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Aan mijn familie
aan Maria

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Chapter 1

General Introduction

1. Pathogenesis of atherosclerosis

Atherosclerosis is the underlying pathology responsible for most cardiovascular disease (CVD)-related deaths and as such a leading cause of death worldwide. This chronic, lipid-driven autoimmune-like disease starts already during early adolescence and persists throughout a lifetime. The disease affects primarily the medium and large-sized arteries and through the combination of vascular damage, lipid accumulation and inflammation, atherosclerotic lesions develop. In time these initial lesions progress and may eventually rupture and give rise to clinical manifestations such as myocardial infarction or stroke (Figure 1).[1]

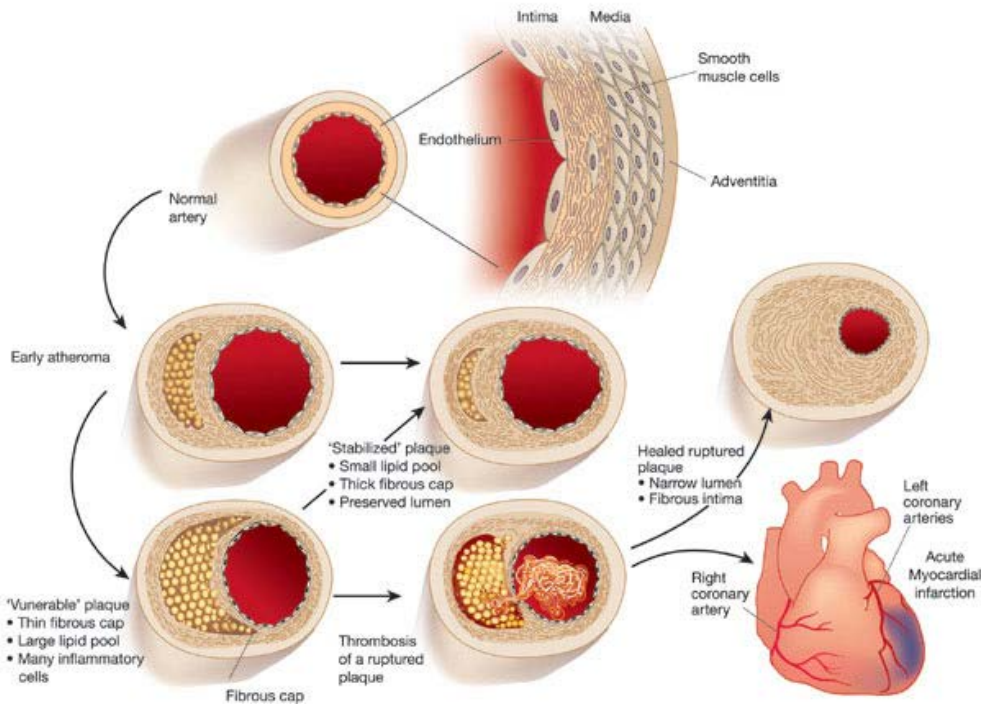

Figure 1. Atherosclerosis development. Lesion initation starts already during adolescence and can progress over the following decades into stable or vulnerable plaques. Subsequent rupture or erosion of the fibrous cap covering the vulnerable plaque can cause thrombus formation, resulting in an acute myocardial infarction or stroke. Libby *et al.* Nature 2002; 420, 868-874

High-fat intake, sedentary lifestyle, hypertension, smoking and stress are a few of the risk factors for atherosclerosis and reflect the high incidence of CVD in Western society.[2] Nowadays, CVD is one of the leading causes of death globally, with an estimated 17.3 million deaths (30% of all reported deaths) in 2008.[3] With the increasing prevalence of traditional risk factors for atherosclerotic cardiovascular diseases (e.g. high cholesterol diet, diabetes, stress) in the developing world, this number is predicted to increase to 23.3 million deaths annually by 2030.[4,5] Current therapies are aimed at modifying these risk factors via pharmacological

interventions and behavioral changes. At later stages of the disease surgical interventions may be necessary such as percutaneous transluminal angioplasty (PTA), stenting and bypass surgery. The most successful interventional therapy so far has been plasma lipid lowering (low-density lipoprotein cholesterol) by means of statin treatment.[6] However, despite an astonishing 30% reduction in symptomatic cardiovascular disease risk in patients receiving statins, a clear residual risk remains and indicates the urgent need for new therapeutic strategies based on a thorough understanding of the disease process and all of its risk factors.

1.1 Lesion initiation

A healthy functional artery is composed of three layers. In direct contact with the blood is the intima, which consists of a monolayer of endothelial cells and subendothelial connective tissue (forming the basement membrane) which covers the second, medial layer of vascular smooth muscle cells. This medial layer is flanked by the internal and external elastic lamina. Outside the external lamina resides the collagen-rich adventitial tissue composed of fibroblasts, connective tissue and perivascular nerves (Figure 2). A healthy endothelium is important to maintain vascular homeostasis, as it mediates vasoconstriction and vasodilation, has anticoagulant properties and affects vascular smooth muscle cell proliferation.[7] The primary arteries affected by atherosclerosis are the coronary arteries, the branching points of the aorta and the carotid arteries at the height of the bifurcations. This observation and the patchy distribution of the early atheroma, made Caro *et al.* already in 1969, suggests an important role for blood hemodynamic forces (e.g. oscillatory and low shear stress) in lesion initiation.[8,9] Over the years, lesion initiation has been a fiercely debated topic and led to several hypotheses. Currently the general consensus is a response-to-injury model in which endothelial damage induced by hemodynamic stresses and atherogenic factors such as high plasma levels of cholesterol-containing low-density lipoprotein (LDL), hypertension and smoking play a central role.[10,11] Activation of the endothelium changes the endothelial permeability resulting in the entry and retention of lipoproteins. Within the vessel wall, these LDL particles undergo modifications (oxidation, glycation and association with proteoglycans), which change the particle's size, charge and lipid content.[12] In parallel, increased endothelial expression of adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1)[13], E- and P-selectin[14] and chemokines such as monocyte chemoattractant protein-1 (MCP-1) results in attraction and attachment of circulating leukocytes to the endothelium and subsequent infiltration into the intima. The recruited monocytes differentiate into macrophages under the influence of local inflammatory cytokines (such as tumor necrosis factor-α (TNF-α), macrophage colony stimulating factor (M-CSF),

interleukin-2 (IL-2) and interferon-γ (INF-γ)) and take up the modified LDL via scavenger receptors.[15] Modified LDL is readily taken up by macrophages but the massive amount of cholesterol cannot be secreted effectively, resulting in the formation lipid-rich foam cells, characteristic of the atherosclerotic lesion.[16,17] The expanding inflammatory response at the site of initial injury results in a further increase in endothelial adhesion molecule expression and chemokine secretion (e.g. MCP-1, CCL5, CXCL10 and CXCL11), which attracts additional monocytes and T lymphocytes (T cells) to the initial lesion, called the 'fatty streak'. Such fatty streaks can already be detected in the arteries of children and young adolescents and while usually asymptomatic these initial lesions may progress in time into more advanced stages of the disease.

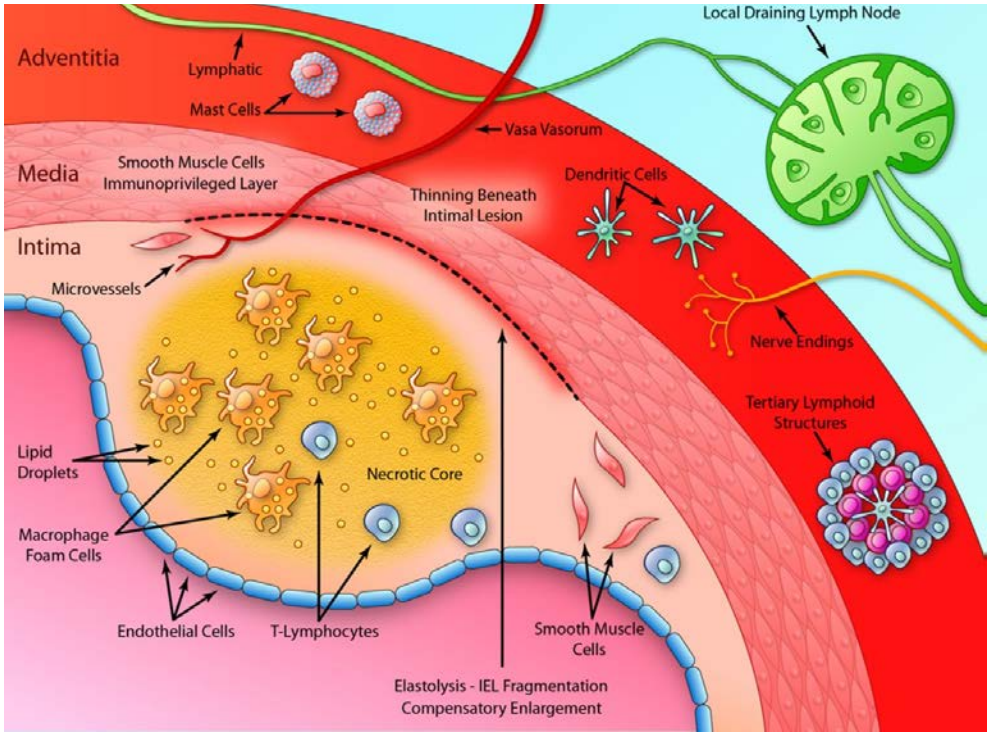

Figure 2. The different layers and players. A characteristic atherosclerotic lesion, with an inflamed intimal layer consisting of a lipid-rich necrotic core covered by a fibrous cap and endothelial monolayer. Multiple cellular players infiltrate the intimal layer and contribute to plaque progression, including cells of the innate and adaptive immune system as well as vascular smooth muscle cells. The internal and external elastic lamina form the boundaries of the tunica media, which consists primarily of vascular smooth muscle cells and extracellular matrix. This layer is immuneprivileged and can facilitate outward remodeling to initially compensate for the decrease in lumen size due to plaque growth. Outside the external elastic lamina resides the adventitia which harbors a network of small blood vessels (the vasa vasorum), lymphatic vessels and nerve fibers, providing nutrients and inflammatory stimuli which contributes to plaque formation and progression. Libby & Hansson Circ Res. 2015 ;116(2):307-311.

1.2 Lesion progression and destabilization

The next stage of atherosclerotic lesion development is characterized by the proliferation and migration of vascular smooth muscle cells (VSMC) from the media into the intima. Here they accumulate subendothelially and produce extracellular matrix components such as collagen and proteoglycans, which form a fibrous cap covering the subendothelial pool of lipids, cholesterol crystals and cellular debris. This so called lipid-rich necrotic core is primarily formed due to apoptosis/necrosis of the lipid-laden macrophages and retention of lipoprotein particles. While the content of the necrotic core is highly thrombogenic, initially the fibrous cap forms an adequate barrier and when thick enough the plaque can remain stable and asymptomatic for years.[18] However, the majority of clinical manifestations such as a myocardial infarction (MI) or a stroke arise upon rupture or erosion of the fibrous cap. In over 70% of the cases, acute MI is attributable to the thrombotic occlusion of a coronary artery caused by the exposure of the thrombogenic content of the necrotic core to the coagulation system of the blood.[19] Changes in the integrity of the fibrous cap occur through a combination of exposure to stressors from the luminal side (e.g. mechanical shear stress from the blood flow) and from within the intima (e.g. inflammation, vascular smooth muscle cell apoptosis and collagen degradation).[20,21] Initially, the reduction in cross-sectional area of the lumen by the growing lesion is compensated by outward remodeling of the artery wall, which normalizes the shear stress.[22] However, as lesion development progresses this compensation is not sufficient and the plaque starts to protrude into the lumen, resulting in local regions of disturbed flow.[23] Pro-inflammatory cytokines and enzymes produced and released within the advanced atherosclerotic lesion have been shown to affect both collagen synthesis by the intimal VSMCs and induce breakdown of collagen. In particular matrix metallo-proteinases (MMPs) locally released from recruited and infiltrated macrophages, T cells, neutrophils and mast cells play an important role in plaque and fibrous cap destabilization.

Although the intima is the centre stage of the disease, accumulating evidence demonstrates the importance of the media[24] and adventitial tissue[25] in atherosclerotic plaque development and progression. In addition to the extracellular matrix and fibroblasts, the perivascular tissue contains a large capillary blood vessel network, the vasa vasorum, sympathetic and autonomic nerves as well as lymphatic vessels, which provide nourishment and facilitate communication and trafficking routes throughout the different layers. Furthermore, populations of macrophages, mast cells, dendritic cells, T- and B-lymphocytes and vascular progenitor cells reside in the adventitia[26] and increased perivascular inflammation strongly correlates with plaque progression and destabilization.[27,28] The adventitial vasa vasorum is the major source of neovessels, which are formed during disease progression due to intimal hypoxia and the local release of angiogenic factors (vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)) from endothelial

cells and infiltrated immune cells. Increased neovascularization and a high vasa vasorum density has been associated with increased inflammation and reduced stability of the plaque. Leakiness of these newly developed intimal capillaries may result in intraplaque hemorrhage (IPH), which further fuels the inflammatory response and increases the stress on the fibrous cap.

