in which lipids and inflammation play major roles[6,7]. The approach to primary prevention of atherosclerosis and CVD is founded on the public health approach that calls for lifestyle changes, including (I) reduced intakes of saturated fat and cholesterol, (II) increased physical activity, and (III) weight control. The clinical approach emphasizes preventive strategies for higher-risk persons. The major risk factors for CVD development and the general therapeutical options will be outlined in this chapter.

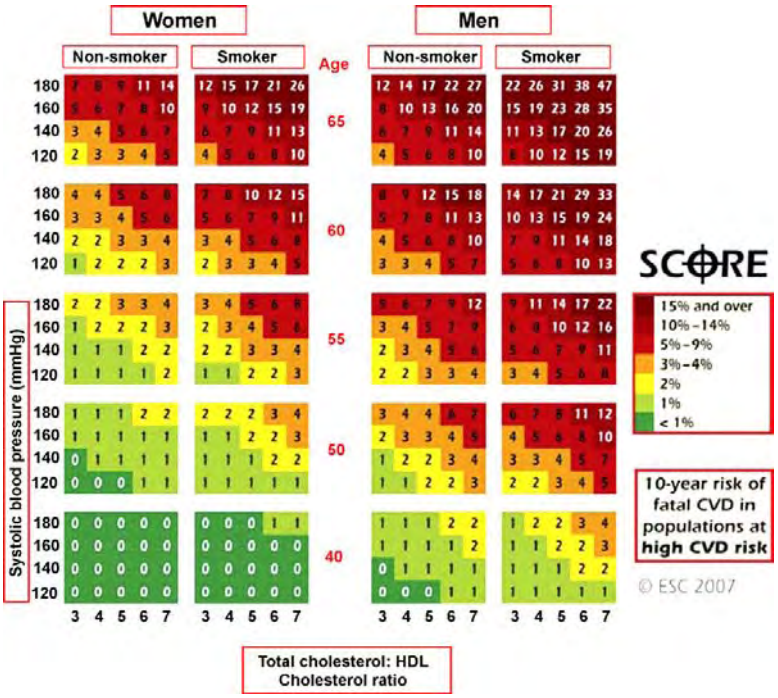

Figure 1 The SCORE estimate for The Netherlands providing a 10-year hazard ratio for fatal CVD[5]

Risk Factors for the development of CVD

Cholesterol

Identified as an important risk factor in SCORE and FHS, also the current guidelines to treat CVD from the Adult Treatment Panel III[8], the American Diabetes Association[9] and American Heart Association[10] emphasize targeting primarily LDL-C. HMG-CoA reductase inhibitors (statins) are widely used to lower LDL-C. In intervention trials using statins substantial reductions in major cardiovascular events in the treated groups were observed[11]. Furthermore, the magnitude of the reduction in events is a function of the amount of LDL-C, with each decrease of 1.0 mmol/L in LDL-C corresponding to a 23% reduction in major cardiovascular events[11]. However, in all the statin trials, substantial residual cardiovascular risk remains, even with very aggressive reductions in levels of LDL-C [11-14]. This indicates that additional treatment is required.

Clinical studies have shown that HDL-C levels, independently of LDL-C, are inversely correlated with the risk of CVD (**figure 2**)[15-18]. In statin treated patients this relationship was also observed among patients with very low LDL-C levels (<1.8 mmol/L) [19]. In the FHS, HDL-C level was more potent as a risk factor for CVD than was the level of LDL-C[20]. An analysis of data from four large studies concluded that each increase of 0.03 mmol/L in HDL-C is associated with a decrease of 2 to 3% in the risk of future CVD[16]. These findings have shifted the attention towards strategies for targeting HDL-C as adjunctive therapy to prevent and treat CVD.

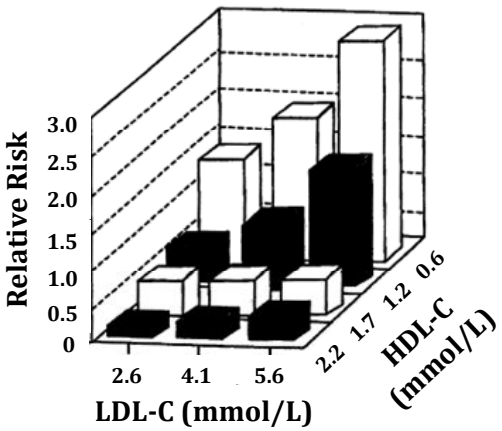

Figure 2 For any given level of LDL-C in the Framingham population, the relative risk of CHD decreases with increasing serum concentrations of high-density lipoprotein cholesterol (HDL-C) [15,21].

Hypertension

Hypertension is considered a 'traditional' risk factor for developing atherosclerosis, which entails a threefold risk over that of normotensive persons of the same age[22]. Hypertension *per se* might facilitate atherosclerosis development by the pressure-induced stretching of the arterial wall, which is a major determinant of arterial mass transport. Therewith it could enhance LDL-C accumulation in the inner media of the vessel wall[23], inducing endothelial activation and vascular inflammation[7]. However, evidence is also accumulating that hypertension may be just a marker and that the underlying mechanism is the risk factor for the development of atherosclerosis. A central role herein is considered for angiotensin II, a key molecule of the renin-angiotensin-aldosterone-system (RAAS), which regulates blood pressure[24-26]. However, in the fast majority of cases no single reason can be found for a patient causing the hypertension, indicating that hypertension is a very complex multifactorial disease, in which different mechanisms are involved.

Diabetes

Diabetes has been identified as a risk factor for CVD since many years and has gained the interest of research because of its increasing prevalence[8,10,27]. Estimates of current and future diabetes prevalence predict more than a doubling of the global burden of diabetes within 25 years from now[28]. In 75% of these subjects with type 2 diabetes mellitus

(T2D) an atherogenic triad will be observed, whereas it also is a common characteristic of patients with insulin resistance and abdominal obesity[29]. The atherogenic lipid triad comprises raised plasma triglycerides (TGs), reduced HDL-C, and a predominance of small dense (sd) LDL, all of which are associated with an increased risk in CVD[27].

Besides inducing dyslipidemia, insulin resistance itself also affects other pathophysiologic mechanisms, which may increase the risk on CVD. Though not all mechanisms are clarified yet, insulin resistance is thought to contribute to the development of hypertension[29,30], to impair thrombolysis[30,31], to case endothelial dysfunction[30] and to induce systemic and vascular inflammation[32], all contributors to the development of atherosclerosis and CVD[7,33].

Atherothrombosis

The main CVD events are MI and stroke, which occur when an atheromatous process precipitates thrombosis that prevents blood flow through the coronary or cerebral artery. Platelets, essential for primary hemostasis and repair of the endothelium, play a key role in the development of acute coronary syndromes and contribute to cerebrovascular events by triggering the acute onset of arterial thrombosis when atherosclerotic lesions rupture. In addition, they participate in the process of forming and extending atherosclerotic lesions. As atherosclerosis is a chronic inflammatory process, inflammation is an important component of acute coronary syndromes[7]. The relation between chronic and acute vascular inflammation is unclear, but platelets are a source of inflammatory mediators, which once activated, are able to activate vascular cells[34,35]. The activation of platelets by inflammatory triggers may be a critical component of atherothrombosis[36].

Pharmaceutical therapies

Cholesterol

The current armamentarium of lipid-lowering drugs includes inhibitors of hydroxy-3-methyl-glutaryl-CoA reductase (statins), PPARα agonists (fibrates), niacin (nicotinic acid), all directly or indirectly inhibiting lipid synthesis in the liver, and selective cholesterol absorption inhibitors (e.g. ezetimibe) and bile acid sequestrants (anion exchange resins), which work in the intestine by inhibiting the cholesterol absorption from food and bile. Next to lipid lowering, statins and fibrates also reduce inflammation via inhibition of NF-κB pathways, whereas lowering LDL-C *per se* also has anti-inflammatory effects[7,37,38].

Statin therapy may be considered as the 'standard' therapy to decrease (V)LDL-C, which has been shown to be very effective by lowering LDL-C by almost 30% in numerous studies and which may increase HDL-C modestly by a few percents[11].

Together with their pleiotropic effects statins are very potent in reducing CVD endpoints[11,39,40]. Fibrates potently reduce VLDL-TG (approx. -40%) and mildly increase HDL-C (approx. +10%) and seem to have most pronounced effect on CVD in obese and diabetic patients[18,41,42]. Niacin is a very powerful (V)LDL-TG lowering compound (approx. -40%) and the strongest HDL-C raising compound currently available (+ 15-30%)[18]. However, due to its side-effect, severe flushing, it is not very well tolerated.

The cholesterol uptake inhibitor ezetimibe mildly lowers LDL-C (approx. -20%)[43], but in combination with a low dose of statin the compounds strongly reduces LDL-C levels (approx. -55%). Bile acid sequestrants also lower LDL-C mildly, which is to a similar extent as ezetimibe, however, these compounds tend to increase TG[18,44].

Future therapies aiming at increasing HDL-C are cholesteryl ester transfer protein (CETP) inhibitors, GPR109A ('niacin receptor') agonists, selective cannabinoid type I receptor (CB1) antagonists, ApoAI mimetics and intravenous infusion of HDL[18]. These latter two therapies with a transient increase of HDL aim at an increased cholesterol efflux from the vessel wall and additionally a reduced the vessel wall inflammation[39].

Hypertension

The RAAS plays an important role in the regulation of blood pressure and body fluid and electrolyte homeostasis and may therefore be targeted to treat hypertension. The synthesis of angiontensin II, the main regulator molecule of RAAS, or the binding of angiotensin II to its receptor can be inhibited by angiotensin converting enzyme (ACE) inhibitors or angiotensin II type I receptor blockers (ARBs), respectively both are frequently used anti-hypertensive treatments. The RAAS also interacts with inflammatory pathways and its inhibition has clear anti-inflammatory effects[26,45]. Vasoconstriction can also be inhibited by blocking the calcium transport into the vascular smooth muscle cells by selective calcium channel blockers (CCBs). Other regularly used anti-hypertensive drugs are β-blockers and diuretics. Collective data of numerous prospective trials showed that anti-hypertensive treatment with any commonly-used regimen reduces the risk of total major cardiovascular events, whereby larger reductions in blood pressure produce larger reductions in risk [46].

Diabetes and insulin resistance

The most prescribed and effective insulin sensitizers are the thiazolidinediones, also referred to as the glitazones, and metformin. The latter compound has been used internationally for decades. Its primary mechanism of action is to suppress gluconeogenesis and to increase glucose uptake in the liver[47]. The glitazones increase peripheral utilization of insulin by acting as ligands of the peroxisome proliferator-activated receptor gamma (PPARγ). This receptor is found in high concentrations in adipose tissue and in the vessel wall, and is involved in the regulation of genes that

control glucose homeostasis, lipid metabolism, and adipose tissue[48]. In order to increase the plasma levels, insulin can be administered, whereas it is also possible to stimulate its secretion by the use of sulfonylureas and meglitinides. However, these compounds exhibit adverse effects of hypoglycemia and weight gain[49]. New and future therapies to improve insulin sensitivity enclose the endocannabinoid system (CB1 receptor antagonists) and gut-hormone regulated routes.

Anti platelet therapies

The anti-platelet drug acetylsalicylic acid (aspirin) has been proven to prevent myocardial infarction and stroke in patients with CVD[50]. However, the major adverse side effect, bleeding, and the large prevalence of aspirin resistance (5-45%) are drawbacks of this drug[51,52]. Another class of anti-platelet agents are the thienopyridines, of which clopidogrel is a member, which act by blocking the adenosine diphosphate (ADP)-mediated pathway of platelet activation. Clopidogrel is at least as effective as aspirin in preventing ischemic stroke, myocardial infarction and vascular death[53]. However, combining the two does not significantly decrease cardiovascular events and may even increase major bleedings[54]. The clinical efficacy of aspirin is based on inhibition of the platelet cyclo-oxygenase-1 (COX-1), inhibiting the generation of platelet thromboxane A_2 (TxA_2), which binds to the thromboxane-prostanoid endoperoxide (TP) receptor and thereby activates the platelet[55]. A third therapeutic route to inhibit platelet activation therefore may very well be direct inhibition of TxA_2 or its TP-receptor, which is present on platelets. No TP-receptor antagonist is currently available; however a new compound terutroban (S 18886) has been developed and is currently in phase III of development.

This thesis will present and discuss a variety of novel pharmaceutical interventions in experimental CVD as new ways to treat elevated lipid levels, blood pressure and conditions with increased risk of atherosclerosis. To study the effect of pharmaceutical intervention therapies on lipid metabolism, atherosclerosis and CVD we used suitable 'humanized' mouse models for hyperlipidemia and atherosclerosis: APOE*3Leiden and APOE*3Leiden.CETP transgenic mice.

Experimental model for hyperlipidemia, atherosclerosis and myocardial infarction

Wild-type mice are resistant to atherosclerosis as a result of high levels of anti-atherosclerotic HDL and low levels of proatherogenic LDL and VLDL, making them not useful for atherosclerosis research. All of the current mouse models for atherosclerosis are therefore based on modulations of lipoprotein metabolism through dietary or genetic manipulations. Among the most widely used mouse models are apolipoprotein

E–deficient mice (apoE$^{-/-}$ mice), the LDL receptor- deficient mice (LDLr$^{-/-}$ mice) and the APOE*3Leiden transgenic mice.

*Apolipoprotein E- deficient (*apoE$^{-/-}$*) mice*

The targeted deletion of the *apoE* gene of the homozygous apoE$^{-/-}$ mice results in a pronounced increase in the plasma levels of LDL and VLDL attributable to the failure of LDLr- and LDLr-related protein (LRP) mediated clearance of these lipoproteins[56,57]. Even on a chow diet they exhibit severe hypercholesterolemia (about 9 mmol/L), which, together with the reduced apoe-mediated cholesterol efflux from macrophages, leads to spontaneous lesion development especially in the aortic arch[58]. Over time these lesions become quite complex, progressing well beyond the fatty streak and they resemble human lesions. This model is suitable to study cellular aspects of lesion development and has been used for years to that end. However, one of the major drawbacks of this model is the lack of responsiveness to pharmaceutical and/or nutritional lipid lowering therapy[64]. This makes the model less suitable for the evaluation of therapeutic interventions in atherosclerosis. The apoE$^{-/-}$ mice may be considered as a severe model for atherosclerosis. Additionally hampering the HDL clearance by cross breeding apoE$^{-/-}$ mice with HDL receptor scavenger receptor class B, type I deficient mice (generating apoE$^{-/-}$/SR-BI$^{-/-}$mice), results in extreme hypercholesterolemia and a dramatically accelerated atherosclerosis, which even leads to spontaneous lipid- and fibrin-rich occlusive coronary arterial lesions, multiple myocardial infarctions, and cardiac dysfunction[59]. These apoE$^{-/-}$/SR-BI$^{-/-}$ mice die prematurely at about 6 weeks of age and can be considered as the most extreme model for CVD.

*LDL receptor- deficient (*LDLr$^{-/-}$*) mice*

The LDLr$^{-/-}$ mice display a modest hypercholesterolemia on a chow diet (about 5 mmol/L), with the cholesterol mainly confined to the LDL. Atherosclerosis develops slowly and is enhanced when these mice are fed a lipid-rich diet[60]. Interestingly, LDLr$^{-/-}$ mice cross bred with ApoB mRNA editing catalytic polypeptide-1 deficient mice (generating LDLr$^{-/-}$/ApoBEC$^{-/-}$ mice)[61] or with human ApoB100 transgenic mice (generating LDLr$^{-/-}$; Tg(ApoB$^{+/+}$) mice)[62] show a large increase in plasma LDL-C and develop atherosclerosis on a low-fat diet. The LDLr$^{-/-}$ mouse represents a more moderate model than the apoE$^{-/-}$ mouse, mainly because of the lower degree of hyperlipidemia. However, their responsiveness to lipid-lowering therapies is not optimal or might even be absent[64].

*APOE*3Leiden transgenic mouse*

A milder model is the APOE*3Leiden transgenic mouse, which develops atherosclerosis upon cholesterol feeding, and is more sensitive to lipid-lowering drugs than apoE$^{-/-}$ and LDLr$^{-/-}$ mice[63,64]. Hyperlipidemic APOE*3Leiden transgenic mice were generated by

introducing a human APOE*3Leiden gene construct, which also contained the APOC1 gene and a promoter element regulating the expression of APOE and APOC1 genes, into wild-type C57Bl/6 mice[63,65]. Although APOE*3Leiden mice still express endogenous apoE protein, the clearance of apoE-containing lipoproteins is impaired, albeit less dramatically than in apoE$^{-/-}$ mice. APOE*3Leiden mice show significant elevations of plasma cholesterol and TG on a regular chow diet and are, in contrast to wild-type mice, highly responsive to fat-, sugar-, and cholesterol-containing diets. This results in a lipoprotein profile similar to that of patients with familial dysbetalipoproteinemia in whom the elevated plasma cholesterol and TG levels are mainly confined to the VLDL/LDL-sized lipoprotein fraction[63]. Plasma lipid levels can easily be adjusted to a desired concentration by titrating the amount of cholesterol and sugar in the diet. As compared with other hyperlipidemic mouse models (e.g. apoE$^{-/-}$ and LDL$^{-/-}$ mice), APOE*3Leiden mice represent a milder mouse model for hyperlipidemia (cholesterol levels on chow are about 2-3 mmol/L and do not exceed 25 mmol/L on a western-type high-cholesterol diet). The development of atherosclerosis strongly correlates with the plasma cholesterol levels and the duration of cholesterol elevation (**figure 3**), and consists of lesions with all the characteristics of human vascular pathology, varying from fatty streak to mild, moderate, and severe lesions[66].

The APOE*3Leiden mice are a suitable model to study the (V)LDL metabolism and, in contrast to apoE-/- and ldlr-/- mice, they respond in a human-like manner to treatment of CVD (e.g. statins, calcium channel blockers, fibrates, angiotensin II receptor blockers, and cholesterol uptake inhibitors [67-73])[64]. However, APOE*3Leiden mice do not respond to HDL raising therapies. This is the consequence of the lack of CETP expression in mice, an important factor in the human HDL metabolism. CETP mediates the transfer of cholesteryl ester from HDL particles to the apoB-containing lipoproteins ((V)LDL) in exchange for triglycerides.

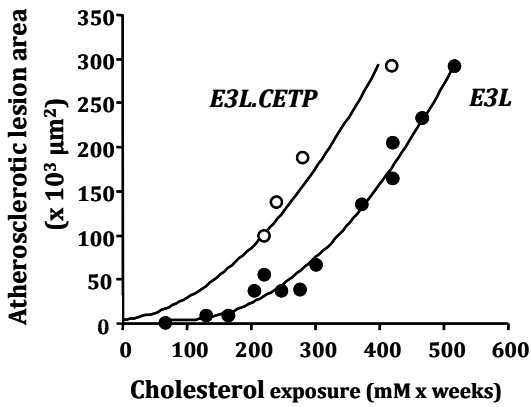

Figure 3 The strong correlation between cholesterol exposure (plasma cholesterol levels in mmol/L times the duration in weeks) and the atherosclerosis development in APOE*3Leiden (black circles) and APOE*3Leiden.CETP (open circles) transgenic mice. Unpublished observations of H.M.G. Princen and P.C.N. Rensen.

Therefore, APOE*3Leiden mice were recently cross-bred with mice expressing the human CETP gene under control of its natural flanking regions, resulting in APOE*3Leiden.CETP mice[74]. These mice display an elevated basal cholesterol level and

a human-like lipoprotein profile. CETP expression in APOE*3Leiden mice shifts the distribution of cholesterol from HDL toward VLDL/LDL, and strongly (7-fold) increases atherosclerosis development (**figure 3**)[74]. Their responsiveness to lipid modulating therapies was even increased, since these mice also respond to the HDL-raising effects of fenofibrate[75], atorvastatin[76], torcetrapib[77] and niacin[78].

Outline of the thesis

This thesis describes a variety of novel pharmaceutical interventions in experimental CVD of APOE*3Leiden mice.

Chapter 2 reviews the effect of current 'standard' therapies for the treatment of two separate risk factors for CVD, *i.e.* hyperlipidemia with atorvastatin and hypertension with the calcium channel blocker amplodipine. Additionally, scientific evidence is collected to determine the possible advantage of combining these two kinds of treatments, potentially leading to additive or synergistic effects in the prevention of CVD.

Although compounds have proven their benefit in the clinic, their exact working mechanism is not always clarified. Niacin (vitamin B3) is one of those compounds. In the early 1950s it was known that niacin decreases LDL-C and concomitantly increases HDL-C. The underlying mechanism however, remained to be elucidated. In **Chapter 3** the mechanism of the HDL-C raising effect of niacin is explored in the APOE*3Leiden.CETP transgenic mouse model, which was proven in this study to be a very suitable model to investigate HDL-raising therapies.

Modulating plasma lipid levels using compounds like niacin, PPARα agonists, or cholesterol uptake inhibitors is of significance to obtain an indication about their effect on atherosclerosis development. In **chapter 4** we evaluate the effect of a new cholesterol uptake inhibitor AVE5530 with regard to its VLDL/LDL-C lowering capacity as well as its anti-atherosclerotic effects in APOE*3Leiden mice. Ezetimibe, a cholesterol uptake inhibitor already used in the clinic, has been used as a reference compound. A major difference between AVE550 and ezetimibe is that nearly 100% of ezetimibe is absorbed in the intestine whereas AVE5530 is not.

As described in chapter 2, combination therapy targeting two risk factors of CVD might have synergistic or additive effects in the prevention of CVD and atherosclerosis. An example of such a study is presented in **chapter 5** where APOE*3Leiden mice are treated with either the anti-hypertensive angiotensin receptor blocker olmesartan or the antihyperlipidemic drug pravastatin, alone or with the combination of both compounds.

Nowadays compounds are developed in order to target two independent risk factors for CVD simultaneously, for instance the dual PPARα/γ agonist tesaglitazar

treating both hyperlipidemia and insulin resistance/diabetes. In **chapter 6** the effect of tesaglitazar is investigated in APOE*3Leiden.CETP with pre-existing atherosclerotic lesions. Such a study design is of more clinical significance, since in humans lesions have already been developed before treatment is started.

A similar design is used for the study presented in **chapter 7** where APOE*3Leiden mice had developed mild lesions before cholesterol-lowering and anti-platelet therapy with a thromboxane prostanoid (TP) receptor antagonist S18886 was started. The effects of thromboxane and its receptor on platelet function and peripheral tissue are not fully clarified yet. However, evidence is accumulating that it interacts with inflammatory pathways and affects atherosclerosis and CVD endpoints. It has been observed that selective cyclooxygenase-2 (COX-2) inhibition by rofecoxib is associated with increased risk of cardiovascular events. We hypothesized that this could be due to a disrupted local TXA_2-PGI_2 balance, which could be prevented by concomitant treatment with TP-receptor antagonist S18886 that might ameliorate possible negative effects. This is investigated in **chapter 8** in APOE*3Leiden mice with ischemia reperfusion injury of the myocardium.

The results obtained in these studies and their clinical relevance are discussed in the **General Discussion.**

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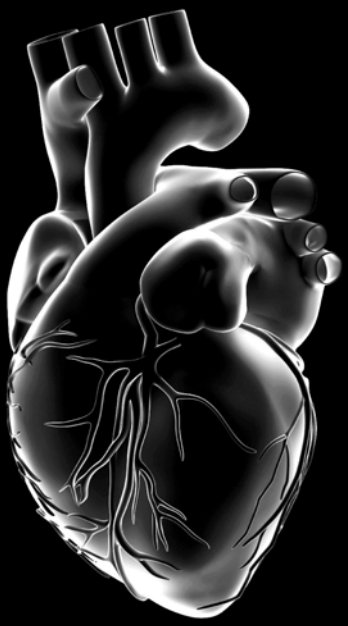

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Amlodipine and Atorvastatin in Atherosclerosis:

A review of the potential of combination therapy

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Expert Opin Pharmacother 2004 Feb;5(2):459-68.

Abstract

Hypertension and hyperlipidemia are major risk factors for the development of atherosclerosis. Calcium channel blockers (CCBs) are used for decades for their established antihypertensive effects, as statins are used for a long time for their potent lipid lowering properties. Amongst others inflammation and oxidation are involved in enhanced progression of atherosclerosis and new lesion development. Therefore research has been initiated focusing on e.g. the antioxidant and anti-inflammatory properties of CCBs and statins, beyond their primary effect in order to evaluate the possible additive effects of combined treatment of CCBs with statins as anti-atherosclerotic therapy.

Clinical studies, amongst others the International Nifedipine Trial on Antiatherosclerotic Therapy (INTACT), have demonstrated that the antiatherosclerotic action of CCBs is limited to attenuation of the first stage of atherosclerogenesis (fatty streak formation or new lesion growth). The lesions that pre-existed the start of CCB therapy did not demonstrate progression or regression on angiography. However, because the mechanisms of action of lipid-lowering drugs and CCBs and their role in preventing the progression of atherosclerosis differ, it is conceivable that these two classes may have an additive or synergic effect, not only on new lesion formation but also on inhibiting the progression of established coronary atherosclerosis. Indeed, this combined effect of lipid-lowering therapy and CCBs on human coronary atherosclerosis has been reported in the Regression Growth Evaluation Statin Study (REGRESS) trial. Researchers observed a significant beneficial effect of CCBs with regard to angiographic progression and new lesion formation in patients treated with a statin, but no similar antiatherosclerotic effect in those treated with CCB alone (placebo group). This beneficial effect as a result of combining CCBs with statins has now been replicated in transgenic atherosclerotic mice, where the combination of amlodipine and atorvastatin produced an additional 60% reduction of atherosclerosis compared with that observed with the statin alone. Serum markers of atherosclerosis and vascular integrity also improved most in the combination group. Recently Mason *et al.* showed a synergistic effect of the combination of atorvastatin and amlodipine on acute NO release/endothelial function, whereas Leibovitz *et al* demonstrated that the combination of amlodipine and atorvastatin had an additive effect in improvement of arterial compliance in hypertensive hyperlipidemic patients.

Collectively, these studies support the clinical anti-atherosclerotic advantages of combination of CCBs and statins and in particular of atorvastatin with amlodipine beyond their established antihyperlipidemic and antihypertensive modes of action.

Introduction

Cardiovascular disease (CVD) is the main cause of death in the Western Society. CVD is mostly caused by atherosclerosis. Established risk factors for developing atherosclerosis are male gender, age, diabetes mellitus, genetic predisposition, high plasma lipoprotein levels, hypertension, obesity and smoking.

Hypertension is considered a 'traditional' risk factor for developing atherosclerosis, which entails a threefold risk over that of normotensive persons of the same age [1]. However, in the fast majority of cases no single reason can be found for a patient causing the hypertension. This indicates that hypertension is a very complex multifactorial disease, in which different mechanisms are involved. The last decade newer 'nontraditional' risk factors for the development of atherosclerosis are identified including inflammation and its markers, like C-reactive protein, homocysteine, oxidative stress and endothelial dysfunction, but also activation of the renin-angiotensin-aldosterone-system (RAAS)[2]. Evidence is accumulating that hypertension may be just a marker and the underlying mechanism is the risk factor for the development of atherosclerosis.

In patients with coronary atherosclerosis, disease progression is one of the main factors that determine clinical prognosis. Patients with progression of coronary atherosclerosis, do significantly worse with regard to clinical event-free survival than patients with attenuated progression[3]. Thus inhibition of the progression of atherosclerosis is almost as important as preventing atherosclerosis development. Lipid-lowering therapy has undoubtedly proven to be an effective therapeutic modality to retard the progression of coronary atherosclerosis[4]. Possible beneficial modes of action of lipid-lowering therapy include: (1) retardation of progression and induction of regression of coronary atherosclerosis[4] (2) atherosclerotic lesion-plaque stabilization[5,6] (3) restoration of endothelial dysfunction[7], (4) decreased thrombotic tendency[8], and immune system modulation [9].

Evidence indicating also that some calcium channel blockers (CCBs), which are established anti-hypertensive drugs, inhibit atherosclerosis is accumulating. Many investigations support the view that a number of key processes in atherosclerosis may be influenced by CCBs. These key processes include: (1) oxidation of circulating lipoproteins, such as LDL[10], (2) binding of monocytes to and transmigration of monocytes through the endothelial cell layer[11],(3) formation of macrophage-derived foam cells, (4) proliferation and migration of VSCMs[12], (5) binding of platelets to the endothelial cells layer and subsequent platelet aggregation[13], and (6) synthesis of matrix components, such as collagen.

In this review first the development of atherosclerosis and hypertension will be briefly described. Secondly mechanisms and effects of lipid-lowering therapy by statins and anti-hypertensive therapy by calcium channel blockers will be described. Because the mechanisms of action of lipid-lowering drugs and CCBs and their role in preventing

the progression of atherosclerosis differ, and it therefore is conceivable that these two classes may have an additive or synergic effect, it is interesting to focus on combination therapy. Most research with regard to combination therapy of CCBs and statins has been performed with amlodipine and atorvastatin and therefore the effects of the combination of these compounds on atherosclerosis will be specially highlighted.

Hypertension and atherosclerosis

In 1996 Meyer *et al.*[14] showed the effects of pressure-induced stretch and convection on low-density lipoprotein (LDL) and albumin uptake in the rabbit aortic wall. It was demonstrated that pressure-induced stretching of the arterial wall is a major determinant of arterial mass transport, and that pressure-driven convection accentuates LDL accumulation in the inner media, which may explain enhanced atherosclerosis in hypertension. Accumulation of atherogenic lipoproteins in the arterial wall is generally considered to be the first step in the development of atherosclerosis. Reactions with reactive oxygen species (ROS) [15] can oxidatively modify the lipid and apoB components of LDL trapped in the subendothelial space. Proinflammatory factors, such as oxidized LDL and ROS stimulate release of cytokines. This leads to the accumulation of mononuclear cells, migration and proliferation of SMCs and formation of fibrous tissue that eventually results in an atherosclerotic plaque.

Most, if not all, of the risk factors that are related to atherosclerosis and cardiovascular morbidity and mortality, were also found to be associated with endothelial dysfunction. Many of these risk factors, including hyperlipidemia, hypertension, diabetes and smoking are associated with overproduction of ROS or increased oxidative stress [16,17]. Measurement of endothelial (dys)function gives a proper indication about the health/condition of the endothelium. In endothelial dysfunction the homeostasis of vasoactive substances is disrupted [18]. The bioavailability of NO, which promotes vasodilation in response to hemodynamic stress, is decreased due to reduced secretion and to interaction with superoxide anion (O_2^-)[19]. The level of the vasoconstrictor factor angiotensin II, promoting the proliferation and influx of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and cytokines, and the level of the potent vasoconstrictor hormone endothelin-1 (ET-1) are increased [20]. These changes in homeostasis lead to changes in vascular structure and function [19,21]. Besides a disturbed balance of vasodilators and vasoconstrictors, endothelial dysfunction also comprises a specific state of 'endothelial activation', which is characterized by a proinflammatory, proliferative, and procoagulatory environment that favors all stages of atherogenesis[16]. As a number of these cellular and inflammatory processes are mediated by disruption of calcium homeostasis, there has been interest in the potential role of calcium channel antagonists (CCBs) as antiatherogenic agents, apart from their anti-hypertensive potential [11].

Calcium Channel Blockers

Mechanism

The mechanisms of the anti-atherosclerotic effect of CCBs, also called calcium antagonists, are not fully understood, however many pathways have been studied last decade, giving more insight in different mechanisms of CCBs. CCBs are essentially used as anti-hypertensive drugs. The primary action of calcium channel blockers is to inhibit calcium ion entry through voltage-gated transmembrane L-type channels, thus decreasing intracellular calcium concentration and inducing smooth muscle relaxation. Several important processes in atherosclerosis may be influenced by CCBs because they require calcium-dependent energy. In vitro studies have shown that CCBs can reduce lipoprotein oxidation and proliferation and migration of smooth muscle cells. However, the anti-atherosclerotic activity of CCBs probably involves many additional properties of the compounds because calcium-independent mechanisms, such as binding of monocytes to the endothelial cell layer, esterification of cholesterol in macrophages, and expression of matrix metalloproteinases in vascular endothelial cells, have also been shown to be inhibited by calcium antagonists[22]. CCB can be separated in two main groups i.e. the dihydropyridine (DHP) and the non-dihydropiridine (non-DHP) CCBs. On average these different CCBs display different anti-atherosclerotic potential, especially some DHP CCBs seem to have anti-atherosclerotic potential.

CCBs and atherosclerosis

Anti-atherosclerotic properties of CCB treatment were discovered in the 1980s. It was demonstrated that plasma membrane calcium transport in the aortic wall of rabbits with experimental atherosclerosis was increased fivefold and that CCBs were able to suppress such experimental atherosclerosis[23]. Since then CCBs have been evaluated for their anti-atherosclerotic effect in humans[24].

In Vitro

Mak *et al.*[10] demonstrated that DHP CCBs had the same protective effect against oxidative damage in bovine aortic endothelial cells like vitamin E. Because endothelial cells do not have receptors for CCBs (the L-type calcium channels are not involved in calcium influx[25]) the molecular mechanism of the antioxidant effect is less clear. Apparently, the cytoprotective effects of the DHP calcium blockers were mediated by a membrane 'chain-breaking' antiperoxidative action similar to that provided by vitamin E[10]. DHP CCB amlodipine has been shown to stimulate NO release from canine coronary microvessels in a dose dependent manner like the ACE inhibitors analaprilat and ramiprilat, whereas DHP CCB nifedipine and diltiazem did not. Amlodipine mediates NO

release and hereby may have antioxidative properties, similar to the mechanism of ACE inhibitors, by modulation of the actions or formation of kinins[26].

Oxidative-modification of LDL contributes to destructive inflammatory processes associated with atherosclerosis. It is characterized by elevations in cholesterol content and increased electro negativity, factors that contribute to aggregation and foam cell formation. Phillips and Mason[27] designed a study to test the effect of the positively charged calcium channel blocker (CCB) amlodipine on the aggregation properties of oxidized LDL lipids. Amlodipine inhibited binding of the oxidized LDL lipids in a dose-dependent fashion, in contrast to other drugs lacking a formal positive charge (including CCBs).

Amlodipine also modulates metabolism of collagens within the extracellular matrix and thus potentially has plaque stabilizing properties. It was demonstrated that CCBs, including amlodipine, specifically increased the proteolytic activity of the 72-kDa type IV collagenase and inhibited the transcription of tissue inhibitor of MMP-2[28]. Amlodipine, but not nifedipine significantly decreased interleukin-1β induced MMP-1 expression in human endothelial cells[29]. Additionally, several studies demonstrated a role for amlodipine in the remodeling of SMC membranes and in the inhibition of SMC proliferation and migration, which are hallmark features of atheroma development [11,12,29].

In vivo

Amlodipine was tested in hyperlipidemic hamsters for is antiatherogenic properties. Male Golden Syrian hamsters were subjected to a hyperlipidemic diet. At intervals ranging from 2 to 14 weeks, the animals were examined for changes in serum constituents and structural modifications of lesion-prone areas like the cardiac valves, coronary arteries and aortic arch. Amlodipine treatment of hyperlipidemic hamsters was assessed. Amlodipine exhibited an atheroprotective effect, acting as antioxidant, reducing the LDL uptake by the vessel wall and consequently, limiting the size and extent of lesion areas[30]. Recently Turgan *et al.*[31]investigated the interactions of amlodipine with major cellular antioxidants in order to elucidate the mechanisms underlying its atheroprotective effects. New Zealand white male rabbits were fed regular chow with or without 1% cholesterol and with or without amlodipine for 8 weeks. Total cholesterol, malondialdehyde and vitamin E concentrations and catalase and superoxide dismutase activities were determined in blood drawn before and after the experimental period. Aortic tissue was examined for atherosclerotic changes and aortic total cholesterol, malondialdehyde, catalase and superoxide dismutase were determined. Amlodipine seemed to exert atheroprotective effects by reducing aortic cholesterol accumulation and blood and aortic lipid peroxidation, enhancing superoxide dismutase activity both in blood and aortic tissue and suppressing the consumption of

vitamin E. On the other hand, the suppression of catalase activity in blood and the aorta interferes with the well-known antioxidant effects of amlodipine.

Atherosclerosis may result in plaque rupture resulting in a myocardial infarction. Calcium dependent factors, including cholesterol-induced changes in membrane calcium permeability and calcium deposition into lesions, may contribute to plaque formation and stability during the early and late stages of atherogenesis. Hoshida et al. evaluated the effect of amlodipine treatment on myocardial infarction size after 30-min coronary occlusion/48-h reperfusion in rabbits fed a diet with or without 1% cholesterol. Infarct size was significantly larger in cholesterol-fed rabbits (72.0 +/- 3.5%, n = 9, mean +/- S.E.M.) than in normal-fed rabbits (47.1 +/- 4.9%, n = 9, $P < 0.05$). Amlodipine treatment effectively reversed the infarct size augmentation in cholesterol-fed rabbits (46.3 +/- 6.3%, n = 9, $P < 0.05$), but did not affect infarct size in normal-fed rabbits (51.0 +/- 4.7%, n = 8). Calcium content and leukocyte accumulation were markedly elevated in the ischemic myocardium of cholesterol-fed rabbits compared with normal-fed rabbits. Amlodipine treatment effectively reversed this elevation. Acetylcholine showed a marked reduction in endothelium-dependent relaxation in the aorta of cholesterol-fed rabbits, which also was reversed by amlodipine treatment[32].

Clinical trails with CCBs, using angiography show different results. The INTACT study[33] showed that nifedipine had no effect on the progression of existing lesion but could reduce the number of new formed lesions in coronary artery diseased patients. The PREVENT study[34] demonstrated in cardiovascular diseased patients that amlodipine treatment was associated with reduction in cardiovascular morbidity, but had no effect on the angiographic progression of atherosclerosis or cardiovascular mortality. The ELSA study[35] compared the progression of atherosclerosis in hypertensive patients treated with β-blocker atenolol or DHP CCB lacidipine. Despite a smaller ambulatory blood pressure reduction, lacidipine was shown to have a greater efficacy on carotid intima-media thickness progression, which predicts cardiovascular events, and number of plaques per patient. Recently, the NICOLE study[36] evaluated the effect of nisoldipine on atherosclerosis progression in patients who had undergone coronary angioplasty. It was found that long acting nisoldipine did not retard the angiographic progression of coronary artery disease and did not affect mortality. However, it reduced the need for coronary revascularisation.

Besides the (clinical) antiatherosclerotic effects found in previous described studies Ghiadoni *et al.* [37] found reduced oxidative stress and increased plasma oxidant capacity hypertensive patients receiving amlodipine or nifedipine.

Statins

Mechanism

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyses the rate-limiting step in cholesterol biosynthesis. Consequently this has been an important target for the development of cholesterol-lowering agents. Inhibition of HMG-CoA reductase leads to an upregulation of LDL-receptors in the liver mediated by the activation of Sterol Regulatory Element-Binding Proteins (SREBPs) and enhanced clearance of LDL from the circulation. HMG-CoA reductase inhibitors, or statins, were first introduced into clinical practice in the 1980s. Pravastatin and lovastatin are produced from fungal metabolites, simvastatin is semi-synthetic and fluvastatin, atorvastatin and rosuvastatin are synthetic statins[38]. Pravastatin and rosuvastatin are hydrophilic agents; other statins are highly lipophillic.

Statins and atherosclerosis

Because lipoproteins infiltrated in the vessel wall are the start of the development of atherosclerotic lesion and oxidized particles may trigger or enhance atherogenicy, statins are relevant in treatment for preventing atherosclerosis. In many clinical trails is proven that treatment with statins reduce morbidity and mortality in CVD patients. Best known, major trials in the 1990s were 4S (1994) [39], CARE (1996) [40] (1996) and LIPID (1998) [41] (1997). The 4S trial was designed to evaluate the effect of cholesterol lowering with simvastatin on mortality and morbidity in patients with coronary heart disease. 4444 Patients with angina pectoris or previous myocardial infarction and serum cholesterol 5.5-8.0 mmol/L on a lipid-lowering diet were randomised to double-blind treatment with simvastatin or placebo. Over the 5.4 years median follow-up period, simvastatin showed a reduction of major coronary events, coronary deaths and overall mortality. Other benefits of treatment included a reduction in the risk of undergoing myocardial revascularisation procedures. This study showed that long-term treatment with simvastatin is safe and improves survival in CVD patients. CARE and LIPID are both trails in which the effect of pravastatin on coronary events and death was investigated in patients with a history of myocardial infarction of unstable angina pectoris. In the CARE study 4159 patients and in the LIPID study 9014 patients participated. The mean follow-up period was 5 (CARE) and 6 (LIPID) years. In both studies was demonstrated that pravastatin reduced the fatal coronary events, myocardial infarction, stoke and overall death.

Statins and inflammation

Over the last years evidence is accumulating that not only the lipid lowering effect of the statins but also their anti-inflammatory properties may be responsible for atheroprotective effects. In 1998 Yamamoto *et al.*[42] demonstrated that fluvastatin, more

than pravastatin inhibited NAD(P)H dependent lipid peroxidation in liver microsomes. In addition fluvastatin scavenged ROS such as hydroxyl radicals and O2-. A reduction of fibrinogen and serum amyloid A was seen in ApoE*3-leiden transgenic mice treated with atorvastatin, which is a synthetic and lipophilic statin like fluvastatin [43].

Recently Hernandez-Presa *et al.*[44] investigated whether simvastatin reduced inflammation in atherosclerosis beyond its hypolipidemic effects. Rabbits with induced femoral injury and on an atherogenic diet were randomized to normolipidemic diet or to continue the atherogenic diet while receiving simvastatin or no treatment for 4 weeks. As compared with no treatment, the normolipidemic diet significantly reduced lipid levels, while simvastatin produced nonsignificant reductions. In spite of this, NF-κB binding activity in peripheral mononuclear cells was reduced in the simvastatin group as compared with no treatment and normolipidemic groups (electrophoretic mobility shift assay). NF-κB activity in the atherosclerotic lesions was also reduced by simvastatin as compared to nontreated animals, while the normolipidemic diet induced only a nonsignificant diminution (Southwestern histochemistry). Similarly, simvastatin decreased macrophage infiltration and the expression of interleukin-8 and MMP-3, while the reduction achieved by normolipidemic diet in all these parameters was again nonsignificant.

In APOE*3Leiden transgenic mice, which develop human-like atherosclerosis, Kleemann *et al*.[45] demonstrated that rosuvastatin treatment reduced atherosclerosis beyond and independent of the reduction achieved by cholesterol lowering alone. In this study mice received a high cholesterol diet with or without rosuvastatin or a normolipidemic diet. It was also shown by in situ hybridization in the aortic root, that the number of TNF-α and MCP-1 positive cells was significant reduced after rosuvastatin treatment. A same reduction was found for Serum Amyloid A and fibrinogen levels. These findings were all lipid independent, demonstrating anti-inflammatory activities of rosuvastatin.

Atorvastatin and inflammation

Several studies showed results regarding the interaction of statins and NF-κB signalling. Atorvastatin reduces activation of transcription factor NF-κB in cultured VSMCs [46] as well as in atherosclerotic lesions in the rabbit[47]. Dichtl *et al*.[48] aimed to characterize the effects of statins on the activation of transcription factors known to regulate inflammation and cell proliferation/differentiation. Simvastatin, atorvastatin, and lovastatin inhibited the binding of nuclear proteins to both the NF-κB and activator protein-1 DNA consensus oligonucleotides in human endothelial cells and VSMCs as assessed by electrophoretic mobility shift assay. Furthermore, statins inhibited DNA binding of hypoxia-inducible factor-1α.

Recently Zhao *et al.*[49] demonstrated that atorvastatin can inhibit IL-6 secretion in rabbit adipocytes, in a dose-dependent manner, possibly through upregulating

peroxisome proliferator-activated receptor (PPAR) gamma. In high cholesterol fed rabbits on two week atorvastatin treatment, IL-6 concentrations in plasma and adipocytes culture supernatant and PPARgamma mRNA expression were measured. Two weeks atorvastatin treatment resulted in significant reduction of circulating IL-6 concentrations, which was associated with IL-6 secretion in adipocytes. Meanwhile mRNA expression of PPARgamma in adipocytes was intimately related to the IL-6 secretion in adipocytes.

Thrombin, a serine protease, plays an important role in inflammation and thus the progression of atherosclerosis. Haloui *et al*. [50] demonstrated that atorvastatin could limit the pro-inflammatory response to thrombin in cultured rat aortic smooth muscle cells. The variations in expression of interleukin-6, heme oxygenase-1, p(22phox) and Mox-1 mRNAs were evaluated. Thrombin activated interleukin-6 secretion and mRNA expression in smooth muscle cells in a dose-dependent manner. The greatest effect on mRNA expression was obtained after 1 h of stimulation. Preincubation of the cells with various concentrations of atorvastatin prevented this effect. Thrombin was without effect on p(22phox) and heme oxygenase-1 mRNA expression but, after 3 hour of stimulation, induced a two-fold increase in that of Mox-1. Preincubation with atorvastatin dose-dependently down regulated this Mox-1 mRNA expression. In addition, thrombin induced NF-kappaB translocation and membrane translocation of RhoA in smooth muscle cells which were both prevented by pre-treatment of the cells by atorvastatin.

Atorvastatin in humans

Inflammation

Adhesion molecules and cytokines are involved in the pathogenesis of intimal injury in atherosclerosis. To investigate their relationship with endothelial function Nawai et al. [51] examined the effects of atorvastatin on soluble adhesion molecules, interleukin-6 (IL-6) and brachial artery endothelial-dependent flow mediated dilatation (FMD) in patients with familial (FH) and non-familial hypercholesterolemia (NFH).

A total of 74 patients (27 FH and 47 NFH) were recruited. Fasting lipid profiles, soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular-cellular adhesion molecule-1 (sVCAM-1), E-selectin, IL-6 and FMD were measured at baseline, 2 weeks, 3 and 9 months post-atorvastatin treatment (FH: 80 mg/day, NFH: 10 mg/day). In both groups, compared to baseline, sICAM-1 levels were significantly reduced at 2 weeks, further reduced at 3 months and maintained at 9 months. The IL-6 levels were significantly reduced at 3 months and 9 months compared to baseline for FH and NFH. In both groups, the FMD at 2 weeks was higher than baseline, with progressive improvement up to 9 months. FMD was negatively correlated with sICAM-1 and IL-6.

Thus both low and high doses of atorvastatin lead to early progressive improvement in endothelial function in patients with primary hypercholesterolemia. sICAM-1 and IL-6 levels do reflect endothelial dysfunction in these patients.

The pleiotropic actions of statins include anti-inflammatory and antioxidant actions. To evaluate whether alternative oxidative pathways are suppressed in vivo after atorvastatin administration was examined by Shishehbor et al.[9]. Hypercholesterolemic subjects with no known coronary artery disease were evaluated at baseline and after 12 weeks of atorvastatin therapy (10 mg/d). Plasma levels of protein-bound chlorotyrosine, NO_2Tyr, dityrosine, and orthotyrosine, specific molecular fingerprints for distinct oxidative pathways up regulated in atheroma, were determined by mass spectrometry. In parallel, alterations in lipoproteins and C-reactive protein were determined. Statin therapy caused significant reductions in chlorotyrosine, NO_2Tyr, and dityrosine (30%, 25%, and 32%, respectively) that were similar in magnitude to reductions in total cholesterol and apolipoprotein B-100 (25% and 29%). Nonsignificant decreases in orthotyrosine and C-reactive protein levels were observed (9% and 11%, respectively). Statin-induced reductions in oxidation markers were independent of decreases in lipids and lipoproteins. Statins promote potent systemic antioxidant effects through suppression of distinct oxidation pathways. The major pathways inhibited include formation of myeloperoxidase-derived and nitric oxide-derived oxidants, species implicated in atherogenesis.

Plaque stability

Beside prevention and regression of atherosclerosic plaque formation, prevention of plaque rupture is another very important aspect. Coronary plaque stabilization by statin therapy in humans was investigated by Takano *et al.* [6]. They evaluated the changes in coronary plaque color and morphology by atorvastatin therapy using coronary angioscopy. Thirty-one patients with coronary artery disease were divided into either the comparison group (n = 16) or the atorvastatin group (n = 15). Before treatment and 12 months after, the color and complexity of 145 coronary plaques were determined according to angioscopic findings. The yellow score of the plaque was defined as 0 (white), 1 (light yellow), 2 (yellow), or 3 (dark yellow), and its disrupted score was defined as 0 (smooth surface) or 1 (irregular surface) and as 0 (without thrombus) or 1 (with thrombus). In each patient, the mean yellow score and mean disrupted score were calculated. Mean LDL cholesterol decreased by 45% in the atorvastatin group, whereas an increase of 9% was seen in the comparison group. The mean yellow score decreased from 2.03 to 1.13 in the atorvastatin group, whereas it increased from 1.67 to 1.99 in the comparison group. There was a good correlation between the change in the mean yellow score and the change in LDL cholesterol levels. The change in the mean yellow score and mean disrupted score differed significantly between the two groups. This is the first report clarifying detailed changes in coronary plaque by statin in humans. This study

indicated that lipid-lowering therapy changes plaque colour and morphology possibly reflecting a more stable plaque phenotype in vivo.

Revascularisation

The AVERT study[52] was designed to determine whether aggressive lipid-lowering therapy with atorvastatin is an alternative to angioplasty or other catheter-based revascularization procedures in patients with significant coronary artery disease. 341 Patients with low-density lipoprotein (LDL) cholesterol ≥ 3 mmol/L and ≥ 1 defined narrowing of a major coronary artery were randomized to atorvastatin or the indicated catheter-based revascularization and conventional care (including lipid-lowering therapy if prescribed). Ischemic events were tracked for 18 months. The primary efficacy parameter was the incidence of an ischemic event, defined as 1 of the following: cardiovascular death, cardiac arrest, nonfatal myocardial infarction, the need for coronary bypass grafting or angioplasty, cerebrovascular accident, and worsening angina verified by objective evidence requiring hospitalization (including unstable angina). Results of this study favour the use of aggressive lipid lowering over percutaneous transluminal coronary angioplasty in patients with mild to moderate coronary disease. Treatment with atorvastatin significantly reduced LDL cholesterol levels, and was associated with a 36% reduction in ischemic events and a significant delay in time to first ischemic event.

Combining Statin and Calcium Channel Blocker therapy

Advances of combination therapy

It has been demonstrated very often that statins and CCBs both have antiatherosclerotic potential via different mechanisms. Gathering their strengths might lead to additive or synergistic antiatherosclerotic effects. Important evidence for an additive effect of CCBs and statin treatment in retarding progression of coronary atherosclerosis was found in a retrospective study on data from the REGRESS-study[4]. Evaluating the effect of pravastatin on progression and regression of coronary artery disease, it was found that the combination of pravastatin and a calcium antagonist was more successful in retarding the progression of atherosclerosis than pravastatin alone. This seemed to be especially true for DHP CCBs, which tended to have stronger antiatherosclerotic properties than non-DHP CCBs [53] (**Figure 1**).

The ENCORE[54] investigators examined the effects of a statin and/or a calcium antagonist on coronary endothelial function in patients with coronary artery disease. In 343 patients the endothelial function was investigated. Thereafter, patients were randomized in a double-blind manner to a 6 month treatment with placebo, cerivastatin, nifedipine, or their combination. In the most constricted segment, nifedipine but not

cerivastatin improved endothelial dysfunction. Patients not taking ACE inhibitors showed a smaller improvement in the placebo group (6.0%), but nifedipine still had an effect (17.0%; P<0.05 versus placebo).

Analysis of all evaluable coronary segments revealed an improved endothelial function in patients receiving nifedipine and cerivastatin (P<0.05 versus placebo). Cerivastatin lowered LDL cholesterol by 35%. The ENCORE II study is designed to detect the effects of nifedipine together with a statin on coronary morphology. This may give more insights in the role of CCB combined with statin in coronary artery disease.

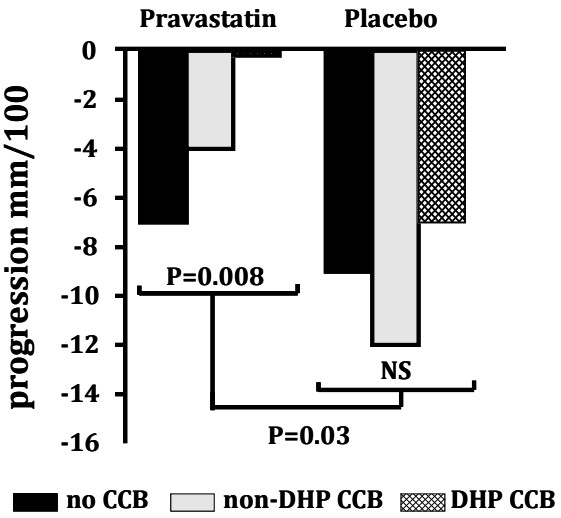

Figure 1 Angiographic progression (change of minimum obstruction diameter) in patients with and without CCB cotreatment, stratified with regard to type of CCB treatment (no CCB, non-dihydropyridine (non-DHP) CCB, and DHP CCB), in the pravastatin group and in the placebo group in the REGRESS study[4,51]. Patients in the pravastatin group had significantly less progression if cotreated with CCBs as compared with no CCB cotreatment (p=0.03), with most striking results for the DHP CCBs (hardly any progression left; p = 0.008), whereas in the placebo (no pravastatin) group no significant effect of any type of CCB treatment was observed.

Atorvastatin and amlodipine

The combination therapy of amlodipine and atorvastatin was tested in APOE*3Leiden transgenic mice, which develop human-like atherosclerosis, by Delsing *et al*[43]. 4 Groups of 15 ApoE*3Leiden mice received a high-cholesterol diet. One group received amlodipine in the diet, which had no effect on plasma cholesterol levels. Another group received atorvastatin, resulting in a decrease of plasma cholesterol by 50% by a reduction in very low density lipoprotein production. The combination group received both amlodipine and atorvastatin. After 28 weeks, atherosclerosis in the aortic root was quantified. Treatment with amlodipine alone had no significant effect on atherosclerotic lesion area, whereas atorvastatin markedly reduced atherosclerosis by 77% compared with the control group. Atorvastatin also reduced inflammation markers. The combination of amlodipine and atorvastatin tended to reduce lesion area by 61% compared with the atorvastatin-only group; this effect just did not reach statistical significance (**Figure 2**). However, after subgroup analysis, the combination therapy did reach significance in animals that respond relatively modestly to atorvastatin. Amlodipine treatment significantly reduced calcification in the lesions, whereas

atorvastatin alone had no effect. The combination of amlodipine and atorvastatin resulted in a near absence of calcium deposits in the lesions. Measurement for Serum Amyloid A resulted in significant lower levels of both in the group receiving both atorvastatin and amlodipine than in the groups receiving no drugs or only one of them. Fibrinogen levels were reduced as compared with the control group, and von Willebrand Factor, a marker for vessel wall integrity, was reduced in the combination group as compared with the group receiving amlodipine alone. Van de Poll *et al*.[55] examined the effects of amlodipine and atorvastatin on advanced atherosclerosis in APOE*3Leiden mice. They showed that the combination treatment of amlodipine and atorvastatin not only reduced lesion size compared to the control group but also reduced the area of existing lesions. The lesion areas of the mice receiving treatment were compared to the lesion area of mice which were sacrificed at the beginning of the experiment. Recently, research by Mason *et al.*[56] demonstrated that the combination of amlodipine and atorvastatin had a synergistic effect on nitric oxide release from human endothelial cells, which in vivo may lead to an improvement of endothelial function.

The ASCOT-LLA study[57] assessed benefits of cholesterol lowering in the primary prevention of coronary heart disease in hypertensive patients who are not conventionally deemed dyslipidemic. Of 19342 hypertensive patients (aged 40-79 years with at least three other cardiovascular risk factors) randomised to one of two antihypertensive regimens in the Anglo-Scandinavian Cardiac Outcomes Trial, 10305 with non-fasting total cholesterol concentrations 6.5 mmol/L or less were randomly assigned additional atorvastatin 10 mg or placebo. These patients, whom at least half were additionally treated with amlodipine, formed the lipid-lowering arm of the study. The primary endpoint was non-fatal myocardial infarction and fatal coronary heart disease. Treatment was stopped after a median follow-up of 3.3 years. A reduction of 36% (p=0.0005) of primary endpoints was seen in the atorvastatin group. This benefit emerged in the first year of follow-up. A 27% reduction of fatal and non-fatal stroke (p=0.024), 21% reduction of total cardiovascular events (p=0.0005), and a 29% reduction of total coronary events (p=0.0005) were also observed. Addition of amlodipine to the atorvastatin treatment may have accounted for these impressive positive effects.

The effects of amlodipine monotherapy and combination therapy of atorvastatin and amlodipine on arterial compliance were investigated on 21 consecutive hypertensive hyperlipidemic patients by Leibovitz *et al.*[58]. Patients were followed every month for 6 months (3 months of amlodipine therapy and 3 months of amlodipine and atorvastatin combination). During the 3 months of amlodipine monotherapy, large and small arterial compliance were improved by 26% and 38%, respectively, and the systemic vascular resistance was reduced by 10%. The addition of atorvastatin during the next 3 months improved small arterial compliance by an additional 42% and decreased the systemic vascular resistance by another 5%, but large arterial compliance

and blood pressure did not change. Thus amlodipine improved large and small arterial compliance, and the beneficial effect of atorvastatin on small arterial compliance was additive to the effect achieved by amlodipine in hypertensive hyperlipidemic patients.

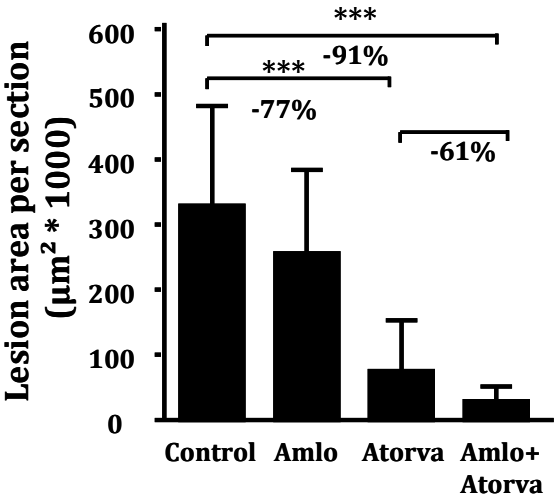

Figure 2 Lesion area per section in the aortic root of APOE*3Leiden mice after treatment with amlodipine, atorvastatin of the combination of both as described by Delsing et al. [42]. Atorvastatin reduced lesion area with 77% as compared with the control group. The combination therapy decreased the lesion area with 91% when compared with the control group and 61% (not significant) when compared to the atorvastatin monotreatment. *** $P < 0.005$.

Conclusion and final remarks

Previous described studies demonstrate the antiatherosclerotic properties of CCBs, especially amlodipine. Of great importance are the anti-oxidative properties[10,26] and the ability to inhibit the aggregation of oxidized LDL particles[56]. Amlodipine was also shown to stabilize plaques and to reduce the ischemic area after myocardial infarction[32]. Statins are used for years to decreased plasma cholesterol levels and thereby reduce atherosclerosis. Huge clinical trails like 4S[39], CARE[40] and LIPID[41] showed that statins reduced myocardial infarction and fatal coronary deaths. Over the last years researchers demonstrated lipid independent antiatherosclerotic properties of statins *e.g.* anti-inflammatory and antioxidative capacities[9,46-51]. It was also angiographically demonstrated that atorvastatin improved plaque stability in humans[6]. High dose treatment of atorvastatin may even be favoured above angioplasty in reducing ischemic cardiac events in patients with stable angina pectoris[52].

Because of the verifiable antiatherosclerotic properties of amlodipine and atorvastatin, it seems a logical step to combine these two types of drugs. This potent combination therapy has now been shown to result in reduced atherosclerosis, by inhibiting the progression of atherosclerosis[24,43] and even reduce lesion size[55], improvement of endothelial function[56] and arterial compliance[58]. Capacities for plaque stabilization and reduction of infarction size are suggested and should be further tested. Outcomes of the ASCOT study[59] are of great interest, since a group of approximately 2500 hypertensive patients is randomised to the combination treatment of atorvastatin

and amlodipine. The lipid lowering arm of the ASCOT study[57] has demonstrated impressive effects of atorvastatin treatment.

These results of obtained from *in vitro*, mice and clinical studies, in which the combination therapy was tested, collectively support the clinical anti-atherosclerotic advantages of combination of CCBs and statins and in particular of atorvastatin with amlodipine beyond their established antihyperlipedemic and antihypertensive modes of action.

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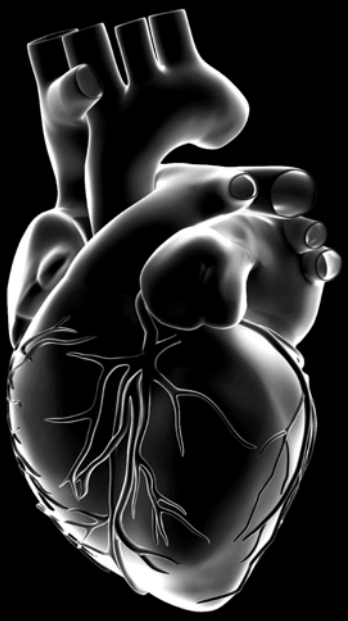

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Niacin Increases HDL by Reducing Hepatic Expression and Plasma Levels of Cholesteryl Ester Transfer Protein in APOE*3Leiden.CETP Mice

Arterioscler Thromb Vasc Biol. 2008, *In Press*

Abstract

Objective: Niacin (nicotinic acid) potently decreases plasma triglycerides and LDL-cholesterol. In addition, niacin is also the most potent HDL-cholesterol increasing drug used in the clinic. In the present study, we aimed at elucidation of the mechanism underlying its HDL-raising effect.

Methods and Results: In APOE*3Leiden transgenic mice expressing the human CETP transgene, niacin dose-dependently decreased plasma triglycerides (up to -77%, $P<0.001$) and total cholesterol (up to -66%, $P<0.001$). At the same time, niacin dose-dependently increased HDL-cholesterol (up to +87%, $P<0.001$), plasma apoAI (up to +72%, $P<0.001$), as well as the HDL particle size. In contrast, in APOE*3Leiden mice that do not express CETP, niacin also decreased total cholesterol and TG but did not increase HDL-cholesterol. In fact, in APOE*3Leiden.CETP mice, niacin dose-dependently decreased the hepatic expression of CETP (up to -88%; $P<0.01$) as well as plasma CETP mass (up to -45%, $P<0.001$) and CETP activity (up to -52%, $P<0.001$). In addition, niacin dose-dependently decreased the clearance of apoAI from plasma and reduced the uptake of apoAI by the kidneys (up to -90%, $P<0.01$).

Conclusion: Niacin markedly increases HDL-cholesterol in APOE*3Leiden.CETP mice by reducing the CETP activity, as related to lower hepatic CETP expression and a reduced plasma (V)LDL pool, and increases HDL-apoAI by decreasing the clearance of apoAI from plasma.

Introduction

Dyslipidemia is an important risk factor for the development of cardiovascular disease (CVD). Although lowering of LDL-cholesterol (C) by e.g. statins reduces CVD risk by approximately 30%, substantial residual cardiovascular risk remains, even with very aggressive reductions in levels of LDL-C.[1-3] Due to clinical studies, which have shown that HDL-C, independently of LDL-C, is inversely correlated with the risk of CVD,[4,5] attention has shifted toward strategies for targeting HDL composition as adjunctive therapy to prevent and treat CVD. Current strategies to mildly increase HDL-C levels include aggressive overall lifestyle modification (*i.e.* exercise, diet, weight loss, and smoking cessation), and modest increases in HDL-C levels are achieved with statins[6] and fibrates (5-10%).[7]

Niacin (nicotinic acid, vitamin B3) has been described to exhibit lipid-modifying capacities already since the 1950s. Since then various (clinical) studies have shown the beneficial effects of niacin on plasma lipid levels. Treatment with niacin alone was associated with a 27% reduction in non-fatal myocardial infarction and it reduced all cause mortality by 11%.[8,9] In combination with colestipol (FATS trial) or simvastatin (HATS trial), niacin reduced cardiac events by as much as 80-90%.[10,11] These potent atherogenic properties of niacin are thought to be due to its marked HDL-elevating effect (+20% to +30%), besides it potent effect on reducing plasma TG (-40% to -50%) and LDL-C (-20%).[7,12] In fact, niacin is currently the most effective therapy for elevating HDL-C.

The mechanism underlying the ability of niacin to reduce the plasma (V)LDL level has been well-studied. By selective binding to GPR109A on adipocytes, niacin suppresses hormone sensitive triglyceride lipase (HSL) activity, resulting in a decreased release of free fatty acids (FFA) from adipose tissue and decreased plasma FFA levels.[13] The resulting reduced supply of FFA towards the liver is believed to bring about a decreased hepatic VLDL-TG production, resulting in reduced VLDL-TG and (V)LDL-C levels.[13,14] In contrast, the mechanism underlying the HDL-C raising effect of niacin has not been elucidated as yet. This is probably related to the lack of suitable animal models that respond in a human-like manner to HDL-raising drug interventions. In wild-type mice and apoE-knockout mice (the classical animal model for hyperlipidemia and atherosclerosis), rats and dogs, niacin only transiently reduced plasma levels of TG but failed to failed to raise HDL-C.[15,16] An HDL-C-elevating effect of niacin has been reported in rabbits, but with 30% ethanol as dosing vehicle and only after 12 weeks of treatment.[17]

Therefore, the aim of this study was to elucidate the mechanism underlying the HDL-C raising effect of niacin. To this end, we used our recently developed APOE*3Leiden (E3L).CETP transgenic mouse model. We have previously demonstrated that E3L mice have a human-like lipoprotein profile in which the elevated plasma cholesterol and TG levels are mainly confined to the (V)LDL-sized lipoprotein

fractions.[18,19] These mice develop atherosclerosis upon dietary cholesterol feeding and respond in a human-like manner to drugs used in the treatment of CVD (e.g. statins, fibrates, cholesterol uptake inhibitors, calcium channel blockers and angiotensin II receptor antagonists[20-23]), but they did not yet respond to HDL-modulating interventions. By cross-breeding E3L mice with mice expressing human CETP under control of its natural flanking regions, E3L.CETP were obtained[24] that respond to the HDL-raising effects of fenofibrate,[25] atorvastatin[26] and torcetrapib.[27] We now fed these mice a Western-type diet without or with increasing doses of niacin to reveal the mechanism underlying its HDL-C raising effect.

Methods

Animals

Hemizygous human CETP transgenic (CETP) mice, expressing a human CETP minigene under the control of its natural flanking sequences[28] were purchased from the Jackson Laboratory (Bar Harbor, ME) and crossbred with hemizygous *E3L* mice[18] at our Institutional Animal Facility to obtain E3L and E3L.CETP littermates.[24] In this study, female mice were used, housed under standard conditions in conventional cages with free access to food and water. At the age of 12 weeks, E3L and E3L.CETP mice were fed a semi-synthetic cholesterol-rich diet, containing 15% (w/w) fat and 0.25% (*E3L*) or 0.1% (E3L.CETP) (w/w) cholesterol (Western-type diet; Hope Farms, Woerden, The Netherlands) for three weeks to obtain similar total cholesterol levels in both strains (about 12-14 mmol/L). After matching based on total plasma cholesterol (TC), triglyceride (TG) levels, and age, mice (n=8 per group) received a Western-type diet without or with 0.03% (~36 mg/kg/day), 0.1% (~118 mg/kg/day), 0.3% (~360 mg/kg/day) or 1% (~1180 mg/kg/day) niacin (Sigma, St. Louis, MO, USA) for at least 3 weeks. These doses correspond well to the doses used in humans, if the 10 times faster metabolism of mice as compared to humans is taken into account. Experiments were performed after 4 h of fasting at 12:00 pm with food withdrawn at 8:00 am, unless indicated otherwise. The institutional Ethical Committee on Animal Care and Experimentation has approved all experiments.

Plasma lipid and lipoprotein analysis

Plasma was obtained via tail vein bleeding as described[24] and assayed for TC, TG and phospholipids (PL), using the commercially available enzymatic kits 236691 and 11488872 (Roche Molecular Biochemicals, Indianapolis, IN, USA) and 'Phospholipids B' (Instruchemie, The Netherlands), respectively. The distribution of lipids over plasma lipoproteins was determined by fast-performance liquid chromatography (FPLC) using a Superose 6 column as described previously.[24] HDL-C was isolated by precipitating the

apoB-containing lipoproteins from 20 µL EDTA plasma by adding 10 µL heparin (LEO Pharma, The Netherlands; 500 U/mL) and 10 µL 0.2 M $MnCl_2$. Mixtures were incubated for 20 min at room temperature and centrifuged for 15 min at 13,000 rpm at 4°C. In the supernatant HDL-C was measured using enzymatic kit 236691 (Roche Molecular Biochemicals, Indianapolis, IN, USA).

Plasma apoAI concentration

Plasma apoAI concentrations were determined using a sandwich ELISA. Here to, rabbit anti-mouse apoAI polyclonal antibody (ab20453; Abcam plc, Cambridge, UK) was coated overnight onto Costar strips (Costar, Inc., New York, NY) (at 3 µg/mL) at 4°C and incubated with diluted mouse plasma (dilution 1:400,000) for 90 min at 37°C. Subsequently, goat anti-mouse apoAI antibody (600-101-196; Rockland Immunochemicals, Inc., Gilbertsville, PA; dilution 1:3000) was added and incubated for 90 min at 37°C. Finally, horse radish peroxidase (HRP)-conjugated rabbit anti-goat IgG antibody (605-4313; Rockland; dilution 1:15000) was added and incubated for 90 min at 37°C. HRP was detected by incubation with tetramethylbenzidine (Organon Teknika, Boxtel, The Netherlands) for 15 min at room temperature. Purified mouse apoAI (A23100m; Biodesign International, Saco, Maine, USA) was used as a standard.

HDL size by native PAGE

The HDL size was determined essentially as described.[29] Total lipoproteins were isolated from plasma by ultracentrifugation (5 h at 541,000 g) as the d < 1.21 g/mL plasma fraction in a TLA 100.3 rotor (Beckman). Lipoproteins (7.5 µg protein) were loaded onto a 4-20% polyacrylamide Tris.HCl gel (BioRad, Hercules, CA, USA) and electrophoresis was performed according to the manufacturer's protocol. Gels were stained with Coomassie Brilliant Blue (Merck) and HDL size was compared with globular protein standards (HMW native marker kit, GE Healthcare).

Plasma lipolysis

Post-heparin plasma from overnight fasted mice was collected from the tail vein at 20 minutes after intraperitoneal injection of heparin (1.0 U/g body weight). Post-heparin plasma triacylglycerol hydrolase activity was determined in the presence or absence of 1 mol/L NaCl to estimate the hepatic lipase (HL) activity, which was calculated as the portion of total triacylglycerol hydrolase activity not inhibited by 1 mol/L NaCl.[30]

Preparation of ^{125}I-apoAI-labeled autologous HDL

ApoAI was radiolabeled at pH 10 with carrier-free ^{125}I according to the ICl method[31], and separated from unbound ^{125}I by Sephadex G50 gel filtration. ^{125}I-apoAI (~75 µg) was incubated with 1.4 mL of plasma from E3L.CETP mice (3 h at 37°C), and ^{125}I-apoAI-HDL

was isolated after density gradient ultracentrifugation. The specific activity was ~15 cpm /ng HDL protein.

In vivo kinetics of ^{125}I-apoAI-labeled HDL

E3L.CETP mice were injected via the tail vein with ^{125}I-apoAI-HDL (40 µg protein) in a total volume of 200 µL PBS. At the indicated time points after injection, blood was collected from the tail vein to determine the plasma decay of ^{125}I-apoAI. The total plasma volumes of the mice were calculated from the equation V (mL) = 0.04706 x body weight (g), as determined from previous ^{125}I-BSA clearance studies.[32] At 6 h after injection, the mice were sacrificed and organs were taken and counted for ^{125}I-activity. Values were corrected for serum radioactivity present in the liver (84.7 µL/g wet weight), kidneys (135.2 µL/g wet weight), skeletal muscle (13.7 µL/g wet weight) and white adipose tissue (16.1 µL/g wet weight).[33]

Hepatic lipid analysis

Liver tissue samples were homogenized in phosphate-buffered saline (approx. 10% wet w/v), and the protein content was measured according to the method of Lowry *et* al. Lipids were extracted, separated by high-performance thin-layer chromatography on silica gel plates and analyzed with TINA2.09 software (Raytest Isotopen Messgeräte, Straubenhardt, Germany), as described before.[34]

Hepatic mRNA expression

Total RNA extraction from liver tissue samples was performed using RNA-Bee (Amsbio, Oxon, UK) according to the manufacturer's instructions. RNA was converted to single-stranded cDNA by a reverse transcription procedure (Promega) according to the manufacturer's protocol using random primers. cDNA levels were measured by real-time polymerase chain reaction (PCR) using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. PCR master mix from Eurogentec was used. Primers and probes were obtained from Biosource (Nivelles, Belgium). The probes were labelled with 3-BHQ1 and 5-FAM or 5-TET. The mRNA levels were normalized to mRNA levels of three housekeeping genes (*i.e.*, cyclophilin, HPRT and GAPDH). Primers and probes used for this study were described previously.[25] The level of mRNA expression for each gene of interest was calculated according to the manufacturer's instructions (Applied Biosystems) as described previously.[35]

CETP mass and activity in plasma

Plasma CETP mass was analyzed by ELISA using kit 'CETP ELISA Daiichi' (Daiichi Pure Chemicals Co, Ltd, Tokyo, Japan). Plasma CETP activity was measured as the transfer of

[^{3}H]cholesteryl oleate ([^{3}H]CO) from exogenous LDL to HDL as described.[36] CETP activity was calculated as µmol CE transfer per mL plasma per hour.

Biliary lipid secretion

The common bile duct of anesthetized mice was ligated, the gall bladder was cannulated, and bile was collected during 90 minutes.[30] Cholesterol, PL and total bile acids in bile were determined using kits '236691' (Roche Molecular Biochemicals, Indianapolis, IN, USA), 'Phospholipids B' (Instruchemie, The Netherlands) and 'Total bile acids assay' (Bio-Stat, UK), respectively.

Fecal excretion of bile acids and neutral sterols

The mice were housed at 3 mice per cage. Feces produced during 2 subsequent periods (48 h each) were separated from the wood shavings by sieving. Aliquots of lyophilized feces were used for determination of neutral and acidic sterol content by gas-liquid-chromatography procedures as described.[30]

Statistical analysis

All data are presented as means ± SD unless indicated otherwise. Data were analyzed parametrically by one-way ANOVA followed by Dunnett to correct for multiple testing. *P*-values less than 0.05 were considered statistically significant. SPSS 14.0 was used for statistical analysis.

Results

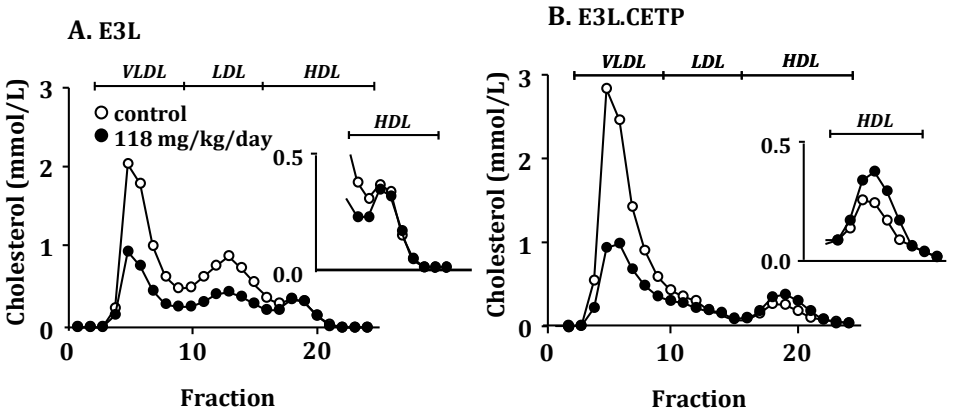

Figure 1. Effect of niacin on lipoprotein profiles. E3L (A) and E3L.CETP (B) mice received a Western-type diet without (open circles) or with (closed circles) niacin (118 mg/kg/day) for 3 weeks. Plasma was pooled per group and the distribution of cholesterol over the individual lipoproteins was determined after separation by FPLC.