2. Risk factors for cardiovascular disease

2.1 Established risk factors and current interventions

Until the late 60's, risk factors for coronary heart disease entailed the patient's age and family history. Large-scale epidemiological studies such as the Framingham Heart Study have added many more risk factors, which are now widely used for risk prediction calculations and therapeutic strategies. Current risk prediction algorithms for developing symptomatic cardiovascular disease (e.g. the Framingham Risk Score or Systematic Coronary Risk Evaluation) take into account gender, age, hypertension, blood cholesterol levels (low and high density lipoprotein (LDL and HDL) levels), smoking and diabetes mellitus.[29,30] While the original Framingham Risk Score could only be applied to predict the risk of coronary heart disease, extensions of this study and others have led to new prediction models, which also include other cardiovascular diseases, such as cerebrovascular disease (stroke), peripheral arterial disease and deep vein thrombosis. Based on these predictions patients are classified in low, intermediate or high risk categories of developing CVD within 10 years and offered cholesterol and blood pressure lowering drugs and urged to change behavioral risk factors, such as an unhealthy diet, smoking, physical inactivity. An alternate means of representing and communicating an individual's cardiovascular risk is vascular age, which represents an individual's risk matched with the age at which the risk is equivalent but all other risk factors are at ideal levels (Figure 3). Especially in young- and middle-aged individuals in which the 10-year risk of CVD is generally low, vascular age may have a bigger impact and result in better compliance with medication schemes and suggested lifestyle changes. However, the precise definition of vascular age is still ambiguous and needs further application and validation.[31]

The initial knowledge of (modifiable) risk factors for CVD were mainly based on Caucasian study results. As cardiovascular disease is a leading cause of death globally the INTERHEART study was set up as a first step to assess the worldwide association of risk factors for myocardial infarction. This case-control study in over 15.000 cases and almost 15.000 control subjects confirmed the strong associations for the major risk factors (e.g. smoking, abnormal lipid levels, hypertension, diabetes, abdominal obesity) and importantly these were consistently adverse in all countries and ethnic groups included in the investigation.[32] The INTERHEART

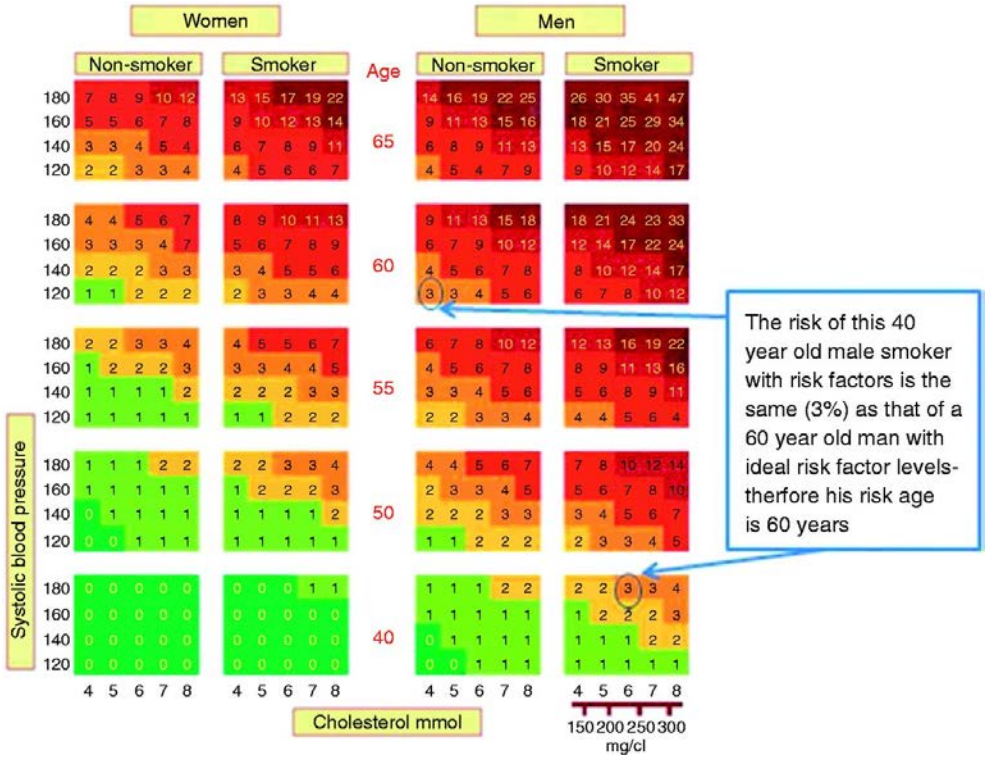

Figure 3. An example of a vascular age determination. Vascular age represents an individual's risk matched with the age at which the risk is equivalent but all other risk factors are at ideal levels. This concept may improve risk communication with the patient, especially for patients with a low 10-year risk of CVD, and may prove valuable as a tool to improve cardiovascular risk prediction. Groenewegen *et al.* European Journal of Preventive Cardiology 2015

study also evaluated additional factors such as socioeconomic status (education and income) and psychosocial factors (depression, locus of control, perceived stress, and life events) and their association with risk of myocardial infarction.[2] The results of this study corroborated results from previous prospective studies demonstrating positive associations between levels of work stress[33,34], marital stress[35] and perceived mental stress[36] and the incidence of coronary heart disease. Importantly, this stress-induced excess risk of acute MI was shown to be consistent across geographic location, age and gender and remained significant when adjusting for other cardiovascular risk factors.

2.2 Psychological stress as risk factor for CVD

The stress response is aimed at readying an organism to fight or flight. Therefore, two major pathways are activated, the limbic hypothalamus-pituitary-adrenal (HPA-axis) and the sympathetic adrenomedullary (SAM) axis. The first primarily leads to the production and secretion of corticosteroids and the second to catecholamine release, an increased heart rate and peripheral vasoconstriction.

Together these systems are aimed at accessing and mobilizing the bodies energy's stores and enhance the immune system in anticipation and response to a threat. Chronic exposure to stress and / or its main hormones has been shown affect the development and progression of many diseases, including atherosclerosis.[37] On the acute timescale, (severe) stress exposure has been demonstrated to act as a trigger for acute cardiovascular events, such as myocardial infarction or stroke. The many facets of the stress response and its implications for cardiovascular disease are covered in more detail in **Chapter 2**. With atherosclerosis being a chronic inflammatory disease, special interest goes out to the immune modulatory actions of the various hormones and neuropeptides released upon stress exposure.[38] In addition to the well-known immunosuppressive effects of glucocorticoids, pituitary hormones and neuropeptides released from peripheral nerve endings, such as prolactin, corticotrophin releasing hormone (CRH), adrenocorticotrophic hormone (ACTH), neuropeptide Y (NPY), substance P (SP) and opioids can affect cellular and humoral immune responses implicated in atherosclerosis development and progression.

3. Plaque inflammation the immune cells involved and their responses to stress

The immune responses in all layers of the vessel wall play an important role in the development and progression of atherosclerosis. In general the immune system can be divided into innate and adaptive immunity. The innate immune response is mediated by monocytes and macrophages, neutrophils and mast cells as well as dendritic cells, basophils, eosinophils, natural killer (NK) cells and NK T cells, which provide a general, non-specific, first line of defense against infection and tissue damage. The adaptive immune response, facilitated by T- and B-lymphocytes on the other hand is antigen-specific and initiated by antigen presentation by specialized antigen presenting cells (APCs), such as dendritic cells (figure 4).

3.1 Innate immunity

In order to respond quickly to invading pathogens and tissue damage, cells of the innate immune system are either highly abundant in the circulation or, in the case of the mast cell, reside in tissues in close contact with the outside environment. Recognition of pathogens and danger signals (e.g. extracellular nucleic acids) occurs primarily via membrane bound pattern recognition receptors, such as the Toll-like receptor (TLR) family, NOD-like receptors and C-type lectin and scavenger receptors. Upon activation these cells produce and secrete pro-inflammatory cytokines and chemokines to kill the pathogen and attract other immune cells to the site of inflammation. Phagocytes, such as monocytes, macrophages, dendritic cells and neutrophils efficiently internalize pathogens and cellular debris, which is subsequently degraded in the lysosomal and endosomal compartment.

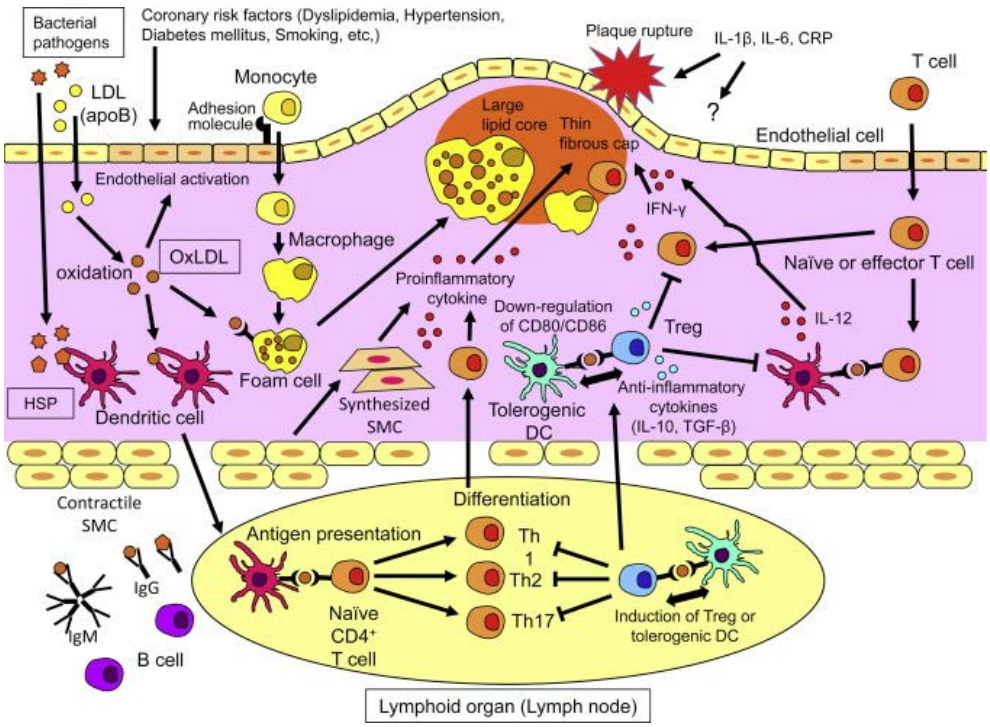

Figure 4. Immune responses in the atherosclerotic plaque. In general (ox)LDL and other atherosclerotic risk factors activate the endothelium, increasing its permeability and adhesion molecule expression level. Monocytes subsequently adhere to the endothelium and transmigrate to the subendothelial space, where they differentiate into macrophages, take up oxLDL, and become foam cells. Self or foreign antigen processing and presentation by macrophages and dendritic cells in the plaque and draining lympnodes, combined with co-stimulatory and cytokine signals result in the differentiation of naïve T cells into the different pro- and anti-atherosclerotic T cell subsets observed in the atherosclerotic lesion. Yamashita *et al.* J Cardiol. 2015.

Small antigenic parts can be subsequently presented on the outside of the cell by MHC molecules and initiate an antigen-specific immune response. The psychological stress response is aimed at preparing an individual for possible injury and thus has potent immunomodulatory properties on both innate and adaptive immune function.

3.1.1 Monocytes and macrophages

The most abundant immune cell type within the atherosclerotic lesion is the monocyte/macrophage. Derived from hematopoietic stem and progenitor cells (HSPC) in the bone marrow and residing outside the bone marrow in the splenic reservoir, two distinct populations of monocytes have been identified.[39,40] The non-classical monocyte population, characterized by surface expression of $CD14^{low}CD16^{+}$ in humans and $Ly6C^{-}CX3CR1^{high}CCR2^{-}$ in mice, have been demonstrated to patrol and crawl along the endothelium. Upon PAMP or DAMP

recognition, these cells secrete pro-inflammatory cytokines and chemokines to recruit other immune cells.[41] The classical monocyte subset ($CD14^{high}CD16^{-}$ in human and $Ly6C^{+}CX3CR1^{low}CCR2^{+}$ in mice) is subsequently among the first to be recruited to the site of inflammation, in a chemokine receptor (CCR2, CCR5 and CX3CR1) -dependent manner.[42] There they produce large amounts of inflammatory mediators such as TNFα, IL-6, IL-1β, IL-12 and infiltrate in the vessel wall. Under the influence of differentiation factors like macrophage-colony stimulating factor (M-CSF), infiltrated monocytes mature into macrophages and monocyte-derived dendritic cells. Via a scavenger receptor-mediated process plaque macrophages and dendritic cells take up the oxidized LDL, become foam cells and present specific antigens which further fuel the ongoing inflammatory response.[43] Similar to monocytes, different functional subpopulations of tissue macrophages have been identified. Under the influence of local concentrations of cytokines, bacterial moieties (e.g. LPS) but also lipid mediators, macrophages can differentiate into a classically activated M1 or alternatively activated M2 phenotype.[44] M1 macrophages induced by LPS, INFγ, IL-1β, TNFα, and oxLDL are considered pro-inflammatory and have increased phagocytic and bacterial killing capacity via pro-inflammatory cytokine release and the production of nitric oxide and reactive oxygen species (ROS). Alternative activation, resulting in an anti-inflammatory macrophage type, occurs via PPARγ receptor activation and by stimulation with the anti-inflammatory cytokines IL-4 and IL-13.[45] Atherosclerosis severity has clearly been linked to the presence and amount of lesional M1 macrophagesref! and polarization of the macrophage phenotype to M2 macrophages, for example by IL-13 or thioredoxin-1 administration, results in reduced macrophage numbers in the atherosclerotic lesion, a less vulnerable lesion type or even a reduction in lesion development.[46,47]

The effects of stress mediators on monocyte and macrophage functioning has primarily focused on monocyte progenitor release into the circulation and subsequent trafficking to the atherosclerotic plaque. Chronic stress exposure in mice and humans was recently shown to enhance HSPC release from the bone marrow via sympathetic nervous system-induced reduction of the retention factor CXCL12. The resulting increase in circulating leukocytes and inflammatory monocytes significantly enhances atherosclerotic lesion development.[48] On the other hand, in wound healing models, stress seems to increase neutrophil recruitment without affecting macrophage infiltration, while decreasing both macrophage and neutrophil pro-inflammatory mediator release and microbicidal capacity.[49]

3.1.2 Mast cells

Another important effector cell of the innate immune system is the mast cell. Equipped with various damage- and pathogen-associated molecular pattern recognition receptors, such as TLRs and immunoglobulin receptors, mast cells are involved in the first line of defense against bacteria, viruses and parasites. To fulfill this function these relatively long-lived cells reside in tissues which are in close proximity to the outside world, such as various mucosal tissues, the skin and perivascular tissue. Bone marrow-derived mast cell precursor cells migrate to these tissues by chemotactic gradients, for instance CCL11, and maturate under the influence of locally produced stem cell factor (SCF) and IL-3. Mast cell activation occurs via multiple pathways. In allergy and asthma, antigen-induced crosslinking of IgE molecules bound to membrane FcεR results in massive degranulation of preformed mast cell granules in the surrounding tissue. Mast cell granules contain a plethora of pro-inflammatory cytokines, mast cell specific proteases, such as tryptases, chymases and carboxypeptidase A3 and vasoactive amines like histamine. Although mast cells had been discovered in the atherosclerotic plaque in the 1990s[50,51], their participation in lesion progression and destabilization was conclusively demonstrated only a few years ago.[52,53] In human atherosclerotic lesions mast cells are found both in the intima and adventitia[54], while murine mast cells reside only in the perivascular tissue. In both species however, mast cells have been shown to accumulate during disease progression and actively contribute to the severity and vulnerability of the plaque.[55] Mast cell derived TNFα and IL-6 was sufficient to enhance lesion development in apoE$^{-/-}$ mice[52] and mast cell-derived chymase and tryptase induced neovessel leakiness, increased the incidence of intraplaque hemorrhages and decreased the overall stability of atherosclerotic lesions.[56,57] Mast cells express various receptors, including TRLs, complement receptors, scavenger receptors and different neuropeptide receptors. Mast cell activation by complement factors (e.g. C5a)[58], plaque-derived phospholipids (e.g. LPA)[59] and TLR ligands[60] results in selective release of different pro-inflammatory mediators and as such can have different effects on the atherosclerotic plaque. Within the perivascular tissue mast cell reside in close proximity to peripheral nerves[61], which via the local release of neuropeptides may contribute to mast cell activation. For example, the sensory neuropeptide substance P (SP) is a potent mast cell activator and local administration of this peptide caused intraplaque hemorrhaging in a SP-receptor (NK1R) -dependent manner.[62]

The abundance of mast cells in the skin and lungs makes this cell type a key player in allergic skin conditions and asthma. Interestingly, stress has been shown to contribute to sudden exacerbations of asthma and allergic responses[63,64] as well as impair intestinal barrier function in inflammatory bowel disease[65] in a mast cell-dependent manner. In contrast to atopic disease, limited literature exist on

stress-induced mast cell activation in cardiovascular disease.[66] Outside the HPA-axis, peripherally released corticotrophin releasing hormone (CRH) may be an important factor involved in stress-induced cardiac mast cell activation.[67] However, various other factors released upon chronic and acute stress exposure, including corticosteroids, catecholamines, neurotensin and neuropeptide Y are capable of modifying mast cell responses and require additional research.

3.1.3 Neutrophils

In the circulation, the neutrophil is the most abundant white blood cell type. However, due to their short life span and rare and difficult detection in the atherosclerotic lesion, they have received little attention with respect to their role in lesion initiation and progression. Recent technical advances in imaging and tracing techniques however have shown neutrophils to be recruited to the atherosclerotic lesion and, like mast cells, contribute to local inflammation by the release of preformed granules containing ROS, myeloperoxidase (MPO), multiple pro-inflammatory cytokines and matrix degrading enzymes.[68] Ly6G staining (expressed by murine neutrophils) localizes the neutrophil primarily to highly inflammatory and vulnerable shoulder regions of the plaque and depletion of neutrophils by Ly6G antibody injections reduced lesion development in apoE$^{-/-}$ mice.[69] Neutrophils attach and are captured at the site of lesion initiation in a similar way as monocytes. E- and P-selectin are involved in the initial retention of circulating neutrophils. Chemokine-chemokine receptor interactions, such as CCL5, binding to CCR1 and CCR5 and the CXCL1-CXCR2 and CXCL12-CXCR4 axis facilitate neutrophil recruitment and attachment to the endothelium.[46,70,71] Macrophage-derived CCL3, another chemokine binding to CCR1 and CCR5, was also demonstrated to be a potent chemotactic factor for neutrophils and to reduce their turnover, both contributing to atherosclerosis.

Chronic stress induced hematopoietic progenitor proliferation in the bone marrow and the observed reduction in CXCL12 expression significantly increased neutrophil numbers in the circulation and the atherosclerotic plaque.[48] Stress, via epinephrine and norepinephrine release, rapidly increases circulating levels of all leukocyte populations. Driven mainly by corticosterone these cells subsequently traffic to sites of inflammation or return to the bone marrow or lymphoid organs or die. In contrast, neutrophil numbers keep increasing during a 2h lasting experimental stressor, indicating the need for secondary (inflammatory) cues to contribute to the stress-induced exacerbation of inflammation (figure 5).[72]

3.1.4 Dendritic cells

While plaque macrophages can present antigens and activate antigen-specific CD4^{+} T cells, the most potent antigen presenting cell type is the dendritic cell (DC). Different DC subsets are derived from a common monocyte-DC precursor, which

subsequently may give rise to a committed DC precursor, however the precise branching points in the developmental pathway are still under debate.[73] These precursors differentiate into plasmacytoid dendritic cells (pDCs) or exit the bone marrow as immature or pre-DCs. These immature DCs patrol the circulation and pick up antigens at sites of inflammation, like the atherosclerotic lesion, upon which they maturate into classical DCs and upregulate antigen-presenting molecules (major histocompatibility (MHC) class I and II) and co-stimulatory molecules (CD40). Subsequently they either migrate under the influence of pro-inflammatory cytokines and chemokines towards lymphoid organs or directly present the processed antigens to naive and memory T cells in the adventitia.[74,75] Depending on the type of antigen presentation and co-stimulatory molecule expression both pro- and anti-inflammatory adaptive responses can be induced. DCs have been shown to accumulate during atherosclerosis progression and experiments with CD11c deficient mice suggest a contributing role for particularly monocyte-derived DCs in lipid retention in the lesion.[76] In contrast, the potent suppressive effect of tolerogenic DCs via the induction of anti-inflammatory regulatory T cells (Tregs) may prove a promising therapeutic avenue. For instance, both transfer of oxLDL-pulsed mature DCs77 and injection of oxLDL-induced apoptotic DCs[78] markedly upregulated the number of splenic and circulating Tregs, which resulted in reduced inflammatory monocyte levels and lesion development in $LDLr^{-/-}$ mice. Similar to its effects on monocyte and macrophage migration stress exposure results in increased aortic levels of $CD11b^{+}$ $CD11c^{+}$ DCs in $apoE^{-/-}$ mice both under normal and hypercholesterolemic conditions.[79] Acute stress may actually act as an endogenous adjuvant during immunization by enhancing maturation and migration of dendritic cells resulting in increased T cell activation in the draining lymphodes.[80] Similar human results were obtained for antibody titer production upon influenza vaccination. Both exercise and mental stress in close temporal proximity to the immunization ensued lasting high antibody titers, especially in women.[81] Chronic stress however seems to inversely correlate with the antibody response to influenza vaccination in a strain-dependent manner.[82]

3.2 Adaptive immunity

In contrast to innate immunity, adaptive immunity is characterized by specific recognition of an antigen by membrane B cell receptors and immunoglobulin receptors on B cells and T cell receptors (TCR) on T cells. Antigen presentation by macrophages and dendritic cells and subsequent recognition by T and B cells drives the clonal expansion and differentiation in effector cells with pro-inflammatory properties directed against the source of the antigen. To protect against randomly generated TCRs that recognize self-molecules, additional signals in the form of cytokines and costimulatory molecules (e.g. CD40L) produced by innate immune cells are necessary for T cell survival. In addition, regulatory T

cells are another form of a tolerogenic mechanism to limit autoimmunity.[83] In atherosclerosis several exogenous and endogenous antigens have been identified, resulting in detectable antibody titers against pathogens such as Heliobacter pylori and Cytomegalovirus, but also endogenous proteins, including heat-shock proteins and modified self-proteins such as oxLDL.

3.2.1 T-lymphocytes

Like the innate immune cells, T cells are recruited to the atherosclerotic lesion and contribute to lesion development. Most T cells in the atherosclerotic plaque are $CD4^+$ T cells and adoptive transfer of these cells in immunodeficient $apoE^{-/-}$scid/scid mice resulted in accelerated atherosclerosis.[84] Priming and clonal expansion of naïve T cells occurs primarily in the secondary lymphoid organs where $CD8^+$ T cells (cytotoxic T cells) and $CD4^+$ T cells (T helper cells) recognize antigens presented by APCs on MHC class I and MHC class II molecules, respectively. Further activation occurs upon a secondary encounter with the antigen in combination with additional activation signals. Depending on the cytokine milieu present $CD4^+$ T cells differentiate further into pro-atherogenic T helper 1 (Th1) cells, which secrete IL-1, IL-2, INF-γ, TNF-α, IL-12 and IL-18 and stimulate plaque macrophages and other immune cells or anti-inflammatory T helper 2 (Th2) cells, producing IL-4, IL-5 and IL-13. Vaccination against IL-12 resulting in reduced Th1-mediated immunity significantly attenuated experimental atherosclerosis.[85] The precise involvement of Th2 cells in atherosclerosis remains controversial as both pro- and anti-inflammatory effects of the cells have been observed and during disease progression a shift toward Th2-mediated immunity occurs.