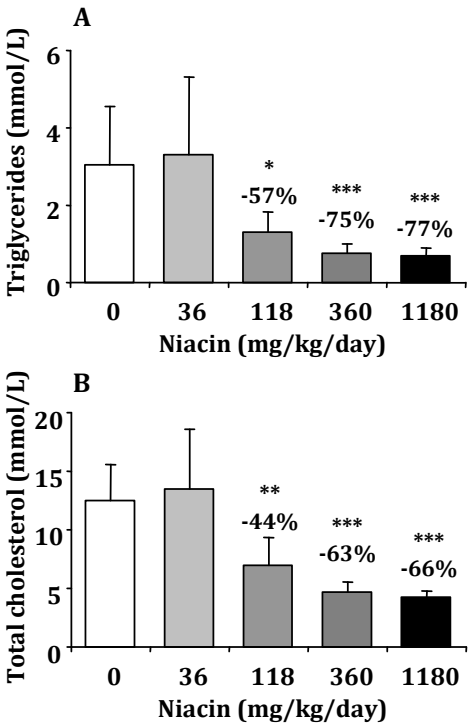

Figure 2. Dose-dependent effect of niacin on plasma triglycerides and total cholesterol. E3L.CETP mice received a Western-type diet without or supplemented with incremental doses of niacin for 3 weeks. Plasma triglycerides (A) and total cholesterol (B) were determined. Values are means ± SD (n=8 per group). **P*<0.05, ***P*<0.01, ****P*<0.001.

Niacin decreases plasma lipids in both E3L and E3L.CETP mice, but increases HDL only in E3L.CETP mice

No adverse clinical signs were observed with increasing dosages of niacin as indicated by absence of differences in weight gain and plasma ALT levels between treatment groups and the control. Treatment of E3L mice with niacin (118 mg/kg/day) caused a sustained reduction in plasma TG by -26% (1.4±0.6 mM vs 1.9±0.6 mM; *P*<0.05) and in plasma TC by -35% (9.2±3.4 mM vs 14.2±4.5 mM; *P*<0.05). Lipoprotein fractionation by FPLC showed that the reduction in cholesterol was confined to the apoB-containing lipoproteins (V)LDL, whereas HDL-C was not affected (**figure 1A**). An equal dose of niacin even more potently reduced plasma TG (-57%, *P*<0.05) and TC (-44%, *P*<0.01) in E3L.CETP mice. As in E3L mice, the TC-decreasing effect of niacin in E3L.CETP mice was caused by a reduction of (V)LDL-C. However, whereas niacin did not affect HDL levels in E3L mice, it increased HDL-C in E3L.CETP mice (**figure 1B**). In E3L.CETP mice, the effects of niacin on plasma TG and TC levels were dose-dependent as shown in **figure 2**. At the highest dose of 1180 mg/kg/day, niacin reduced TG levels by -77% (*P*<0.001) (**figure 2A**) and TC levels by -66% (*P*<0.001) (f**igure 2B**).

The HDL-increasing effect of niacin in E3L.CETP mice is dose-dependent

To investigate whether the HDL-increasing effect of niacin in E3L.CETP mice was also dose-dependent, we determined HDL-C concentrations in whole plasma after precipitation of apoB-containing lipoproteins by heparin/ $MnCl_2$. Indeed, niacin appeared to decrease (V)LDL-C levels up to -79% (*P*<0.001) (f**igure 3A**), and to increase HDL-C up to +87% (*P*<0.001) (**figure 3B**), both in a dose-dependent fashion. We next evaluated whether niacin also affects apoAI, the main apolipoprotein constituent of HDL.

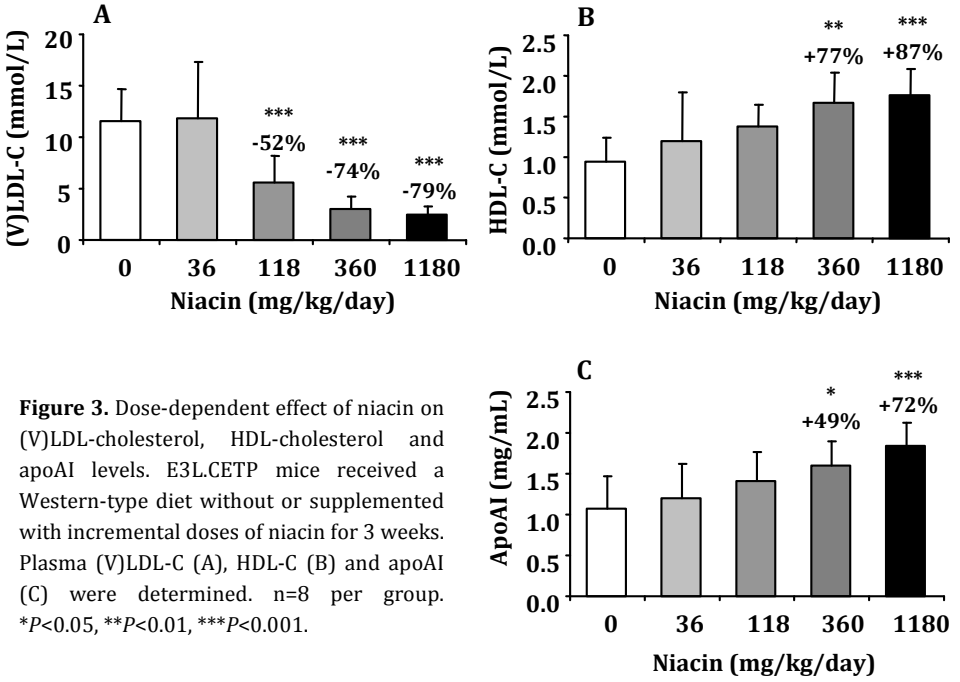

Figure 3. Dose-dependent effect of niacin on (V)LDL-cholesterol, HDL-cholesterol and apoAI levels. E3L.CETP mice received a Western-type diet without or supplemented with incremental doses of niacin for 3 weeks. Plasma (V)LDL-C (A), HDL-C (B) and apoAI (C) were determined. n=8 per group. **P*<0.05, ***P*<0.01, ****P*<0.001.

Indeed, niacin dose-dependently increased apoAI up to +72% (*P*<0.001) (**figure 3C**). Whereas niacin thus increases both HDL-C and apoAI, the effects on HDL-C at the various doses are somewhat more pronounced than on apoAI, suggesting that niacin increases the lipidation of apoAI. This was reflected by a modest increase of the HDL particle size as determined by native PAGE (**figure 4**). Further analyses of the pooled HDL fractions showed a decrease in triglycerides (-45%) and an increase in cholesteryl ester (+56%) and phospholipids (+66%) (data not shown). Niacin did not seem to affect the hepatic synthesis or clearance of HDL, at least judged from unchanged hepatic mRNA expression of genes involved in HDL synthesis (*apoa1*, *abca1*) or clearance (*sr-b1*) (data not shown). Hepatic *pltp* mRNA expression was slightly increased upon niacin treatment (data not shown). In plasma niacin did decrease the HL activity, albeit that the effect was not dose-dependent (maximal reduction of -47% at 118 mg/kg/day; *P*<0.05).

Niacin increases the residence time of apoAI in plasma

To evaluate whether the dose- dependently increased plasma apoAI level as induced by niacin-treatment was caused by decreased clearance of apoAI from plasma, we determined the effect of niacin on the plasma kinetics of intravenously injected ^{125}I-apoAI-labeled HDL (**figure 5**). Indeed, niacin dose-dependently increased the residence of ^{125}I-apoAI in plasma (**figure 5A**). From the mono-exponential decay curves it was calculated that the plasma half-life of ^{125}I-apoAI (3.5±0.1 h) was increased by niacin at 118 mg/kg/day (5.5±1.3 h; *P*<0.01) and 1180 mg/kg/day (6.6±1.3 h; *P*<0.01). This was

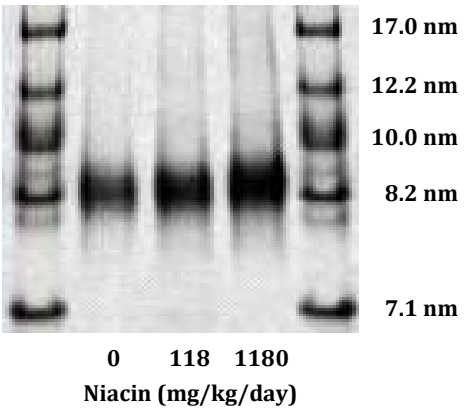

Figure 4. Dose-dependent effect of niacin on the HDL particle size. Total lipoproteins from pooled plasma were subjected to native 4-20% PAGE, and the resulting gel was stained with Coomassie Brilliant Blue.

accompanied by a dose-dependent reduction in the uptake of ^{125}I-activity by the liver (up to -50%; P<0.05) and the kidneys (up to -90%; P<0.01) (**figure 5B**). For comparison, the uptake of [^{3}H]cholesteryl oleoyl ether-labeled HDL by the liver was much larger (approx. 40% of dose/g wet weight), whereas the uptake by the kidneys was undetectable (data not shown).

Niacin reduces the hepatic lipid content

The effects of niacin on plasma lipid metabolism in E3L.CETP mice are consistent with a niacin-induced reduction in CETP activity. Since CETP expression is regulated by the hepatic cholesterol content,[28] we first examined effects of niacin on liver lipids (**figure 6A**). Niacin decreased the hepatic TG content (-38%, P<0.05). This is consistent with the inhibitory effects of niacin on HSL in adipose tissue,[13] thereby reducing the trafficking of FFA to the liver for TG synthesis. Niacin also decreased the hepatic TC content (-21%, P<0.01), which was mainly attributed to a reduction in hepatic cholesteryl esters (-22%, P<0.05). This effect was in line with a compensatory increase in hepatic *Hmgcoared* mRNA expression (+232%, P<0.05) (not shown).

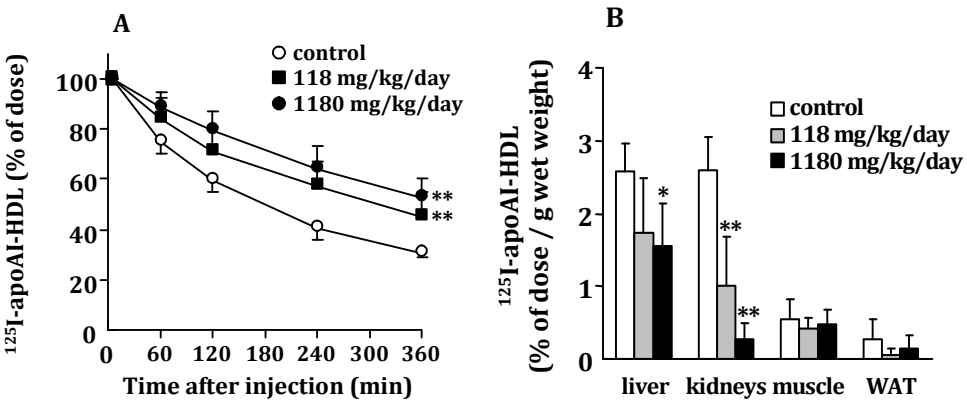

Figure 5. Dose-dependent effect of niacin on plasma apoAI kinetics. E3L.CETP mice were injected with ^{125}I-apoAI-HDL, and plasma ^{125}I activity was determined at the indicated time points (A). After the last time point, mice were sacrificed and ^{125}I activity was determined in the liver, kidneys, skeletal (hindlimb) muscle and white adipose tissue (WAT) (B). n=5 per group. *P<0.05, **P<0.01.

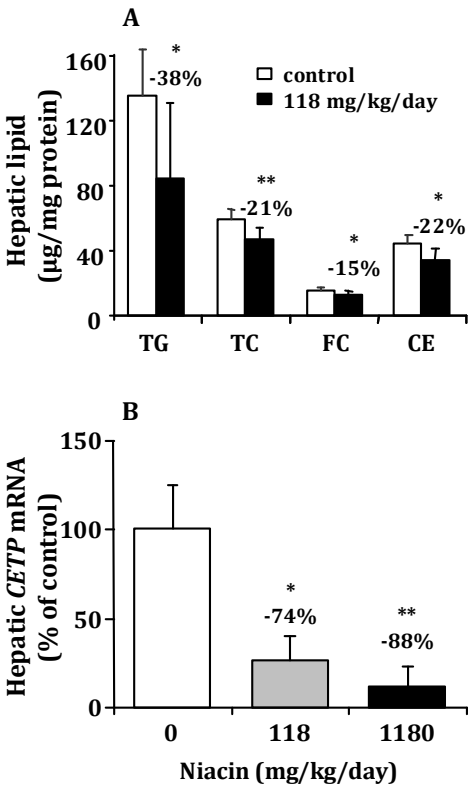

Figure 6. Effect of niacin on hepatic lipid content and *CETP* mRNA expression. E3L.CETP mice received a Western-type diet without (open bars) or with (closed bars) niacin for 3 weeks, and livers were excised. Total lipids were extracted, and triglycerides (TG), total cholesterol (TC), free cholesterol (FC) and cholesteryl esters (CE) were separated by high-performance thin-layer chromatography and quantified (A). Total RNA was extracted, and *CETP* mRNA expression was measured by RT-PCR (B). Values are means ± SD (n=7 per group). **P*<0.05, ***P*<0.01.

Niacin decreases hepatic CETP mRNA expression and plasma CETP levels

The decrease in hepatic cholesterol was indeed accompanied by a dose-dependent reduction in hepatic *CETP* mRNA up to -88% (*P*<0.01) at 1180 mg/kg/day (**figure 6B**). To evaluate whether the niacin-induced decreased hepatic CETP mRNA expression was reflected by reduced CETP levels in plasma, we determined both CETP mass (**figure 7A**) and activity (**figure 7B**). Indeed, niacin dose-dependently decreased plasma CETP mass and CETP activity to a similar extent (up to -45% and -52%; *P*<0.001).

Niacin does not affect biliary and fecal cholesterol output

To evaluate the consequences of the niacin-induced alterations in lipid metabolism on lipid excretion into bile and feces, we determined bile flow, biliary lipids and sterols in stool. Niacin did not affect bile flow or the bile composition (cholesterol, phospholipids and bile acids). The highest dose of niacin (1180 mg/kg/day) did affect the composition of the fecal sterols to some extent, as reflected by a slight non-significant increase in neutral sterols and a decrease in bile acids (-22%; *P*<0.05). However, like the dietary input, total fecal sterol output was not affected by niacin (**table 1**).

Discussion

In this study, we investigated the mechanism(s) underlying the HDL-raising effect of niacin. We demonstrated that CETP plays a crucial role in the niacin-induced increase in plasma HDL-C and apoAI levels in E3L.CETP mice. Niacin reduced CETP dependent transfer of cholesterol from HDL to (V)LDL as related to lower hepatic CETP expression

	Control	Niacin 118 mg/kg/d	Niacin 1180 mg/kg/d
Bile			
Bile flow (µL/min/100g bw)	2.0 ± 0.5	2.0 ± 0.4	2.3 ± 0.7
Bile acid output (nmol/min/100g bw)	50 ± 14	67 ± 18	65 ± 25
Cholesterol output (nmol/min/100g bw)	1.1 ± 0.3	1.1 ± 0.2	1.1 ± 0.2
Phospholipid output (nmol/min/100g bw)	14 ± 3	14 ± 3	16 ± 5
Feces			
Neutral sterols (µmol/100g bw/d)	32.7 ± 2.2	34.8 ± 4.6	36.1 ± 4.5
Bile acids (µmol/100g bw/d)	8.6 ± 1.5	8.4 ± 1.6	6.7 ± 0.9*
Total sterols (µmol/100g bw/d)	41.3 ± 3.1	43.2 ± 5.1	42.7 ± 4.7

Table I. Effect of niacin on biliary and fecal lipid output. *E3L.CETP* mice received a Western-type diet without or supplemented with niacin for 3 weeks. The bile bladder was cannulated, and bile flow and composition were measured during 90 minutes (n=6-7). Feces were collected per cage (3 mice per cage) in two subsequent periods of 48 h each (n=8). Fecal composition was measured by gas-liquid-chromatography and fecal sterol output was calculated. Data are presented as mean ± SD, **P*<0.05.

and a reduced plasma (V)LDL pool. This resulted in an increased lipidation of apoAI, as reflected by an increased HDL particle size, and a reduced uptake of apoAI by the kidneys.

We previously showed that E3L mice are highly susceptible to dietary interventions with respect to modulating plasma lipid levels and that these mice show a human-like response to drug interventions aimed at treatment of CVD (e.g. statins, fibrates, cholesterol uptake inhibitors, calcium channel blockers and angiotensin II receptor antagonists[20-23]) with respect to alterations in the lipoprotein profile and/or atherosclerosis development. This is in sheer contrast with wild-type C57Bl/6 mice and conventional hyperlipidemic mice, such as apoE-deficient or LDL receptor-deficient mice, which show either an adverse response or no response to such interventions.[37] In

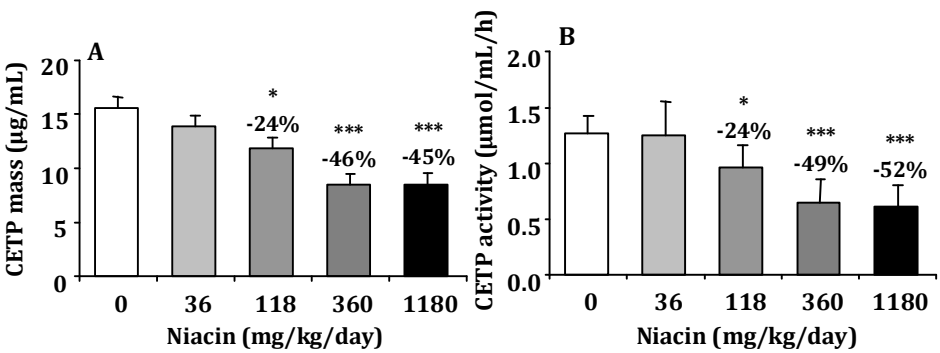

Figure 7. Dose-dependent effect of niacin on plasma CETP mass and activity. E3L.CETP mice received a Western-type diet without or supplemented with incremental doses of niacin for 3 weeks. Plasma CETP mass (A) and CETP activity (B) were determined. Values are means ± SD (n=8 per group). **P*<0.05, ****P*<0.001.

particular, administration of niacin to wild-type mice or apoE-deficient mice did show a transient decrease in plasma TG and FFA levels, but failed to increase plasma HDL-C in these mice.[13,16] Likewise, we now showed that niacin lowered TG and cholesterol within apoB-containing lipoproteins in E3L mice, but did not affect HDL-C levels.

Recently, we showed that introduction of the human CETP gene in *E3L* mice results in a mouse model which also shows a human-like response with regard to raising HDL-C after treatment with fenofibrate,[25] atorvastatin[26] and torcetrapib.[27] Since the introduction of CETP permits cross-talk between (V)LDL and HDL metabolism via the exchange of neutral lipids, we reasoned that the E3L.CETP mouse would also be an excellent mouse model to study the effects of niacin on HDL metabolism.

First, we observed that niacin dose-dependently reduced VLDL-TG and (V)LDL-C levels. The primary action of niacin is inhibition of HSL activity in adipose tissue after binding to the GPR109A receptor that is selectively expressed by adipocytes. This results in a decreased liberation of FFA from adipose tissue, and a decreased flux of albumin-bound FA to the liver, which is required for substrate-driven hepatic TG synthesis and VLDL production.[13] As a consequence we thus observed a concentration-dependent drop in VLDL-TG and (V)LDL-C levels. In addition, we observed that niacin reduced the hepatic cholesterol content. This may be caused by reduced input of cholesterol from plasma into the liver, since plasma (V)LDL-C concentrations are reduced and cholesterol-enriched HDL is formed from which cholesteryl esters are presumably not being delivered efficiently to the liver. The decreased hepatic cholesterol content cannot be explained by differences in biliary sterol output, since the excretion of bile acids and cholesterol remained unchanged. Alternatively, niacin may reduce the endogenous hepatic synthesis of cholesterol.

Second, we showed that niacin dose-dependently raised HDL-C levels in E3L.CETP mice, but not in E3L mice, as paralleled by a less pronounced raise in apoAI. The presence of CETP thus plays a crucial role in the HDL-raising effect of niacin, and we reasoned that niacin may dose-dependently inhibit CETP activity. It is well-known that VLDL-TG is a driving force for CETP activity, and the relative proportions of VLDL and HDL have been shown to play a determinant role in CETP activity. It has been demonstrated that the capacity of apoB-containing lipoproteins to accept CE from HDL is closely correlated with the relative TG content of the lipoprotein acceptor particles.[38-41] By decreasing VLDL levels, niacin may thus reduce CETP activity simply by decreasing the availability of VLDL-TG as substrate for CETP.

Our data corroborate recent observations from Hernandez *et al*.[15,42] who showed that niacin increased HDL-C levels in CETP mice and APOB.CETP mice, but not their CETP-deficient wild-type littermates. In fact, they speculated the reduced VLDL levels to be the main mechanism underlying the HDL-raising effect of niacin. However, we observed that niacin not only reduced plasma CETP activity, but also dose-dependently reduced plasma CETP mass to a similar extent, suggesting that niacin reduces the

synthesis of CETP leading to less CETP protein being released in plasma as reflected by similar reductions in CETP mass and activity. Indeed, niacin dose-dependently reduced hepatic *CETP* mRNA expression. It has been reported that hepatic cholesterol determines the hepatic *CETP* mRNA expression in CETP transgenic mice,[28] presumably via an LXR responsive element in the CETP promoter.[43] Therefore, it is likely that niacin decreases the hepatic CETP mRNA expression as a result of the observed decreased cholesterol content of the liver upon niacin treatment.

Besides increasing HDL-C, niacin also dose-dependently increased plasma apoAI levels. Niacin has been shown to inhibit the uptake of HDL-apoAI (but not HDL-CE) by cultured hepatocytes,[44] which we now confirmed *in vivo*. This may partly contribute to the increased apoAI levels. Such a potential effect of niacin should be independent of GPR109A, since expression of this receptor has not been detected in hepatocytes.[13,45,46] Together with our observations that hepatic mRNA expression of genes involved in HDL synthesis (*apoa1*, *acba1*) and clearance (*sr-b1*) were not affected by niacin, and an increase of PLTP would rather lead to a decrease in HDL-C levels [30,47], it is most likely that the raise in apoAI is explained directly by the niacin-induced decreased CETP activity, which prevents cholesteryl ester transfer from HDL to (V)LDL. This leads to increased lipidation of apoAI, resulting in larger and cholesteryl ester-enriched HDL particles, and thus decreased glomerular filtration and excretion of lipid-poor apoAI via the cubulin/megalin receptor complex.[48] Indeed, we demonstrated a clear dose-dependent reduction in the uptake of ^{125}I-apoAI by the kidney.

Based on our collective data, we thus propose the following mechanism by which niacin reduces TG and (V)LDL-C and concomitantly raises HDL-C, as summarized in **figure 8**. By inhibiting HSL in adipose tissue upon binding of the niacin receptor GPR109A, niacin decreases TG lipolysis and thereby the supply of FFA to the liver, required for lipid synthesis. The consequently reduced hepatic lipid content results in a lower VLDL production and thus lower (V)LDL levels. In addition, reduction in hepatic cholesterol results in reduced hepatic expression of CETP, as well as diminished release of CETP into the plasma. Additionally, HL activity is reduced which may contribute to reduced remodelling of HDL in plasma, resulting in decreased clearance of HDL. The HDL particles become CE enriched, and less lipid-poor apoAI is cleared by the kidney. Niacin thus increases HDL-C and apoAI levels by 1) reducing levels of (V)LDL, the acceptor of CETP-mediated HDL-CE transfer, 2) decreasing CETP expression, 3) decreasing HL activity, and 4) decreasing the clearance of apoAI.

As concluded from a many clinical trials using statins, lowering LDL-C alone is not longer regarded to be sufficient to treat CVD. Therefore, comprehensive lipid management, in which raising HDL-C is an important target, is becoming a new standard.[4,7] Niacin (at dosages of 2-4 g/day) is unsurpassed in raising HDL-C. We show that niacin (in a clinical relevant range if we take into account the 5-10 times faster metabolism of mice) significantly improves the plasma lipid levels in E3L.CETP mice, *e.g.*

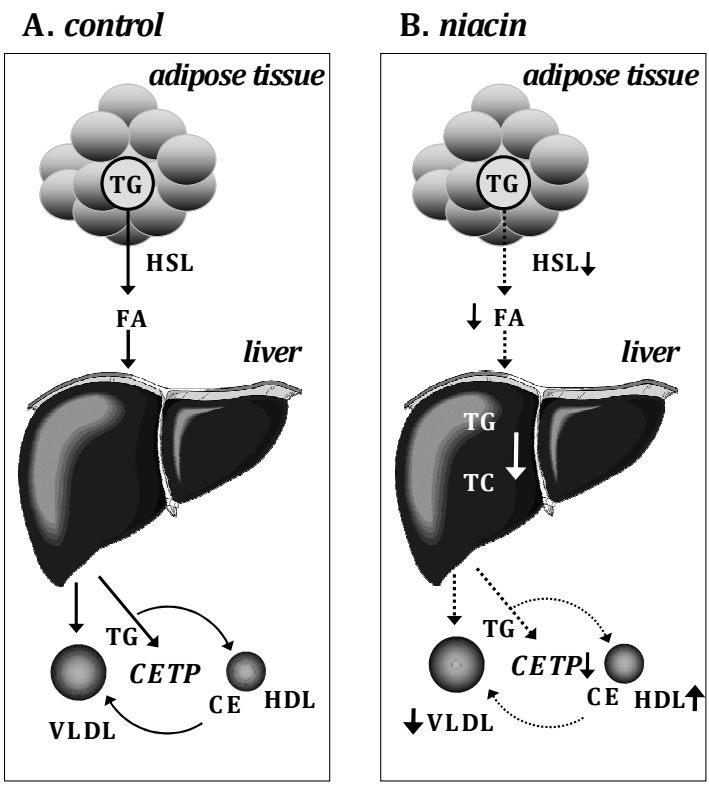

Figure 8. Proposed mechanism underlying the HDL-raising effect of niacin. For explanation see text. CE, cholesteryl ester; FA, fatty acids; HSL, hormone sensitive lipase; TC, total cholesterol; TG, triglycerides.

reduces TG and (V)LDL-C and increases HDL-C, albeit that total fecal sterol output is unaffected. Whether this will lead to improved HDL function and HDL-related reductions in CVD in the clinic still remains to be investigated.

Niacin has not been a very successful drug thus far because of its side-effect: severe flushing. Niacin is nowadays produced as an extended release (ER) compound, which enhances the tolerability. Clinical trails AIM-HIGH[49] and ARBITER-6 (HALTS)[50] evaluating the secondary prevention of CVD by ER niacin treatment are currently running. Post-hoc analysis of a subgroup of ARBITER-2, a randomized, placebo-controlled trial, showed increases in HDL-C upon daily intake of ER niacin (+20%), which were related to reduced progression of carotid intima-media thickness in the setting of both normal glycemic status and diabetes mellitus.[51,52] Since the flushing effects of niacin appeared to be prostaglandin D_2 (PGD_2) receptor mediated,[53] a combination therapy is currently being evaluated combining ER niacin and PGD_2 receptor antagonist laropiprant, which is better tolerated than ER niacin alone.[54] Currently one trail evaluating effects of this combination drug on hard clinical endpoints, as myocardial infarction, stroke or revascularisation (HPS2-THRIVE) is underway.

In conclusion, our results show that niacin increases HDL-C by reducing the hepatic *CETP* expression and plasma CETP protein and CE transfer activity in *E3L.CETP* mice. Therefore, we postulate that reduction of *CETP* expression contributes to the

increase in HDL that is found in human subjects treated with niacin, which should be subject of further investigation.

Acknowledgements
We thank M.E.A. Bekkers, R. van den Hoogen, A. van Nieuwkoop, E.H. Offerman and M. Voskuilen for their excellent technical assistance.

Sources of Funding
This work was supported by the Leiden University Medical Center (Gisela Thier Fellowship to P.C.N.R.), the Netherlands Organization for Scientific Research (NWO grant 908-02-097 and NWO VIDI grant 917.36.351 to P.C.N.R.; NWO grant 903-39-291 to L.M.H.), the Netherlands Heart Foundation (NHS grant 2003B136 to P.C.N.R.), and the Center for Medical Systems Biology (project 115 to L.M.H.). J.W.J. is an established clinical investigator of the Netherlands Heart Foundation (2001 D032).

Disclosures
None.

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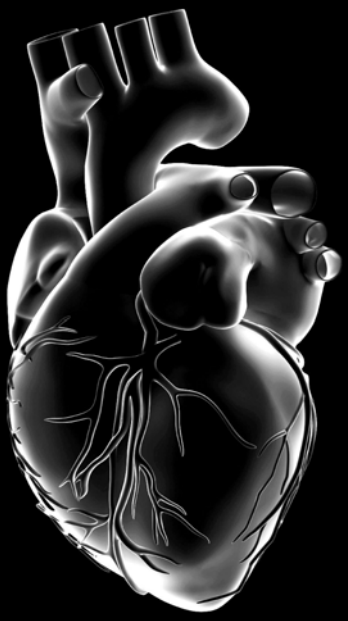

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New cholesterol absorption inhibitor AVE5530 is more effective in preventing atherosclerosis than ezetimibe in APOE*3Leiden mice

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Submitted

Abstract

Objectives The cholesterol uptake inhibitor ezetimibe is currently used as a cholesterol lowering drug mainly in combination with statin therapy. Ezetimibe is nearly 100% absorbed in the intestine and is active on cholesterol transport in macrophages. The consequence of this systemic activity is unclear. The novel cholesterol absorption inhibitor AVE5530 which is being developed for the treatment of dyslipidemia is poorly absorbed in the intestine. The aim of this study was to compare the anti-atherosclerotic activities of AVE5530 and ezetimibe.

Methods and Results APOE*3Leiden mice were fed a cholesterol-raising diet alone or either supplied with AVE5530 or ezetimibe (both 0.3 mg/kg bw/day). Effects on plasma lipids, levels of pro-inflammatory biomarkers and atherosclerosis were assessed after 20 weeks of treatment. AVE5530 and ezetimibe lowered plasma cholesterol as compared to control (-64% and -33%, respectively; $p<0.001$) and both had favorable effects on inflammation markers, as indicated by reduced plasma levels of SAA (by 70% and 69%), MCP-1 (by 38 % and 31%), E-selectin (by 30% and 29%), VCAM-1 (by 24% and 16%, all $P<0.01$, respectively). Additionally, AVE5530 also reduced fibrinogen (by 32%) levels and reduced hepatic cholesterol content (by 69%, $P<0.05$). Ezetimibe did not affect fibrinogen levels and reduced hepatic cholesterol content to a less extent. AVE5530 strongly inhibited atherosclerosis development: it decreased lesion size (by 93%), the number of lesion (by 61%), and additionally improved the quality of lesions. The percentage of severe lesions was also decreased (by 58%) and the undiseased segments were 7-fold increased (all $P<0.001$). Ezetimibe only reduced the quantity of atherosclerosis by decreasing the lesion size by 59% ($P<0.001$), but did not have significant effects on the other three parameters. AVE5530 differed significantly from ezetimibe in all atherosclerosis parameters.

Conclusions We showed that AVE5530 markedly reduced plasma cholesterol levels and therewith decreased the systemic and local vessel wall inflammation. Together these effects resulted in a more potent prevention of atherosclerosis development than ezetimibe.

Introduction

Dyslipidemia is an important risk factor for the development of cardiovascular disease (CVD). Therefore current guidelines to treat CVD emphasize targeting primarily Low-Density Lipoprotein- cholesterol (LDL-C)[1]. Since the introduction of statins, which became the gold standard of cholesterol lowering therapy, a large reduction of plasma LDL-C levels can be achieved[2]. However, there is still an important percentage of patients who do not reach their treatment goals or are statin intolerant[3]. Therefore, other lipid lowering drugs, used alone or in combination, are of great clinical significance.

Ezetimibe is the first of a new class of selective cholesterol absorption inhibitors[4]. Ezetimibe or rather its phenolic glucuronide selectively inhibit cholesterol absorption in the intestine at the brush border membranes of small intestine enterocytes, confining the cholesterol to the intestinal lumen for subsequent excretion[5,6]. The working mechanism of ezetimibe has been investigated extensively, and both Niemann–Pick 1 like 1 protein (NPC1L1) as well as aminopeptidase N (or CD13), both expressed in a.o. the intestine and macrophage, were suggested to be its molecular targets[6,7]. Ezetimibe is rapidly and completely absorbed and metabolized in the intestine and liver to its phenolic glucuronide, then it is excreted into the bile and delivered back to its site of action[8]. The compound has been shown to reduce atherosclerosis development in apoE knock-out mice[9]. Clinical trials have demonstrated the lipid-lowering properties of ezetimibe as a single agent and its additive cholesterol-lowering effects when combined with a statin[10,11]. However, unexpected and still unexplained were the results of the recently published ENHANCE trial, in which patients with familial hyperlipidemia were treated with simvastatin alone or in combination with ezetimibe. Despite lower levels of LDL-C and C reactive protein with the combination therapy, no additional protective effects in retarding on intima-media thickness were measured[12].

AVE5530 is a new cholesterol absorption inhibitor, which, in contrast to ezetimibe, is very poorly absorbed[13]. AVE5530 has similar targets as ezetimibe[7], and lowers LDL cholesterol in a similar manner, however, it is not systemically available[13].

The aim of the present study was to compare AVE5530 with ezetimibe regarding their effects on plasma lipid levels and atherosclerosis development. To this end, we used female APOE*3Leiden transgenic mice, which are a well-established mouse model for hyperlipidemia and atherosclerosis[14]. These mice have a lipoprotein profile similar to the profile of patients with familial dysbetalipoproteinemia in which the elevated plasma cholesterol and triglyceride levels are mainly confined to the VLDL/LDL-sized lipoprotein fraction. In addition, in contrast to other mouse models for dyslipidemia and/or atherosclerosis[15], these mice respond in a human-like manner to treatment of CVD (e.g. statins, cholesterol uptake inhibitors, calcium channel blockers, fibrates and, angiotensin II receptor antagonists[16-23]).

Methods

Mice and treatments

Female heterozygous APOE*3Leiden transgenic mice (11 to 16 weeks of age), characterized by ELISA for human apoE[14], were used. During a 3 week run-in period, all animals received a semi-synthetic western-type diet (WTD) containing 40.5% sucrose, 15% cacao butter and 0.75% (w/w) cholesterol. After matching into 3 groups, based on age, plasma cholesterol and triglyceride levels, the mice received WTD diet either alone (control group) or supplemented with either ezetimibe (0.3 mg/kg bw/day) or with AVE5530 (0.3 mg/kg bw/day). Both compounds were provided by Sanofi Aventis Deutschland GmbH. EDTA blood was drawn at week 2, 4, 9, 12, 16 and 20 of the study, and was assayed for lipids. After 20 weeks, mice were sacrificed and the hearts and livers were isolated to assess atherosclerosis and hepatic cholesterol content. The animals received food and water *ad libitum*. Body weight and food intake were monitored during the study. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Scientific Research (TNO). Animals were bred by TNO.

Lipid and lipoprotein analysis and plasma inflammation markers

After a 4-hour fasting period from 9 a.m. to 1 p.m., EDTA plasma was collected (Sarstedt, Nümbrecht, Germany) and lipoproteins were separated by FPLC[24]. Total hepatic cholesterol content was determined after homogenization of the liver tissue[25]. Total cholesterol (TC) (No-1489437, Roche Diagnostics, USA) and triglyceride (TG) (1488872, Roche Diagnostics, USA), levels were measured. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically using a Reflotron system (Roche Diagnostics, USA). Fibrinogen (FBG) (as described before [18]), serum amyloid A (SAA) (Biosource International, Belgium), monocyte chemoattractant protein (MCP)-1, endothelial (E) -selectin, vascular cell adhesion molecule (VCAM)-1 (all R&D systems Inc, USA), were determined by ELISA.

Histological assessment of atherosclerosis

After the 20-week treatment period, the mice were sacrificed. The hearts with aortic root were dissected, formalin fixed and embedded in paraffin. Serial cross sections (5 µm thick, spaced 50 µm apart) throughout the entire aortic valve area were used for histological analysis. Sections were stained with haematoxylin-phloxine-saffron (HPS). Per mouse, 4 sections with intervals of 50 µm were used for quantification and qualification of the atherosclerotic lesions. For determination of severity of atherosclerosis, the lesions were classified into 5 categories as described before[17,18,20] I) early fatty streak, II) regular fatty streak, III) mild lesion, IV) moderate lesion, V) severe

lesion. Per mouse the percentages of all lesions found in the respective categories were calculated. The total lesion area was calculated per cross-section. In each segment used for lesion qualification the number of monocytes adhering to the endothelium was counted and the macrophage area was measured after immunostaining with AIA31240 (1:3000, Accurate Chemical and Scientific, USA). Smooth muscle cells (SMCs) were immunostained with mouse anti-human actin (DAKO, Denmark), which cross reacts with mouse actin. The collagen was stained by a Sirius Red staining. The lesion content of the different compounds was quantified morphometrically. All analyses were performed by the same operator, who was blinded for experimental group allocation.