Stress has been shown to result in mobilization of both cytotoxic T cells and T helper cells and increase their migration to target tissues, including the inflamed endothelium.[72,86]

3.2.2 B-lymphocytes

Although relatively few B cells can be detected in the atherosclerotic lesions and originally confined to their role in humoral immunity, recent advances in the identification of different B cell subsets indicate both atheroprotective and atherogenic responses by these different subsets.[87] B cell depletion by means of splenectomy in $apoE^{-/-}$ mice aggravates atherosclerotic plaque development and this effect is reversed by transfer of educated B cells from older $apoE^{-/-}$ mice.[88] Interestingly, this atheroprotective effect seems to be attributable to the B1a B cell subset, while B2 B cells are highly atherogenic.[89,90] Both B cell subsets have been shown to produce and secrete different amounts and types of immunoglobulins directed against atherosclerosis-related antigens (e.g. oxLDL), which may explain their differential effects on lesion progression.[91] Limited data exist on the effects of stress on B cell function. Chronic restraint stress was shown

to decrease B cell maturation in response to tetanus toxin in mice and in rats, while 2 hours of immobilization stress significantly decreased mitogen-induced B cell proliferation.[92,93]

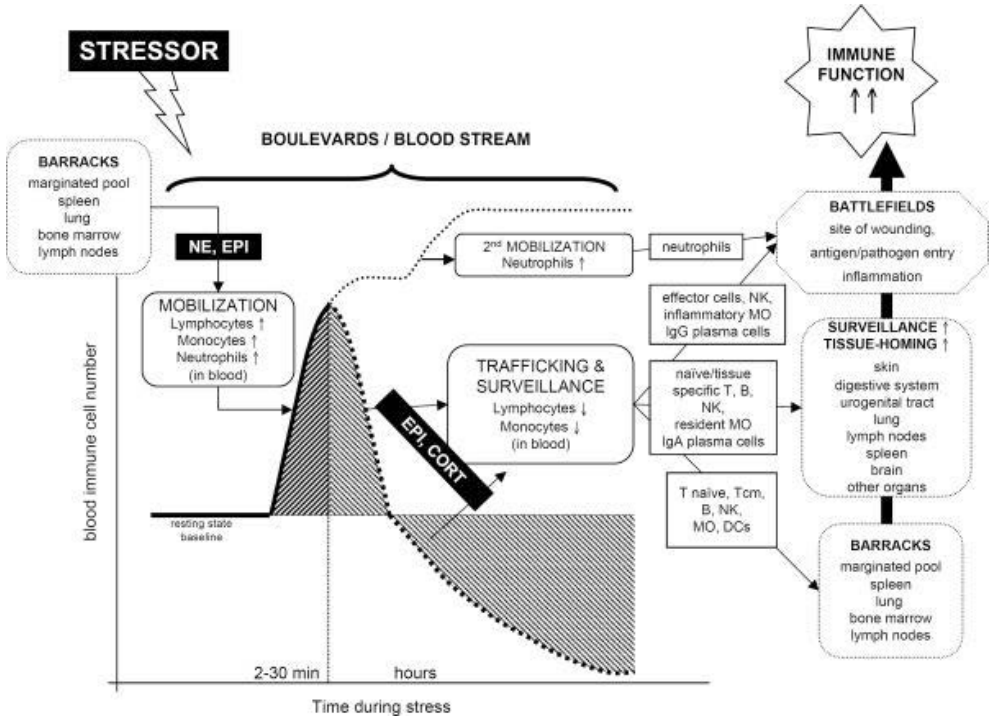

Figure 5. Schematic overview of acute stress-induced leukocyte redistribution mediated by the major stress hormones norepinephrine, epinephrine and corticosterone. Dhabhar *et al.* 2012 Psychoneuroendocrinology Sep; 37(9): 1345–1368

3.3 Atherothrombosis – Platelets and the coagulation system

In addition to the large lipid and inflammatory content of the plaque, the atherosclerotic lesion contains a variety of procoagulant and thrombogenic factors. Atherothrombosis, the process of (occlusive) thrombus formation upon rupture or erosion of the fibrous cap, is the major cause of acute coronary syndromes. Upon disruption of the vessel wall both platelet activation and aggregation and the fibrin-producing coagulation system generate a clot to restore the vascular integrity.[94] Two major blood coagulation pathways exist, the contact (intrinsic) pathway and the tissue factor (extrinsic) pathway, which both converge into a common pathway resulting in the conversion of prothrombin to thrombin. In turn thrombin facilitates the conversion of fibronectin to soluble fibrin, which becomes stabilized and forms the eventual thrombus.[94,95] Regulation of coagulation, resolution of the clot and restoration of blood flow is normally facilitated by inhibitory factors, such as the Tissue Factor Pathway Inhibitors (TFPI-1 and 2), anti-thrombin formation

and activation of the blood fibrinolytic system. In short, activation of the pro-enzyme plasminogen into plasmin results in plasmin-mediated degradation of the fibrin clot into fibrin degradation products (FDP). Plaque derived tissue factor (TF), collagen, fibrinogen and FDPs as well as innate immune cells play a key role in atherothrombosis. For instance, direct interactions between neutrophils, monocytes, mast cells and platelets have been shown to both potentiate and inhibit thrombus formation and platelet activation in atherothrombosis.[96,97]

Like its effects on the immune system, aberrant HPA and SAM activity as seen with chronic stress results in haemostatic complications. Platelet abnormalities, increases in procoagulant molecules (i.e. fibrinogen) and endothelial dysfunction together result in a procoagulant status of the blood in depressed patients and subjects experiencing chronic work-related stress.[98,99] As acute stress activates both the coagulation and fibrinolysis system, the observed triggering of acute cardiovascular events upon severe stress exposure may reflect a combined action of immune cell activation and changed coagulant status of the blood.

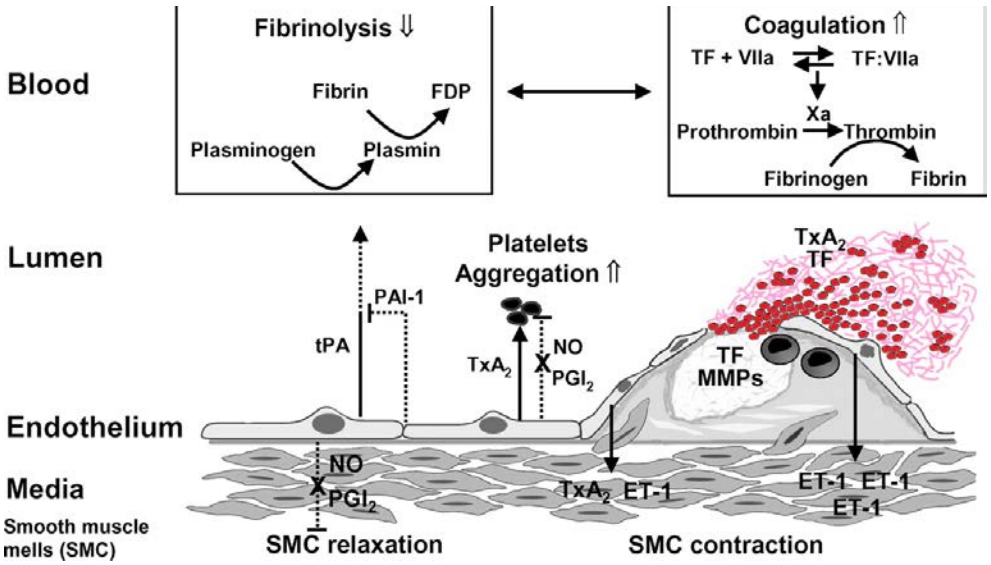

Figure 6. Blood coagulation and fibrinolysis pathways in atherothrombosis. Viles-Gonzalez *et al.* Eur Heart J. 2004 ;25(14):1197-207.

4. Research models

Atherosclerosis is a complex multifactorial disease limiting the usefulness of in vitro models, in which single cell types can be studied. Although these in vitro models are supportive when studying a specific immunological response to for example an atherogenic plaque component, the use of animal models is indispensable. The ability to generate specific gene knockout mice has been and is highly valuable to study the mechanisms underlying atherosclerotic disease susceptibility, progression and for preclinical therapeutic screening of

(pharmacological) interventions. Unfortunately, unlike humans, wild-type mice do not develop atherosclerosis and are highly resistant to atherogenic stimuli. Genetic knockout of the LDL receptor, apolipoprotein E or insertion of a transgene with the ApoE*3-Leiden mutation, has resulted in three hypercholesterolemic and atherosclerosis susceptible mouse strains, which have been extensively used for preclinical atherosclerosis research. Still, only apoE$^{-/-}$ mice develop lesions on a normal chow (low cholesterol) diet. LDLr$^{-/-}$ mice and ApoE*3-Leiden mice develop atherosclerosis when fed a western-type (high cholesterol) diet.[100] A new strategy inducing hypercholesterolemia by means of adenoviral expression of PCSK9 circumvents germline genetic engineering and provides temporal control of disease induction, which may be very useful in disease models for which no LDLr$^{-/-}$ or apoE$^{-/-}$ strains exist.[101]

4.1 Mouse models of atherosclerosis and thrombosis

Similar to the human situation, atherosclerotic plaques develop in mice at sites of disturbed blood flow, which include the aortic arch and its bifurcations, the brachiocephalic artery, innominate artery and the aortic root at the level of the tricuspid valve (figure 7A). However, lesion growth at different sites varies greatly and is relatively slow.[9] To induce rapid site-specific lesion formation several strategies have been developed. Semi-constrictive cuff or collar placement around the femoral arteries or carotid arteries (figure 7B) considerably accelerates atherosclerosis development and progression of the lesions into more advance and vulnerable stages.[102,103] Endothelial denudation, by means of wire-injury, and vein graft (vena cava) placement in the carotid artery, results in a neointima like lesion in which vascular smooth muscle proliferation plays an important role. An additional benefit of the cuff and collar-induced atherosclerosis and vein graft models is the ability to locally manipulate the perivascular environment. For instance, pluronic gel placement with cytokines or viral vectors have proven useful in elucidating specific effects of atherogenic and atheroprotective agents at different stages of the disease.[56,58,59,62,104,105] In contrast to the human situation, spontaneous plaque rupture and subsequent thrombus formation does not occur in mice. To study thrombogenesis and the atherothrombotic responses in mice one has to revert to more extreme models such as mechanical disruption of established lesions or laser- and FeCl3-induced vascular injury models.[106,107]

4.2 Mouse models of acute and chronic stress

Like the pathophysiology of cardiovascular disease, an organisms response to stress (both perceived and real) is a complex and multifactorial phenomenon in which endocrine, metabolic and immunologic processes are intricately woven. The use of laboratory animal models has been indispensable in shedding light on the central and peripheral effect of stress and its contribution to various

diseases, including intestinal and skin diseases as well as autoimmune diseases like rheumatoid arthritis and atherosclerosis.

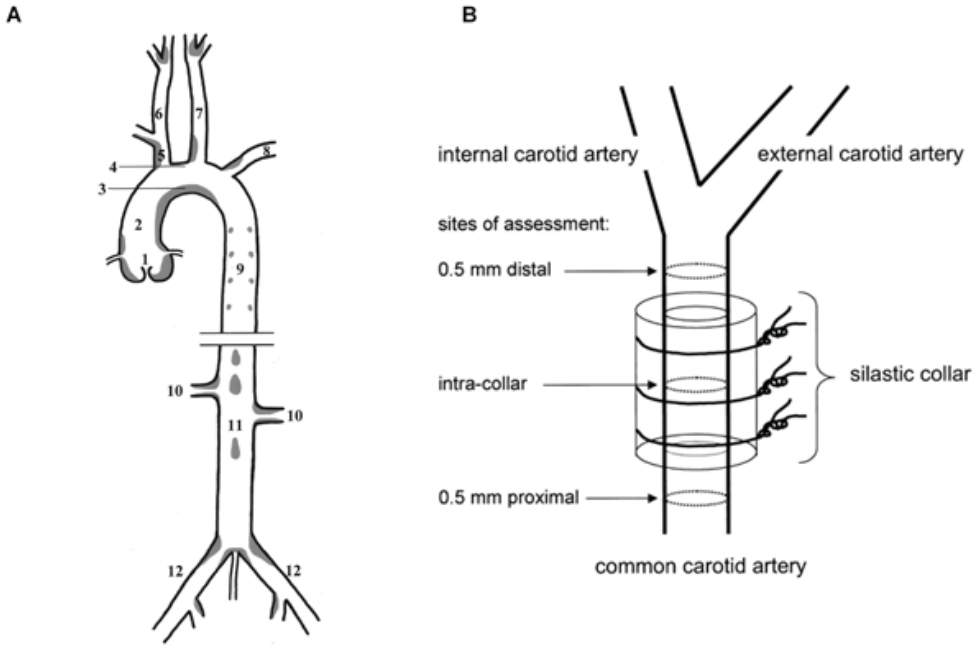

Figure 7. A) Longitudinal representation of the major arterial vasculature illustrating the distribution of atherosclerosis (gray shading) in LDL receptor–deficient mice fed a high-fat atherogenic diet. Adapted and modified from Van der Laan *et al.* Arterioscler Thromb Vasc Biol. 2004;24:12-22. B) Perivascular collar-induced atherosclerosis model in the carotid arteries. Adapted and modified from von der Thusen *et al.* Circulation. 2001 ;102:1164-1170.

The physiological response to stress is largely dependent on the intensity and duration of the stressor. The acute (lasting minutes to hours) and chronic stress response (lasting several hours a day for weeks or months) vary, in their respective hemodynamic and neurohormonal responses as well as the persistence of these effects. Chronic stress exposure is generally assumed harmful due to metabolic, endocrine and immunologic adaptation, which even last after cessation of the stressor. Animal models of chronic stress, mainly rat and mouse models, entail the chronic exposure to (combinations of) physical and psychological stressors. Like humans, rodents exhibit clear age, sex and even individual differences in perception, processing and coping mechanisms and as such many different models have been developed tailored at mimicking specific human conditions, such as depression and post-traumatic stress disorder. To prevent habituation to the stressor most chronic stress paradigms involve multiple stressors in a random order, causing so called chronic unpredictable stress. Although generally immunosuppressive, chronic stress exposure results in maladaptation of the neuroendocrine response and a shifts in leukocyte distribution, which can aggravate atherosclerosis.[108] In contrast

to the immune dysregulation observed with chronic stress, acute stress primarily results in immune enhancement. Depending on the inflammatory or disease status of an organism the result can be detrimental or beneficial. For instance, acute stress has been causally linked to exacerbations of skin diseases and asthma, but also increased immunoprotection during surgery and vaccination.[109] Methods to induce acute stress include immobilization (restraint), cold or heat stress, forced swim, shaking, electric foot shock and the introduction of an (aggressive) intruder. Due to both the physical and psychological aspect of restraint stress and the relative ease and low cost of this stressor, for example in a well-ventilated 50 ml centrifuge tube, restraint stress is most often used in acute stress experiments.

5. Study aims

Vascular inflammation plays a crucial role in atherosclerotic lesion development and progression and the immunemodulatory properties of the (acute) stress response is among the most potent known to man. Previous research has demonstrated a contributing role for perivascular mast cells in lesion development, progression and destabilization. However the precise triggers of mast cell activation in the context of atherosclerosis remain elusive. Interestingly, perivascular mast cells have been shown to colocalize with nerve fibers innervating the vessel wall, suggesting the possibility of neuronal regulation of mast cell activation. In this thesis we aimed to investigate the potential of the acute stress response and related neurohormonal mediators in activating vascular mast cells and its subsequent effects on atherosclerotic lesion progression and atherothrombotic complications.

6. Outline thesis

Acute cardiovascular syndromes such as myocardial infarction and stroke remain a leading cause of death world-wide. Atherosclerotic lesion stability and the thrombogenic potential of the blood upon plaque rupture are major risk factors. In order to reduce the amount of cardiovascular disease-related deaths, a thorough understanding of all its risk factors is eminent and the immunological response to psychological stress is a underappreciated modifiable risk factors which deserves further attention.

The aim of this thesis is to gain insight in the pro-atherogenic effects of acute stress exposure and in particular investigate the contribution of stress-induced perivascular mast cell activation on atherosclerotic lesion progression and destabilization. **Chapter 2** encompasses a review which focuses on acute and chronic psychological stress as risk factors for atherosclerotic cardiovascular disease and its deadly complications, myocardial infarction and stroke. In **Chapter 3**, we show that acute stress, by means of 2 hours immobilization, significantly activates cardiac mast cells, resulting in a mast cell-dependent

increase in circulating and local pro-inflammatory cytokine release and reduced atherosclerotic lesion stability. **Chapter 4** describes the effects of the acute stressor on the thrombogenic potential of the blood and the contribution of the mast cell herein. Previous results have shown a potent pro-atherogenic response to neuropeptide induced mast cell activation. In **Chapter 5** we investigated the differential expression of neuropeptide Y in stable and unstable human and mouse atherosclerotic plaques, demonstrating NPY accumulation during disease progression. A pro-atherogenic role for NPY was demonstrated by local overexpression of NPY near the atherosclerotic plaque, which resulted in increased lesion development and perivascular mast cell activation. **Chapter 6** provides further insight in the contribution of NPY signaling via its different receptors, Y1, Y2 or Y5. In **Chapter 7**, mast cell derived chemokines were shown to contribute to neutrophil recruitment to the atherosclerotic plaque and accumulation within the intima. In vitro and in vivo migration and influx studies confirmed a mast cell-dependent role for the CXCL1-CXCR2 axis in neutrophils migration. The results of all the studies described in this thesis, as well as future prospects are discussed in **Chapter 8**.

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Chapter 2

Acute and chronic psychological stress as risk factors for cardiovascular disease: Insights gained from epidemiological, clinical and experimental studies

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Abstract

Cardiovascular disease (CVD) remains a leading cause of death worldwide and identification and therapeutic modulation of all its risk factors is necessary to ensure a lower burden on the patient and on society. The physiological response to acute and chronic stress exposure has long been recognized as a potent modulator of immune, endocrine and metabolic pathways, however its direct implications for cardiovascular disease development, progression and as a therapeutic target are not completely understood. More and more attention is given to the bidirectional interaction between psychological and physical health in relation to cardiovascular disease. With atherosclerosis being a chronic disease starting already at an early age the contribution of adverse early life events in affecting adult health risk behavior, health status and disease development is receiving increased attention. In addition, experimental research into the biological pathways involved in stress-induced cardiovascular complications show important roles for metabolic and immunologic maladaptation, resulting in increased disease development and progression. Here we provide a concise overview of human and experimental animal data linking chronic and acute stress to CVD risk and increased progression of the underlying disease atherosclerosis.

Introduction

Cardiovascular diseases (CVD) remain one of the leading causes of death and disability worldwide[1] and their high prevalence in Western society has been attributed to major changes in work and eating habits as well as income, educational and physical activity levels.[2] The primary underlying pathology of CVD is atherosclerosis, a chronic inflammatory disease of the vessel wall of large- to medium-sized arteries. Atherosclerosis is characterized by the retention of cholesterol, packaged into low-density lipoproteins (LDL), in the artery wall. The subsequent modification, primarily oxidation, of these LDL particles is considered the key process driving the inflammatory response. Recruited macrophages take up modified LDL via scavenger receptors and become lipid-laden foam cells and further fuel local inflammation. With disease progression, cross-talk between innate and adaptive immune cells as well as the resident cells of the vessel wall eventually leads to the formation of a plaque consisting of an a-cellular debris-rich necrotic core covered by a fibrous cap. Degradation or erosion of the cap as a consequence of hemodynamic forces and inflammatory processes can cause exposure of the thrombogenic content to the blood, triggering a thrombotic response which may subsequently lead to occlusion of the vessel and acute cardiovascular syndromes (ACS) associated with CVD, such as myocardial infarction and stroke.[3]

Traditional risk factors for atherosclerosis are dyslipidemia, inflammation, obesity, hypertension, diabetes, genetic predisposition and several behavioral parameters, including smoking, alcohol intake and physical inactivity. Often disregarded are psychosocial factors such as job-strain, anxiety, depression and personality characteristics. Early work by Rosenman *et al.* provided solid evidence for a correlation between type A behavior, illustrated by time urgency, hostility, and achievement striving and the risk of developing coronary heart disease, demonstrating higher serum cholesterol, increased blood coagulation and an increased incidence of clinical heart disease in these subjects compared to subjects with the converse behavior type B.[4,5] More recently, results from the INTERHEART study, a large case-control study evaluating over 11,000 patients with a first myocardial infarction (MI) and over 13,000 age- and sex-matched controls from 52 countries world-wide, presented convincing associations between psychosocial factors and risk of MI.[6,7] For example, in participants who had suffered from a MI, more than twice as many reported permanent stress at the workplace (odds ratio 2.12 [99%CI, 1.68-2.93]) compared with controls. Also, significantly more patients in the cohort reported to be exposed to two or more acute stressful life events (e.g. loss of a loved one, divorce, loss of job or business failure) in the previous year (odds ratio 1.48 [99%CI, 1.33-1.64]).[7] However, inherent difficulties of delineating the pathological effects of different stressors as well as individual variations in the perception of stress make it difficult to implement psychosocial factors as a causal risk factor and therapeutic target.

Despite these difficulties, much progress has been made over the last decades by means of epidemiological, clinical and experimental studies evaluating various psychosocial stressors (e.g. work stress, job insecurity, anxiety, social isolation) and their respective effect on CVD risk.[8–11] Furthermore, the relative new field of psychoneuroimmunology, which addresses the multi-directional interactions between the central nervous system, the endocrine system and the immune system, is now providing mechanistic insight into ways by which (perceived) stressors can be translated into physiological changes.[12]

In this review we aim provide an overview of the contribution of acute and chronic stress to clinical and experimental atherosclerosis development and discuss the contribution of the stress response in triggering ACS, such as myocardial infarction and stroke.

Stress and cardiovascular disease: the major systems involved

The original concept of stress, first described in medical literature by Selye, was the non-specific (neuroendocrine) response of the body to any noxious stimulus.[13] Later this concept was refined by distinguishing between stressor and stress response and evaluating stress as the body's response to environmental demands that exceed its natural regulatory capacity. The main systems activated by psychological stressors and mediating the stress response are the hypothalamus-pituitary-adrenal (HPA) axis, the sympathetic adrenomedullary (SAM) system, the renin-angiotensin-aldosterone system (RAAS) and the cholinergic system, which are aimed at accessing and mobilizing the body's energy stores and modulating the immune system in anticipation and response to a threat. A perceived threat is relayed through the limbic system (amygdala, hippocampus and prefrontal cortex) to the hypothalamus, resulting in corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) release from the paraventricular nucleus. CRH and AVP in turn trigger the synthesis of pro-opiomelanocortin (POMC) and the release of its cleavage product adrenocorticotropic hormone (ACTH) by the anterior pituitary. ACTH then acts on the adrenal cortex to synthesize and secrete the glucocorticoid (GC) hormone cortisol (in humans) or corticosterone (in rodents).[14] In addition, the hypothalamus also activates the adrenal medulla via the sympathetic nervous system (SNS), resulting in the release of the catecholamines adrenalin and noradrenalin. ACTH, CRH and glucocorticoids (GCs) in turn provide negative feedback to dampen and cease the signaling cascade and return to homeostasis.[15,16] In addition, activation of the autonomic nervous system, both centrally and via afferent pathways, activates efferent neuronal circuits, including the cholinergic anti-inflammatory reflex.[17] Although this systems has potent immune-modulatory properties, its direct implication in atherosclerosis development and progression remains unresolved.[18-21] Nevertheless, the immune reflex and neuroendocrine control of metabolic pathways are clearly contributing factors in stress-mediated

pathologies, as for example reviewed by Kibler *et al.*[22]

Stress research can be roughly subdivided in the effects and mechanisms of chronic and acute stress. Chronic psychological stress in early life and adulthood has been demonstrated to result in maladaptive changes in both the HPA-axis and the sympathetic nervous system, causing elevated local and circulating levels of GCs, cathecholamines and several (growth) hormones and neuropeptides. As mentioned above, modulation of immune responses by stress is mediated via complex multi-directional interactions between the central nervous system, the endocrine system and the immune system. Although these interactions are designed to adapt the body to deal with threats, chronic exposure to stress-induced factors have been shown to either enhance or even wear down the immune system, thus enhancing the risk of developing infectious diseases and prolong illness episodes. While acute and time-limited stressors seem to result in adaptive redistribution of all major leukocyte subpopulations to for example a site of inflammation[23,24], more persisting stressors cause suppression of the (Th1-mediated) cellular immune response and a shift towards a (Th2-mediated) humoral response.[25,26] As a consequence, chronic stress renders the individual susceptible to infection and cancer[27,28] and increases a person's vulnerability to autoimmune and allergic diseases.[29]

In the context of cardiovascular diseases, psychological stress exposure is known to activate specific brain regions leading to the activation of the HPA axis and the sympathetic system, which can directly affect the vessel wall (for review see Pereira et al., Golbidi et al.)[30,31], by increasing heart rate and blood pressure, but also by causing endothelial dysfunction, which is actually one of the primary triggers for the development of atherosclerosis. Stress-mediated induction of the sympathetic nervous system and the HPA-axis results in an enhanced activation or the RAAS system, which directly results in endothelial activation and damage as indicated by increased adhesion molecule expression. This in turn mediates the recruitment, adhesion and transmigration of inflammatory cells across the endothelial layer, thus initiating atherosclerotic plaque formation. Furthermore, stress may accelerate atherosclerosis progression by facilitating a chronic low-grade inflammatory response, characterized by increased levels of for example plasma C reactive protein (CRP), Interleukin (IL)-6 and tumor necrosis factor-α (TNFα). This low grade inflammatory response can further enhance heart rate and blood pressure via regulation through the central autonomic network[32], potentially leading to a vicious circle that will culminate in disease progression. Along that line, the increased inflammatory response, which may be mediated by stress-induced NFκB[33], can reduce endothelial nitric oxide synthase (eNOS) expression by the already damaged endothelium, and induce the release of reactive oxygen species (ROS), resulting in a pro-oxidative milieu that can cause lipid oxidation. Finally, chronic stress can via the induction of the endocannabinoid system result

in insulin resistance and metabolic syndrome, which is yet another risk factor for the development of CVD (Figure 1).