Statistical analysis

Data are presented as means ± SD unless indicated otherwise. Statistical differences were assessed using the non-parametrical Kruskal-Wallis test followed by Mann Whitney U test. $P<0.05$ was considered significant. In tables and figures: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Results

Effect of AVE5530 and ezetimibe on plasma lipid levels

Treatment with AVE5530 or ezetimibe did not affect food intake or body weight gain. As presented in **figure 1** feeding the Western type-diet induced hyperlipidemia in the mice, giving plasma cholesterol levels of 17.5 ± 4.1 mmol/L and triglyceride levels of 3.0 ± 0.9 mmol/L at the start of treatment (week 0). Inhibiting the cholesterol uptake by AVE5530 resulted in a strong reduction of plasma cholesterol after two weeks and a 64% ($p<0.001$) reduction at the end of the study and decreased plasma triglycerides by 20% ($p<0.05$) at the later time points in the study. Ezetimibe was less potent as compared to AVE5530 treatment resulting in a 33% ($p<0.001$) reduction of plasma cholesterol levels without affecting the triglycerides. The reductions in plasma cholesterol upon AVE5530 and ezetimibe treatment were confined to apoB-containing lipoproteins as measured after plasma separation by FPLC (**figure 1C**).

		Control			AVE			reduction	EZE			reduction
FBG	mg/ml	2.9	±	1.1	2.0	±	0.6	-32%*	2.4	±	0.8	
SAA	µg/ml	9.2	±	5.3	2.8	±	2.6	-70%***	2.8	±	1.8	-69%***
MCP-1	pg/ml	162.8	±	51.8	100.8	±	36.4	-38%**	111.9	±	44.4	-31%**
E-Selectin	ng/ml	64.0	±	13.9	44.9	±	4.2	-30%***	45.1	±	13.2	-29%**
V-CAM	µg/ml	3.1	±	0.4	2.4	±	0.3	-24%***	2.6	±	0.6	-16%**

Table 1. The effect of AVE5530 and ezetimibe (EZE) on plasma inflammation markers. The parameters were measured at the end of the study after a 20 week treatment period. Values are means ± SD (n=15-16 per group). *$p<0.05$,**$p<0.01$ ***$p<0.001$ as compared to control.

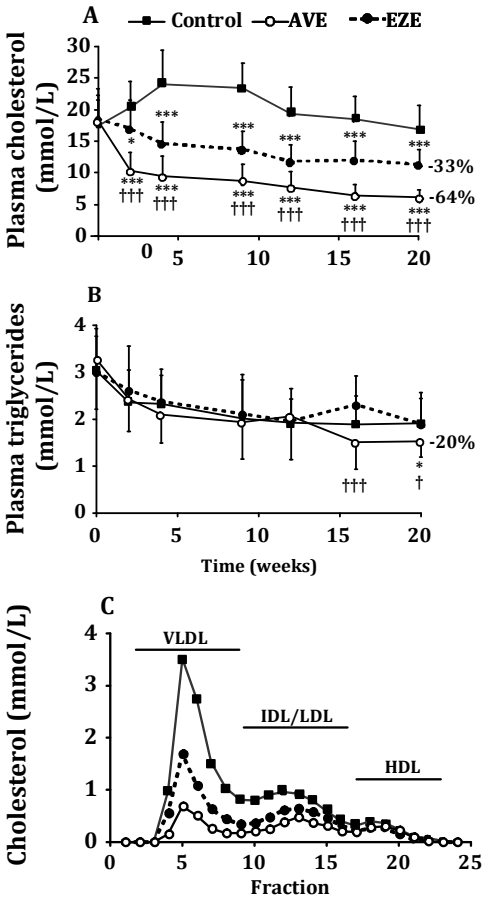

Figure 1. The effect of AVE5530 (AVE) and ezetimibe (EZE) on plasma lipid levels. The plasma cholesterol (A) and triglyceride levels (B) were measured throughout the study. After 16 weeks of treatment the lipoproteins were separated by FPLC and the cholesterol was measured in the fractions (C). *P<0.05, ***P<0.001 as compared to control and †P<0.05, †††P<0.001 as compared to ezetimibe.

Effect of AVE5530 and ezetimibe on liver and inflammatory parameters

As elevated cholesterol has an important impact on liver condition and plays a major role in enhancing inflammation[17,26], we measured hepatic cholesterol content and also plasma markers for systemic and vessel wall inflammation (**table 1)**. The relative liver weights (as percentage of body weight) tended to be reduced in the animals treated with AVE5530 (5.6 ± 1.1% vs 6.2 ± 0.9% in the control group, P=0.1 Kruskall-Wallis followed by P=0.03 Mann-Whitney), which was not observed in the ezetimibe treated group (5.9 ± 1.1%, N.S.). Concomitantly AVE5530 significantly reduced the cholesterol content of the liver by 69% (17.7 ± 5.5 vs 57.6 ± 5.5 mg/ g liver in the control group, P<0.001). Ezetimibe decreased liver cholesterol content by only 35% (to 37.2 ± 12.8 mg/ g liver, P<0.001), which was significantly less than the reduction observed after AVE5530 treatment (P<0.001). Plasma ALT and AST levels were not affected by both treatments. Additionally, treatment with AVE5530 resulted in reduced levels of liver derived acute phase proteins SAA (by 70%, P<0.001) and FBG (by 32%, P<0.05), chemokine MCP-1 (by 38%, P<0.01), and vessel wall inflammation markers E-selectin and VCAM-1 (by 30% and 24%, both P<0.01, respectively) as compared to control. Treatment with ezetimibe brought about similar reduction of inflammation markers as AVE5530, however, without affecting FBG as compared to control.

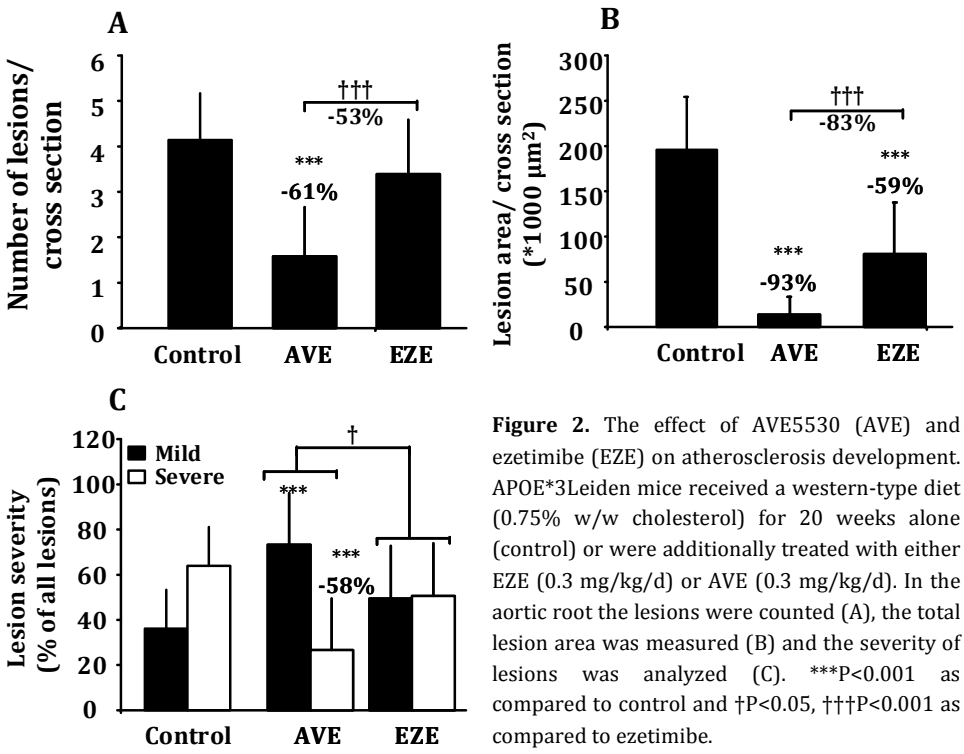

Figure 2. The effect of AVE5530 (AVE) and ezetimibe (EZE) on atherosclerosis development. APOE*3Leiden mice received a western-type diet (0.75% w/w cholesterol) for 20 weeks alone (control) or were additionally treated with either EZE (0.3 mg/kg/d) or AVE (0.3 mg/kg/d). In the aortic root the lesions were counted (A), the total lesion area was measured (B) and the severity of lesions was analyzed (C). ***P<0.001 as compared to control and †P<0.05, †††P<0.001 as compared to ezetimibe.

Effect AVE5530 and ezetimibe on atherosclerosis development

To determine the effect of AVE5530 and ezetimibe treatment on atherosclerosis development we measured the amount of lesions, the lesion area and we qualified the severity of these lesions in the aortic root (**figure 2)**. The control group had on average 4.1 ± 1.0 lesions per cross section in the aortic root with a total area of 196 ± 59 *1000 µm^2; 64 ± 17% of these lesions were severe type IV-V lesions. AVE5530 strongly reduced lesion number by 61% (1.6 ± 1.1, P<0.001), the lesion area by 93% (14 ± 19 *1000 µm^2, P<0.001) and the percentage of severe lesions (down to 27 ± 23%, P<0.001) as compared to control. AVE5530 at the same dosage was more potent in preventing atherosclerosis development than ezetimibe. This was reflected by an absence in reduction of the amount of lesions in the ezetimibe group, whereas the decrease in total lesion area was less as compared to the AVE5530 (to -59%, 80 ± 58 *1000 µm^2, P<0.001). Also the lesion severity was not affected by ezetimibe treatment. For all atherosclerosis parameters AVE5530 had significantly stronger effects than ezetimibe treatment. Representative pictures of the lesions are shown in **figure 3**. In line with these latter results we observed relatively more undiseased segments after either treatment, whereby again AVE5530 was more potent than ezetimibe (from 7 ± 15% in the control up to 54 ± 23% in the AVE5530 group and to 21 ± 25% in the ezetimibe group, P<0.001, **figure 4**).

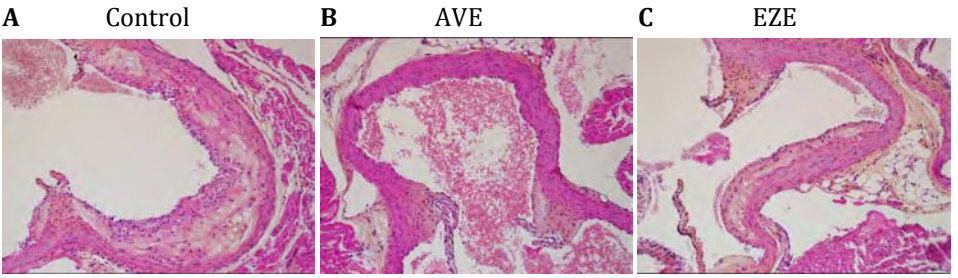

Figure 3. After 20 weeks of intervention the amount of atherosclerosis in the aortic root was measured. Representative pictures of atherosclerotic lesions of the control group (A), AVE5530 (AVE) (B) and ezetimibe (EZE) (C) are presented.

To assess the vulnerability to rupture of the lesions we measured the amount of collagen and SMCs, which can be considered to stabilize the lesions, and the amount of macrophages, known to be a destabilizing factor, in the lesions (**figure 5A**). 40 ± 13% of the lesion content in the control group was collagen. Treatment with AVE5530 or ezetimibe resulted in an equal increase in the collagen content to 65 ± 20% and 68 ± 11%, respectively (P<0.001). No change in the amount of SMCs was found (control 1.2 ± 1%). The macrophage content of the lesions in the control group was 30 ± 11%. Treatment with AVE5530 reduced this amount to 14 ± 14% (P<0.01). Ezetimibe was comparably effective and decreased it to 21 ± 9% (P<0.05). AVE5530 thereby tended to be more potent in reducing the macrophage content than ezetimibe (P=0.077).

As both the macrophage content of the lesions and the plasma markers for vessel wall inflammation were reduced by both the treatments, we also investigated the amount of monocytes adhering to the activated endothelium of the aortic root, which is considered as the first step in lesion development and a functional parameter for the extent of vessel wall inflammation. In the control group on average 7.9 ± 4.1 monocytes per cross section adhered to the activated endothelium (**figure 5B**). This was 75% reduced upon AVE5530 treatment (to 2.0 ± 1.1, P<0.001), while ezetimibe inhibited adhesion of monocytes less effectively, but still noteworthy by 35% (to 5.1 ± 2.8, P<0.05). Also for this parameter AVE5530 had significantly more effect than ezetimibe (P<0.001).

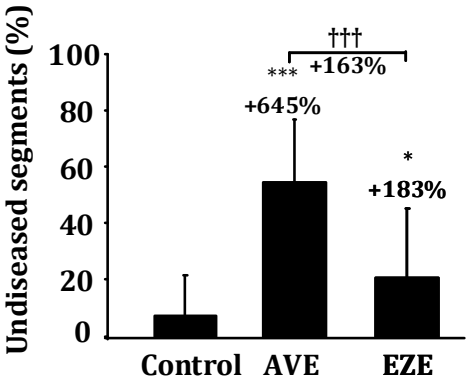

Figure 4. The effect of AVE5530 (AVE) and ezetimibe (EZE) on the percentage of undiseased (healthy) segments in the aortic root. *P<0.05, *** P<0.001 as compared to control and †††P<0.001 as compared to ezetimibe.

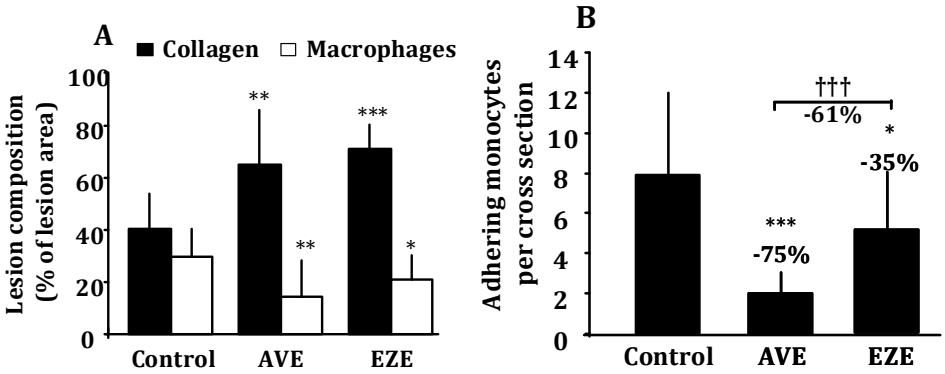

Figure 5. The effect of AVE5530 (AVE) and ezetimibe (EZE) the vessel wall. The lesion composition was analyzed by measuring the collagen content and the macrophage content (A). In the aortic root the number of adhering monocytes was counted per cross section (B). *P<0.05, ** P<0.01, *** P<0.001 as compared to control and †††P<0.001 as compared to ezetimibe.

Discussion

In this study we showed that the new low-absorbable, non-systemically acting cholesterol absorption inhibitor AVE5530 is more potent in cholesterol lowering than ezetimibe, resulting in less hepatic and vascular inflammation and in a stronger prevention of atherosclerosis development in APOE*3Leiden transgenic mice.

The present study was designed to investigate the efficacy of AVE5530 to reduce plasma lipid levels and to inhibit the development of atherosclerosis as compared to ezetimibe. To this end we used APOE*3Leiden transgenic mice, which are highly susceptible to dietary and pharmacological interventions with respect to modulating plasma lipid levels. Moreover, APOE*3Leiden mice show a human-like response to interventions aimed at treatment of CVD (*e.g.* statins, cholesterol uptake inhibitors, fibrates, calcium channel blockers and angiotensin II receptor antagonists [17-23]) with respect to alterations in the lipoprotein profile and/or atherosclerosis development at clinically relevant dosages. AVE5530 and ezetimibe both reduced plasma cholesterol; however, at an equal dose AVE5530 was markedly more effective than ezetimibe. Inhibition of cholesterol absorption with AVE5530 also resulted in reduced plasma triglycerides at the later time points, which was not observed in the ezetimibe treated animals. The reductions in cholesterol were mainly confined to the apoB-containing lipoproteins, similarly as observed previously in APOE*3Leiden mice on a low cholesterol diet and after treatment with statin, fibrates and ACAT inhibitors[17,20,21,26].

There is ample evidence that hypercholesterolemia (*i.e.* elevated plasma levels of (V)LDL) is a major causative factor in atherogenesis, but that an inflammatory component, thought to drive the initiation and progression of the disease, is also

required[27,28]. It has been shown that hypercholesterolemia and inflammation are not separate factors in diet induced hypercholesterolemia in APOE*3Leiden mice, but closely related features of the same trigger, hepatic cholesterol content[26]. We showed that inhibition of cholesterol absorption with either AVE5530 or ezetimibe reduced circulating inflammation markers mostly to the same extent. Difference was found for the liver derived acute phase protein fibrinogen, which was only reduced after AVE5530 treatment. Additionally AVE5530 strongly reduced the cholesterol content of the liver and was therewith significantly more potent than ezetimibe. Both hepatic cholesterol content and inflammation are considered as indirect measures for the amount of cholesterol absorption[26,29]. Therefore, it is likely that the strong anti-inflammatory effects of the two cholesterol absorption inhibitors are secondary to their cholesterol lowering properties. Similar anti-inflammatory and liver protective effects were observed previously in APOE*3Leiden mice treated with sphingolipids, which protect the liver from fat- and cholesterol induced steatosis[22]. Cholesterol and inflammation activate the endothelium of the vessel wall, which in turn expresses adhesion molecules facilitating the adherence and infiltration of monocytes, which is considered to be the first step in lesion development[27]. Therefore we investigated the effect of both treatments on a functional parameter of vessel wall inflammation: monocyte adherence. Whereas we did not observe differences in circulating levels of vessel wall derived inflammation markers (*i.e.* E-selectin and VCAM-1), we found less monocyte adherence to and macrophage accumulation in the arterial wall, both indicative for reduced local inflammation in the vessel wall.

Atherosclerosis development was strongly prevented by both AVE5530 and ezetimibe treatment, in line with previous reports on the anti-atherosclerotic effects of ezetimibe in apoE knock-out and apoE/eNOS double knock-out mice[9,30]. However, while AVE5530 reduced lesion area very potently (-93%, $P<0.001$) and additionally reduced the lesion number and prevented the progression of lesion severity, the effect of ezetimibe was restricted to a reduction of the lesion area (-59%, $P<0.001$) as compared to control. At an equal dose ezetimibe was markedly less efficacious as compared to AVE5530 for most atherosclerosis related read-out parameters. The reason for this marked difference in atherosclerosis development between AVE5530 and ezetimibe is likely related to the difference in potency to lower plasma cholesterol levels. However, an additional explanation may be provided by recent reports showing that NPC1L1 is not only present in the intestinal epithelial cells, but also in the liver and in monocytes and macrophages[31-33]. Herein is the protein involved in transport of cholesterol and modified lipoproteins. In macrophages in cell culture ezetimibe blocked the uptake of oxidized LDL and repressed the induction of cholesterol transporter genes ABCA1, ABCG1 and apoE[31]. Moreover, the drug has been reported to interfere with raft assembly in monocytes and to reduce the surface expression of raft-associated proteins involved in the cellular uptake of modified lipoproteins or phagocytosis[32]. What this

consequently means for the macrophage function *in vivo* remains unanswered, however, it can be hypothesized that macrophage activation, differentiation and efflux capacity are at least influenced.

Since its approval by the FDA in October 2002 many studies have confirmed the effective cholesterol lowering capacity of ezetimibe, especially when being co-administered with other lipid lowering drugs[34]. Surprisingly, the results of the ENHANCE trial[35] showed no benefit of ezetimibe/statin treatment over statin alone on intima media thickening, a surrogate endpoint for CVD, in patients with familial hypercholesterolemia, despite of a stronger decrease in LDL-cholesterol in the co-treated group. In fact, a worsening of intima media thickening in the group treated with the combination of drugs was found. Whether this also translates into differences in hard clinical endpoints, need to be investigated, *e.g.* in the currently ongoing larger IMPROVE-IT trial[36]. Also long-term safety data for ezetimibe have not been established yet. Generally ezetimibe is well tolerated, demonstrating a favorable safety profile [37]. Side effects have been infrequently reported, mainly in combination treatment with statins, concerning myopathy, liver damage and pancreatitis[38]. This might hypothetically be a consequence of the similar metabolism of the two compounds, as both statins and ezetimibe are glucuronidated by the uridine 5'-diphosphate glucuronosyltransferase isoenzymes[39,40]. However, more research is required to elucidate the consequence of the systemic availability and metabolism of ezetimibe, *e.g.* also whether this drug is active on cholesterol transport in macrophages *in vivo*[31,32]. Since AVE5530, in contrast to ezetimibe, is barely systemically available, it may have less adverse side effects in clinical use. This, however, remains to be investigated in clinical studies.

In conclusion, we showed that the new low-absorbable, non-systemically acting cholesterol absorption inhibitor AVE5530 is more effective in lowering plasma lipid levels and development of atherosclerosis than ezetimibe in APOE*3Leiden mice.

Acknowledgements

We thank Erik Offerman and Anita van Nieuwkoop (TNO-BioSciences, Leiden, The Netherlands) for their excellent technical assistance. This study was sponsored in part by a grant from Sanofi-Aventis Deutschland GmbH.

Disclosures

H.O.H. and H.L.S. are employees of Sanofi-Aventis DeutschlandGmbH, Frankfurt am Main, Germany.

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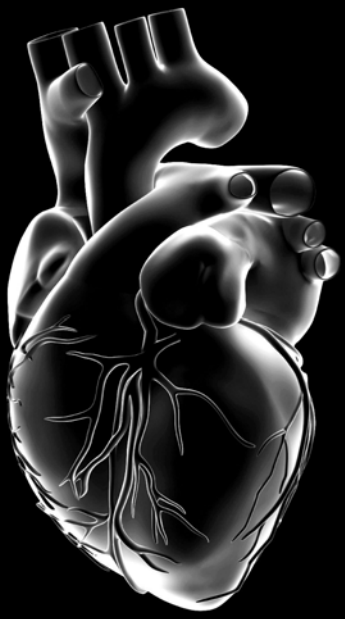

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Olmesartan and Pravastatin Additively Reduce Development of Atherosclerosis in APOE*3Leiden transgenic mice

J Hypertens . 2007 Dec;25(12):2454-62

Abstract

Objective This study was designed to investigate the effect of the angiotensin II receptor blocker olmesartan alone, or in combination with standard treatment with a statin, pravastatin, on atherosclerosis development in APOE*3Leiden transgenic mice.

Methods and Results Four groups of 15 mice received an atherogenic diet alone (plasma cholesterol 17.4 ±2.7 mM) or supplemented with either 0.008% (w/w) olmesartan (9.3 mg/kg/d) (plasma cholesterol 16.4 ±3.9 mM), 0.03% (w/w) pravastatin (35 mg/kg/d) (plasma cholesterol 14.6 ±2.6 mM), or the combination of both (plasma cholesterol 14.5 ±2.9 mM) for six months. Treatment with olmesartan or pravastatin reduced the development of atherosclerosis as compared to the control group (-46% and -39%, respectively). Pravastatin also reduced the severity of the lesions. As compared to control the combination of both treatments almost fully prevented atherosclerosis (-91%, $p<0.001$) and strongly reduced lesion number (-69%), lesion severity (-79%), number of macrophages (-89%) and T lymphocytes (-86%) per cross-section. Treatment with olmesartan alone and in combination with pravastatin inhibited the adhesion of monocytes to the vessel wall (-22%; $p<0.05$ and -25%; $p<0.01$, respectively), and reduced the relative quantity of macrophages in the lesions (-38%; $p<0.05$ and -26%; N.S., respectively) as compared to control.

Conclusion Olmesartan reduced atherosclerosis development mainly by decreasing monocyte adhesion and the relative amount of macrophages, whereas pravastatin inhibited the progression of atherosclerosis to more advanced lesions, reflecting different anti-atherosclerotic modes of action of the two drugs. Combination therapy with olmesartan and pravastatin additively reduced atherosclerosis development, resulting in less and less severe lesions.

Introduction

Atherosclerosis is a complex disease in which foam cell formation and vascular remodeling, next to oxidation and inflammation, play an important role[1]. Since atherosclerosis is considered to be a multifactorial disease, there is broad consensus that medical treatment should have different approaches. Cholesterol accumulation in macrophages, which leads to foam cell formation, is a crucial stage in the development of atherosclerotic lesions. Therefore, reduction of high plasma cholesterol appears to be the first choice approach for medical treatment in preventing atherosclerosis development. Reduction of plasma cholesterol levels by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, is a widely used therapy in primary and secondary prevention of cardiovascular disease[2]. Angiographic clinical trials, like REGRESS[3] and MAAS[4], clearly demonstrate that statins significantly reduce progression of coronary atherosclerosis and decrease the occurrence of new cardiovascular events in patients with coronary artery disease. Large clinical trials like CARE[5], WOSCOPS[6], LIPID[7] have shown significant benefit from pravastatin in both primary and secondary prevention of coronary events. There is growing evidence that statins, independent of their cholesterol lowering capacities, have anti-inflammatory activity as well[8-10].

Angiotensin II, the major effector molecule in the Renin-Angiotensin-System (RAS), is known to play a pivotal role in the regulation of blood pressure and electrolyte homeostasis. Besides its vasoconstrictive effect by binding to the angiotensin II type I receptors (AT1) on vascular smooth muscle cells (VSMCs), angiotensin II has proinflammatory actions by stimulating the production of cytokines and of reactive oxygen species. These can activate nuclear factor-κB (NF-κB) resulting in its translocation into the nucleus where it regulates the transcription of genes encoding for cytokines, chemokines and adhesion molecules, which are all involved in the recruitment of monocytes/macrophages and leukocytes to sites of inflammation in the vascular wall[11,12]. It is known that angiotensin II plays an important role in the development of atherosclerosis[13,14]. Strong links between hypercholesterolemia and the production and expression of angiotensin II and AT1 have been described[15,16]. Clinical intervention studies like SAVE[17] and SOLVD[18] with angiotensin-converting enzyme (ACE) inhibitors and ELITE[19] with an angiotensin II receptor blocker (ARB), showed a reduction in myocardial infarction and sudden cardiac death. In clinical studies it was observed that olmesartan is a very potent anti-hypertensive drug with minimal adverse effects[20]. Olmesartan also significantly reduced vascular microinflammation in patients with essential hypertension[21].

The purpose of this study was to investigate whether the angiotensin II receptor blocker (ARB) olmesartan has additional or synergistic anti-atherosclerotic effects, when it is used together with the HMG-CoA reductase inhibitor pravastatin in APOE*3Leiden transgenic mice. APOE*3Leiden mice are a well-established model for

hyperlipidemia and atherosclerosis[9,22,23]. The mice have a human-like lipoprotein profile in which, upon feeding a cholesterol-containing diet, elevated plasma cholesterol and triglyceride levels are mainly confined to the VLDL/LDL-sized lipoprotein fraction. In contrast to other mouse models for hyperlipidemia, i.e. LDL receptor deficient[24] and ApoE deficient mice[25], APOE*3Leiden mice have relatively mildly increased plasma cholesterol levels, and respond well to statin treatment by reduction of both the apoB-containing lipoproteins and atherosclerosis. In this mouse model the human atherosclerotic situation can be mimicked both with regard to the development of atherosclerosis as well as to the response on therapy[9,22,26-28].

Methods

Mice

Female heterozygous APOE*3Leiden transgenic mice (16 to 18 weeks of age), characterized by ELISA for human apoE[23], were used. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Scientific Research (TNO). Animals were bred by TNO.

Diets

During a 3 week run-in period, all animals received a semi-synthetic high fat cholesterol diet (HFC) containing 40.5% sucrose, 15% cacao butter and 0.5% (w/w) cholesterol. After randomization into 4 groups on the basis of age, body weight, plasma cholesterol and triglyceride levels, the mice received HFC diet alone (control group) or supplemented with either 0.008% (w/w) olmesartan (9.3 mg/kg/d), with 0.03% (w/w) pravastatin (35 mg/kg/d), or 0.008% (w/w) olmesartan plus 0.03% (w/w) pravastatin. The mice receiving olmesartan in the diet showed a small reduction in food intake, probably causing the slight decrease in plasma cholesterol levels. After 18 weeks of treatment, both diets containing olmesartan were adjusted to 0.005% (w/w) (5.8 mg/kg/d) to reduce the hypotensive effect and increase the food intake. The pravastatin concentration was raised to 0.04% (w/w) (47 mg/kg/d) to obtain equal cholesterol exposures (i.e. plasma cholesterol levels x total time in weeks) between the control and olmesartan groups and between the pravastatin and combination groups. Since the olmesartan group had a significant lower total cholesterol exposure after 24 weeks of treatment, this group was sacrificed 2 weeks later. Olmesartan and pravastatin were provided by Sankyo Company, Ltd. The animals received food and water *ad libitum*. Body weight and food intake were monitored during the study.

Lipid and lipoprotein analysis and plasma SAA

After a 4-hour fasting period from 9 a.m. to 1 p.m., EDTA plasma was collected (Sarstedt,

Nümbrecht, Germany). Total plasma cholesterol (Roche Diagnostics, No-1489437) and triglyceride (Roche Diagnostics, No-1488872) levels were measured. Lipoprotein profiles were obtained by FPLC[23]. Serum amyloid A was determined by ELISA (Biosource International, Nivelles, Belgium)[9,28].

Systolic blood pressure

To evaluate the effect of olmesartan, the systolic blood pressure was measured in all groups after 4, 13 and 20 weeks of treatment using the Blood Pressure System for Rats and Mice (RTBP1001, Harvard Apparatus, Holliston, MA, USA). The mice were trained every day, seven days before measurement. For each mouse the blood pressure was measured three times during one session[27].

Histological assessment of atherosclerosis

After the six-months treatment period, the mice were sacrificed after anesthetizing and blood collection[23]. Formalin fixed and paraffin embedded sections of the entire aortic root area were haematoxylin-phloxine-saffron stained for atherosclerosis measurement[29]. For determination of severity of atherosclerosis, the lesions were classified into 5 categories[9,27,28]: I) early fatty streak, II) regular fatty streak, III) mild plaque, IV) moderate plaque, V) severe plaque. Per mouse the percentages of all lesions found in the respective categories were calculated. The total lesion area was calculated per cross-section.

In each segment used for lesion qualification, the number of monocytes adhering to the endothelium was counted. Mouse monocytes were immunostained with AIA31240 (1:3000, Accurate Chemical and Scientific, New York, USA). Macrophage area was measured after immunostaining with anti mouse CD68 (1:100, Serotec Ltd, UK). The number of T lymphocytes was counted after immunostaining with mouse anti human CD3 (Serotec Ltd, UK), cross reacting with mouse CD3, a marker for all T cell subtypes. Collagen content of the plaque was quantified morphmetrically after Sirius Red staining. Mouse smooth muscle cells were immunostained with mouse anti-human alpha actin (1:800, DAKO, Denmark), which cross reacts with mouse alpha actin. smooth muscle cells were counted in the superficial part of the lesions (e.g. the cap) in the type III, IV and V lesions. Proliferating smooth muscle cells in the cap were immunostained with anti PCNA (1:180, Calbiochem, Merck, Germany). All analyses were performed by the same operator, who was blinded for experimental group allocation.

Statistical analysis

Significance of differences was calculated by using the non-parametric Mann-Whitney U test. Each group was compared to control and the combination group was additionally compared to the pravastatin group. Differences in lesion area were corrected for differences in blood pressure, using analysis of variance and analysis of covariance. To

ensure normality, lesion area was transformed using a square-root transformation, which was used as dependent variable. The treatment group was used as the independent variable and difference in blood pressure was the covariate. P<0.05 was considered significant. All data are presented as mean ± SD.

Results

Plasma lipids and blood pressure

As presented in **table 1** the control group had a cholesterol exposure of 420 ± 34 mM*weeks, which was equal to the olmesartan group. Cholesterol exposure was decreased by 18% (p<0.001) in the pravastatin group and by 17% (p<0.001) in the combination group, as compared to the control group. Average plasma cholesterol levels were 17.5 ± 2.7 mmol/L for the control group. Although at any time point the olmesartan group did not differ from the control group, the average overall cholesterol level was slightly decreased (p<0.05). In the pravastatin and the combination group a 17% (p<0.001) reduction of the plasma cholesterol level was observed. The average plasma triglyceride level for the control group was 1.47 ± 0.47 mmol/L. Olmesartan lowered plasma triglyceride levels by 13% (p=0.001) and pravastatin by 37% (p<0.001). In the combination group triglyceride levels were decreased by 39% (p<0.001). Average systolic blood pressure, measured after 4 and 13 weeks of treatment, the blood pressures were 101 ± 6 mmHg for the control group, 83 ± 6 mmHg for olmesartan group (-18%, p<0.001), 104 ± 7 mmHg for the pravastatin group and 89 ± 4 mmHg for the combination group (-11%, p<0.001). After diet adjustment (at t=18 weeks), the blood pressures were measured again after 20 weeks of treatment (shown in **table 1**). Olmesartan alone or in combination with pravastatin decreased systolic blood pressure by 14% as compared to control and pravastatin treatment.

	Total cholesterol exposure (mM*weeks)	**Average plasma cholesterol** (mmol/L)	**Average plasma triglyceride** (mmol/L)	**Systolic blood pressure** (mmHg)
Control	420 ± 34	17.5 ±2.7	1.47 ±0.47	104 ± 4
Olmesartan	415 ± 74	16.4 ± 3.9*	1.27 ± 0.48*	90 ± 2*#
Pravastatin	346 ± 48*†	14.6 ± 2.6*	0.93 ± 0.42*	105 ± 4
Olmesartan + Pravastatin	348 ± 49*†	14.5 ± 2.9*	0.89 ± 0.45*	89 ± 3*#

Table 1 The effect of olmesartan, pravastatin and the combination of both on plasma lipids and systolic blood pressure after twenty weeks of treatment. (*p<0.05 compared to control; †p<0.05 compared to olmesartan; #p<0.05 compared to pravastatin)

Atherosclerosis evaluation: Lesion number, lesion area and lesion severity

Representative photomicrographs of atherosclerotic lesions found in the different groups are shown in **figure 1**. The number of lesions per cross-section in the control group was 5.9 ± 1.1 (**figure 2A**). A significant decrease of 31% was found in the olmesartan group (P<0.005) and of 34% in the pravastatin group (P<0.001). Combination treatment reduced lesion number by 69% (P<0.001), which was also significantly different from the olmesartan group (-56%; P=0.001) and pravastatin group (-54%; P<0.001). The total lesion area per section for the individual groups is shown in **figure 2B**. For the control group the total lesion area was 164.4 ± 68.8 μm^2*1000. Olmesartan significantly reduced lesion area by 46% (P<0.05) and

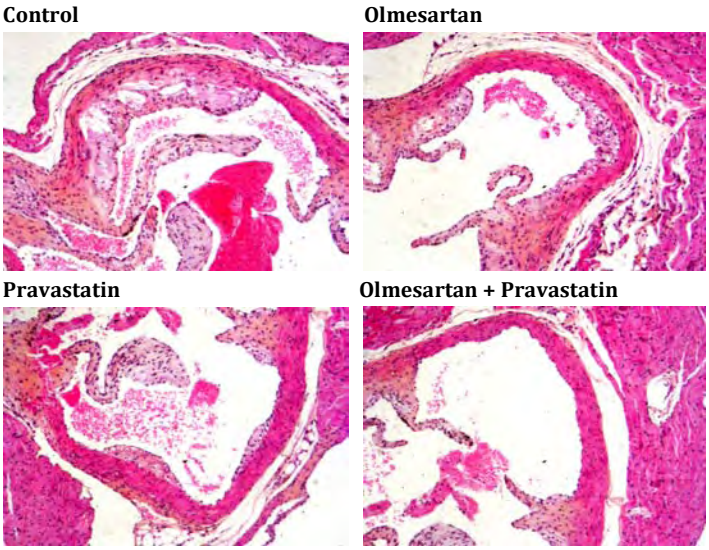

Figure 1 Representative photomicrographs of atherosclerotic lesions found in the different groups (haematoxylin-phloxine-saffron staining). The control example shows a severe lesion (type V). For both the olmesartan and pravastatin group mild and moderate lesions (type II, III and IV) are presented. The example for the combination group shows a small fatty streak (type I).

pravastatin by 39% (P<0.05). The combination therapy further inhibited the development of atherosclerosis by 91% which was highly significant compared to the control (P<0.001), the olmesartan (-83%; P=0.001) and pravastatin (-85%; P<0.001) group. For each animal the lesion severity was analyzed and the percentages of lesions belonging to the respective lesion categories were calculated. **Figure 2C** shows the percentages of type 0-III lesions (no lesions, fatty streaks and mild plaques) and type IV-V lesions (moderate and severe plaques). About 70% of lesions in the control group were type IV or type V lesions, which was 47% (N.S.) in the olmesartan group, 38% (P<0.01) in the pravastatin, and only 15% (P<0.001) in the combination group. This finding indicates that treatment with olmesartan, alone or in combination with pravastatin, interferes with the progression of lesion development, resulting in less advanced lesions.

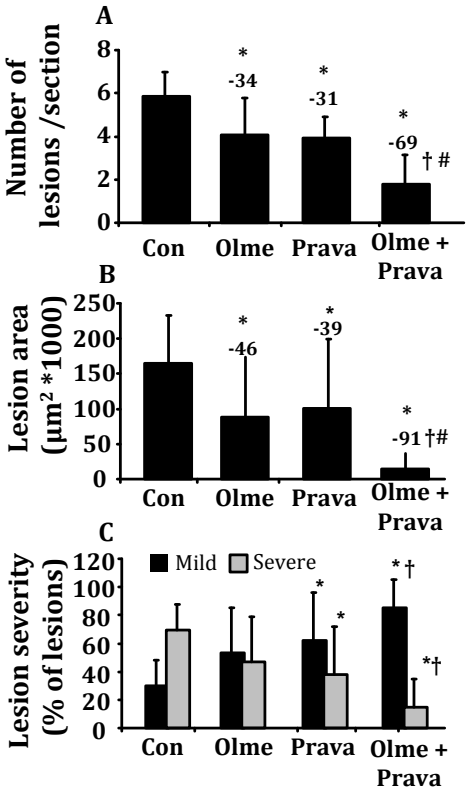

Figure 2 Effect of olmesartan, pravastatin and the combination of both on atherosclerosis. The number of lesions (A) and lesion area per cross-section (B). Severity of the atherosclerotic lesions (C) as determined by the percentages of lesions classified as mild (absence of lesions + type I-III lesions) and severe (type IV-V lesions). P<0.05 compared to control; †P<0.05 compared to olmesartan; #P<0.05 compared to pravastatin.