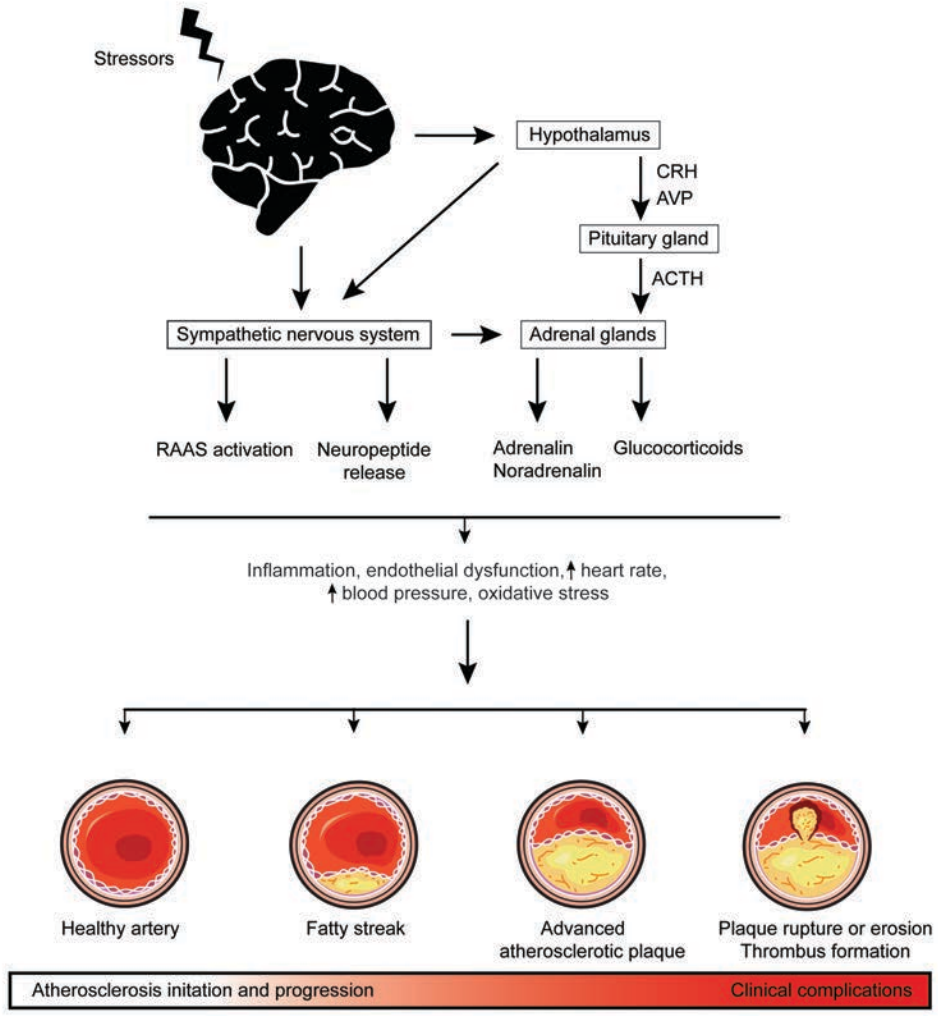

Figure 1: Schematic overview of the most prominent effects of the stress response on atherosclerosis development and progression. Stress perception culminates via activation of the hypothalamic-pituitary-adrenal axis in the production and release of glucocorticoid hormones and via the sympathetic-adrenal-medullary in synthesis of the catecholamines adrenalin and noradrenalin. Furthermore, it leads to activation of the renin-angiotensin-aldosterone system (RAAS), while neuropeptides are released from peripheral nerve endings. Together, these factors can affect e.g. the inflammatory response, induce endothelial dysfunction, increase heart rate and blood pressure, and increase the levels of oxidative stress. These processes in turn can impact atherosclerosis development and progression at each stage of the disease, eventually resulting in acute cardiovascular events due to plaque rupture or erosion. CRH = corticotrophin releasing hormone, AVP = arginine vasopressin, ACTH = adrenocorticotropic hormone.

The actual clinical outcomes such as MI or stroke, however, are most likely to be triggered by an acute event in people with high plaque burden in combination with an inflammatory and pro-coagulant status. The body's response after exposure to a sudden and severe stressor such as bereavement[34], an earthquake[35,36] or a terrorist attack[37,38] has been demonstrated to fulfill these criteria and associates with increased incidence of ACS.

Chronic stress and cardiovascular disease

The onset of atherosclerosis starts already early in life, with the development of foam cell-rich fatty streaks in the vessel wall detectable in childhood.[39] However, the disease progresses relatively slow and clinical symptoms, primarily caused by flow limiting stenosis, often do not manifest itself until middle or older age. Many risk factors for atherosclerosis act at multiple time points on the pathogenic process[40] and stress may influence disease progression and other risk factors across the life course. Chronic stress, both in early life (sexual abuse, parental disease, poor socioeconomic status)[41] and adulthood (job-strain, social isolation, caregiver syndrome) have been linked to increased coronary heart disease (CHD) risk[42]. The largest body of epidemiological evidence comes from work-related stressors such as job-strain – high job demands combined with low control - and effort-reward imbalance. For instance, the Whitehall II prospective cohort study demonstrated job insecurity in a population of British civil servants to be associated with a 1.38-fold (95% CI, 1.01-1.88) risk of CHD even after adjustment for physiological and behavioral cardiovascular risk factors[9] and similar results were obtained in the Copenhagen City Heart Study[43] and the Primary Prevention Study.[44] Although two recent meta-analysis indicated smaller risk-ratios of 1.2-1.3 due to publication and reverse causation bias[8,45], work-related stress seems a robust risk factor and to be accountable for a significant proportion of CHD in the working population.

Other chronic psychological conditions investigated in relation to cardiovascular morbidity are depression, anxiety, post-traumatic stress disorder (PTSD) and the before mentioned personality types. The prevalence of a major depression has been demonstrated to be high in patients with cardiovascular disease and consistent associations with cardiovascular morbidity and mortality rates have been established.[46] The first reports of a higher incidence of CHD-related deaths in patients suffering from major depression originate from 1930.[47] More recent epidemiological evidence from the Baltimore cohort of the Epidemiologic Catchment Area Study[48] and the Nord-Trondelag Health Study[49] reported an odds ratio of 4.45 (95% CI, 1.65-12.44) and a multi-adjusted hazard ratio of 1.41 (95% CI, 1.07-1.87) for major depression associated heart failure, respectively. In the Nurses' Health Study II, trauma exposure and high PTSD symptoms were shown to associate with an increased risk of CVD in women.[50] Furthermore, progression of sub-clinical atherosclerosis as well as elevated blood pressure and

systemic increases in inflammatory markers have been studied in this context. Depressive symptoms have been associated with greater carotid intima-media thickness[51,52] and aortic and coronary calcifications, although some contradicting reports also exist.[53,54] This discrepancy could possibly be explained by differences in the assessment of atherosclerosis and failure to assess the persistence of the depressive symptoms.[55]

As mentioned, atherosclerotic plaque development begins already at a very young age and increasing recognition is given to the adverse effects of early life experiences on cardiovascular health later in life. These early life challenges include exposure to toxins and diet but also situations which induce behavioral and emotional stress, anxiety, fear and discomfort. While children can be exposed to a wide variety of chronic psychological stressors, most research in this area has focused on socioeconomic disadvantage and parental maltreatment.[41] The Adverse Childhood Experiences (ACE) Study was one of the first studies describing the long-term relationship between childhood experiences and medical and public health problems.[56] This large-scale project assessed childhood maltreatment (abuse, neglect and household dysfunction) retrospectively in over 17,000 adults. Clear dose-dependent correlations (by exposure to 0 up to 4 of the investigated types of ACE) were demonstrated for multiple leading causes of death in adults, including liver disease[57], skeletal fractures, but also ischemic heart disease[58] and stroke. Furthermore, the ACE Study provided strong evidence that the different adverse childhood events often co-occur and that these events are interrelated, which thus may have implications for other studies performed in this research area, evaluating a specific category of ACE.[59] In line with these results, a recent meta-analysis examining the health consequences of childhood maltreatment in a combined cohort of 48,000 individuals showed similar correlations with neurological (migraines), musculoskeletal (arthritis, broken bones) as well as cardiovascular complications, such as myocardial infarction and stroke[60], indicating the important role of early adverse events in life in affecting adult health risk behavior, health status and diseases, including cardiovascular disease. Another factor associated with cardiovascular health is socioeconomic status (SES). Low SES during adulthood is associated with a wide range of health issues including cardiovascular disease. Low SES is characterized by low environmental quality such as poor housing, air and water quality and limited food options, which can lead to adverse psychological effects like hyperactivity and aggression, but also to a sedentary life style, a poor diet and an increased susceptibility to infection, thus increasing the risk of CVD. Interestingly, recent data indicate that low childhood SES also seems to be a good predictor of adult cardiovascular morbidity and mortality independent of adult SES status, further stressing the importance of early life conditions on adult health.[61] Family status, neighborhood crime, violence, deprivation of healthcare and substance use can have negative effects during

childhood, leading to psychological dysregulation early in life, which can affect cardiovascular health as an adult.

Table 1: Clinical inflammatory risk markers associated with acute and chronic stressors

Types of stressors	Inflammatory risk markers	References
Chronic stressors		
Work-related stressors		
high demands - low control, burnout	TNFα, IL-4, CRP, fibrinogen, IL-10	68-70, 84, 85
Affective disorders		
Depression	CRP, IL-6 (after additional stress exposure)	54, 71
Anxiety disorders	CRP, IL-6, TNFα,	72
Post Traumatic Stress Disorder	CRP, TNFa, IL-4	64-67
Low socioeconomic status	CRP, IL-6 (after additional stress exposure), sICAM, ET-1	73, 74, 75
Caregiver stress	IL-6, CRP, D-dimer	73, 76, 77, 78
Loneliness / social isolation	IL-6, CRP, sICAM-1	79, 80, 81
Early life stress / childhood maltreatment	CRP, fibrinogen	82, 83
Acute stressors		
Population-wide		
Natural disasters	CRP	120
Sporting events	ET-1, sCD40L, sVCAM, MCP-1, TNFα,	121
Individual		
Bereavement	IL-6, IL-1RA, sE-selectin	122, 123
Anger outburst	CPR, IL-6	124, 125
Acute laboratory stressors	IL-1β, TNFα, IL-2, IL-4, IL-10, CRP	126

As mentioned above, chronic stress may contribute to atherosclerosis progression by inducing a chronic low-grade inflammatory response. CRP is an acute-phase reactant that is commonly used as a marker for systemic inflammation, and is also used as a determinant for the risk of cardiovascular disease. Interestingly, plasma CRP levels were generally found to be enhanced upon chronic psychosocial stress, as determined in a literature survey.[62] For example, caregivers of a family member with dementia, who experienced daily stressors, were seen to have enhanced levels of systemic inflammation compared to noncaregiving controls, as plasma CRP as well as IL-6 levels were elevated.[63] Similarly, CRP levels were higher in patients with PTSD compared to controls without PTSD.[64] In fact, a positive association between PTSD and elevated inflammatory markers has been observed in a number of studies.[65-67] Work-related stressors such as a burnout have also been demonstrated to correlate with inflammatory markers.[68-70] Higher levels of total burnout symptoms in schoolteachers associated with increased TNFα levels[68], while in another study females with a burnout showed elevated levels of CRP compared to healthy controls.[69] In that study, males suffering from a depression were seen to display elevated levels of CRP, and a relation between depression and inflammation has been observed in other studies as well.[71-72] A literature study established that low SES associated with higher plasma CRP levels.[73] Evidence illustrating stress-induced inflammation and its specific markers are summarized in Table 1[65-83], together clearly establishing that a variety of psychosocial disorders, such as burnout, depression, loneliness or PTSD, affect

the inflammatory status of the patient, mostly demonstrated by increased levels of plasma CRP and IL-6. Both plasma CRP and IL-6 have been associated with an increased risk of unstable cardiovascular diseases, and as such chronic stress may contribute to the cardiovascular disease burden. Indeed, e.g. depression was proven to be predictive for cardiovascular mortality, which was at least partly explained by the contribution of inflammation (i.e. CRP and IL-6 levels).[84,85]

Effects of chronic stress on experimental atherosclerosis

Human studies have clearly identified chronic stress as a risk factor for ACS, however these studies are in principle correlative in nature and do not prove causality or insight in the underlying mechanism of disease. This mechanistic insight into the multifaceted neurohormonal response induced by chronic stress is necessary to better understand its impact on the cardiovascular system and to identify new therapeutic targets. Over the years many small and larger animal models have been developed to investigate the processes involved in atherogenesis, which also involved effects of chronic stress on lesion development. Landmark studies on the effects of psychological stress were performed by Ratcliffe *et al.* and Kaplan *et al.* who demonstrated accelerated coronary atherosclerosis development in separated swines[86] or in cynomolgus monkeys living in unstable hierarchies, independently of plasma lipid levels.[87] Currently, hyperlipidemic mouse strains, such as the LDL receptor ($LDLr^{-/-}$) or apolipoprotein E ($apoE^{-/-}$) knockout mice which develop human-like atherosclerotic lesions when fed a high fat (Western-type) diet, are the mainstay for atherosclerosis studies.[88] Furthermore, experimental protocols to mimic (aspects of) the human stress response and related diseases such as depression have been developed. Again a clear distinction between models aimed at eliciting an acute or chronic stress response can be made. While an acute trigger rapidly increases circulating GC and cathecholamine concentrations, which return to baseline quickly after cessation of the stressor, chronic exposure may result in lasting and variable neuroendocrine responses depending on the type of stressor and habituation to the stress protocol (Table 2a;b).

A number of studies have been performed to establish the effects of a single chronic stressor on the development and progression of atherosclerosis. For example, social isolation by means of individual housing of male $apoE^{-/-}$ mice significantly elevated plasma cholesterol and triglyceride levels and resulted in an increase in atherosclerotic plaque development in the innominate artery, suggesting dyslipidemia induced by the stressor as a driving force.[89] However, another form of chronic stress, induced by introduction of an intruder mouse, also resulted in accelerated atherosclerosis in the aorta but without effects on lipid homeostasis.