We also analyzed whether olmesartan had anti-atherosclerotic properties beyond its blood pressure lowering qualities. We calculated, using a univariate analysis of variance (with blood pressure as covariate), that the differences in lesion area remained significant after statistical correction for differences in blood pressure (P<0.01). This shows that olmesartan had an additional beneficial effect independent of its blood pressure lowering effect. The nature of these effects was explored in more detail below.

Systemic inflammation: plasma serum amyloid A

The liver-derived plasma inflammation marker serum amyloid A (SAA), which reflects the overall systemic inflammatory state, was measured at the beginning of the study and at sacrifice (**figure 3**). Levels at sacrifice were 32.2 ± 19.2 µg/mL in the control group, which was significantly higher (P=0.01) when compared to the levels at the start of the treatment (11.6 ± 3.5 µg/mL). As compared to control, SAA was reduced by 68% (p<0.001) in the olmesartan group, by 72% (p<0.001) in the pravastatin group, and by 64% (P<0.001) in the combination group. The pravastatin group showed a significant 22% reduction (p<0.05) in SAA levels as compared to the levels at the beginning of the study. These data emphasize the anti-inflammatory properties of both drugs.

Inflammatory cells: Monocyte adhesion and macrophage and T lymphocyte abundance

As inflammation is an important process in atherogenesis, the presence of pro-inflammatory cells was measured. In the same four sections of the aortic root used for measurement of lesion number, size and severity, the monocytes adhering to the activated endothelium were counted. In the control group on average 18.1 ± 3.2 adhering monocytes per cross-section were present (**figure 4A**). A significant reduction

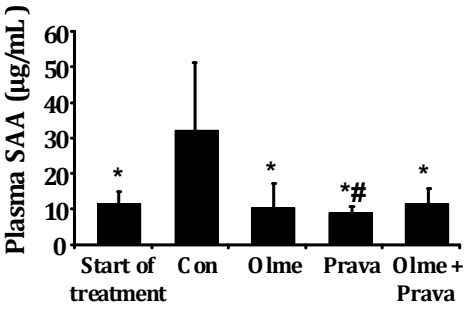

Figure 3 Effect of olmesartan, pravastatin and the combination of both on plasma levels of serum amyloid A (SAA). *P<0.05 compared to control; #P<0.05 compared to start of treatment.

of 22% (p<0.05) was observed in the olmesartan group, whereas the pravastatin group had an equal number of adhering monocytes as compared to the control group. The combination group showed a similar reduction as the olmesartan group, which was significantly different from the control group (-25%; P<0.01) and the pravastatin group (-27%; P<0.01). A resembling pattern was seen for the relative amount of macrophages in the total lesion area (**figure 4B**), in which a 38% (P<0.05) decrease was observed in the olmesartan group. No reduction was observed in the pravastatin group and there was a 26% (N.S.) decrease in the combination group. The total number of macrophages in the lesions showed a pattern comparable to the lesion area (compare **figure 4C** with **figure 2B**). In the control group on average 209 ± 67 macrophages were present per cross-section. Significant decreases of 46% (P<0.05) and of 51% (102 ± 113, P<0.01) were found in the olmesartan group and pravastatin group, respectively. An 89% (P<0.001) reduction was observed in the combination group, which was significantly lower than the olmesartan (P<0.01) and pravastatin group (p<0.001).

In the control group on average 2.1 ± 0.4 T-lymphocytes were present per cross-section (**figure 4D**). This number was lowered by 62% (P<0.05) in the olmesartan group, by 76% (P<0.01) in the pravastatin group, and by 86% (P<0.001) in the combination group. Taken figures 4A to 4C together, our data indicate that treatment with olmesartan decreases activation of the endothelium and results in less foam cell rich plaques as compared with pravastatin treatment.

Lesion composition: Collagen and smooth muscle cell content

To obtain an indication of plaque stability, the collagen content of the lesions and smooth muscle cell area in the cap of the lesions were measured. The average collagen content was 30.8 ± 10.9% in the control group. A 30% increase (39.8 ± 14.0%; N.S.) was seen in the olmesartan group, 40% (43.3 ± 10.7%; P<0.01) in the pravastatin group and 48% (45.6 ± 22.2%; N.S.) in the combination group. Smooth muscle cell area in the cap was measured and expressed as percentage of the lesion area, in those lesions that contained fibrous caps (type III, IV en V) (**figure 4E**). It was found that 1.7 ± 1.2% of the lesion area was smooth muscle cell area in the control group. A 2.6-fold (P<0.01) increase was measured in the olmesartan group. Pravastatin treatment resulted in a 3.1-fold increase (P<0.001) and a 5-fold (P<0.01) increase was observed in the combination

group. To obtain an indication about the state of differentiation of these smooth muscle cells the sections were stained for alpha-SMactin, as a marker of the differentiated, contractile phenotype and for PCNA as marker of proliferation. In all groups hardly any proliferation of smooth muscle cells was found in the cap area (data not shown). This suggests that the smooth muscle cells in the cap area are differentiated contractile smooth muscle cells, which hardly proliferate. The enhanced smooth muscle cell area per lesion area suggests an increased stability of the plaques.

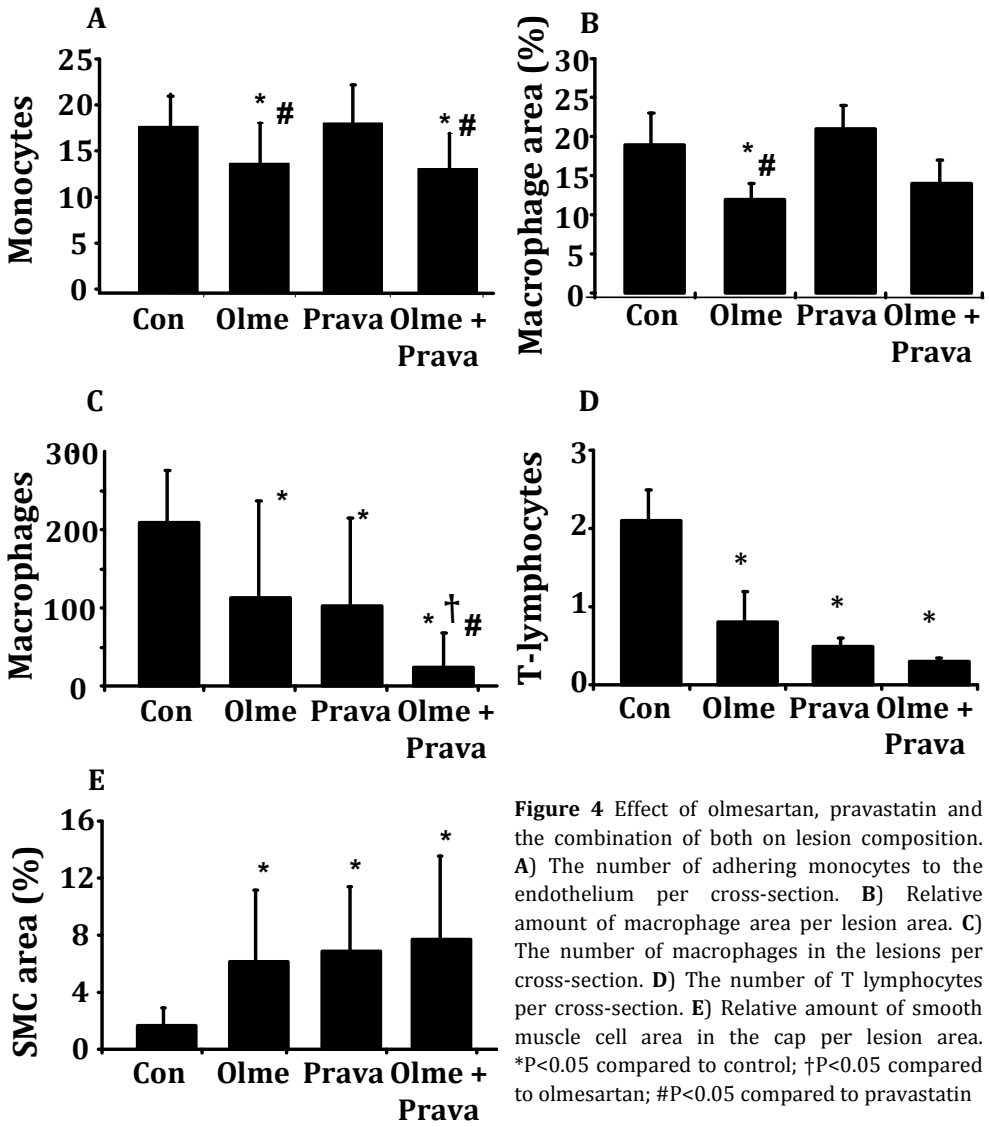

Figure 4 Effect of olmesartan, pravastatin and the combination of both on lesion composition. **A**) The number of adhering monocytes to the endothelium per cross-section. **B**) Relative amount of macrophage area per lesion area. **C**) The number of macrophages in the lesions per cross-section. **D**) The number of T lymphocytes per cross-section. **E**) Relative amount of smooth muscle cell area in the cap per lesion area. *P<0.05 compared to control; †P<0.05 compared to olmesartan; #P<0.05 compared to pravastatin

Discussion

The present study was designed to evaluate and characterize the nature of the effect of the ARB olmesartan alone or in combination with statin treatment, the latter of which can be considered as standard therapy for patients suffering from cardiovascular disease, on the development of atherosclerosis. To create a human-like condition APOE*3Leiden transgenic mice were used, since these mice respond to statins with cholesterol-lowering just as humans[9,22,26-28] and develop atherosclerotic lesions akin to their human counterparts with respect to morphological, histological and immunohistochemical characteristics[23]. In this study treatment with olmesartan resulted in an 11 to 18% reduction in blood pressure, resembling the human response (-10 to -17%) to ARB treatment[30]. Pravastatin reduced plasma cholesterol levels by 17%, which is also comparable to the about 20% decrease achieved in clinical trials[31].

Mono treatment with olmesartan inhibited atherosclerosis development, beyond and independent of the reduction achieved by its antihypertensive action alone. This finding is in line with previous studies in monkeys[32] and in apoE deficient mice[33], in which olmesartan reduced atherosclerosis but did not affect blood pressure. We investigated whether olmesartan exerts this additional anti-atherosclerotic effect via an anti-inflammatory activity by measuring plasma levels of the liver-derived inflammation marker SAA. SAA is a risk factor for cardiovascular disease, which reflects the overall inflammatory state[34]. Treatment with olmesartan reduced SAA levels to initial levels of healthy control animals (*i.e.* before the atherogenic diet was started). The decrease was observed even under conditions of increased plasma cholesterol levels, which are known to increase SAA levels[9,28,29]. In addition, histological analysis of the lesions showed anti-inflammatory features of olmesartan as characterized by reductions in the number of pro-inflammatory adhering monocytes, macrophages, and T-cells per cross-section and by a decrease in total macrophage area in the lesions. Since lesion formation starts by monocyte adherence to the activated endothelium, the above data indicate that olmesartan has an inhibiting effect on the early phase of lesion formation. The reduction in the 'soft' macrophage area, known to be prone to plaque rupture, together with the increased smooth muscle cell area of the contractile phenotype covering the lesions, suggests that olmesartan has plaque stabilizing effects.

Besides blood pressure and inflammation, elevated plasma triglyceride levels are an independent risk factor for cardiovascular disease[35]. In this study it was observed that olmesartan slightly but significantly decreased plasma triglyceride levels. A similar effect combined with an improved insulin sensitivity was observed in olmesartan-treated, fructose-fed rats[36]. Another angiotensin II receptor blocker, telmisartan, was recently shown to reduce triglyceride levels and to improve insulin sensitivity in insulin resistant rats and humans[37]. These effects were attributed to its peroxisome proliferator-activated receptor-γ (PPAR-γ) modulating abilities. PPAR-α and PPAR-γ are

expressed in the cells of the cardiovascular system and have been shown to participate in the regulation of cell growth and migration, and oxidative stress and inflammation[38]. For olmesartan no PPAR-γ activating capacity has been detected[39] until now and it needs to be investigated whether the effect on triglyceride levels and the anti-inflammatory effects of olmesartan are due to modulation of PPAR-α activity.

Mono treatment with pravastatin inhibited the progression of atherosclerosis resulting in less severe and less advanced lesions. This can not solely be attributed to the reduction in plasma lipid levels by pravastatin treatment, but also to its anti-inflammatory properties. These were exhibited in the liver as was visible by reduced plasma SAA levels to even lower concentrations than at the start of the study and histologically in the vessel wall by a reduced number of macrophages and T-cells. SAA, macrophages and T cells are considered to participate in pro-atherogenic processes of early lesion evolution and promote lesion development[40,41]. The reductions in these parameters were all comparable to the decreases achieved by olmesartan mono therapy. Pravastatin did not affect the number of adhering monocytes and the macrophage containing area in the plaques. However, it may stabilize the lesions by increasing the amount of differentiated contractile alpha-SMactin positive smooth muscle cells in the fibrotic cap like olmesartan does.

When olmesartan was combined with pravastatin the anti-atherosclerotic and anti-inflammatory activities of both drugs appeared to be additive, resulting in a significant reduction of 85% when compared to the pravastatin mono treatment. Combination treatment lowered the number of adhering monocytes and T-cells like olmesartan mono therapy, but further reduced the lesion severity and the number of macrophages. No further reduction by combination treatment was found for plasma SAA, which already was decreased by olmesartan or pravastatin alone to levels found at the start of treatment. In agreement with the present data combination treatment with candesartan/rosuvastatin[42], or valsartan/fluvastatin[43] reduced atherosclerosis to a greater extent than treatment with each drug alone in ApoE$^{-/-}$ mice. However, treatment with telmisartan plus atorvastatin did not show any additional effects on atherosclerotic progression and stability in this mouse model[44]. Our data in APOE*3Leiden transgenic mice are in line with a recent report on the effect of combination treatment in rabbits[45] and extend the latter observation of reduced atherosclerosis development by providing a more mechanistic explanations for the additive effect of both drugs.

In conclusion, the current data show that olmesartan interferes with the initiation of lesion formation, whereas pravastatin inhibits lesion progression, and both drugs have vascular and hepatic anti-inflammatory properties. The clinical study EUTOPIA[21] also points to anti-inflammatory activity of olmesartan in humans by reducing the plasma levels of C-reactive protein, interleukin-6 and tumor necrosis factor-α in patients

with essential hypertension and microinflammation. Co-treatment with pravastatin resulted in further reductions of these levels, whereas pravastatin alone did not affect these inflammatory factors. SAA levels were not measured in the latter study.

The effect of combination treatment of olmesartan with pravastatin on cardiovascular endpoints in humans has not yet been studied. However, combination therapy of atorvastatin with an ACE inhibitor was shown to be more effective in reducing cardiovascular events than statin treatment alone in a post-hoc analysis of the patients of the GREASE[46] study[47]. Similar synergistic effects of atorvastatin and anti-hypertensive treatment with the calcium channel blocker amlodipine and ACE inhibitor perindopril were recently reported in the ASCOT study[48]. The present study and the results of the mentioned clinical trails provide evidence that combination treatment of olmesartan and pravastatin may be more effective in the prevention of atherosclerosis than treatment with statins alone.

Acknowledgements

H. van der Boom, W. van Duyvenvoorde, A. Jie, A. Maas, E. Offerman, H. Putter, K. Toet and L. Verschuren are thanked for their outstanding technical assistance. J.W.J. is an established clinical investigator of the Netherlands Heart Foundation (2001 D032). The Netherlands Organization for Scientific Research NWO supported this work (grant VENI 016.036.061 to R.K.) Sankyo Company Ltd. (Tokyo, Japan) is gratefully acknowledged for supporting this study in part by an unconditional grant.

Disclosures

None.

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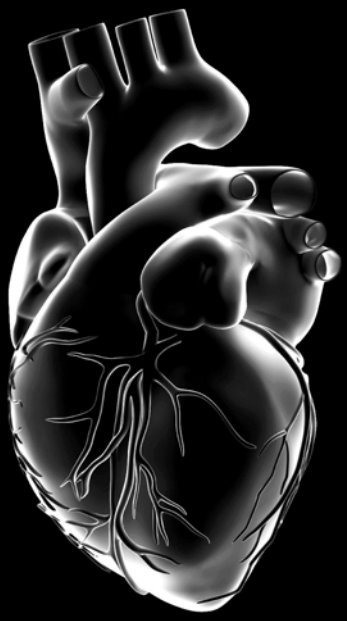

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Dual PPARα/γ agonist tesaglitazar blocks progression of pre-existing atherosclerosis in APOE*3Leiden.CETP transgenic mice

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Submitted

Abstract

Objective To evaluate the effect of the PPARα/γ agonist tesaglitazar on the progression of pre-existing atherosclerotic lesions in APOE*3Leiden.CETP (E3L.CETP) transgenic mice.

Methods and Results After feeding E3L.CETP mice a high cholesterol (HC) diet for 11 weeks to induce atherosclerosis, mice were fed a low cholesterol (LC) diet for 4 weeks to obtain a human-like plasma total cholesterol level of ~10 mmol/L. Thereafter, Mice were subdivided into three groups. Then, one group was sacrificed ("baseline group") and two groups were continued to feed for another eight weeks with LC diet, one group without ("control group") and one group with 10 μg/kg/day tesaglitazar ("tesaglitazar group"). Atherosclerosis development was assessed in the aortic root. Tesaglitazar significantly reduced plasma triglycerides (-71%), total cholesterol (-55%; mainly by reducing (V)LDL-C), CETP mass (-42%) and CETP activity (-56%), and increased HDL-C (+38%). In the baseline group, substantial atherosclerosis had developed (lesion area ~136,000 μm^2) as reflected by mainly moderate and severe lesions. During the 8 weeks LC diet period, atherosclerosis had progressed in the control group with respect to lesion area (+54%) and severity, whereas tesaglitazar inhibited lesion progression during this period. In fact, tesaglitazar reduced local inflammation in the vessel wall as reflected by decreased monocyte adhesion (-48%) and macrophage area (-85%) and introduced lesions with more stabilized phenotype , as evident from an increased smooth muscle cell content in the cap (+64%) and collagen content (+97%).

Conclusion Dual PPARα/γ agonism with tesaglitazar markedly reduced (V)LDL-C and increased HDL-C and turned down cholesterol-induced vessel wall activation. These combined actions resulted in complete inhibition of progression and even stabilization of pre-existing atherosclerotic lesions in E3L.CETP transgenic mice.

Introduction

A doubling of the global burden of diabetes within 25 years from now has been predicted[1]. Patients with obesity, insulin resistance, or type 2 diabetes (T2D) are prone to develop the atherogenic triad, as characterized by raised plasma triglycerides (TG), reduced high-density lipoprotein cholesterol (HDL-C) and a predominance of small dense low-density lipoprotein (sdLDL)[2]. Besides insulin resistance (IR) and high glucose levels, these lipid abnormalities are all associated with an increased risk of cardiovascular diseases (CVD)[3]. HMGCoA-reductase inhibitors (statins) effectively lower plasma LDL cholesterol (LDL-C) but do not optimize the other lipid abnormalities associated with increased CVD risk. Therefore, additional therapies are required to further improve the atherogenic dyslipidemia typically associated with insulin resistance.

Peroxisome proliferator–activated receptors (PPARs) are nuclear hormone receptors that control the expression of genes involved in both carbohydrate and lipid metabolism, and could be valuable as additional drug targets. Stimulation of PPARα by fibrates 1) inhibits hepatic TG production, 2) increases lipoprotein lipase (LPL)-mediated TG lipolysis 3) provides a higher affinity of remnants for the LDL receptor (LDLr), 4) enhances human apoAI and apoAII synthesis[4], and 5) reduces the expression of the cholesteryl ester transfer protein (CETP)[5]. These concomitant effects of fibrates result in a significant on average 36% decrease in TG and a 10% increase of HDL-C in humans[6]. Fibrates reduce atherosclerosis in mice independent of their cholesterol-lowering effect[7], which may be related to their anti-inflammatory capacities[8]. They also reduce cardiovascular events in humans, which is most evident in obese and insulin resistant patients[9,10]. Thus, PPARα agonism is beneficial by its effect on the lipoprotein profile and its inflammation-reducing properties. On the other hand, PPARγ agonists (glitazones) improve insulin sensitivity and induce glycemic control in diabetic animals as well as in patients with T2D[11]. Independent of their metabolic action, they also reduce inflammatory markers such as C-reactive protein (CRP), matrix metalloproteinase-9 (MMP-9), serum amyloid A (SAA), soluble CD40 ligand (sCD40L), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor α (TNFα), which may contribute to their anti-atherogenic properties[8,12].

Because of their additive modes of action, dual PPARα/γ agonists (glitazars) improve both lipid and glucose abnormalities in animal models and human subjects with insulin resistance and/or T2D. For example, in patients with T2D, tesaglitazar treatment reduced TG, apoB, total cholesterol (TC), nonHDL-C and VLDL-C, and increased HDL-C[13]. In obese dyslipidemic patients, tesaglitazar improved dyslipidemia on top of atorvastatin, and was more effective in glucose modulation than the PPARγ agonist pioglitazone[14]. However, its clinical development was discontinued in May 2006 following phase III clinical trial results as its benefit-risk profile did not provide a significant advantage over existing anti-diabetic therapies. End point studies using

tesaglitazar or other dual PPARα/γ agonists in humans have thusfar not been performed.

We have previously shown that tesaglitazar improved the HOMA-IR index, lowered plasma TC and TG levels and lowered inflammation markers in APOE*3Leiden (E3L) mice[15]. These mice display a lipoprotein profile comparable to that of patients with dysbetalipoproteinemia (with plasma TC and TG are mainly confined to (V)LDL[16]) and do respond to various hypolipidemic drugs in a similar way as humans[17]. Tesaglitazar did not increase HDL in E3L mice, probably by the fact that mice naturally lack expression of CETP, which is an important factor for human HDL metabolism. In these mice, tesaglitazar reduced atherosclerosis development in a prevention design, in which pharmacological treatment was started before the onset of atherosclerosis[15].

The aim of the present study was to evaluate the effect of tesaglitazar on atherosclerosis development in a more clinically relevant design, in which pharmacological treatment with tesaglitazar was started after atherosclerosis had been developed. To this end, we used our recently developed E3L.CETP transgenic mouse model[18], a more humanized animal model that has been shown to respond to both lipid-lowering interventions[17] and HDL-raising interventions[5 19,20].

Methods

Mice and diets

Human *CETP* transgenic mice which express CETP under control of its natural flanking regions (strain 5203)[21] were obtained from Jackson laboratories (Bar Harbor, MC) and crossbred with *E3L* mice[22] in our local animal facility at TNO to obtain heterozygous E3L.CETP mice[18]. Forty-seven female E3L.CETP mice (on average 18 weeks of age) received a semi-synthetic high cholesterol (HC) western-type diet, containing 40.5% sucrose and 15% cacao butter, supplemented with 0.3% (w/w) cholesterol for 11 weeks to induce hypercholesterolemia and atherosclerosis development. After 11 weeks, the diet was replaced by a low cholesterol (LC) western-type diet containing 0.1% (w/w) cholesterol for another four weeks to reduce total cholesterol (TC) towards a more 'human-like' level (approx. 10 mmol/L). Thereafter, the animals were divided into three groups, after matching based on age, body weight and plasma TC and TG levels. Subsequently, the mice were fed the LC diet without tesaglitazar ('control group'; n=16) or with tesaglitazar ('tesaglitazar group', 0.25 μmol / kg diet ~ 10 μg/kg body weight/ day; n=16) to reduce TC levels by about 50% (dose determined in a pilot study). To quantify the extent of atherosclerosis at the start of the drug intervention, a third group of mice was sacrificed immediately after matching, which was considered a reference for the baseline atherosclerosis level ('baseline group', n=15). The animals received food and water *ad libitum*. Body weight and food intake were monitored during the study.

Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Scientific Research (TNO).

Plasma lipids and apolipoproteins

After a 4-hour fasting period from 9 a.m. to 1 p.m., blood was collected from the tail vein into EDTA-coated tubes (Sarstedt, Nümbrecht, Germany). Plasma was assayed for TC (No-236691, Roche Diagnostics, USA) and TG (No-1488872, Roche Diagnostics, USA), and for apoAI and (human) apoE using sandwich ELISAs as described previously[18]. The distribution of cholesterol of the various lipoproteins was determined after separation of lipoproteins by fast-performance liquid chromatography (FPLC) using a Superose 6 column[18]. HDL-C was also quantified in plasma after precipitation of apoB-containing lipoproteins. Hereto, 10 µL heparin (LEO Pharma, The Netherlands) and 10 µL 0.2 M $MnCl_2$ were added to 20 µL plasma and mixtures were incubated for 20 min at room temperature and centrifuged for 15 min at 13,000 rpm at 4°C.

Plasma CETP mass and CET activity

CETP mass was determined using the DAIICHI CETP ELISA kit (Daiichi Pure Chemicals Co, Japan) according to manufacturer's instructions. Cholesteryl Ester Tranfer (CET) activity was measured exactly as described, and calculated as nmol/mL/h[5].

Plasma inflammation markers

SAA (Biosource International, Belgium), adiponectin, E-selectin, MCP-1, and vascular cellular adhesion molecule-1 (VCAM-1) (all R&D Systems Inc, USA) were determined by ELISA according to the manufacturers' instructions. Fibrinogen was measured with an in-house procedure as described previously[23].

Histological assessment of atherosclerosis

The various mouse groups were sacrificed either at the start of LC treatment ("baseline group") or after the 8-week treatment period with LC diet ("control group") or LC diet supplemented with tesaglitazar ("tesaglitazar group"). The hearts were dissected, formalin-fixed and embedded in paraffin. Serial cross sections (5 µm) throughout the entire aortic valve area were used for histological analysis. Per mouse, 4 sections with intervals of 50 µm were used for quantification and qualification of the atherosclerotic lesions after staining with hematoxylin-phloxin-saffron (HPS). For determination of the severity of atherosclerosis, the lesions were classified into 5 categories as described[24]: I) early fatty streak, II) regular fatty streak, III) mild lesion, IV) moderate lesion, and V) severe lesion. Per mouse the percentages of all lesions found in the respective categories were calculated. The total lesion area was calculated per cross section. In each segment used for lesion qualification, monocytes were immunostained with AIA31240 (1:3000, Accurate Chemical and Scientific, USA) and the number of monocytes adhering to the

endothelium was counted[25]. Macrophage area was measured after immunostaining with anti-mouse Mac-3 (BD Pharmingen, the Netherlands). Smooth muscle cell (SMC) area was measured after immunostaining with mouse anti-human actin (DAKO, Denmark) that cross-reacts with murine actin. The collagen content of the lesions was quantified morphometrically after Sirius Red staining. SMC area in the cap was measured and calculated as percentage of the total lesion area. All analyses were performed by the same operator, who was blinded for experimental group allocation.

Statistical analysis

Data are presented as means ± SD unless indicated otherwise. Statistical differences were assessed using the non-parametrical Kruskal-Wallis test followed by Mann Whitney U test. $P<0.05$ was considered statistically significant.

Results

Tesaglitazar decreases (V)LDL-C and increases HDL-C

To induce the development of atherosclerosis, E3L.CETP mice were fed a HC diet for 11 weeks, resulting a relatively high plasma TC level (19.9 ± 4.9 mmol/L) and a moderate TG level (2.3 ± 1.3 mmol/L) (**figure 1**). Thereafter, the mice were fed a LC diet for 4 weeks to obtain more 'human-like' TC levels (10.8 ± 4.9 mmol/L) without reducing TG levels (2.2 ± 0.9 mmol/L) at the start of the intervention period. One mouse group was subsequently sacrificed ("baseline group"), and the other groups were treated with either LC diet ("control group") or the LC diet supplemented with tesaglitazar ("tesaglitazar group") for 8 weeks. As compared to the control group,

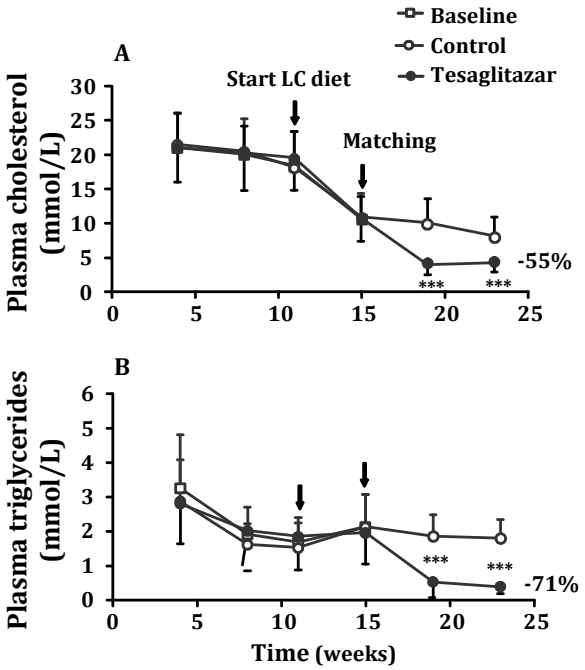

Figure 1. Effect of dietary cholesterol reduction and tesaglitazar on plasma cholesterol and triglyceride levels. E3L.CETP mice received a high cholesterol diet for 11 weeks followed by a low cholesterol diet for 4 weeks. Subsequently, the mice were matched and either sacrificed ("baseline"; squares) or continued to be fed the LC diet without tesaglitazar ("control"; white circles) or with tesaglitazar ("tesaglitazar"; black circles) for 8 weeks. Blood samples were taken and plasma was analyzed for total cholesterol (A) or triglycerides (B). ***$P<0.001$ compared to the control group.

tesaglitazar reduced TC by -55% (4.1 ± 1.4 mmol/L vs 9.1 ± 3.2 mmol/L; P<0.001) (**figure 1A**) and TG by -71% (0.5 ± 0.3 mmol/L vs 1.9 ± 0.6 mmol/L; P<0.001) (**figure 1B**). Lipoprotein fractionation by FPLC showed that the TC-lowering effect of tesaglitazar was accounted for by a large reduction in (V)LDL-C (~-80%), whereas tesaglitazar increased HDL-C (~+45%) (**figure 2A**). The increase in HDL-C was confirmed by direct measurement of HDL-C in plasma after precipitation of apoB-containing lipoproteins (+38%; P<0.01) (**figure 2B**). No differences were observed in plasma concentrations of apoAI (1.4 ± 0.4 mg/mL vs 1.5 ± 0.5 mg/mL) and human apoE (0.22 ± 0.08 mg/mL vs 0.21 ± 0.08 mg/mL).

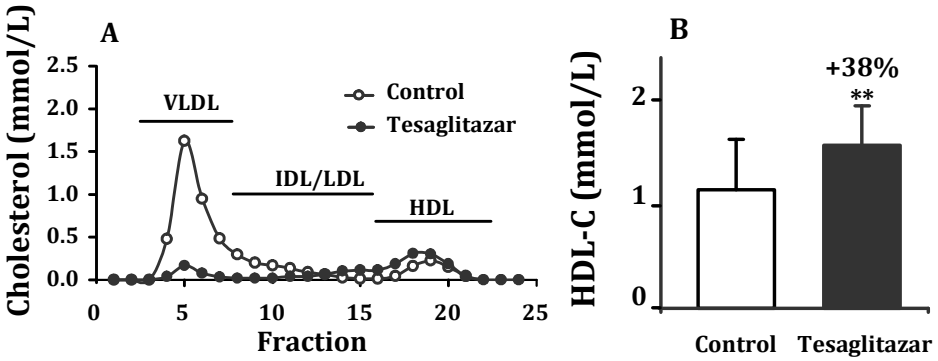

Figure 2. Effect of tesaglitazar on plasma lipoproteins. After matching, *E3L.CETP* mice were fed the LC diet without (white symbols) or with (black symbols) tesaglitazar for 8 weeks. The distribution of cholesterol over the individual lipoproteins in pooled plasma was determined after separation of lipoproteins by FPLC (A). Plasma HDL-C levels were also measured individually after precipitation of apoB-containing lipoproteins (B). **P<0.01 as compared to the control group.

Tesaglitazar decreases CETP mass and CET activity

Since the HDL-raising effect of tesaglitazar may have resulted from decreased CETP expression, plasma CETP mass and CETP activity were determined after the 8 weeks LC treatment (**figure 3**). As compared to the control group, tesaglitazar lowered CETP mass by -42% (8.2 ± 4.5 vs 14.1 ± 4.4 µg/mL; P<0.001) and reduced CETP activity by -56% (21.2 ± 13.8 vs 48.5 ± 18.6 nmol/mL/h, P<0.001).

Tesaglitazar blocks progression of atherosclerosis development

Eleven weeks of feeding the HC diet followed by 4 weeks on the LC diet, resulted in a lesion area per cross section of 136 ± 87 x10^3 µm^2 as determined in the baseline group (**figure 4A**). Prolonged feeding of the mice with the LC diet without tesaglitazar for another 8 weeks increased the lesion area by +54% (210 ± 84 x10^3 µm^2; P<0.05). This diet-induced increase in lesion area was fully blocked by tesaglitazar (140 ± 97 x10^3 µm^2; NS vs baseline). These changes in lesion area were also reflected by changes in

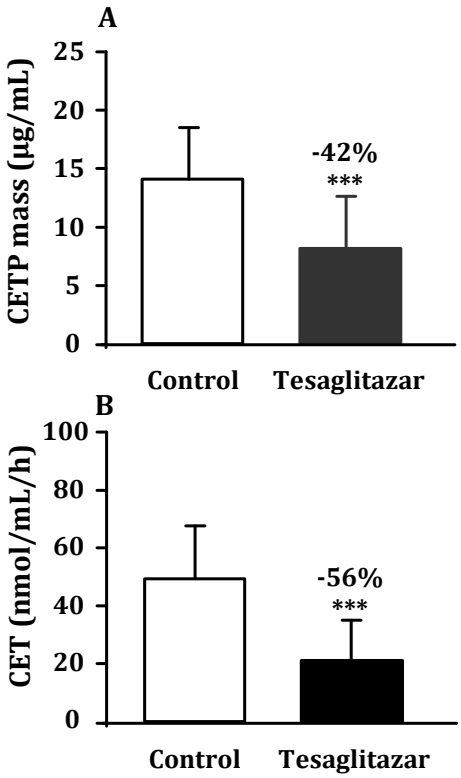

Figure 3. Effect of tesaglitazar on CETP mass and CET activity. After matching, *E3L.CETP* mice were fed the LC diet without (white bars) or with (black bars) tesaglitazar for 8 weeks. Plasma CETP mass (A) and CET activity (B) were determined. Values are means ± SD (n=16 per group). ***P<0.001 as compared to the control group.

lesion severity. Whereas prolonged feeding the LC diet without tesaglitazar increased the percentage of severe lesions (type IV-V, 69 ± 17% vs 47 ± 23% at baseline; P<0.01) at the expense of mild (type I-II) and moderate (type III) lesions, the LC diet with tesaglitazar did not result in an increase in type IV-V lesions (59 ± 24%) or reduction in type III lesions (40 ± 24% vs 43 ± 23% at baseline, NS) as compared to baseline (**figure 4B**).

Tesaglitazar reduces inflammation in the arterial wall

To investigate the contribution of inflammation to the observed effects of tesaglitazar, we measured inflammation markers. At baseline, plasma markers for systemic inflammation such as adiponectin (5.0 ± 0.9 µg/mL), SAA (1.0 ± 0.6 µg/mL), and fibrinogen (1.8 ± 0.3 mg/mL) were in the physiological range, as were the vascular inflammation markers E-selectin (43.5 ± 7.2 ng/mL), VCAM-1 (1.1 ± 0.3 µg/mL) and macrophage-derived chemokine MCP-1 (75.0 ± 20.1 pg/mL). These markers in plasma were not differentially changed in the control group and the tesaglitazar group as compared to baseline levels, albeit that we observed a slight increase in E-selectin levels upon tesaglitazar treatment (51.0 ± 7.2 ng/mL; P<0.05).

Local inflammation in the vessel wall was histologically assessed by measuring the adhesion of monocytes to the endothelium (**figure 5A**) and the macrophage content of the lesions (**figure 5B**). As compared to baseline, the number of monocytes adhering to the endothelium (3.1 ± 2.2 vs 3.2 ± 2.2 per cross section) and the macrophage content of the lesions (6.4 ± 3.3% vs 5.2 ± 3.7%) were not affected in the control group. Tesaglitazar, however, potently reduced inflammation in the vessel wall as compared to baseline, as reflected by a 48% decrease in adhesion of monocytes (1.6 ± 1.7 vs 3.2 ± 2.2;

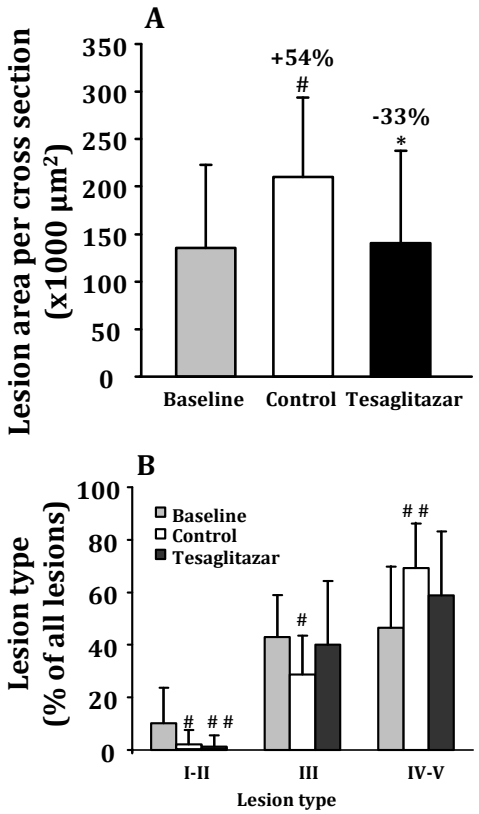

Figure 4. Effect of tesaglitazar on atherosclerosis development. Mice were sacrificed at baseline (grey bars) or after 8 week intervention with LC diet without (white bars) or with (black bars) tesaglitazar. In the aortic root the lesion area was measured per cross section (A) and the severity of lesions was analyzed (B). #P<0.05, ##P<0.01 as compared to the baseline group.

P<0.05) and a strong -85% reduction in macrophage content of the lesions (0.8 ± 1.2% vs 5.2 ± 3.7%; P<0.001).

Tesaglitazar increases the SMC and collagen content of the atherosclerotic lesions

To evaluate the effect of tesaglitazar on parameters reflecting lesion stability, we measured the collagen content (**figure 5C**) and SMC area in the cap (**figure 5D**). As compared to baseline, the lesions in the control group were more severe and contained therefore more collagen (54 ± 10% vs 33 ± 11%, p<0.001), but had a similar amount of SMCs in the cap per lesion area (4.8 ± 1.9% vs 3.4 ± 2.6%). Tesaglitazar not only blocked lesion development (**figure 4A**), but also enhanced the induction of a more stable lesion phenotype as compared to control, reflected by a further increase in collagen content (to 65 ± 9%, P<0.001 vs baseline, P<0.05 vs control). The increase in SMC area in the cap was +64% increased as compared to the baseline (to 5.6 ± 1.9%, P<0.05), which was not different from the control group.

Discussion

In this study we showed that tesaglitazar, but not dietary cholesterol lowering, fully prevented the progression of pre-existing atherosclerosis in E3L.CETP transgenic mice. Tesaglitazar also reduced inflammation in the vessel wall as reflected by less monocyte adherence and less macrophage area in the lesions, and induced a more stable lesion phenotype as indicated by an increased collagen content and SMC area in the cap.

We previously showed that E3L mice are highly susceptible to dietary interventions with respect to modulating plasma lipid levels. Moreover, E3L mice show a human-like response to drug interventions aimed at treatment of CVD (*e.g.* statins, fibrates, cholesterol uptake inhibitors, calcium channel blockers and angiotensin II

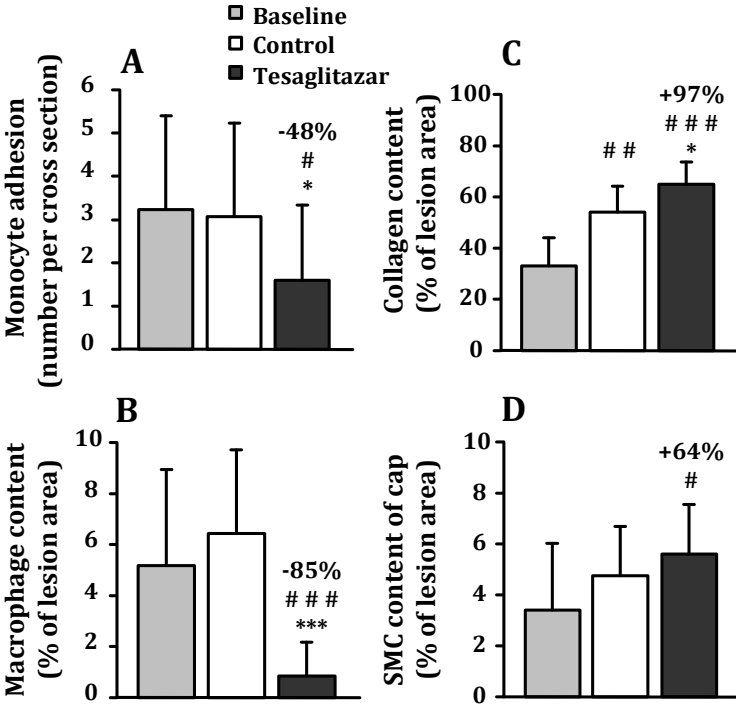

Figure 5. Effect of tesaglitazar on lesion composition. The number of monocytes adhering to the endothelium was counted (A). The macrophage content (B), the collagen content (C), the smooth muscle cell (SMC) content in the cap (D) of the lesions were measured. #P<0.05, ##P<0.01, ###P<0.001 as compared to the baseline group; *P<0.05, ***P<0.001 as compared to the control group.

receptor antagonists[7,23,25-27]) with respect to alterations in the lipoprotein profile and/or atherosclerosis development. This is in sheer contrast with wild-type C57Bl/6 mice and conventional hyperlipidemic mice, such as apoE-deficient or LDLr-deficient mice, which show either an adverse response or no response to such interventions[17]. In particular, administration of tesaglitazar to LDLr-deficient mice did not affect plasma lipid levels[28], while we demonstrated that tesaglitazar lowered TG and cholesterol within apoB-containing lipoproteins in E3L mice [15] similarly as in humans[13,14].

Recently, we showed that introduction of the human *CETP* gene in E3L mice results in a mouse model which also displays a human-like response with regard to raising HDL-C after administration of fenofibrate[5], atorvastatin[19], niacin (Van der Hoorn et al, unpublished) and torcetrapib[20]. In the present study, we confirm that treatment of E3L.CETP mice with tesaglitazar reduced (V)LDL-C and strongly reduced TG similarly as observed previously in E3L mice. In addition, we now demonstrate that tesaglitazar also raised HDL-C levels in E3L.CETP mice similarly as reported in clinical intervention trials [13,14]. The presence of CETP thus plays a crucial role in the HDL-C-raising effect of tesaglitazar. In fact, tesaglitazar markedly decreased CETP mass in plasma. This is most probably due to the PPARα-agonistic effect of tesaglitazar, since we have recently observed that treatment of E3L.CETP mice with the PPARα agonist fenofibrate reduced hepatic CETP expression as well as the plasma CETP mass and CETP activity[5]. The

observation that plasma CETP activity is reduced to a larger extent than CETP mass may relate to the large reduction in plasma TG, as the capacity of apoB-containing lipoproteins to accept CE from HDL is closely correlated to its plasma level[29-32]. The tesaglitazar-induced increase in HDL-C was not accompanied by a raise in apoAI, suggesting that tesaglitazar increases the particle size of HDL rather than the amount of HDL particles, which was indeed confirmed by FPLC profiling.

To establish the effect of tesaglitazar on atherosclerosis in a setting mimicking the clinical situation, we first induced atherosclerosis in E3L.CETP mice by feeding a high cholesterol-containing western-type diet. Then, plasma cholesterol was lowered by limiting the dietary cholesterol before treatment with tesaglitazar was started. Interestingly, lowering cholesterol alone did not prevent further atherosclerosis development. More specifically, vessel wall inflammation was not attenuated by just cholesterol lowering and the atherosclerosis had even progressed with respect to total lesion area and lesion severity. In contrast, tesaglitazar inhibited further atherosclerosis development and stabilized lesions. This can be ascribed to a further reduction of atherogenic (V)LDL-C, an increase in HDL-C as well as to local anti-inflammatory effects of tesaglitazar in the vessel wall, since atherosclerosis is a dynamic lipid- and inflammation-driven process.

As evidenced by the largely reduced adherence of monocytes and macrophage content of the lesion, we demonstrated that tesaglitazar turned down the local vessel wall inflammation induced by the high cholesterol diet, whereas cholesterol lowering alone did not, These observations confirm our previous observations in E3L mice, which showed that tesaglitazar reduced NF-κB-positive areas in the atherosclerotic lesions, indicating reduced local inflammation. This was accompanied by less ICAM-1 and MCP-1 positive areas[15], which explains decreased monocyte adherence and transmigration, and thus decreased macrophage content of the lesions. Similar anti-inflammatory effects regarding endothelium activation have been shown for both single PPARα and PPARγ agonists[8,33]. It has recently been shown that PPARγ activation may program mononuclear precursor cells toward an M2 phenotype *in vivo*, leading to generation of a macrophage population with enhanced anti-inflammatory properties[34,35]. The anti-inflammatory effect of tesaglitazar is local and not systemic, since tesaglitazar did not decrease the (physiologic) plasma levels of inflammation markers in either E3L or E3L.CETP mice.

Furthermore, we observed that tesaglitazar stabilized the lesions by increasing the SMC content of the lesion cap as well as the collagen content of the lesion. It has been shown that inflammation not only enhances lesion development and progression, but also destabilizes lesions by stimulating the release of metalloproteinases (MMPs) from macrophages, which are able to degrade collagen. Contrary, tesaglitazar may thus increase the collagen content secondarily to the reduction in macrophage accumulation in the lesion. In fact, stimulation of PPARγ by rosiglitazone in humans stabilized lesions

in the carotid artery, which was ascribed to a reduced expression of MMP-3, MMP-8 and MMP-9[36]. These observations indicate that, in established atherosclerotic lesions, the anti-inflammatory actions of dual PPARα/γ activation not only inhibit lesion progression but also induce a more stable lesion phenotype.

To date, all dual PPAR a/g agonists including tesaglitazar, have been discontinued either at a preclinical stage or during clinical development and thus no dual PPARα/γ agonist has yet been approved for clinical use[37]. Long term clinical effects can therefore only be extracted from clinical studies using either single PPARα or PPARγ agonists. The effect of PPARα-agonistic fibrates on cardiovascular morbidity and mortality was studied in primary (HHS[38] and FIELD[39]) and secondary (BIP[40], VA-HIT[41] and FIELD[39]) cardiovascular prevention studies. The results from these trials suggest that fibrate therapy reduces CVD and is most efficacious in overweight individuals with insulin resistance and chronic inflammation. Whereas PPARγ agonists have been demonstrated to be efficient in the management of insulin resistance and T2D in a number of prospective clinical trials, their effects on CVD are still under debate[42-44]. While pioglitazone reduced the incidence of myocardial infarction and acute coronary syndrome in patients with T2D (PROactive study[45]), rosiglitazone has been noted to increase the CVD risk (ADOPT[46] and DREAM[47] study). The contradicting results of these clinical trials using different compounds to agonize PPARγ might just reflect the complex and meticulous balance in PPAR pathways and mechanisms involved in either their beneficial or detrimental effects. Likewise, whereas the dual PPARα/γ agonist tesaglitazar has atheroprotective effects in E3L.CETP mice, as shown in the present study, and in other mouse models for hyperlipidemia and atherosclerosis[15,28], the dual PPARα/γ agonist 'compound q' aggravated inflammation and atherosclerosis in apoE-deficient mice[48].

In conclusion, we showed that the dual PPARα/γ agonist tesaglitazar halted progression of pre-existing atherosclerosis and stabilized lesions in E3L.CETP mice, as related to reduction of (V)LDL-C, increase of HDL-C, and of local anti-inflammatory properties in the vessel wall. Despite the clinical failure of tesaglitazar, these data clearly demonstrate a potential therapeutic window for PPARα/γ agonists for treatment of diabetic cardiovascular complications.

Acknowledgements

We thank Ria van den Hoogen, Anita van Nieuwkoop, Erik Offerman and Marijke Voskuilen for their brilliant technical assistance. This work was supported by the Leiden University Medical Center (Gisela Thier Fellowship to P.C.N.R.), the Netherlands Organization for Scientific Research (NWO grant 908-02-097 and NWO VIDI grant 917.36.351 to P.C.N.R.; NWO grant 903-39-291 to L.M.H.), the Netherlands Heart Foundation (NHS grant 2003B136 to P.C.N.R.), and the Center for Medical Systems

Biology (project 115 to L.M.H.). J.W.J. is an established clinical investigator of the Netherlands Heart Foundation (2001 D032). AstraZeneca, Mölndahl, Sweden is gratefully acknowledged for supporting this study in part by an unconditional grant.

Disclosures

E.L. and G.C. are employees of AstraZeneca, Mölndahl, Sweden

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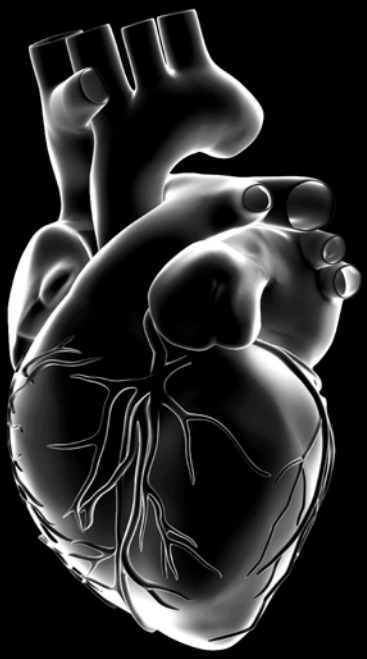

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The thromboxane prostanoid receptor antagonist S18886 (terutroban), combined with dietary cholesterol-lowering, blocks the progression of atherosclerosis in APOE*3Leiden mice

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Abstract

Objective To evaluate the effect of Thromboxane Prostanoid (TP) receptor inhibition by increasing doses of the antagonist S18886 (terutroban) in combination with dietary cholesterol-lowering on existing atherosclerotic lesions in APOE*3Leiden transgenic mice.

Methods and Results APOE*3Leiden mice were fed an atherogenic diet resulting in plasma cholesterol levels of 27.6 mM to develop atherosclerosis. At 10 weeks, 15 mice were sacrificed to assess atherosclerosis development. Then cholesterol in the diet was reduced to reach plasma cholesterol levels of 5-6 mM and the remaining mice were treated with: vehicle (control), S18886 (10, 30 or 60 mg/kg bw/d) or ADP-receptor antagonist clopidogrel (3.8 mg/kg bw/d). After 12 weeks all groups were sacrificed and atherosclerosis was measured.Cholesterol-lowering alone decreased levels of inflammation markers SAA (-95%), MCP-1 (-70%) and E-selectin (-36%;all $p<0.001$), lessened the number of lesions (-20%;$p<0.05$) and improved the lesion stability. The latter was revealed by a reduced macrophage content (-95% $p<0.001$) and by a 7-fold increase of smooth muscle cells and collagen areas as compared to baseline. Supplementary treatment with S18886 reduced additionally and dose-dependently the lesion area (up to -55%), the progression of lesion severity (up to -43%) and the monocyte adherence (up to -70%) when compared to control (all $P<0.01$). Clopidogrel did not add to the effect of cholesterol-lowering on atheroclerosis development.

Conclusion In APOE*3Leiden mice dietary cholesterol-lowering decreased the systemic and vascular inflammatory status and improved lesion stability. S18886 demonstrated additional atheroprotective effects indicated by a dose-dependently reduced lesion size and severity and endothelial monocyte adherence.

Introduction

Atherothrombosis is a multifactorial disease responsible for most cardiovascular events. The reduction of high plasma cholesterol by nutritional and/or pharmaceutical intervention is the first choice approach for treatment in preventing atherosclerosis development. However, different studies have shown that substantial residual cardiovascular risk remains, even with very aggressive reductions in levels of LDL cholesterol[1]. More recently, it has been evidenced that circulating platelets, when activated, are not only involved in thrombus formation leading to clinical complications, but are also able to activate vascular cells. Accumulating evidence has shown that they are involved in the initiation and the progression of atherosclerosis[2], and inhibition of platelet adhesion reduces leukocyte infiltration and atherosclerosis in hypercholesterolemic mice[3]. Therefore, platelet inhibition not only leads to a significant decrease in cardiovascular events but could also result in a slower progression of atherosclerosis.

The most widely prescribed anti-platelet treatment is aspirin, which clinical efficacy is based on inhibition of the platelet cyclo-oxygenase-1 (COX-1), thus inhibiting the generation of platelet thromboxane A_2 (TxA_2,), which binds to the thromboxane-prostanoid endoperoxide (TP) receptor[4] and triggers platelet aggregation. TP receptors are widely expressed by vascular cells (smooth muscle cells, endothelial cells) and by circulating leucocytes, and their activation leads to vasoconstriction, inflammation and cell proliferation, phenomena widely involved in atherosclerosis initiation and progression. Aspirin treatment has clearly shown a beneficial effect in the secondary prevention of cardiovascular diseases (CVD), but is less accepted for the primary prevention. This is mainly caused by the increased incidence of bleeding and the very modest non-significant CVD risk reduction in individuals at low risk[5-7]. Additionally, aspirin has controversial effects on vasoconstriction, endothelial dysfunction or vascular wall proliferation[8,9] known to be of major importance in the atherosclerotic process.

Platelet inhibition can also be achieved by blocking TP receptors with specific antagonists which have advantages over aspirin. These compounds not only block the effects of TxA_2, but also inhibit the binding of non specific TP receptor ligands, mostly isoprostanes and HETEs, formed in response to oxidative stress and able to exert deleterious effects[10,11]. Furthermore, in contrast to aspirin, TP receptor antagonists do not affect the synthesis of prostacyclin (PGI_2) by the endothelial cells, which is a endogenous antiplatelet and vasodilatory prostanoid[10].

S18886 (terutroban) is a selective TP receptor antagonist developed for the secondary prevention of atherothrombotic complications in patients[12,13]. It has been shown to be a well-tolerated and powerful antiplatelet agent, able to inhibit

thrombus formation more efficiently than aspirin [14,15]. Previous studies in animal models have shown that S18886 displays anti-inflammatory properties combined with a decreased expression of adhesion molecules and a reduced recruitment of monocytes/macrophages within the arterial wall, resulting in the inhibition of atherogenesis [16].

A third approach to inhibit platelet activation is antagonizing the adenosine diphosphate (ADP) receptor on the platelet with compounds like clopidogrel, which, like aspirin, is a generally applied treatment in the clinic. Clopidogrel has been shown to be at least as effective as aspirin in preventing ischemic stroke, myocardial infarction and vascular death in patients suffering from CVD[17].

The purpose of this study was to evaluate the effects of inhibition of TP receptors by incremental doses of S18886 on existing atherosclerotic lesions in combination with dietary cholesterol intake lowering. For this study APOE*3Leiden transgenic mice were used, which is a well-established model for hyperlipidemia and atherosclerosis[18,19]. These mice become hyperlipidemic upon dietary cholesterol and respond to various hypolipidemic drugs in a similar way as humans[20] As a control for anti-platelet therapy an additional group of mice was treated with the ADP receptor antagonist clopidogrel.

Methods

Mice

Eighty-three female heterozygous APOE*3Leiden transgenic mice (16 to 18 weeks of age), characterized for human apoE[18], were used. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Scientific Research (TNO). Animals were bred by TNO.

Diets

To induce atherosclerosis development, all animals received a semi-synthetic western type diet (WD)[18] supplemented with 0.75% (w/w) cholesterol and 0.05% (w/w) cholate for ten weeks. Thereafter (on t=0 weeks) on the basis of age, body weight and plasma cholesterol and triglyceride levels, the mice were matched into five groups (n=13-14): The control group, fed WD + 0.025% (w/w) cholesterol only and four treatment groups, fed the same diet as the control group supplemented with either clopidogrel (3.8 mg/kg bw/d) or S18886 (10, 30 or 60 mg/kg bw/d) for 12 weeks (compounds provided by Institut de Recherches Internationales Servier, France). To determine the amount of atherosclerosis at the start of treatment 15

mice were sacrificed after matching at t=0 weeks and were considered the reference for baseline levels. The animals received food and water *ad libitum*.

Lipid and lipoprotein analysis and plasma SAA, MCP-1, E-selectin and VCAM-1
After a 4-hour fasting period, EDTA plasma was collected (Sarstedt, Germany). Total plasma cholesterol and triglyceride levels were measured (Roche Diagnostics, No-1489437 and No-1488872). Lipoprotein profiles were obtained by FPLC. Serum amyloid A (SAA) (Biosource International, Belgium), MCP-1, E-selectin and VCAM-1 (all R&D systems Inc, Minneapolis, USA) were determined by ELISA[19,21].

Histological assessment of atherosclerosis
After the 10-week induction and the 12-week treatment period the mice were sacrificed the hearts were dissected, formalin-fixed and embedded in paraffin. Per mouse, 4 serial aortic root sections (5µm, 50µm spaced) were used for quantification and qualification of the atherosclerotic lesions after staining with hematoxylin-phloxin-saffron (HPS). For determination of severity of atherosclerosis, the lesions were classified into 5 categories as described before[19,21]: I) early fatty streak, II) regular fatty streak, III) mild lesion, IV) moderate lesion, V) severe lesion. Per mouse the percentages of all lesions found in the respective categories were calculated. The total lesion area was calculated per cross-section. Collagen content of the lesion after Sirius Red staining, macrophage area after immunostaining with anti-mouse Mac-3 (BD Pharmingen, the Netherlands) and smooth muscle cells (SMCs) area after immunostaining with mouse anti-human actin (DAKO, Denmark), cross reacting with mouse actin, were quantified morphometrically. Also the number of monocytes adhering to the endothelium was counted after immunostaining with AIA31240 (Accurate Chemical and Scientific, New York, USA). All analyses were performed by the same operator, who was blinded for experimental group allocation.

Statistical analysis
Significance of differences was calculated by using parametric t-test for comparison of baseline levels at the start of the study with the control group. ANOVA tests, followed by Dunnett test were applied for comparison of the intervention treatment groups with the control group. A parametric Pearson's correlation was calculated for dose dependency. The Extreme Studentized Deviate method was used to identify and exclude outliers. $P<0.05$ was considered significant. All data are presented as mean ± SD.

Results

Plasma lipid levels

During the first 10 weeks of study, when the mice were fed the high cholesterol diet prior to intervention, average plasma cholesterol and triglyceride levels were 27.6 ± 7.3 mM and 2.3 ± 0.6 mM, respectively. As aimed for, these values decreased significantly to 5.3 ± 1.0 mM and 1.5 ± 0.3 mM ($p<0.001$) when the dietary cholesterol intake was reduced during the 12-week intervention period. The reduction was confined to the apoB-containing lipoproteins (data not shown). The total cholesterol exposure (plasma cholesterol level x duration of study) did not differ between all treatment groups and the control group. **Figure 1** represents the plasma cholesterol levels.

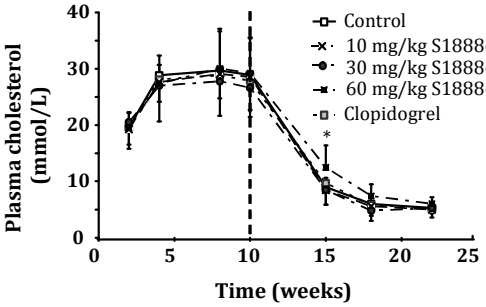

Figure 1 Mean plasma cholesterol levels over time. APOE*3Leiden mice were fed an atherogenic diet for 10 weeks to induce atherosclerosis development. Thereafter dietary cholesterol was lowered and supplementary intervention was started. *P=0.014; 60 mg/kg S18886 vs control.

	SAA (μg/mL)	**MCP-1** (pg/mL)	**E-Selectin** (μg/mL)	**VCAM-1** (μg/mL)
Baseline	13.3± 7.3	81 ± 34	188 ± 26	2.5 ± 0.3
Control	2.5 ± 0.8$^{\lozenge}$	24 ± 7$^{\lozenge}$	120 ± 10$^{\lozenge}$	2.3 ± 0.2
S18886 (10 mg/kg)	2.6 ± 1.0	26 ± 12	124 ± 16	2.5 ± 0.5
S18886 (30 mg/kg)	2.7 ± 0.7	21 ± 5	133 ±15	2.4 ± 0.4
S18886 (60 mg/kg)	3.4 ± 1.7	19 ± 8	127 ± 15	2.6 ± 0.4
Clopidogrel	3.0 ± 1.1	14 ± 5*	115 ± 19	2.5 ± 0.4

Table 1. Plasma levels of systemic inflammation marker Serum Amyoid A (SAA) and vascular inflammation markers MCP-1, E-selectin and VCAM-1 at baseline (t=0) and after 12 weeks of intervention. Cholesterol-lowering with or without additional treatment resulted in a significant decrease of SAA, MCP-1 and E-selectin levels. $^{\lozenge}$P<0.001 compared to baseline, *P<0.001 compared to control group.

Cholesterol-lowering reduces inflammation markers

Inflammation plays a major role in the development of atherosclerosis and is described to be influenced by lipid lowering[22-24]. Therefore we investigated the effect of dietary cholesterol-lowering and the additional treatments on plasma levels of the liver-derived plasma inflammation marker serum amyloid A (SAA), which reflects the overall systemic inflammatory state, and VCAM-1, MCP-1 and E-selectin, as markers for endothelial activation. Cholesterol-lowering from 27.6 to 5.3 mM by itself had clear anti-inflammatory effects as demonstrated by decreased plasma levels of inflammation markers SAA (from 13.3 ± 7.3 to 2.5 ± 0.8 μg/mL; $p<0.001$), MCP-1 (from 81 ± 34 to 24 ± 7 pg/mL; $p<0.0001$) and E-selectin (from 188 ± 26 to 120 ± 10 μg/mL; $p<0.0001$) (**table 1)**. Additional treatment with S18886 did not add to this effect, while clopidogrel significantly decreased MCP-1 levels (14 ± 5 pg/mL; $P<0.001$). Plasma VCAM-1 levels were not affected by cholesterol-lowering or supplementary treatments.

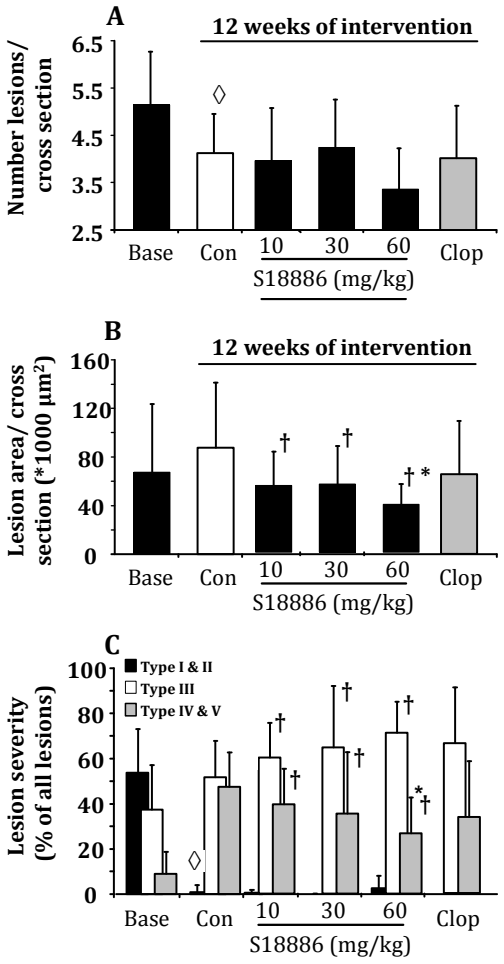

Figure 2. Effect of S18886 and clopidogrel (Clop) on the number of lesions per cross sections (A), the lesion area per cross section (B, † P_{trend} =0.006) and the lesion severity (C). Per lesion type the percentage of all lesions was calculated. ◊p<0.05 compared to baseline (Base) and *P<0.0001 compared to control (Con). † P_{trend} =0.011 type III and P_{trend} =0.007 type IV & V (compared to control).

Treatment with S18886 in combination with cholesterol-lowering inhibits progression of pre-existing atherosclerosis

Ten weeks of high cholesterol diet resulted in the development of a moderate amount of atherosclerosis in the aortic root, as measured in the mice sacrificed at t=0 weeks (baseline: 5.1 ± 1.1 lesions and 67.6 ± 56.0*1000 μm² lesion area per cross section; **figure 2A and 2B**). The

lesions were mainly foam cell rich lesions**.** As presented in **figure 2C,** 54 ± 19 % of the lesions were fatty streaks, consisting of only foam cells (type I and II), 37 ± 20 % were mild lesions, made up of foam cells covered by a fibrous cap (type III) and the remaining 9 ± 10% were severe lesions, which are infiltrated into the media and contain necrosis and cholesterol clefts (type IV and V). Despite a significant reduction in the number of lesions per cross section (from 5.1 ± 1.1 to 4.1 ± 0.8; $p<0.05$), lowering of plasma cholesterol alone during 12 weeks failed to inhibit the progression of atherosclerosis, as reflected by the progression of lesion area (87.5 ± 53.8 *1000 μm^2 in the control group vs 67.6 ± 56.0*1000 μm^2 at baseline, NS). Furthermore, a major shift in the distribution of lesion types as compared to baseline was observed. Almost all fatty streaks (type I and type II) had disappeared (1% ± 3 %; $p<0.0001$) and both mild type III lesions (52 ± 16 %; $p<0.05$) and severe type IV-V lesions (47 ± 16 %; $p<0.0001$) were present, reflecting a progression of atherosclerosis severity.

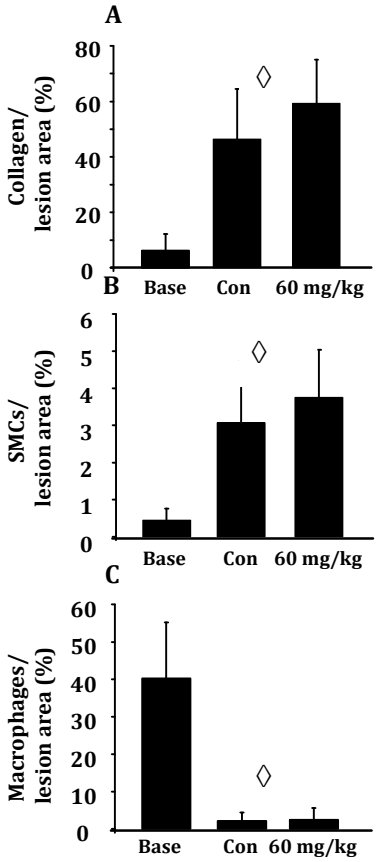

Figure 3 Effect of dietary cholesterol lowering and S18886 on lesion composition. The collagen content of the lesions as percentage of lesion area (A). The SMC area as percentage of total lesion area (B). The macrophage area as percentage of total lesion area per group (C). ◊P<0.001 compared to baseline. Base = baseline, Con = control.

Additional treatment with increasing doses of S18886 showed a dose-dependent reduction of the amount of atherosclerosis as compared to the control group ($p=0.006$ for Pearson's correlation coefficient) as reflected by the decreased lesion area. Whereas the doses of 10 and 30 mg/kg bw/d tended to reduce the lesion area by 37 % and 36 % respectively (NS), the highest dose of 60 mg/kg bw/d resulted in a significant decrease of 55 % ($p=0.001$). Moreover, the progression of the lesions towards more severe lesions as observed in the control group was partly prevented with increasing doses of S18886 ($p=0.011$ and 0.007 for Pearson's correlation coefficient for the increase in type III lesions and the decrease in type IV-V lesions, respectively). The highest dose of 60 mg/kg bw/d S18886 displayed 42% less severe type IV-V lesions as compared to the control group (27 ± 16 % vs 47 ± 16 %). Treatment with

clopidogrel failed to reach a statistically significant effect, either in lesion size, number or severity, when compared to control group.

Cholesterol-lowering stabilizes lesions

To assess whether platelet inhibition with S18886 together with cholesterol-lowering had additional effects next to the reduction of lesion size and severity, the lesion composition was analyzed. To this end the amounts of collagen, SMCs (both assumed to stabilize lesions) and macrophages (considered as an instable component) of the lesions were measured (**figure 3A-C)**. At baseline only 6.3 ± 5.9% of the total lesion area was filled with collagen, and SMCs were nearly absent with a presence of 0.5 ± 0.3% of the total lesion area. The major component of these lesions was macrophage foam cells (40.3 ± 14.9% of the total lesion area). Dietary cholesterol-lowering increased both the collagen and SMC content of the lesions about a 7-fold (46.4 ± 18.0%, $p<0.001$ and 3.1 ± 1.7%, $p<0.005$ respectively), and concomitantly dramatically decreased the amount of macrophages by 95% ($p<0.001$), resulting in more stable lesions. Supplementary treatment with S18886 did not add to this improved lesion composition induced by dietary cholesterol-lowering.

S18886 combined with cholesterol-lowering reduces monocyte adhesion

The onset of lesion development is considered to be the adhesion of monocytes to the activated endothelium followed by infiltration into the media and maturation to macrophages and foam cells. Feeding a high cholesterol diet resulted in 4.4 ± 2.9 adhering monocytes per cross section at baseline (**figure 4)**. Cholesterol-lowering did not prevent monocyte adhesion (3.7 ± 2.8 monocytes per cross section). As compared to the control, treatment with S18886 also reduced the adherence of monocytes to the endothelium in a dose-dependent manner ($p=0.007$ for Pearson's correlation coefficient) with only 1.1 ± 0.9 adhering monocytes per cross section in animals receiving the highest dose of S18886 ($p<0.05$). Treatment with clopidogrel tended to decrease the number to 1.7 ± 2.0 monocytes per cross section, but not significantly.

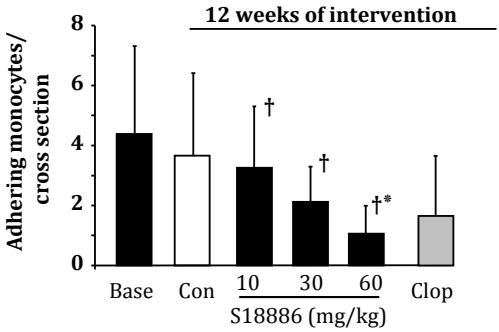

Figure 4. Effect of S18886 and clopidogrel (Clop) on the number of monocytes adhering to the endothelium per cross-section. *P<0.05 compared to control. †P_{trend} =0.007 (compared to control). Base = baseline, Con = control.

Discussion

This study shows that in a relevant model of advanced atherosclerosis in APOE*3Leiden mice the TP receptor antagonist S18886 together with cholesterol lowering in the diet reduced additionally and dose-dependently the atherosclerotic lesion area, the progression of lesion severity and the amount of adhering monocytes as compared to control.

In patients with increased risk for cardiovascular diseases, cholesterol-lowering by treatment with mainly statins is the first choice approach to reduce or prevent the progression of atherosclerosis or to even regress it. In the present study, dietary cholesterol was reduced and this resulted in lowering of plasma cholesterol to human-like levels of 5-6 mM within 4-8 weeks. Positive effects of cholesterol-lowering on atherogenesis were reflected by a strong decrease in systemic and vascular inflammation markers (SAA, MCP-1 and E-selectin) and by a reduction in lesion number (-20%). A decrease in macrophage content (-95%) and a 7-fold increase in SMC and collagen content of the lesions indicated a shift toward more stable lesions. Nevertheless, despite the strong decrease of plasma cholesterol levels in the intervention phase, the development of atherosclerosis continued. Albeit that a decreased number of lesions was observed, lesions kept on growing as demonstrated by an increase in lesion size (NS) and, more strikingly, by a massive progression from mild towards severe advanced lesions (when comparing control group to baseline levels). These observations revealed that in the present model, dietary cholesterol-lowering was unable to fully inhibit atherosclerosis progression and are in accordance with previous studies in APOE*3Leiden mice in which dietary cholesterol reduction was less efficient than drug-induced reduction by hypolipidemics[19,21].

A second line of defence in treatment of clinical atherosclerosis complications is platelet inhibition. Although not all physiological functions of platelets are fully clear yet, there is accumulating evidence that they go beyond aggregation and actively participate in atherosclerotic processes[25,26]. It has been described that platelets can oxidize LDL particles and stimulate their accumulation in monocytes and even stimulate CD34+ cells to differentiate into foam cells[27,28]. Moreover, activated platelets have been reported to increase the adhesive properties of monocytes[29] via the expression of adhesion molecules and the release of thromboxane A_2, a prostanoid that acts as a pro-inflammatory and chemotactic agent. This contention is supported by the observation of atheroprotection in

animals in which platelet inhibition was achieved[16,30,31] and of aggravating atherosclerosis after platelet activation[32] .

In the present study, platelet inhibition by S18886 was in part responsible for the decreased progression of atherosclerosis and reduced vascular inflammation. Treatment of the APOE*3Leiden mice with incremental doses of the selective TP-receptor antagonist S18886 resulted in dose-dependent beneficial effects on important parameters of the development of atherosclerosis. S18886 inhibited lesion progression, as represented by a reduction in lesion size (up to -55%) and lesion severity with a reduced progression towards more complicated lesions. This observation is of critical importance as complicated lesions are more prone to trigger clinical events after rupture. The adhesion of monocytes to the endothelium, a primary step in the formation of atherosclerotic lesions, was dose-dependently decreased by S18886 (up to -70%) as compared to the control group.

To our knowledge this is the first study that demonstrates that S18886 has additional atheroprotective effects on top of cholesterol-lowering. In different studies in apoE$^{-/-}$ mice[16], apobec1/LDLR DKO mice[33] and rabbits[34], it was shown that S18886 reduced the progression of atherosclerosis in a prevention design, in which the animals were treated during progression of atherosclerosis. Regarding the effect of S18886 on pre-existing atherosclerotic lesions, which is of more therapeutic relevance, inconsistent data were found. Without concomitant cholesterol lowering S18886 showed no beneficial effect in apobec1/LDLR DKO mice[33]. In rabbits the compound induced regression of pre-existing lesions as observed by MRI analysis in the absence of cholesterol-lowering[35] and showed no effect when cholesterol levels were lowered concomitantly[34]. In agreement with our data, S18886 was also shown to increase the stability of lesions as reflected by a decreased content in pro-inflammatory macrophages and lytic enzymes such as MMP[34,35].

Limited and ambiguous data on the effects of the platelet inhibitor clopidogrel on progression of atherosclerosis in animal models have been reported. In rabbits the compound showed atheroprotective and anti-inflammatory effects[31] at a similar dose as used in the present study, whereas it did not hamper atherosclerosis development in apoE deficient mice[36] at a higher dose. In accordance with the latter report, no atheroprotective effects of clopidogrel in combination with cholesterol-lowering on pre-existent atherosclerosis was observed.