Table 2a: Rodent models of chronic single stress-induced atherosclerosis development and progression

	Type of stressor (and duration)	Animal / Strain	Investigated site(s)	Effects / Measurements	Reference
Single stressors	Social isolation (individual housing for 20 wks)	Male ApoE$^{-/-}$ mice on chow diet	Thoracic aorta (cross sections) Innominate artery (*en face*)	↑ plaque size in the innominate artery; ↑ total plasma cholesterol; ↑ plasma tryglycerides; ↑ plasma G-CSF; ↑ salt appetite; ↑ locomotor activity; ↓ systolic blood pressure; No significant differences in urinary CORT; HR; MAP; plasma INF-γ, IL-2, IL12, TNF-α, IL-4, IL-5, IL-10, MCP-1	89.
	Social disruption (dominant intruder for 2h/day for 3-4 days/wk for 12 wks)	Male ApoE$^{-/-}$ mice, diet not specified	Aorta (*en face)* Aortic root (cross sections) Innominate artery (*en face* and cross sections)	Plaque size in the aorta correlated with stress severity; ↑ plasma CORT; ↑ serum IL-6, CXCL1, IL-10 No significant differences in lesional CD68, VCAM-1, CD3, I-A^{b}, IL-6, total serum cholesterol, urinary CORT one day after stress	90.
	Intermittent cold exposure (4°C for 4h/day for 8 wks)	Male ApoE$^{-/-}$ mice on chow diet	Aortic root (cross sections)	↑ plaque size in the aortic root; ↑plaque macrophages; ↑ plaque lymphocytes; ↓ plaque VSMC; ↓ plaque collagen content ↑ plaque MMP-2, 9 and 14 expression; ↓ TIMP-1 expression No significant differences in total plasma lipids (TC, TG, LDL, HDL)	91.
	Emotional stress (7x social defeat by rat for 4h/7days) for 8 wks Physical stress (3x 10 days water limitation 2x day + 3 days water deprivation) for 8 wks Both protocols combined for 8 wks	Male ApoE$^{-/-}$ mice on chow diet	Brachiocephalic atery (cross sections)	Emotional stress or combined protocol: ↑ plaque size; ↑plaque macrophages and T-cells; ↓ plaque VSMC; ↑ calcifications; ↑ calcifications; necrotic cores ↑ firbous caps and plaque ruptures; ↓ bodyweight; ↑ Total cholesterol and tryglycerides	92.
	Cold stress (1cm iced water for 1h/day for 5 days/wk for 4wks)	Male mixed background (C57BL / Sv129) ApoE$^{-/-}$ mice on lard-containing diet	Brachiocephalic atery (cross sections)	↑ advanced lesions with less stable phenotype: necrotic cores, intraplaque hemorrhages, neovascularization, thin fibrous caps; ↑ urinary CORT; ↑ neuropeptide Y levels in platelet-rich plasma No significant difference in level of stenosis	93.

In this study a significant increase in the pro-inflammatory cytokine IL-6 and the chemokine CXCL1 was observed directly after stress induction, which can lead to faster disease development.[90] A recent study applying a chronic intermittent cold stress paradigm demonstrated increased lesion formation in the aortic root with features of increased plaque vulnerability, indicated by decreased vascular smooth muscle cell (VSMC) and collagen levels and an increased macrophage and lymphocyte content. Furthermore, the expression ratio of matrix degrading enzymes and their inhibitors was less favorable in the chronically stressed mice.[91] Interestingly, emotional stress alone or combined with a physical stressor, but not the physical stressor by itself seemed to contribute to lesion progression and destabilization in apoE$^{-/-}$ mice.[92] Here, social defeat stress induced by exposing mice to a rat was sufficient to increase atherosclerotic lesion development and this effect was even potentiated when combined with the physical stress of water withdrawal. Lesions in the mice exposed to emotional stress or combined emotional and physical stress combined exhibited a significant increase in CD68^{+} macrophages and CD3^{+} T cells and a decrease in α-SMA^{+} VSMCs, suggesting a less stable phenotype. Najafi *et al.* recently combined a chronic stress regimen with the stress susceptible mixed background (C57BL/Sv129) apoE$^{-/-}$ mice on a lard diet, which resulted in a highly advanced plaque phenotype with large necrotic cores, thin fibrous caps, intraplaque hemorrhages and inflammatory infiltrates, which may be a useful model to evaluate therapeutic interventions aimed at plaque stabilization.[93]

Table 2b: Rodent models of chronic multiple stressor-induced atherosclerosis development and progression

	Type of stressor (and duration)	Animal / Strain	Investigated site(s)	Effects / Measurements	Reference
Multiple stressors	Restraint stress or exposure to rat odor (20-60 minutes /day for 3 days/wk for 12wks)	Male and female ApoE$^{-/-}$ mice, diet not specified	Aorta (*en face*)	↑ plaque size; ↓ bodyweight; ↑ plasma CORT	96.
	Severe chronic unpredictable stress regimen (2 x 10 different stressors for 14 days	Male ApoE$^{-/-}$ mice (Balb/c background) on chow diet	Brachiocephalic atery (cross sections)	↑ plaque size; ↓ locomotor activity; ↓ bodyweight; ↑ serum cholesterol, triglycerides and LDL; ↓ HDL; ↑plaque macrophages; ↑ plaque lymphocytes; ↓ plaque VSMC; ↑intimal VCAM-1 and ICAM-1 expression; ↑ serum CRP, IL-6, soluble VCAM-1 and ICAM-1	97.
	Electric foot shock + noise stimulation (1h/day for 4wks) with or without i.p. LPS injection (1mg/kg, 2x/wk)	Male ApoE$^{-/-}$ mice on high-fat diet	collar-induced carotid artery (cross sections)	↑ less stable phenotype: plaque disruptions, thin fibrous caps; ↑ intimal macrophages; ↑ intimal lipids; ↓ intimal collagen; ↑ plaque MMP-12 expression; ↑ serum norepinephrine ↑ heart rate; ↑ blood pressure; ↑ plasma fibrinogen No significant difference in plaque area; bodyweight; serum lipids (TC, TG, HDL, LDL) Combined LPS treatment aggrevated (almost) all stress-induced effects	98.
	One of 5 different stressors: balance stress, restraint stress, rat odor stress, rat odor + restraint or air-jet stress (2h/day for 5 days/wk for 12wks)	Female ApoE$^{-/-}$ mice, diet not specified	Thoracic aorta (cross sections) Innominate artery (*en face*)	↑ urinary CORT; ↓ bodyweight gain No significant difference in plaque area in throracic aorta or aortic root; plasma CORT; adrenal gland weight; HR; MAP; TC; TG; HDL; plaque collagen or macrophage staining	99.
	Chronic variable stress regimen (6 different stressors for 6 weeks) with or without β_3-adrenoreceptor blocker	Female ApoE$^{-/-}$ mice, diet not specified	Aortic root (cross sections)	↑ hematopoeitic system activity; ↑ plaque protease levels; ↑ intimal neutrophils, monocytes and macrophages; ↑ necrotic core area; ↓ firbous cap thickness No significant difference in plaque size; bodyweight; total cholesterol Treatment with a β_3-adrenoreceptor blocker reduced the stress-induced plaque inflammation	138.

As repeated exposure to a single type of stressor has been shown to cause habituatization, and thus a different (pathological) phenotype[94], various protocols inducing chronic unpredictable and variable stress have been developed. These protocols usually consist of exposure of animals to multiple randomly assigned stressors including restraint, forced swim, food or water deprivation, exposure to rat odor, housing in damp bedding, cage tilt, dark-light cycle changes and social isolation for several weeks. These models have been shown to cause robust increases in GC secretion, sensitization of the HPA-axis to new stressors, reduced body weight and behavioral changes in immobilization and anxiety tests, indicating the many central systems affected.[95] Kumari *et al*. demonstrated a dose-dependent atherogenic effect of such a chronic stress protocol in apoE$^{-/-}$ mice.[96] Bodyweight was significantly reduced in the chronically stressed mice, while displaying a 10-fold increase in plasma corticosterone levels and three times more atheroma in the aorta compared to their unstressed littermates, however no direct mechanistic insights were provided. In line with these results, another chronic unpredictable stress regimen with ten different stressors induced heightened plasma lipid and inflammatory cytokines levels (CRP and IL-6) and accelerated atherosclerotic plaque formation.[97,98] Lesions in the brachiocephalic artery of the stressed mice exhibited a less stable phenotype, with high numbers of infiltrated macrophages and T cells and a decrease in VSMCs. Experiments performed by Bernberg *et al*. however contradict a general pro-atherogenic response upon stress exposure. Although chronic exposure of apoE$^{-/-}$ mice to five different physical stressors induced a clear stress response, evidenced by acute increases in heart rate,

blood pressure and plasma corticosterone levels, neither significant differences in cholesterol levels nor disease progression in the thoracic aorta or aortic root were observed.[99] It must be mentioned however, that differences in stress protocol and site of atherosclerosis measured may affect the outcome of the studies.

Rodent models that approximate human early life stress involve maternal separation or decreased maternal care protocols during early postnatal life. During this stress hypo-responsive period, when neural development is still ongoing, repeated episodes of maternal separation have been demonstrated to induce long-lasting effects on cognitive functions and to change the responsiveness of the HPA axis and sympathetic nervous system under basal and stressed conditions. Although the described effects are quite diverse and sometimes species-specific, generally an increase in basal corticosterone levels and exaggerated ACTH and corticosterone responses upon exposure to novel stressors are observed.[100] While such early life stress models have been used extensively to study its neuroendocrine and behavioral implications in the context of depression and cognitive function, no direct experimental results on atherosclerosis susceptibility and progression are available.

Activation of the HPA-axis upon stress exposure culminates in the central and peripheral release of mineralocorticoids (MCs) and GCs. These steroid hormones have been utilized for their anti-inflammatory actions for decades. However, chronic exposure to elevated levels of GCs introduces a state of GC resistance, reducing the anti-inflammatory actions and actually increasing local concentrations of pro-inflammatory cytokines.[101] Furthermore, a prolonged or enhanced exposure to pro-inflammatory mediators, such as to IL-1 or IL-6, can induce a reduction in GC receptor function and even impairment, as illustrated by a reduced sensitivity for GCs[102,103], which further enhances the pro-inflammatory response that can contribute to the progression of cardiovascular disease. In fact, patients with CVD were recently seen to exhibit a reduction in GC receptor expression and sensitivity, while showing increased inflammation.[104] In addition, prolonged GC excess, as seen in patients with Cushing's syndrome, correlates with cardiovascular complications. In animal studies however, GCs primarily demonstrate an athero-protective effect. For example administration of physiological levels of corticosterone in APOE*3-Leiden-CETP mice, which are atherosclerosis-prone and have human-like lipoprotein metabolism, resulted in significantly less atherosclerotic lesion development.[105] Furthermore, removal of endogenous GCs by adrenalectomy in LDLr$^{-/-}$ mice resulted in increased atherosclerotic plaque formation and this effects was reversed by adrenal transplantation.[106] However, when combined with high fat diet, GC excess promoted fat accumulation in the APOE*3-Leiden-CETP[106], while enhancing plasma lipid and insulin levels in C57BL/6 mice[107], which all have potential negative cardiovascular implications. A similar experiment in apoE$^{-/-}$ mice treated with low dose corticosterone combined with a Western-type diet resulted

in 77% larger aortic root lesions compared to control mice. Furthermore, this increase was accompanied by a significant reduction in white blood cell count and serum IL-1β concentration, but elevated levels of LDL and very low-density lipoprotein (VLDL), suggesting clear strain-specific differences.[108] Taken these animal studies together, exposure to chronic stress, either via a single stressor or by a combination of different stressors, results in enhanced development of atherosclerosis and in plaque destabilization as indicated by increased intraplaque inflammation and necrosis. These effects are generally caused by modulation of lipid homeostasis and an increased immune responses (Table 2c).

Table 2c: Rodent models of chronic stress-induced atherosclerosis development and progression

	Type of stressor (and duration)	Animal / Strain	Investigated site(s)	Effects / Measurements	Reference
CORT supplementation / adrenalectomy/ adrenal transplantation	Adrenalectomy and adrenal transplantation (4 wks)	Female LDLr$^{-/-}$ mice on Western-type diet (1.0% cholesterol)	Aortic root (cross sections)	Adrenalectomy: ↑ plaque size; ↓ plasma CORT; ↓ plasma cholesterol and triglycerides; ↓ plasma VLDL; ↑ white blood cell count; ↑ plasma MCP-1 No significant difference in bodyweight Adrenal transplantation restored (almost) all effects to levels observed in SHAM-operated mice	106.
	Corticosterone (50µg/mL) in drinking water for 5 or 17 wks	Female APOE*3-Leiden.CETP mice on Western-type diet (1.0% cholesterol)	Aortic root (cross sections)	↓ plaque area; ↓ plaque macrophage area; ↑ plasma CORT; ↓ adrenal gland weight; ↑ bodyweight; ↑ gonadal and subcutaneous white adipose tissue; ↑ adipose tissue CD68 and F4/80 and ↓ TNF-α expression; No significant difference in plasma TC; TG; LDL; HDL or M-CSF	105.
	Corticosterone in drinking water for 13 wks (intake approx. 8.4 - 3.8 mg.kg bodyweight/day during the experiment)	Male ApoE$^{-/-}$ mice on high-fat diet (21% cholesterol)	Aortic root (cross sections)	↑ plaque size; ↑ skeletal muscle atrophy; ↓ white blood cell count; ↓ serum IL-1β; ↑ Total cholesterol, LDL, VLDL, sd-LDL; ↑ white blood cell count; No significant difference in bodyweight; food intake; serum IL-6, CCL2; HDL, TG and chlyomicrons	107.