Other positive effects of platelet inhibition on atherosclerosis were recently reported with the novel dual TP-receptor antagonist and thromboxane synthase inhibitor BM-573, by hampering the progression of pre-exisiting lesions in LDLr$^{-/-}$

mice[37]. No effect on inflammation markers was described in the latter study. In contrast, anti-inflammatory properties of S18886 were reported *in vitro* as well as *in vivo* in mice and rabbits[16,34]. It is possible that these anti-inflammatory effects of S18886 on plasma parameters are not visible in the present study, since they may be overshadowed by the strong effect of dietary cholesterol-lowering. However, S18886 demonstrated clear anti-inflammatory activity as evidenced by a marked reduction in monocyte adhesion, which is considered as a functional marker of activation of the endothelium *in vivo*. In conclusion, the present and reported data provide evidence for an anti-inflammatory capacity of S18886, ultimately resulting in inhibition of lesion progression.

In humans, the anti-platelet drug aspirin is widely accepted to reduce the risk of CVD. However, the major adverse side effect bleeding and the large prevalence of aspirin resistance (5-45%) are drawbacks of this drug[7,38]. In the large CAPRIE trial[17] clopidogrel was shown to be at least as effective as aspirin in preventing ischemic stroke, myocardial infarction and vascular death. Combining the two in the MATCH[39] and CHARISMA[40] study did, however, not significantly decrease cardiovascular events and may even increase major bleedings. Besides their proven benefit in patients with CVD, the variety in response to these anti-platelet compounds is considerable, this might be called 'resistance', possibly reflecting the unravelled complexity of mechanisms affected by these treatments[41]. Analysis of urine samples of aspirin treated patients of the HOPE study demonstrated that thromboxane B_2 levels were predictive for the risk of myocardial infarction and vascular death, providing evidence that inhibition of thromboxane production or activity might be protective for cardiovascular death[42]. This hypothesis was confirmed in the DAVID study[43], in which picotamide, a dual inhibitor of thromboxane A_2 synthase and receptor, was shown to be more effective in reducing all cause mortality in diabetic patients as compared to aspirin. Previously picotamide was shown to inhibit the progression of plaque growth in the carotid artery in diabetic patients[44]. These data suggest that antagonizing the TP-receptor may indeed have clinical benefits.

In conclusion, this study demonstrates that the TP-receptor antagonist S18886 combined with dietary cholesterol-lowering prevents the progression of established atherosclerosis lesions towards more advanced lesions. The atheroprotective effect of S18886 is suggested to stem from the combination of platelet inhibition and its additional effects resulting from TP antagonism in vascular and inflammatory cells, as reflected by a dose-dependent reduction in adhesion of activated monocytes. Data from ongoing clinical trials will indicate

whether treatment with the TP receptor antagonist S18886 in humans is also successful in the secondary prevention of atherosclerosis and its complications.

Acknowledgements

Ria van den Hoogen, Erik Offerman and Marijke Voskuilen are thanked for their superb technical assistance. Prof. Dr J.W. Jukema is an established clinical investigator of the Netherlands Heart Foundation (grant 2001D032). This study was supported by Institut de Recherches Internationales Servier (I.R.I.S.), Courbevoie, France.

Disclosures

L.C. is an employee of I.R.I.S., Courbevoie, France.

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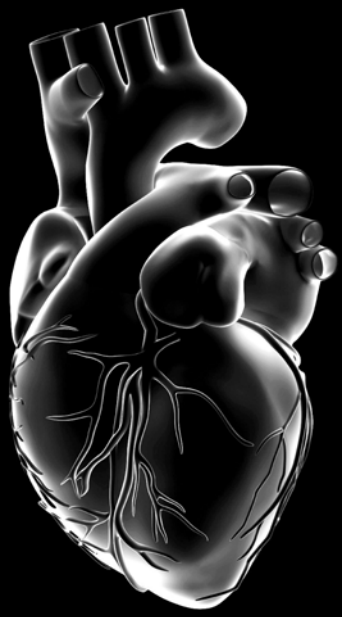

8

Negative effects of rofecoxib treatment on cardiac function after ischemia-reperfusion injury in APOE*3Leiden mice are prevented by combined treatment with thromboxane-prostanoid receptor antagonist S18886 (terutroban)

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Crit. Care Med. *2008 Sep;36(9):2576-82*

Abstract

Objective Selective COX-2 inhibition by rofecoxib was associated with increased risk of cardiovascular events. We hypothesized that concomitant treatment with thromboxane prostanoid receptor antagonist S18886 might ameliorate possible negative effects. We evaluated the effects of S18886, rofecoxib, and the interaction of both compounds in a combined treatment on myocardial infarct (MI) size and cardiac function after experimental ischemia/reperfusion injury in hyperlipidemic APOE*3Leiden transgenic mice.

Methods and Results After four weeks of feeding an atherogenic diet, MI was induced by a 30-min ligation of the left anterior descending coronary artery, followed by reperfusion. Oral compound treatment was initiated 90 minutes prior to MI, and continued daily by gavage for seven days. Four treatment groups (n=12, each) were studied: solvent (Control), S18886, rofecoxib, and S18886 plus rofecoxib. One week after MI, the mice were anesthetized and cardiac function was quantified by left ventricular (LV) pressure-volume relationships obtained by miniature pressure-conductance catheters. No significant differences in infarct size were found between groups as measured by morphometry. Compared to Control, treatment with S18886 did not affect heart function whereas the rofecoxib group had significantly lower cardiac output (4.5±0.8 vs. 3.2±1.1 mL/min, $p<0.01$), lower ejection fraction (40±8 vs. 27±11%, $p<0.005$), and increased end-systolic volume (18.6±5.7 vs. 28.6±9.0 µL, $p<0.05$). The group with combined (S18886+rofecoxib) treatment was not different from Control. Statistical analysis showed significant interactive effects between S18886 and rofecoxib indicating that negative effects of rofecoxib on cardiac function were prevented by S18886 treatment.

Conclusion Rofecoxib treatment reduced global and systolic LV function after ischemia-reperfusion injury in APOE*3Leiden mice. These negative effects are prevented by combined treatment with thromboxane prostanoid-receptor antagonist S18886.

Introduction

Cyclooxygenases are the rate-limiting enzymes in prostaglandin (PG) synthesis. They metabolize arachidonic acid to PGH_2, which is followed by cell-specific synthase and isomerase enzymes (**figure 1**). Two cyclooxygenase iso-enzymes are characterized: COX-1 and COX-2. COX-1 is constitutively expressed in most cells to mediate physiological responses and regulate homeostasis. COX-2 is expressed in a few organs including the central nervous system, kidneys and the gonads in a constitutive manner similar to COX-1[1], but shows increased expression in pathological conditions such as inflammation and ischemia and acts as an inflammatory mediator[2]. Based upon these findings, a subclass of nonsteroidal anti-inflammatory drugs, designed to selectively inhibit COX-2 has been developed for the treatment of chronic inflammatory diseases. However, their use has been under debate since an increased risk of cardiovascular events with the COX-2 inhibitor rofecoxib was noted in the Vioxx Gastrointestinal Outcomes Research study (VIGOR)[3] and was also observed in subsequent studies[4,5]. The Adenomatous Polyp Prevention on Vioxx (APPROVe)[6] study also showed a significant increase in the risk of myocardial infarction and atherothrombotic events, leading to the market withdrawal of rofecoxib. Two other large randomized controlled trials assessing the safety of celecoxib [7] and valdecoxib found the same side effects[8]. A recently published meta-analysis based on nine case-control studies and two cohort studies showed an relative risk of 1.35 for serious cardiovascular events with rofecoxib treatment[9]. To explain these observations, the hypothesis has been advanced that COX-2-selective inhibitors shift the balance in the vascular system between the prostanoid thromboxane A_2 (TXA_2) and prostaglandin I_2

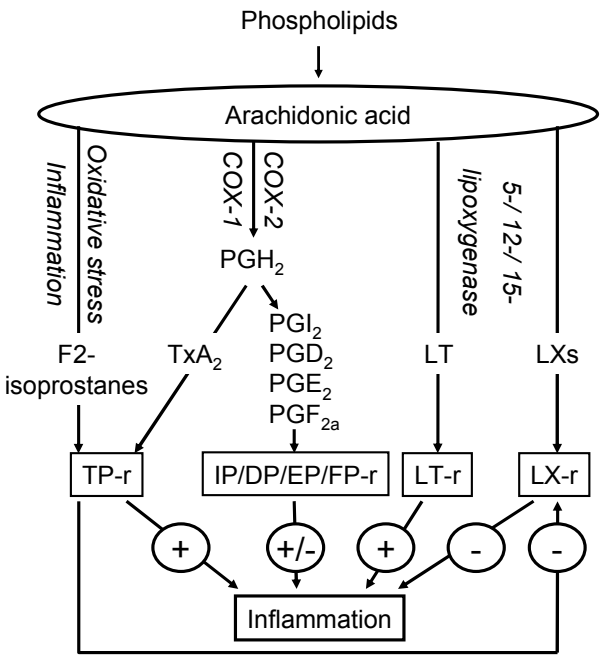

Figure 1. Schematic overview of the production arachidonic acid metabolites. The COX-derived PGs are mediators of inflammation, whereas TXA_2 and the LTs are mainly pro-inflammatory. LXs have anti-inflammatory effects. COX = cyclooxygenase, LT = Leukotriene, LX = lypoxin,PG = prostaglandin, xP receptor = prostanoid receptor, TXA_2 = Thromboxane A_2.

(PGI_2) by suppressing the synthesis of (endothelial) COX-2-derived PGI_2, without changing the (platelet) COX-1-derived TxA_2 synthesis[10]. Increased TXA_2 release has been shown during myocardial ischemia, possibly not only derived from aggregating platelets but also from macrophages and other immunologically reactive cells[11]. Since TXA_2 is responsible for platelet aggregation, vasoconstriction of coronary arteries, and has pro-inflammatory and cytotoxic effects[12], it can be speculated that TXA_2 exerts a deleterious effect during ischemia and reperfusion.

S18886 is a potent and selective antagonist of the thromboxane-prostanoid (TP) endoperoxide receptor[13]. The compound is being developed for the secondary prevention of ischemic events in patients with atherosclerosis. S18886 inhibits platelet aggregation and vasoconstriction induced by the agonists of the TP receptor, without affecting the production of prostacyclin by endothelial cells and other vessel wall cells[14]. We thus hypothesized that concomitant treatment with S18886 might ameliorate negative effects (if any) of COX-2 inhibition on the cardiovascular system, by redressing the functional balance between inflammatory mediator PGI_2 and the pro-inflammatory TXA_2.

The goal of the present study was to further investigate the previously reported negative effects of the COX-2-selective inhibitor rofecoxib on myocardial infarct size and on heart function and to test the hypothesis that concomitant treatment with thromboxane prostanoid receptor antagonist S18886 might ameliorate the anticipated negative effects. We used APOE*3Leiden transgenic mice on a standardized Western type diet - an established mouse model for hyperlipidemia and pharmaceutical interventions[15,16] - and induced myocardial injury by ischemia-reperfusion in order to mimic the human situation with regards to elevated plasma lipid levels and relatively limited infarct size due to early reperfusion by PTCA or thrombolysis.

Methods

Animals

Twelve-week-old male heterozygous APOE*3Leiden transgenic mice backcrossed into a C57BL6/J background and characterized by an ELISA for human apoE[15], were used. The mice were housed in a clean-conventional animal room at the Gaubius Laboratory TNO. Housing conditions were: relative humidity 50-60%, temperature ~21˚C, light cycle 6 am to 6 pm. Mice were housed in macrolon cages. Food and water were supplied *ad libitum*. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Scientific Research TNO, and conformed to the rules and regulations set forth by The Netherlands Animal Experiments Act.

Study design

Fifty APOE*3Leiden mice were fed a standardized Western type diet, containing 15% (w/w) cacao butter, 1.0% (w/w) cholesterol and 0.05% (w/w) cholate[17]. After three weeks, mice were randomized into four groups of 12 or 13 mice each, matched for body weight and plasma cholesterol level. One week later, myocardial ischemia and reperfusion was induced, essentially as described by Michael et al.[18]. One hour prior to the induction of anesthesia (approximately 90 min before the ischemic period), mice were treated orally (100 µL/10 gram bw) with vehicle (1.0% hydroxyethylcellulose) for the control group, 10 mg S18886/kg bw, for the S18886 group, or 10 mg rofecoxib/kg bw for the rofecoxib group. The S18886 plus rofecoxib combination group received 10 mg S18886/kg plus 10 mg rofecoxib/kg (given separately, each in 50 µL/10 gram body weight). Subsequently, drugs or solvent were administered daily by gavage until sacrifice seven days later. The S18886 dose was based on previous studies[13,19,20] showing effective TP receptor antagonism with 5-10 mg/kg/day. Likewise rofecoxib dose was chosen in line with previous studies[21-23] mimicking human use and taking into account approximately 10x higher metabolism in the mouse.

Body weight was determined weekly. Seven days after ischemia-reperfusion, heart function was assessed by LV pressure-volume relationships (as described in detail below), and the hearts were harvested to measure the infarct size. Before excision of the heart, intracardiac blood was drawn and serum was prepared (30 min 37ºC, 30 min 4ºC, 2000 x g for 15 min) and stored at -70ºC.

Induction of myocardial ischemia.

The mice were anesthetized by intraperitoneal injection of a mixture of fentanyl (0.8 mg/kg bw), fluanison (25 µg/kg bw) and midazolam (12.5 µg/kg bw) (FFM). An intratracheal tube was inserted, and the animals were artificially ventilated, using a dedicated mouse ventilator (UNO, Zevenaar, The Netherlands). After left lateral thoracotomy, the left anterior descending coronary artery (LAD) was ligated just distal to the left atrial appendix. After thirty minutes of ischemia (evidenced by blanching in the LV), reperfusion was initiated by removing the ligature. The thorax was closed, and the mouse was allowed to recover on a heating pad[18]. Oxygen support and an analgesic (Nubaine) were given as required.

Hemodynamic measurements

Seven days after myocardial ischemia-reperfusion, the mice were re-anesthetized with FFM, intubated, ventilated, and instrumented to assess LV function by pressure-volume loop analysis. The jugular vein was cannulated for infusion of hypertonic saline to determine parallel conductance. Via the carotid artery a miniaturized pressure-conductance catheter (SPR-839, Millar Instruments, Houston, TX) was positioned into

the LV. The abdomen was opened just below the diaphragm to enable temporary preload reductions by directly compressing the inferior vena cava. The pressure-conductance catheter was connected to a Sigma-SA signal processor (CD Leycom, Zoetermeer, The Netherlands) for on-line display and registration of LV pressure and volume signals. All data were acquired at 2000Hz using Conduct-NT software (CD Leycom) and analyzed off-line by custom-made software (CircLab). LV pressure-volume signals were acquired in steady state to quantify general hemodynamic conditions: heart rate (HR), stroke volume (SV), cardiac output (CO), end-diastolic volume (EDV), end-systolic volume (ESV), ejection fraction (EF), end-diastolic pressure (EDP), and end-systolic pressure (ESP). Stroke work (SW) was determined as the area of the pressure-volume loop, and the maximal and minimal rate of LV pressure change, dP/dt_{MAX} and dP/dt_{MIN}, were obtained. Effective arterial elastance (E_A) was calculated as ESP/SV. Relaxation time τ was calculated as the time-constant of mono-exponential pressure decay during isovolumic relaxation[24]. To obtain load-independent indices of LV function, we determined pressure-volume relations by recording pressure-volume loops during a gradual preload reduction. The slopes of end-systolic pressure-volume relation (end-systolic elastance, E_{ES}) and end-diastolic pressure-volume relation (end-diastolic stiffness, E_{ED}) quantify systolic and diastolic function, respectively[25]. This approach is illustrated in **figure 2**.

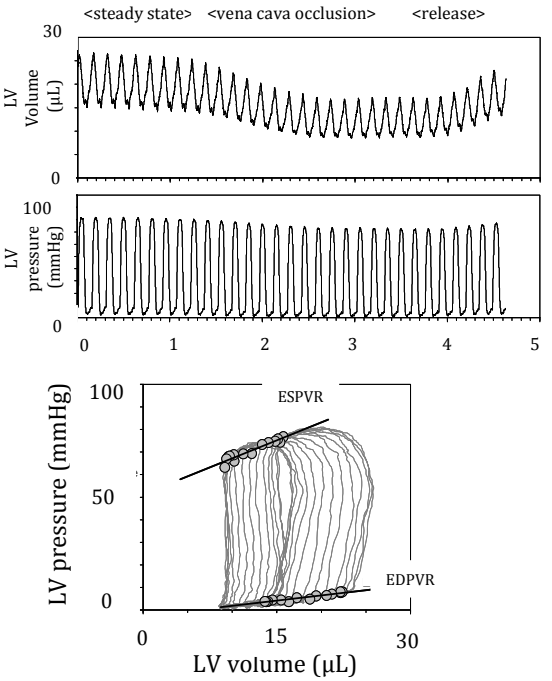

Figure 2. Typical pressure-volume loops to illustrate our methodology. End-systolic and end-diastolic pressure-volume relations (ESPVR, EDPVR) were determined from pressure-volume loops obtained during gradual preload reduction by vena cava occlusion. The slopes of these relations (E_{ES} and E_{ED}) are load-independent parameters of systolic and diastolic LV function. Steady state hemodynamic parameters were derived from pressure-volume loops just prior to vena cava occlusion.

Infarct area, morphometry and immunohistochemistry

After hemodynamic measurements, a cannula was inserted into the aorta, and the heart was stopped in diastole by injection of 0.1M cadmium chloride, followed by perfusion of the heart with sodium nitroprusside (0.1mg/mL saline) for two minutes and perfusion fixation using 4% p-

formaldehyde. The heart was then removed, fixed overnight in p-formaldehyde, and cut into six 1 mm-thick slices, perpendicular to the long axis of the heart. These slices were flat-embedded in paraffin and 3 µm-thick sections were prepared. Sections were stained by the haematoxylin-phloxin-saffran (HPS) method for general histology. To delineate the infarcted area, sections were stained immunohistochemically with a mouse monoclonal antibody against cardiac Troponin T (Lab Vision Co, Fremont, CA; NeoMarkers cat # MS-295-P), and with Sirius red F3B for collagen to delineate the infarcted area. On paraffin sections from the six 1 mm-slices the infarct area and the total LV wall area (including the septum) were measured by morphometry (Leitz Qwin system). The LV area was measured on HPS-stained sections. The infarcted area was measured on Sirius red stained sections. Sirius red staining and Troponin T-immunohistochemical staining were generally fully overlapping, but delineation was easier with Sirius-Red staining and therefore preferred for infarct quantification. The infarct area was expressed as percentage (vol/vol) of the LV wall volume. The volumes were calculated as measured area multiplied by slice thickness. COX-2 immunoreactivity was visualized immunohistochemically[26], using an affinity-purified polyclonal antibody against COX-2 (Cayman Chemical Co, Ann Arbor, MI, cat # 160126).

Plasma assays

At randomization and at sacrifice total cholesterol (Roche Diagnostics, No-1489437) and total triglycerides (Roche Diagnostics, No-1488872) were determined.

Statistical analysis

Differences between groups were analyzed by one-way ANOVA followed by post-hoc Bonferroni correction for multiple comparisons using SPSS for Windows (version 12.0, SPSS Inc, Chicago IL). Differences were considered significant at $p<0.05$. All data are presented as mean±SD (unless otherwise indicated). To specifically test whether the effect of rofecoxib was significantly altered by the absence or presence of S18886 we applied a full factorial univariate linear regression model. If the S18886*rofexocib interaction term included in this model reached significance, this indicated that presence of S18886 significantly influenced the effect of rofecoxib (or *vice versa*).

Results

Body weight and plasma values

Body weights increased during the dietary study phase, and slightly decreased postoperatively, most likely due to the ischemic insult. Plasma cholesterol and triglyceride levels were equal in all groups both at surgery (week 3) and at sacrifice (week 5) (**table 1**).

		Control	S18886	ROF	ROF + S18886
At randomization					
Body weight	(gram)	22.9 ± 1.9	23.0 ± 1.6	24.2 ± 2.5	23.6 ± 2.1
Cholesterol	(mmol/L)	12.4 ± 2.8	13.2 ± 7.1	11.6 ± 4.2	12.7 ± 4.7
Triglycerides	(mmol/L)	2.1 ± 0.6	1.9 ± 0.8	1.8 ± 0.7	2.1 ± 0.9
At sacrifice					
Body weight	(gram)	22.4 ± 1.4	23.0 ± 1.6	24.9 ± 2.2	23.8 ± 1.7
Cholesterol	(mmol/L)	11.9 ± 3.5	14.3 ± 3.0	11.8 ± 6.5	12.1 ± 2.1
Triglycerides	(mmol/L)	1.9 ± 0.6	2.0 ± 0.5	1.8 ± 0.6	2.1 ± 0.5

Table 1. Body weight and plasma lipids. Body weight and plasma cholesterol and triglycerides were measured at randomization and at sacrifice. No significant differences between groups were observed at any time point.

Ventricular volume and infarct size

LV myocardial volume and infarct size did not differ between groups (**figure 3**). In the control group, the 7-day-old infarct constituted 9.0±3.9% of the LV wall volume.

Cardiac function

The results of the cardiac function measurements are shown in **table 2**. ANOVA indicated significant differences between groups for CO, ESV and EF. SW just failed to reach statistical significance (P=0.067). These indices are presented as bar graphs in **figure 4** (left panels). Between-group comparisons showed that the rofecoxib group had significantly lower CO, higher ESV and lower EF compared to the control group, indicating reduced global and systolic LV function. No other significant effects were present, except for a significantly higher CO in the S18886+rofecoxib group compared to the rofecoxib group. The finding that neither

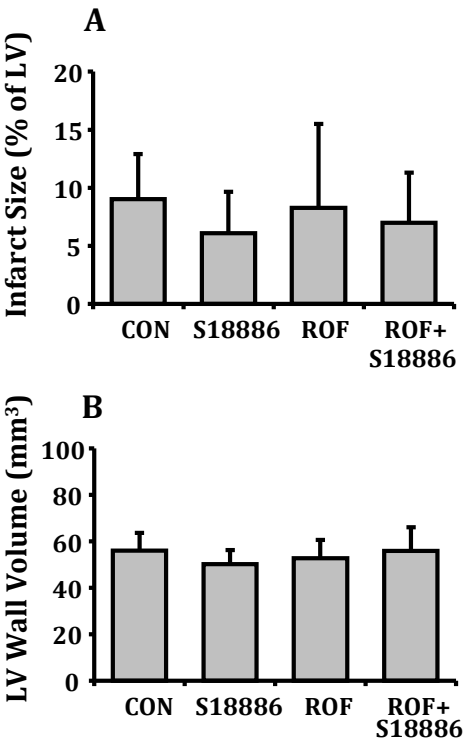

Figure 3. The effect of S18886, rofecoxib (ROF), and their combination on infarct size and LV wall volume. No differences were observed between the groups. Data are presented as mean ± SD.

the S18886 nor the group with combined S18886+rofecoxib treatment showed significant differences with the control group suggests an interactive effect by which the negative effects of rofecoxib are prevented by combined treatment with S18886. To specifically test the rofecoxib-S18886 interactive effects we applied a univariate analysis. The results are presented in **table 2** and for the indices that showed significance also displayed in **figure 4** (right panels). The results show significant interaction for CO, SW and EF, and marginally significant interaction (p=0.074) for ESV. The significant interaction indicates that the effect of rofecoxib is different in the absence and presence of S18886. In all cases the significant negative effect of rofecoxib on cardiac function in absence of S18886 (-S18886) was reversed in presence of S18886 (+S18886).

Cardiac function		Groups					Univariate linear		
		Control	S18886	ROF	ROF + S18886	ANOVA P	S18886 effect	ROF effect	Inter-action
General									
HR	(beats/min)	385 ± 51	369 ± 42	350 ± 58	377 ± 62	0.437	0.735	0.372	0.181
CO	(mL/min)	4.5 ± 0.8	3.9 ± 1.3	3.2 ± 1.1*	4.6 ± 1.3#	**0.017**	0.254	0.393	**0.004**
SW	(mmHg.µL)	800 ± 160	668 ± 260	584 ± 294	819 ± 232	0.067	0.464	0.651	**0.012**
E_A	(mmHg/µL)	6.2 ± 1.3	7.2 ± 3.7	7.8 ± 3.7	5.9 ± 1.9	0.345	0.616	0.860	0.084
Systolic									
ESV	(µL)	18.6 ± 5.7	23.2 ± 10.7	28.6 ± 9.0*	24.4 ± 7.0	**0.044**	0.909	**0.025**	0.074
ESP	(mmHg)	70.3 ± 8.7	64.9 ± 16.4	61.9 ± 13.8	66.5 ± 9.3	0.533	0.900	0.354	0.175
EF	(%)	40.4 ± 8.1	33.4 ± 9.4	27.0 ± 11.0**	35.2 ± 7.5	**0.008**	0.810	**0.032**	**0.006**
dP/dt_{max}	(mmHg/ms)	6.0 ± 1.9	4.7 ± 1.7	4.5 ± 2.2	5.5 ± 2.3	0.263	0.799	0.527	0.063
E_{ES}	(mmHg/µL)	2.1 ± 0.8	1.8 ± 0.6	2.0 ± 1.0	1.7 ± 0.5	0.510	0.158	0.650	0.794
Diastolic									
EDV	(µL)	29.8 ± 6.5	33.3 ± 13.0	37.1 ± 10.1	35.8 ± 8.8	0.293	0.705	0.092	0.396
EDP	(mmHg)	8.4 ± 3.2	9.5 ± 5.3	9.9 ± 5.4	6.7 ± 1.8	0.280	0.400	0.618	0.091
$-dP/dt_{min}$	(mmHg/ms)	4.8 ± 1.1	3.9 ± 9.6	3.8 ± 1.4	4.3 ± 1.3	0.195	0.650	0.442	**0.049**
τ	(ms)	11.5 ± 2.1	12.9 ± 1.4	13.3 ± 3.1	13.3 ± 3.0	0.269	0.353	0.139	0.345
E_{ED}	(mmHg/µL)	0.43 ± 0.20	0.49 ± 0.25	0.47 ± 0.31	0.33 ± 0.15	0.315	0.532	0.376	0.125

Table 2. Cardiac function: general, systolic and diastolic indices. * p<0.05 vs Control group; ** p<0.005 vs Control group; # p<0.05 vs Rofecoxib group (ROF). HR = heart rate; CO = cardiac output; SW = stroke work; E_A = arterial elastance (afterload); ESV = end-systolic-volume; ESP = end-systolic-pressure; dP/dt_{MAX} = maximal rate of pressure increase; E_{ES} = end-systolic elastance; EDV = end-diastolic-volume; EDP = end-diastolic-pressure; $-dP/dt_{MIN}$ = maximal rate of pressure decline; τ = relaxation time constant; E_{ED} = diastolic stiffness.

Immunohistochemistry of COX-2

Immunohistochemical staining for COX-2 showed minor or no reactivity in the myocardium remotely from the infarct area, but was upregulated in cardiomyocytes and vascular smooth muscle cells adjacent to an infarcted area (**figure 5**). Occasionally COX-2 was expressed in scattered cells in the infarcted area (most clearly in conjunction with signs of recent inflammatory activity). No differences were observed between the groups in the present study, as could be expected because infarct size was similar.

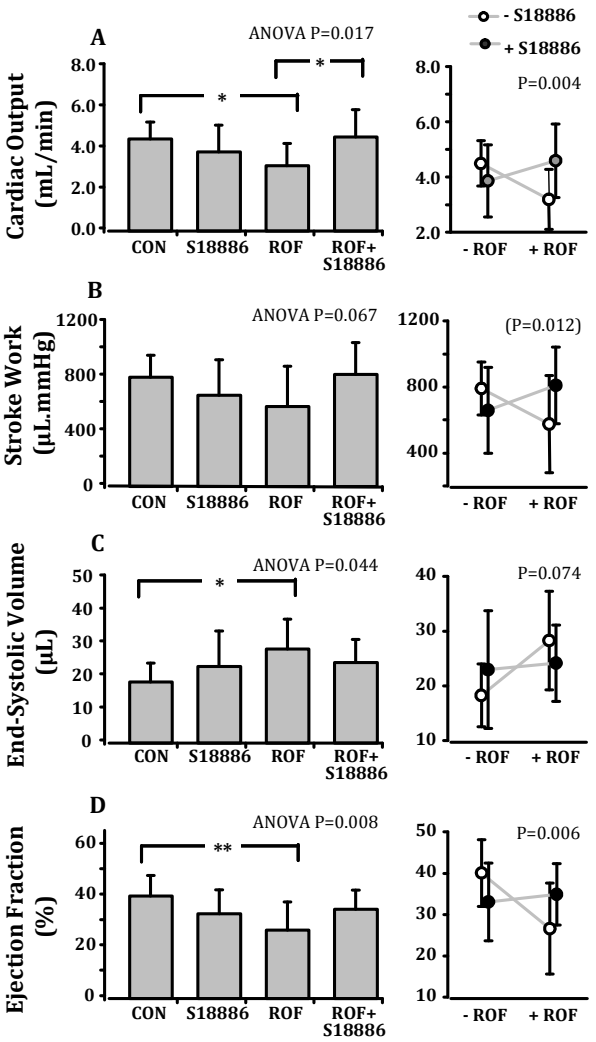

Figure 4. Cardiac output (A), stroke work (B), end-systolic volume (C) and ejection fraction (D) in control (CON) mice and in mice treated with S18886, rofecoxib (ROF), or ROF+S18886. The bar graphs in the left panels show the results for the four groups with ANOVA statistics. The figures in the right panels display the same data from the same four groups but focus on the interactive effects to illustrate the statistical method as described in detail in the text. The P-values refer to the significance of the interactive effect. Full statistics are provided in table 2.

Discussion

In line with previous studies, the present study showed significant negative effects of daily rofecoxib treatment on global and systolic LV function in mice seven days after ischemia reperfusion injury. The main new aspect of our study is the finding that these effects may be prevented by combining the rofecoxib treatment with S18886. The groups did not show differences in infarct size indicating that the hemodynamic effects were not primarily resulting from altered infarct size.

Beneficial effects of TP receptor antagonism have previously been reported in experimental studies of ischemia-reperfusion injury in several species[27,28]. However, the mechanism by which TP receptor antagonists exert their cardioprotective effect is not clear. It does not appear to be the consequence of increased coronary blood flow into ischemic region[29], nor does it appear to be the consequence of a reduced neutrophil accumulation[30]. A hypothesis is that TXA_2 released during reperfusion could result in vasoconstriction and platelet aggregation, contributing to a

no-reflow phenomenon to parts of ischemic myocardial tissue, despite a completely restored blood flow in the large coronary vessels. Other explanations for the benefits of TP-receptor antagonism are an enhanced blood flow during reperfusion, which is related to the increase in tissue viability, an improved utilization of oxygen in myocardial cells[31] or a reduced formation of free radicals[32]. Additionally TP receptor antagonist S18886 has shown anti-inflammatory actions in different murine models of hyperlipidemia and atherosclerosis[19,20,33,34]

In the present study we aimed for 'human like' conditions with regard to plasma lipid levels and ischemia-reperfusion mimicking clinical revascularization by early PTCA or thrombolysis rather than permanent coronary occlusion. Therefore we used animals with slightly elevated plasma lipid levels and obtained a relatively small infarct size. The area-at-risk after ligation of the LAD coronary artery was not measured, because the 7-day interval between the induction of the infarct and sacrifice made such a measurement of little relevance. In previous experiments (unpublished data) we acutely measured an area-at-risk after identical ligation of the LAD of about 38-40 % (vol/vol) of the LV. This would mean that the infarcts typically constituted about 23% of the area-at-risk, suggesting that the ischemic insult was less severe (but more clinically relevant) than in many previous murine myocardial infarction studies, which limits a direct comparison. In the present study, S18886 at 10 mg/kg/day seemed to be associated with a smaller infarct size than control but the difference was not statistically significant (6.1±3.6 vs. 9.0±3.9%, p=NS). Since it has been shown at least in rats[35] that relatively small infarcts (13% of the LV) do not result in reduced global heart function per se, it was not surprising that S18886 did not influence heart function. In fact, if anything, there appeared to be some tendency for depressed cardiac function, which however did not show statistical significance. Rofecoxib did not affect infarct size by itself, but did induce a significant cardiodepression evident from an increased ESV and a

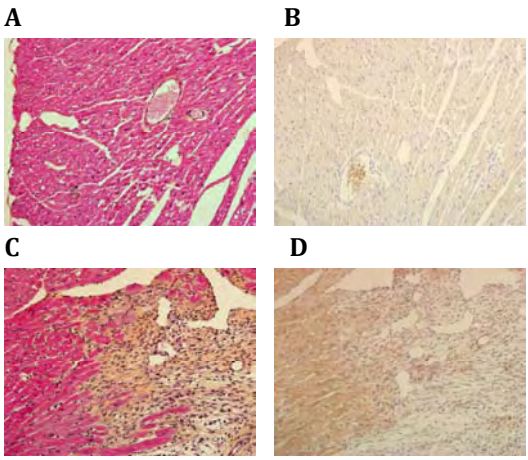

Figure 5. The upper panels show HPS staining (A) and immunohistochemical staining for COX-2 (B) of a section remote from the infarct area. The lower panels show staining for a section adjacent to the infarct. The remote cardiomyocytes do not express COX-2 (B), but the cardiomyocytes adjacent to the infarct area show clear COX-2 reactivity (D). Some COX-2 expression was present in scattered cells in the infarcted area. This example was from the Control group. No between-group differences were observed.

reduced EF and CO and a tendency to reduce SW. When rofecoxib treatment was combined with S18886 these effects were fully prevented. This provides evidence for S18886 to have a significant beneficial interactive effect when added to rofecoxib treatment, which was supported by statistical analysis of the interactive effects.

The use of COX-2 selective inhibitors in animal models of ischemic heart disease has been reported to be both beneficial and deleterious. Recent studies have demonstrated that COX-2 acts as a cardioprotective protein in the late phase of ischemic preconditioning. IP-receptor deficiency led to an increased infarct area[36] and COX-2 expression was associated with a cardioprotective effect after ischemic preconditioning[37]. Recently, impaired systolic function upon COX-2 inhibition was also found in a pig model of MI, with infarct size and EF comparable to our study[38]. Conversely, some studies with murine models of MI[22,23] and chronic heart failure[21] showed improved cardiac function in the group treated with the selective COX-2 inhibitors, without affecting the infarct size. However, in these MI studies the LAD was permanently ligated, which is not comparable to our study. Scheuren *et al*.[37,39] demonstrated, in a rat model of myocardial infarction, that a 4-day treatment with rofecoxib (3 mg/kg/day) resulted in reduced influx of inflammatory and fibroblast-like cells into infarcted tissue, without affecting the infarct size. In line with previous findings[2], we found presence of COX-2 immunoreactivity in heart muscle after ischemia-reperfusion injury suggesting that a change in local PGI_2 production might be involved in the changes in LV function after myocardial infarction. The favorable effects of S18886 may thus be due to restoring the local TXA_2-PGI_2 balance. However, it is also possible that antagonism of TP-receptors inhibits their activation by ligands such as TXA_2 and isoprostanes and subsequently prevents the induction pro-inflammatory pathways. As presented in **figure 1**, the metabolic pathway of arachidonic acid is a balanced and complex network of different cascades. It has been shown that agonizing the TP-receptor may also result in a reduced expression of the lipoxin (LX)-receptor[40], inhibiting the anti-inflammatory effects of the LXs. This might also contribute to the protective effect of S18886.

We conclude that COX-2 inhibition by daily rofecoxib treatment has deleterious effects on cardiac function after ischemia-reperfusion injury in APOE*3Leiden mice. These negative effects on ESV, CO, EF and SW are prevented by concomitant treatment with thromboxane prostanoid-receptor antagonist S18886. This study proves beneficial interaction of S18886 and rofecoxib, and might provide clues to further elucidate the mechanism underlying the increase in cardiovascular risk associated with COX-2 inhibiting treatments.

Acknowledgements
Ria van den Hoogen, Annemarie Maas and Erik Offerman are thanked for their excellent

technical assistance. Dr. Hein Putter is thanked for his assistance in the statistical analyses. Institut de Recherches Internationales Servier (Courbevoie, France) is gratefully acknowledged for supporting this study in part by an unconditional grant.

Disclosures

S.C. is an employee of I.R.I.S., France.

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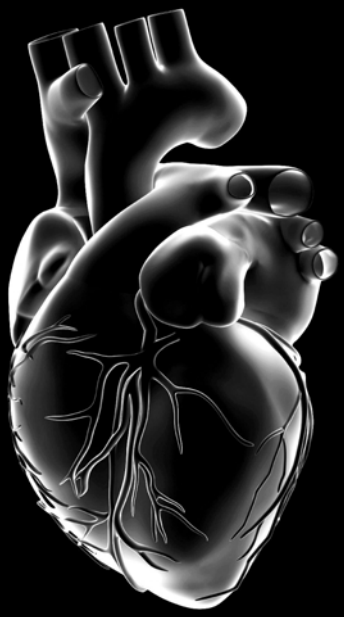

9

General Discussion and Future Perspectives

Cardiovascular disease (CVD) is the number one cause of death globally and is expected to remain the leading cause of death according to figures of the World Health Organization[1]. The single most important contributor to the growing burden of CVD is atherosclerosis, a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. Atherosclerosis is considered as a multifactorial disease in which lipids and inflammation play major roles[2-4]. This thesis presented and discussed a variety of novel pharmaceutical interventions in experimental CVD.

In pre-clinical stages of the development of new anti-atherosclerotic compounds appropriate animal models are a major necessitate. In the current thesis we have made use of APOE*3Leiden transgenic mice as an animal model for hyperlipidemia and atherosclerosis[5]. The APOE*3Leiden mouse model has been proven to be representative for the human situation regarding very low density lipoprotein (VLDL) and LDL metabolism, its responsiveness to hypolipidemic drugs (like statins, fibrates etc.) and nutrition. In contrast, wild type mice as well as apoE deficient and LDL receptor deficient mice do not or only partially respond to these hypolipidemic drugs[6]. In addition, depending on the level of plasma cholesterol, APOE*3Leiden mice develop atherosclerotic lesions in the aorta resembling those found in humans with respect to cellular composition and morphological and immunohistochemical characteristics. Furthermore, the atherosclerosis development in these mice is accelerated upon introducing low grade systemic inflammation, for instance by feeding a high-caloric western-type diet, containing saturated fat and cholesterol.

Like wild type mice, the APOE*3Leiden mice do not express cholesteryl ester transfer protein (CETP), a protein that is very crucial in high density lipoprotein (HDL) metabolism. Consequently, APOE*3Leiden mice are not suitable for studying (the regulation of) HDL metabolism, specifically. In order to render the APOE*3Leiden mouse also suitable for studying HDL metabolism, we recently crossbred our APOE*3Leiden mouse with the human CETP transgenic mouse (APOE*3Leiden.CETP mouse). In these APOE*3Leiden.CETP mice the HDL levels are indeed enhanced by hypolipidemic drugs, like statins and fibrates, comparable to what is observed in humans[7-9].

Therefore, we used the APOE*3Leiden.CETP mouse model also to clarify the mechanistical background of the HDL-cholesterol (HDL-C) raising effect of niacin in **chapter 3.** Niacin is known since the 1950s to beneficially modulate lipid levels and lipoprotein composition. Whereas it is the most potent HDL-C increasing compound currently available, only its TG and (V)LDL-C decreasing effects are well understood. We demonstrated that CETP plays a crucial role in the niacin induced increase in plasma HDL-C and apoAI levels. Niacin reduced the CETP dependent transfer of cholesterol from HDL to (V)LDL due to a lower hepatic CETP expression as well as a reduced plasma (V)LDL pool. It should be taken into account, however, that the HDL raising effect of niacin is most likely secondary to its TG and (V)LDL-C lowering effect brought about by inhibiting hormone sensitive lipase activity in the adipose tissue[10]. Despite its very

potent lipid modulating effect, niacin has not been a very successful drug thus far, due to its side-effect: severe flushing. This problem might be overcome by the current use of an extended release form of niacin or analogues like acipimox, by combining niacin with prostaglandin D_2 (PGD_2) receptor antagonists, which receptor mediates this cutaneous flushing.

Currently two trials evaluating effects of this combination drug on lipid lowering (HPS2-THRIVE) and carotid intima-media thickness (ACHIEVE) are underway. Post-hoc analysis of a subgroup of ARBITER-2, a randomized, placebo-controlled trial, showed increases in HDL-C upon daily intake of the extended release form of niacin on top of statin treatment (+20%), which were associated with reduced progression of carotid intima-media thickness in the setting of both normal glycemic status and diabetes mellitus[11]. Clinical trials evaluating the secondary prevention of CVD by treatment with the extended release form of niacin are currently underway[12,13]. Recently, the niacin receptor GPR109A has been identified, which now is a new target for novel niacin analogues in order to increase HDL-C[10]. This has gained much interest because of the recent negative results of CETP inhibition by torcetrapib in the ILLUMINATE trial[14]. Whereas torcetrapib had been shown to raise HDL-C in humans, it was unexpectedly ineffective in reducing atherosclerosis and it even increased clinical event rate when given on top of atorvastatin treatment. In order to clarify these results we recently simulated this trial in a study in APOE*3Leiden.CETP transgenic mice, focusing on the possible mechanism on the adverse effect[15]. We showed that torcetrapib alone reduced the progression of atherosclerosis, but did not enhance the anti-atherosclerotic potency of atorvastatin. In addition, as compared to atorvastatin, torcetrapib caused a more pro-inflammatory and unstable lesion phenotype, which could explain the unexpected results of the ILLUMINATE trial. Assuming that novel CETP inhibitors do not have these pro-inflammatory adverse effects and that the effects observed with torcetrapib are compound and class related, we speculate that CETP inhibition *per se* is still a valid strategy to reduce cardiovascular risk.

Decreasing plasma VLDL and LDL cholesterol by reducing their production and increasing their clearance (statins and fibrates) or by increasing HDL (niacin, CETP inhibition) is currently the major route to treat the lipid component in CVD. However, CVD still occurs after these treatments. Therefore, other routes of cholesterol-lowering are explored. For instance compounds that inhibit cholesterol absorption in the intestine have been developed, like ezetimibe, which was the first of a new class of selective cholesterol absorption inhibitors[16]. Ezetimibe selectively inhibits cholesterol absorption in the intestine by inhibition of the cholesterol uptake transporters Niemann-Pick C1 Like1, confining the cholesterol to the intestinal lumen for subsequent excretion. Clinical trials have demonstrated the lipid-lowering properties of ezetimibe as a single agent, which was merely -18% due to a compensatory increase in cholesterol synthesis in the liver. Strong additive cholesterol-lowering effects were demonstrated when ezetimibe was combined with a statin[17,18]. Surprisingly, however, additional protective effects in

retarding surrogate endpoints were not observed when ezetimibe was added to simvastatin treatment in the ENHANCE trial, despite a significant lower LDL-C after the combination treatment[19]. The reason for this unexpected result remains to be investigated. For instance, it is not known whether this was a consequence of the systemic availability of ezetimibe, which is nearly 100% absorbed, and which may have effect on cholesterol transport in the other cells, for instance macrophages. New cholesterol uptake inhibitors, which are not or poorly absorbed, are under development, like AVE5530.

In **chapter 4** the effect of this new cholesterol uptake inhibitor was investigated with regards to its LDL-C lowering capacity as well as its anti-atherosclerotic effects in APOE*3Leiden transgenic mice. It was compared to an equal dose of ezetimibe. We showed that AVE5530 was more potent in reducing cholesterol levels, resulting in less systemic and vascular inflammation and less atherosclerosis as compared to ezetimibe. The effect of ezetimibe on true clinical endpoints will be investigated in the currently ongoing large IMPROVE-IT trial[20]. Whereas ezetimbe is generally well tolerated, side effects have been infrequently reported, mainly in combination treatment with statins. This might hypothetically be a consequence of the similar metabolism of the two compounds, as both statins and ezetimibe are glucuronidated by the uridine 5'-diphosphate glucuronosyltransferase isoenzymes. Taking together our results and the unexpected and unexplained absence of beneficial effects in the ENHANCE trial, poorly absorbed cholesterol uptake inhibitors like AVE5530, entering phase III in the second half of 2008, may have advantages over the almost totally absorbed ezetimibe.

Since atherosclerosis is a multifactorial disease, it may be of clinical advantage to lower cholesterol and on top of that co-treat other risk factors of CVD development, *e.g.* hypertension. Analysis of data from 29 randomized trials (n=162,341) showed that anti-hypertensive treatment with any commonly-used regimen reduces the risk of total major cardiovascular events, and that larger reductions in blood pressure lead to larger reductions in risk[21].

In **chapter 2** we describe the potential advantage of combining atorvastatin treatment with anti-hypertensive treatment using the calcium channel blocker (CCB) amlodipine. This hypothesis was later on confirmed in the Anglo-Scandinavian Cardiac Outcomes Trial–Blood Pressure Lowering Arm (ASCOT-BPLA) study, a randomized controlled trial (n=19,257), demonstrating that amlodipine prevented major cardiovascular events more efficiently than atenolol (β-blocker) and a diuretic after a 5.5 year follow-up period[22]. Additionally, a potential interaction between blood-pressure-lowering treatments and statins was reported in the Lipid Lowering Arm of the ASCOT study (ASCOT-LLA) in favor of amlodipine/ atorvastatin treatment above atenolol/ atorvastatin treatment.

In **chapter 5** we designed a study to evaluate and characterize the nature of the effect of the anti-hypertensive angiotensin II receptor blocker (ARB) olmesartan alone

or in combination with statin treatment, on the development of atherosclerosis in APOE*3Leiden mice. We aimed at mimicking the clinical situation with respect to treatment response (regarding the magnitude of lipid lowering as well as blood pressure lowering). Strikingly, olmesartan and pravastatin were equally potent in reducing the atherosclerosis development in the mice. While olmesartan had anti-inflammatory actions which inhibited the onset of lesion development by decreasing monocyte adhesion to the endothelium and the relative amount of macrophages in the lesions, pravastatin inhibited the progression of atherosclerosis in terms of less advanced lesions. Therefore, combination therapy with olmesartan and pravastatin additively reduced atherosclerosis development, resulting in fewer and less severe lesions. The effect of combination treatment of olmesartan with pravastatin on cardiovascular endpoints in humans has not yet been studied. However, combination therapy of atorvastatin with an angiotensin converting enzyme (ACE) inhibitor was shown to be more effective in reducing cardiovascular events than statin treatment alone in a post-hoc analysis of patients of the GREACE study[23,24], which is in line with the effects of atorvastatin and anti-hypertensive treatment in the mentioned ASCOT-LLA study. These latter trials clearly demonstrate additional beneficial effects of combining anti-hypertensive treatment with anti-hyperlipidemic treatment. We showed that, independent of its blood pressure lowering effect, ARB treatment leads to prevention of atherosclerosis via anti-inflammatory routes. Therefore, we suggest that combining the anti-hypertensive treatment with olmesartan and anti-hyperlipidemic treatment with pravastatin may be more effective in the prevention of atherosclerosis than treatment with statins alone.

The studies described so far were evaluating the effect of compounds on the progression of atherosclerosis in a 'prevention' design, which supplies very relevant information about the features and potency of the test substances. However, in the clinic treatment of CVD is mostly started after atherosclerotic lesions have already developed. The established lesions are often accompanied and brought about by a combination of the different risk factors like hypertension, hyperlipidemia and diabetes, all characteristics of the 'metabolic syndrome'. As the metabolic syndrome is a constellation of partly interrelated risk factors, it is strongly correlated with the development of CVD. Its global prevalence has been increasing over the last decades and will probably even double within the coming 25 years. New classes of drugs in development that aim on ameliorating metabolic syndrome (in this case targeting both hyperlipidemia and insulin resistance/ diabetes) and CVD risk are peroxisome proliferator-activated receptorα/γ (PPARα/γ) agonists. In previous experiments we already showed that the newly developed PPARα/γ agonist tesaglitazar improved plasma lipid levels and insulin resistance and inhibited the development of progression atherosclerosis in APOE*3Leiden mice[25]. In **chapter 6** we aimed at investigating the anti-atherosclerotic effect of tesaglitazar in a more clinically relevant experimental design of reduced

progression or atherosclerosis regression, *i.e.* in an animal model with pre-existing atherosclerosis (a 'treatment' design). To this end, we first introduced atherosclerosis in APOE*3Leiden.CETP transgenic mice by feeding a western type diet resulting in elevated plasma cholesterol levels. Thereafter, plasma cholesterol was lowered by reducing dietary cholesterol, and subsequently on top of that by pharmacological treatment with tesaglitazar. The mice showed an similar response to tesaglitazar treatment as human do in terms of reduced VLDL/LDL levels and increased HDL levels. The latter was not observed in mice lacking CETP, emphasizing the key role of CETP in the HDL-raising effects of tesaglitazar. In contrast to dietary cholesterol lowering alone, tesaglitazar fully prevented the progression of pre-existing atherosclerosis and it stabilized lesions, as related to reduction of (V)LDL and local anti-inflammatory action in the vessel wall. Currently, not any dual PPARα/γ agonist has been approved yet for clinical use, since up till now all compounds, including tesaglitazar, have failed in a preclinical stage or in clinical trials. Long term clinical effects can therefore only be extracted from clinical studies using either PPARα or PPARγ agonists. Whereas PPARα agonists have shown beneficial effects on CVD development[26-29], the effects of PPARγ agonists are contradictory[30-33]. This might reflect the complex and careful balance in PPAR pathways and mechanisms involved in their beneficial and detrimental effects. Despite the clinical failure of tesaglitazar due to a decreased creatinine clearance, we demonstrated a therapeutic window for PPARα/γ agonists for treatment of diabetic cardiovascular complications.

Established CVD has been identified as an independent risk factor for fatal CVD and is not included in the SCORE system. Though patients with established CVD are more prone to suffer from CVD events and are considered as high risk persons regardless of the presence or absence of other risk factors. The majority of the patients suffering from CVD events are on an anti-platelet therapy. In **chapter 7** we investigated the effect of anti-platelet therapy with a new thromboxane prostanoid receptor antagonist S18886 (terutroban) on pre-existing lesions in APOE*3Leiden mice, again on top of cholesterol lowering. This new therapy was compared to treatment with the adenosine diphosphate receptor antagonist clopidogrel, a frequently used anti-platelet compound in the clinic. We showed that, on top of aggressive cholesterol-lowering, S18886 completely prevented the progression of established atherosclerosis, which could be addressed to its local anti-inflammatory effects. This was reflected by a dose-dependent reduction in adhesion of activated monocytes to the endothelium, which in part may be related to inhibition of platelet activation by the compound as clopidogrel also tended to decrease the monocyte adhesion. S18886 is currently tested in phase III studies and up till now no thromboxane prostanoid-receptor antagonist has been clinically available. Data from clinical endpoint studies are only available for the combined inhibitor of thromboxane A_2 synthase and receptor, picotamide, in the DAVID study[34], which was reported to be more effective in reducing all cause mortality in diabetic patients as compared to

aspirin. Previously, picotamide was shown to inhibit the progression of plaque growth in the carotid artery in diabetic patients[35]. While all the functions and effects of thromboxane A_2 (TXA_2) and its thromboxane prostanoid receptor on platelets and peripheral tissue are not fully clarified yet, we provide evidence that their anti-atherosclerotic properties are a result of interactions with inflammatory pathways. It also was hypothesized that TXA_2 may be involved in other clinical features of CVD and that S18886 may be a compound with features beyond its anti-platelet aggregating capacities.

In **chapter 8** we further explored the possible deleterious effects of TXA_2 and its receptor on heart function after myocardial ischemia reperfusion injury. The selective cyclooxygenase-2 (COX-2) inhibitor, rofecoxib, has been developed for the treatment of chronic inflammatory diseases like rheumatoid arthritis. Whereas the exact mechanism is unknown, a meta-analysis over nine case-control studies and two cohort studies showed a relative risk of 1.35 for serious cardiovascular events with rofecoxib treatment[36]. It was hypothesized that selective COX-2 inhibitors shift the balance between TXA_2 and prostaglandin I_2 (PGI_2) in the vascular system in favor of TXA_2, by suppressing the synthesis of (endothelial) COX-2-derived PGI_2, without changing the (platelet) COX-1-derived TxA_2 synthesis. Whereas an increased TXA_2 release has been shown during myocardial ischemia, it can be speculated that TXA_2 exerts a deleterious effect during ischemia and reperfusion due to its pro-inflammatory and cytotoxic effects. We thus hypothesized that the deleterious effects of rofecoxib after myocardial ischemia might be eliminated by co-treatment with S18886. To create a hyperlipidemic situation we used APOE*3Leiden transgenic mice on a western type diet and induced myocardial ischemia-reperfusion injury. Whereas treatment with S18886 alone did not affect infarct size or cardiac function, we confirmed the reported deleterious effects of rofecoxib on cardiac function after ischemia-reperfusion injury found in humans. This was reflected by negative effects on cardiac output, ejection fraction and end-systolic volume. Concomitant treatment with S18886 prevented these negative effects. This study provided clues to further elucidate the mechanism underlying the increase in cardiovascular risk associated with selective COX-2 inhibitors. Co-administration of thromboxane prostanoid receptor antagonists might be tool to the reduce risks related to COX-2 inhibiting treatments, which are of major need for the patients suffering from chronic inflammatory diseases.

In conclusion, we have shown a variety of approaches to treat atherosclerosis and CVD. Lowering LDL-C remains the primary target to treat according to current guidelines[37]. Whereas statins have become the 'golden standard', new compounds are being developed aiming at more efficacy and more safety (*e.g.* by developing non-absorbable compounds like AVE5530). Low HDL-C has been identified as a risk factor and especially since low HDL-C is one of the characteristics of the increasing prevalent metabolic syndrome it has become a target of interest for the prevention of CVD. Because safety

(torcetrapib, tesaglitazar) and side effects (niacin) of the new and current HDL-C increasing compounds have limited their use in the clinic, this target has gained the interest of industry, mainly by inhibiting CETP and agonizing the GPR109A (niacin) receptor. As evidence is accumulating that, like the hypertension *per se,* the mechanism beyond hypertension also is a risk factor for the development of CVD, new anti-hypertensive compounds may focus on these underlying effects. The contribution of the renin-angiotesin-aldosteron-system in regulating these different pathways needs to be further elucidated, supplying clues to design novel intervention strategies. An equal scenario is true for platelets. So far, research has focused on their aggregating capacities, whereas their other functions are still partly unclear. Whereas we showed that platelets also modulate inflammation, these additional functions need to be identified.

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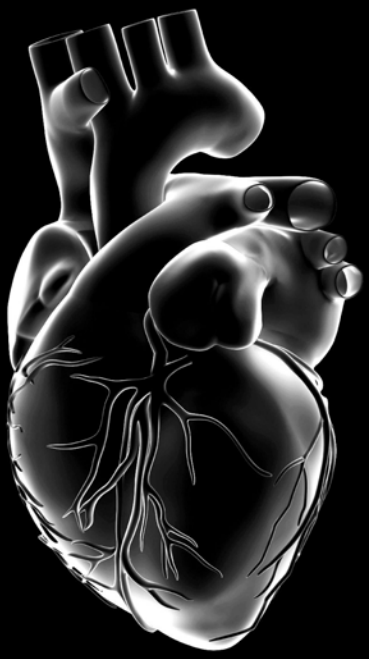


Summary

Figures of the World Health Organization demonstrate that cardiovascular disease (CVD) is the current and future number one cause of death worldwide. The single most important contributor to the growing burden of CVD is atherosclerosis, a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. Novel pharmaceutical therapies to prevent CVD are targeting various risk factors, of which a number is examined in this thesis.

In **Chapter 2** we reviewed the effect of current 'standard' therapies for the treatment of two separate risk factors for CVD, *i.e.* hyperlipidemia with atorvastatin and hypertension with the calcium channel blocker (CCB) amplodipine. In clinical studies monotherapy with CCBs showed attenuation of the first stage of atherosclerosis, meaning that the lesions that pre-existed at the start of CCB therapy did not demonstrate progression or regression on angiography. Statins have shown to reduce CVD events and mortality in numerous clinical trials. Since both kinds of drugs act via different mechanisms to protect against the development of CVD, it was hypothesized that the combination of both may have synergistic beneficial effects. Scientific evidence of *in vitro* and *in vivo* studies and clinical trials was collected in **chapter 2** to illustrate the possible advantage of combining these two kind of treatments. Recently, a potential interaction between blood-pressure-lowering treatments and statins were reported from clinical studies with regard to amlodipine/ atorvastatin treatment.

Although some compounds have proven their benefit in the clinic, their exact working mechanism may not always be clarified yet. Niacin (vitamin B3) is one of those compounds. In the early 1950s already it was known to strongly decrease TG and LDL-C levels and to increase HDL-C, which together results in a remarkable reduction of CVD risk. Whereas niacin even is the most potent HDL-C increasing drug currently available, the mechanism underlying its HDL-raising effect is not fully understood. In **chapter 3** we aimed at elucidation of this HDL-C raising capacity of niacin. To this end, we treated *APOE*3Leiden.CETP* mice with incremental doses of niacin. We observed a markedly increase of HDL-C, which was brought about by a reduction of transfer of cholesterol from HDL to (V)LDL This reduced cholesterol transfer is due to a lower hepatic CETP expression and a reduced plasma (V)LDL pool.

In **chapter 4** we compared the anti-atherosclerotic potential of a newly developed and poorly absorbable cholesterol uptake inhibitor AVE5530 with that of the clinically available drug ezetimibe in APOE*3Leiden transgenic mice. In contrast to AVE5530, ezetimibe is nearly 100% absorbed in the intestine and might also be active on cholesterol transport in macrophages. The consequence of this systemic activity is unclear and remains to be investigated. We showed that AVE5530 was

more efficient in lowering (V)LDL-C than ezetimibe and in addition, AVE5530 had stronger anti-inflammatory effects. As a consequence AVE5530 was clearly more potent in preventing atherosclerosis development in APOE*3Leiden transgenic mice than ezetimibe.

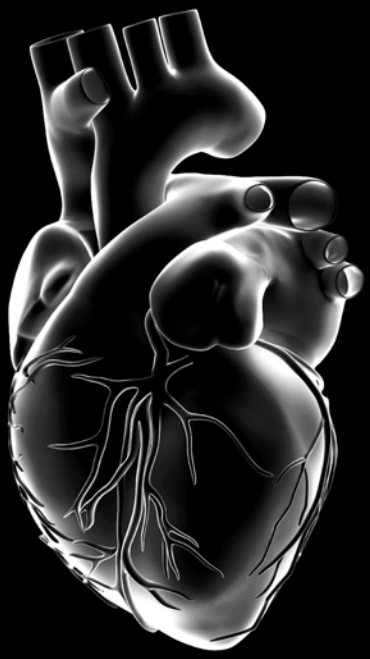
As described in **chapter 2**, combination therapy targeting two risk factors of CVD might have synergistic or additive effects in the prevention of CVD and atherosclerosis. An example of such a study was presented in **chapter 5**, where APOE*3Leiden mice were treated with either the anti-hypertensive angiotensin II receptor blocker (ARB) olmesartan or the antihyperlipidemic drug pravastatin alone or with the combination of both compounds. Treatment with olmesartan or pravastatin reduced the development of atherosclerosis to a similar extent as compared to the control group, albeit via different anti-inflammatory and anti-atherosclerotic mechanisms. Whereas olmesartan inhibited the onset of lesion development, pravastatin hampered the lesion progression towards more severe lesions. Combining and thereby gathering the strength of the two drugs additively reduced atherosclerosis development, resulting in both fewer and less severe lesions.

In **chapter 6** again two risk factors for CVD were targeted, *i.e.* hyperlipidemia and insulin resistance. The effect of a dual PPARα/γ agonist, tesaglitazar, was investigated on pre-existing atherosclerotic lesions in APOE*3Leiden.CETP transgenic mice, a study design of more clinical significance, since in the clinical setting lesions will have developed before treatment is started. We showed that the presence of CETP was crucial for the HDL-raising effect of tesaglitazar, which also markedly reduced (V)LDL-C. Additionally, tesaglitazar turned down the cholesterol-induced vessel wall-specific inflammation. These combined actions resulted in complete inhibition of progression and stabilization of pre-existing atherosclerotic lesions in *E3L.CETP* transgenic mice. This is in contrast to dietary cholesterol lowering alone, which could not halt lesion progression.

A similar study design was used for the experiment presented in **chapter 7** where APOE*3Leiden mice had developed mild lesions before cholesterol-lowering with or without additional anti-platelet therapy was started. We evaluated the effect of incremental doses of the thromboxane-prostanoid receptor antagonist S18886 (terutroban). Adenosine diphosphate (ADP) receptor antagonist clopidogrel was used as an anti-platelet control, a compound which is already used in the clinic. Cholesterol-lowering alone decreased levels of inflammation markers, reduced the number of lesions and improved the lesion stability. Simultaneous treatment with clopidogrel did not significantly add to the effect of cholesterol-lowering. In contrast, S18886 reduced additionally and dose-dependently the lesion area, the

progression of lesion severity and the amount of adhering monocytes when compared to the control. This suggests anti-inflammatory effects of S18886.

Not all functions and effects of thromboxane A_2 (TXA_2) and its receptor on platelets and peripheral tissue are fully clarified yet. However, evidence is accumulating that they indeed interact with inflammatory pathways and affect atherosclerosis and CVD endpoints. It has been observed that selective cyclooxygenase-2 (COX-2) inhibition by rofecoxib is associated with increased risk of cardiovascular events. We hypothesized that this could be due to a locally disrupted TXA_2 - prostacyclin (PGI_2) balance and that this may be prevented by concomitant treatment with the thromboxane-prostanoid receptor antagonist S18886, which then may ameliorate possible negative effects. This was investigated in **chapter 8** in APOE*3Leiden mice with ischemia reperfusion injury of the myocardium. After myocardial infarction, the mice were daily treated with either S18886, rofecoxib or with the combination of both. After one week the cardiac function was quantified and the ischemic area was measured. Whereas none of the treatments affected the infarct size, the heart function was different between the groups. As observed in human studies rofecoxib worsened the heart function. This was measured by a decreased cardiac output and ejection fraction, and an increased end-systolic volume. These negative effects were prevented when S18886 was co-administered; suggesting that indeed the local TXA_2-PGI_2 balance plays a role in the deleterious effects of rofecoxib treatment.



Samenvatting

Cijfers van de Wereld Gezondheid Organisatie (World Health Organization) laten zien dat hart- en vaatziekten wereldwijd doodsoorzaak nummer één zijn en waarschijnlijk zullen blijven in de toekomst. De belangrijkste veroorzaker van hart- en vaatproblemen is atherosclerose (ook wel 'aderverkalking' genoemd), een progressieve ziekte gekarakteriseerd door stapeling van vetten (lipiden) en fibreus weefsel in de grote arteriën. Nieuwe farmaceutische therapieën ter voorkoming van hart- en vaatziekten richten zich op de verschillende risicofactoren. Een aantal van deze therapieën is geëvalueerd in dit proefschrift.

In **hoofdstuk 2** hebben we het effect van de huidige 'standaard' therapieën voor de behandeling van twee risico factoren voor hart- en vaatziekten bekeken: cholesterolverlaging met een cholesterolsynthese remmer (statine) en bloeddrukverlaging met een calciumkanaal blokker. Behandeling met een calciumkanaal blokker alleen heeft in klinische studies een vermindering van progressie van atherosclerose laten zien. De lesies die reeds aanwezig waren bij de start van de therapie vertoonden noch progressie noch regressie na angiografische analyse. In een groot aantal klinische studies is inmiddels aangetoond dat statines hart- en vaatziekten al dan niet met sterfte tot gevolg, verminderen. Omdat beide medicijnen beschermen tegen hart- en vaatziekten via verschillende onderliggende mechanismen, is het mogelijk dat de combinatie van deze geneesmiddelen synergistisch werken. In **hoofdstuk 2** hebben wij wetenschappelijke data verzameld om zo de mogelijke voordelen van deze combinatietherapie in kaart te brengen. Uit recent *in vitro*, dierexperimenteel en klinisch onderzoek blijkt een synergistische werking van bloeddrukverlagende therapieën en behandeling met statines inderdaad te bestaan. Deze studies hadden vooral betrekking op de combinatie amlodipine/ atorvastatine.

Hoewel van sommige medicijnen de effectiviteit al is bewezen in de kliniek, is het werkingsmechanisme niet altijd even duidelijk. Niacine (vitamine B3) is een van die stoffen. Al in de jaren '50 was bekend dat deze stof een sterke triglyceride en (V)LDL-cholesterol verlagende werking heeft en tegelijkertijd het HDL-cholesterol verhoogt. Tezamen resulteert dit in een aanzienlijke verlaging van het risico op hart- en vaatziekten door dit medicijn. Ondanks het feit dat niacine de meest potente HDL-cholesterolverhoger is in de kliniek, is het werkingsmechanisme van deze stof is nog steeds onbekend.

In **hoofdstuk 3** hebben we geprobeerd het mechanisme van deze HDL-verhogende capaciteit op te helderen. Daartoe hebben we APOE*3Leiden.CETP transgene muizen behandeld met oplopende doseringen van niacine. We zagen een zeer significante stijging van het HDL-cholesterol, die werd veroorzaakt door een verlaging van het CETP-afhankelijke cholesterol transport van HDL naar (V)LDL. Dit

werd deels veroorzaakt door een verlaagde CETP expressie in de lever en deels door een daling in het plasma (V)LDL niveau. In deze studie kwam duidelijk naar voren dat CETP een sleutelrol speelt in het HDL-verhogende effect van niacine.

Verlaging van het plasma cholesterol kan ook verkregen worden door de absorptie van cholesterol in de darm te remmen. In **hoofdstuk 4** hebben we in APOE*3Leiden transgene muizen de cholesterolverlagende en anti-atherosclerotische werking van een nieuw ontwikkelde remmer van de cholesterol absorptie (AVE5530) bestudeerd en vergeleken met de absorptieremmer ezetimibe, wat een al geregistreerd geneesmiddel is. In tegenstelling tot AVE5530 wordt ezetimibe bijna volledig geabsorbeerd in de darm en kan het daarom ook werkzaam zijn op het cholesterol homeostase in de macrofagen in de circulatie en in de lesie. Wij hebben laten zien dat AVE5530 effectiever het (V)LDL-cholesterol verlaagt dan ezetimibe. Daarnaast had het ook een sterker anti-inflammatoir effect. AVE5530 was ook aanzienlijk meer potent in het remmen van de atherosclerose-ontwikkeling in de APOE*3Leiden transgene muizen dan ezetimibe. Of deze verbeterde anti-atherosclerotische werking van AVE5530 ten opzichte van ezetimibe een gevolg is van de niet- systemische beschikbaarheid van AVE5530 is nog niet duidelijk en zal verder onderzocht moeten worden.

Zoals beschreven is in **hoofdstuk 2**, een combinatie therapie, welke zich richt op meerdere risico factoren voor de ontwikkeling van hart- en vaatziekten, kan synergistisch of additief werken in de preventie van atherosclerose. Dergelijk onderzoek is uitgevoerd in **hoofdstuk 5**, waarin het effect is beschreven van de behandeling van APOE*3Leiden transgene muizen met de nieuwe bloeddrukverlager (een angiotensine II receptor blokker) olmesartan, de cholesterolverlager pravastatine, of met de combinatie van beide medicijnen. Behandeling met alleen olmesartan of alleen pravastatine remde de ontwikkeling van atherosclerose in vergelijking met de controle groep even effectief. Echter, deze remming werd veroorzaakt door een verschillend anti-inflammatoir en anti-atherosclerotisch werkingsmechanisme van beide medicijnen. Olmesartan remde vooral het ontstaan van de lesies in de beginfase van het atherosclerotisch proces, terwijl pravastatine juist de progressie van de ernst van de lesies remde. Door beide stoffen te combineren werden de krachten gebundeld, wat additief werkte en leidde tot nog meer reductie van de atherosclerose-ontwikkeling. In deze combinatie groep werd een nog kleiner aantal lesies en nog minder ernstige lesies gemeten in vergelijking met monotherapie.

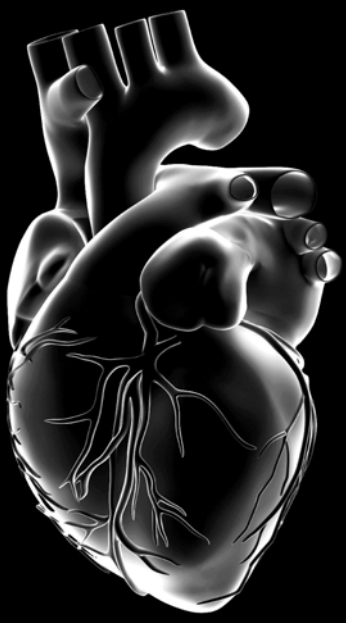
In **hoofdstuk 6** werd ook een combinatie therapie toegepast, die gericht was tegen twee risicofactoren, namelijk hyperlipidemie en insuline resistentie of diabetes type 2. In deze studie werd het effect van de duale PPARα/γ agonist

tesaglitazar onderzocht in APOE*3Leiden.CETP transgene muizen met reeds bestaande atherosclerotische lesies. De opzet van deze studie was klinisch relevanter dan de studie beschreven in **hoofdstuk 5**, omdat ook in de kliniek atherosclerose al ontwikkeld is vóórdat de patiënt behandeld wordt. In deze studie lieten we zien dat CETP een cruciale rol speelt in het HDL-verhogende effect van tesaglitazar, wat tegelijkertijd ook triglycerides en (V)LDL sterk verlaagt. Daarbij verlaagde tesaglitazar ook de cholesterol geïnduceerde vaatwandactivatie. Deze acties resulteerden in complete remming van atherosclerose progressie en leidde zelfs tot stabieler fenotype van de bestaande lesies. Cholesterolverlaging alleen remde de lesie voortgang niet.

Eenzelfde studieopzet gebruikten wij in de studie gepresenteerd in **hoofdstuk 7**. APOE*3Leiden muizen met reeds mild ontwikkelde atherosclerose ondergingen een cholesterolverlagende therapie door middel van cholesterolverlaging in het dieet, al dan niet gecombineerd met bloedplaatjesremmende therapie. We bestudeerden het effect van oplopende doseringen van de thromboxane-prostanoid receptor antagonist S18886 (terutroban). De adenosine diphosphate (ADP) receptor antagonist clopidogrel, een plaatjesremmer die vaak toegepast wordt in de kliniek, werd gebruikt als controle stof voor de plaatjesremming. Cholesterolverlaging alleen verlaagde de ontstekingsmarkers in het plasma, verminderde het aantal lesies en verbeterde de lesie-stabiliteit. Wanneer bovenop de cholesterolverlaging clopidogrel werd gegeven, vond er voor geen van de parameters een extra verandering plaats. Additieve therapie met S18886 daarentegen, verminderderde dosisafhankelijk de lesie-oppervlakte, de lesie-ernst en het aantal monocyten dat aangehecht was aan het vaatwandendotheel. Deze resultaten duiden op anti-inflammatoire effecten van S18886.

De functies van thromboxaan A_2 (TXA_2) en de thromboxane-prostanoid receptor op bloedplaatjes en in de perifere weefsels zijn nog niet helemaal duidelijk. Echter, er komen steeds meer aanwijzingen dat beide een interactie hebben met ontstekingsroutes en dat ze atherosclerose en het vóórkomen van hart- en vaatziekten beïnvloeden. Uit klinische studies is gebleken dat het gebruik van de selectieve cyclooxygenase-2 (COX-2) remmer rofecoxib, een ontstekingsremmer, geassocieerd is met een toegenomen risico op hart- en vaatziekten. In theorie zou dit het gevolg kunnen zijn van een verstoorde lokale TXA_2 - prostacycline (PGI_2) balans. Indien dit het geval is, zouden de negatieve effecten van rofecoxib verholpen kunnen worden door tegelijkertijd een thromboxane-prostanoid receptor antagonist zoals S18886 toe te dienen. Deze hypothese werd onderzocht in **hoofdstuk 8**, waarin APOE*3Leiden transgene muizen ischemie-reperfusie schade in het hart toegebracht kregen. Na dit myocardinfarct, werden de muizen dagelijks

behandeld met ofwel S18886, of rofecoxib, of met de combinatie van beide stoffen. Een controle groep bleef onbehandeld. Na een week werden de hartfunctie en het infarctoppervlak gemeten. Terwijl de infarctgrootte niet veranderde door welke van de drie behandelingen dan ook, was er wel een verschil in hartfunctie tussen de groepen. Zoals waargenomen in de humane studies verslechterde rofecoxib de hartfunctie ook in muizen. Dit werd veroorzaakt door een verminderde hartcapaciteit en ejectiefractie en een toegenomen eind-systolisch volume. Deze negatieve effecten van rofecoxib konden worden voorkomen door de muizen tegelijkertijd met rofecoxib S18886 te geven. Dit suggereert dat de lokale TXA_2-PGI_2 balans inderdaad een rol speelt bij de nadelige effecten van rofecoxib behandeling.



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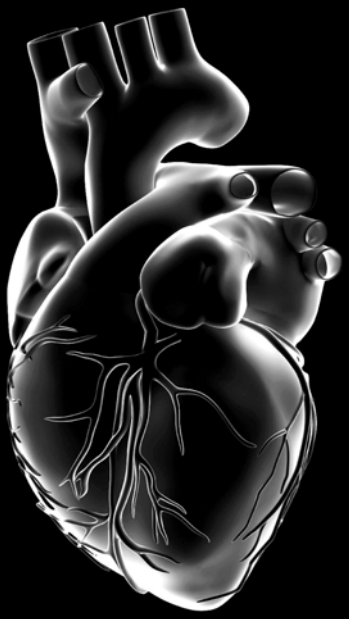
In preparation



Curriculum Vitae

José van der Hoorn werd geboren op 15 januari 1981 in Hoogmade (gemeente Woubrugge). In juni 1999 behaalde zij haar VWO diploma aan het Bonaventura college (Aquino scholengemeenschap) in Leiden. In september van datzelfde jaar startte zij met de opleiding Biomedische Wetenschappen aan de Universiteit Leiden.

In het kader van het doctoraal examen legde zij in oktober 2001 tot maart 2002 haar eerste stage af aan de afdeling Nierziekten van het Leids Universitair Medisch Centrum (LUMC), waarin zij onderzoek deed naar de aanwezigheid en de rol van auto-antilichamen tegen mannose-binding lectin (MBL) in patiënten met systemische lupus erythematosus (SLE) onder begeleiding van Drs. M.A.J. Seelen en Dr. A. Roos. Van september 2002 tot juli 2003 deed zij haar hoofdvakstage bij TNO Kwaliteit van Leven (KvL) in Leiden, waarin zij de effecten van cholesterol en/ of bloeddruk verlagende therapieën op atherosclerose onderzocht in APOE*3Leiden muizen, onder supervisie van Dr. H.M.G. Princen en Prof. Dr. A. van der Laarse. Daarnaast schreef zij een scriptie over de bloeddruk verlagende therapieën en de effecten op hart- en vaatziekten onder supervisie van Prof. Dr. J.W. Jukema.

Na haar afstuderen in augustus 2003 werd José aangesteld als promovenda bij de afdeling Hartziekten van het LUMC. Voor haar promotieonderzoek werd zij gedetacheerd op de afdeling *Vascular and Metabolic Diseases* van TNO KvL in Leiden, onder de supervisie van Prof. Dr. J.W. Jukema, Prof. Dr. Ir. L.M. Havekes en Dr. H.M.G. Princen. Vanaf september 2006 heeft zij naast haar promotieonderzoek een functie als projectleider vervuld bij TNO. Het promotieonderzoek, waarvan de resultaten zijn beschreven in dit proefschrift, is afgerond in maart 2008. Aansluitend is José aangesteld als voltijd projectleider/wetenschappelijk medewerker op de afdeling *Vascular and Metabolic Diseases* van TNO Kwaliteit van Leven, *BioSciences* in Leiden.