Acute stress and acute cardiovascular syndromes

Chronic stress exposure increases cardiovascular disease susceptibility and progression through maladaptation of the neuroendocrine pathways involved. In contrast, acute stress-induced disorders are generally caused by the sudden increase of the major stress mediators in the context of a vulnerable background. Well known are stress-induced exacerbations of dermatologic and respiratory diseases, in which stressors can be directly linked to changes in skin barrier function and exaggerated immune responses in the lung.[16,109] Following this line of thought, stress may contribute to cardiovascular morbidity and mortality through induction of acute cardiovascular events such as unstable angina, myocardial infarction, or sudden cardiac death. The key pathological events that causes most ACS are rupture of the thin fibrous cap or erosion of the intima of an advanced and vulnerable plaque, which then no longer shield the thrombogenic content of the plaque. Exposure to the blood's coagulation system can subsequently lead to the formation of an occlusive thrombus resulting in a myocardial infarction or stroke. The risk of an ACS thus depends on the combination of the vulnerability of existing atherosclerotic plaques, the coagulant status of the blood and plaque erosion or rupture.[110]

Both acute physical (surgery, trauma and extreme physical exertion) and

psychological (anger, fear, depression) stressors may be implicated in the incidence of acute cardiovascular events. Furthermore, a distinction can be made between population-based studies evaluating the effects of an acute stressor such as a terrorist attack or natural disaster on cardiovascular risk and studies evaluating individual stressors in patients who have had an ACS. By comparing the incidence of ACS after a disaster with a similar event free time-period, for example the week or year before, associations have been established between the risk of ACS and the stressed caused by earthquakes, war, terrorist attacks as well as sporting events.[111] For example, the threat of annihilation in the Israeli civilian population during the initial phase of the Gulf war seems to be responsible for the observed sharp rise in acute myocardial infarction and sudden cardiac death cases[112] and hospital records from New Jersey and Brooklyn demonstrated a significant increase in the proportion of various cardiac diagnosis directly after the 9/11 terrorist attacks.[37,38] Another population-based trigger which has been used to evaluate the correlation between acute stress and ACS are earthquakes. Analysis of all deaths after the 1994 earthquake in the Los Angeles area showed a significant increase in sudden deaths from cardiac causes of 24 on the day of the earthquake compared with an average of 4.6 per day in the week before. Similar results were obtained after the 1995 earthquake in the Kobe region of Japan[113] and more recently after the two earthquakes that struck Christchurch, New Zealand.[114] Acute psychological stressors at the individual level such as anger, fear and acute work-related stressors and their association with the incidence of ACS are best evaluated by case-crossover studies comparing the trigger period just before the onset of MI with the similar period the day before in the same individual. This method eliminates differences in the cardiovascular risk profiles between patients and controls and interpretation biases.[115] Using such as study design, Mittleman *et al*. interviewed over 1600 patients within 4 days after acute MI and evaluated the occurrence of anger within the 2 hour period before the onset of symptoms with the same period 24 hours before and with the frequency in which such episodes occurred within the previous year. The relative risk of acute MI in the 2 hours after an episode of anger was 2.3 (95% CI, 3.2-1.7).[116] A recent review and meta-analysis including additional cardiovascular events, such as ischemic stroke, ventricular arrhythmia, and ruptured intercranial aneurysm, demonstrated an increased risk for all those events in the hours after an anger outburst.[117] In addition to the previously discussed chronic stress experienced by patients with depression, acute negative emotions can also act as a sudden trigger of ACS. Both acute work-related stressors and acute depressed mood were shown to increase the relative risk of ACS onset.[118,119] Similar to the response to chronic stress, acute stress exposure has been demonstrated to result in a quick rise in plasma interleukins and inflammatory markers (Table 1[120-126]), such as IL-6, IL-1β and sICAM[127,128], which have all been demonstrated to be pro-atherogenic.

Furthermore, the rise in plasma IL-6 was seen to associate with fibrin formation and pro-coagulant markers[129], which may lead to an enhanced risk of thrombosis upon plaque rupture. Similarly, plasma CRP levels increase upon an acute mental stressor, and this response is even enlarged in patients with coronary artery disease.[130] Combined, the current data clearly indicate an important role for emotional triggering of cardiac events and warrants further research to gain mechanistic insight and into preventative measures.

Effect of acute stress on experimental cardiovascular diseases

Atherosclerotic lesions in LDLr$^{-/-}$ or apoE$^{-/-}$ mice have many of the characteristics of human advanced and rupture-prone plaques, however, they hardly ever result in an acute MI or stroke. To model acute MI in mice one has to revert to surgical models, such as (transient) ligation of the left anterior descending coronary artery (LAD) or the ex vivo Langerdorf perfusion model.[131] Mouse models of acute stress include single sessions of restraint, forced swim, shaking, electric foot shock or exposure to an (aggressive) intruder.[132] Up to date, animal studies on the effects of acute stress on the cardiovascular system have primarily focused on cardiac function and injury (Table 3). Acute stress-induced release of adrenalin and noradrenalin almost instantaneously affects heart rate and blood pressure resulting in changes in cardiac output and regional blood flow.[133] Furthermore, stress-induced cardiomyocyte cell death, resulting in reduced cardiac function, was shown in multiple animal models and elevated levels of cardiac enzymes (e.g. creatine kinase, aspartate aminotransferase and lactate dehydrogenase) can be readily detected in plasma after stress. Interestingly, administration of β-blockers completely protected against the leakage of these enzymes in a water-immersion stress model, strongly suggesting adrenergic pathways to be involved in cardiac cell death induced by stress.[134]

Table 3: Rodent models of acute stress-induced cardiovascular complications

Type of stressor (and duration)	Animal / Strain	Diet	Investigated tissue(s)	Effects / Measurements	Reference
Restraint stress (2h)	Male C57BL/6 mice	Food and water *ad libitum*	Blood	↑ heart rate; ↑ blood pressure; ↑ cardiac output; ↑ regional blood flow	133.
Water immersion stress (6h)	Male Sprague-Dawley rats	18hr food deprived before exp.	Blood	↑ plasma creatine phosphokinase, lactic dehydrogenase, glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase activity; ↑ plasma urea nitrogen and glucose levels; ↑ plasma adrenalin and noradrenalin levels	134.
Restraint stress (15-120 minutes)	C57BL/6 and ApoE$^{-/-}$ mice	Food and water *ad libitum*	Heart; Blood	Stress duration dependent ↑ plasma CORT; ↓ heart histamine level; ↑ serum histamine level; ↑ mast cell degranulation	135.
Restraint stress (15-120 minutes)	C57BL/6 and ApoE$^{-/-}$ mice	Food and water *ad libitum*	Blood	Stress duration dependent ↑ plasma CORT and serum IL-6 levels; Stress-induced IL-6 release partly inhibited by cromolyn and CRH receptor antagonist	136.
Myocardial infarction (permanent left coronary artery ligation) or Stroke (intraluminal middle cerebral artery occlusion)	ApoE$^{-/-}$ mice	High cholesterol diet and water *ad libitum*	Blood; bone marrow; aortic root	↑ plaque size; ↑ hematopoeitic system activity; ↑ plaque protease levels; ↑ intimal neutrophils, monocytes and macrophages; ↑ necrotic core area; ↓ firbous cap thickness; ↑ plaque monocyte inflammatory gene expression No significant difference in bodyweight; total cholesterol	137.

Atherosclerotic plaque disruption is thought be the key event precipitating acute MI and stroke. However, experimental data on the effects of acute stress on atherosclerotic lesion progression and destabilization is scarce and concentrates on immune modulation by the stress hormones and neuropeptides. A single bout of acute restraint stress in apoE$^{-/-}$ mice was demonstrated to increase cardiac histamine and IL-6 levels in a mast cell-dependent manner, which was suggested to be mediated by the stress hormones CRH and urocortin, as the peptide CRH receptor antagonist Astressin was shown to significantly inhibit the stress-induced IL-6 release.[135,136] Unfortunately, no additional analysis of atherosclerotic lesion composition after acute stress exposure was reported. Interestingly, Dutta et al. recently demonstrated the contribution of acute MI on atherosclerotic lesion progression and risk of re-infarction. They showed in mice that the anxiety and pain as a result of an acute MI activates the SNS, which subsequently triggers an innate immune response aimed at repairing the injury. However, the increase in production and release of hematopoietic stem and progenitor cells (HSPC) from the bone marrow and increased myelopoiesis in the spleen did not only aid in restoring the heart, but also fueled the inflammatory process in the atherosclerotic plaque, as demonstrated for example by increased inflammatory monocyte accumulation in the aorta.[137] Pretreatment with a β3-adrenoreceptor blocker significantly reduced the progenitor release and lesional immune cell influx after MI, possibly by affecting the expression of retention factors such as CXCL12, angiopoietin and stem cell factor. These results further stress the important role the brain-immune connection can play in atherosclerosis. In line with an increase in HSPC release after acute MI, exposure of apoE$^{-/-}$ mice to a chronic stress paradigm resulted in similarly elevated levels of circulating progenitor cells and increased inflammatory status of the atherosclerotic plaque.[138]

While distribution of immune cells over the different hematopoietic compartments is known to be crucial for effective immune surveillance and function, the redistributing effect stress can have on the immune system has been relatively underappreciated. Time-course studies in rats recently demonstrated the individual contribution of the principal stress hormones (corticosterone, epinephrine and norepinephrine) on acute redistribution of leukocytes. Early during stress mobilization of monocytes, neutrophils and lymphocytes from the spleen, bone marrow, lung and marginated leukocyte pool was observed resulting in a quick rise in numbers of these cells in the blood. During the second phase (>30 minutes) a drop in blood leukocyte numbers suggest trafficking of those cells to sites of inflammation, for instance the atherosclerotic plaque, or recirculation to spleen, bone marrow, lung or lymph nodes.[139] Interestingly, while all the major hormones act in concert to achieve efficient redistribution, epinephrine and norepinephrine were primarily responsible for the initial mobilization, while epinephrine and corticosterone mediated trafficking towards the target tissues, thus implying

different types of responses to different types of stressors. Taken together, the animal studies described here demonstrate that acute stress effectively activates the immune system, resulting in an enhanced inflammatory response, which can be directed towards the area of disease, such as an ischemic heart or the atherosclerotic plaque.

Therapeutic modulation of psychosocial risk factors in patients with CVD

Despite increased public awareness of its risk factors and improvements in healthcare and treatment options, cardiovascular disease and its clinical complications remain a leading cause of death worldwide. In addition to traditional risk factors, such as dyslipidemia, inflammation, smoking, diabetes and high blood pressure, psychological stress is receiving increased recognition as both a clinically important contributor to cardiovascular morbidity and mortality and as a therapeutic target.

A large body of clinical and experimental research demonstrates correlations between chronic stress exposure during early life as well as adulthood and cardiovascular disease risk. Together, a number of human association studies have firmly established that patients with psychological disorders or people subjected to chronic stress are at risk for the development of CVD. Experimental studies have contributed to the elucidation of the underlying mechanisms. The experimental studies generally have established that chronic stress enhances progression of atherosclerosis predominantly by negatively affecting lipid and immune homeostasis, and by increasing inflammatory mediators, such as CRP and IL-6, phenomena that are also seen in patients with psychosocial disorders and are associated with the development of atherosclerosis (Table 1). Maladaptation, due to the chronic activation of the immune system, results in a low-grade inflammatory response which fuels the development and progression of the atherosclerotic plaque, and thus increases the risk of acute cardiovascular syndromes. Also acute stress may contributes to the incidence of acute cardiovascular disorders, as experimental studies have established that acute stress results in direct effects on heart rate and blood pressure, as well as on circulating inflammatory markers and cardiac cell death. These effects can act in concert to induce adverse cardiac events in patients with established atherosclerosis. Together, data from these clinical and experimental studies have established a clear connection between stress exposure and CVD, but also provide potential therapeutic options to prevent acute cardiovascular events. The emerging field of behavioral cardiology tries to address the expanding number of psychosocial risk factors in clinical practice. Clinical management of negative emotions, chronic stress and social dysfunction by psychotherapy and psychological interventions all aim to promote healthy behavior and improve psychosocial functioning.[140] Accumulating evidence shows that the positive counterparts (e.g. optimism and strong social integration) of

risk factors such as pessimism, depression and social isolation correlate with an array of cardiovascular benefits, including reduced heart failure.[141,142] Changing behavior or emotional status of patients suffering from psychological disorders often requires pharmacologic treatment options. As such, treatment of depression during cardiac rehabilitation has been assessed in multiple trials, including the SADHART (Sertraline AntiDepressant Heart Attack Trial), ENRICHD (Enhancing Recovery in Coronary Heart Disease) and MIND-IT (Myocardial Infarction and Depression Intervention Trial) trials. In these randomized trials, although the depression was adequately treated, limited to no beneficial effect on cardiovascular disease morbidity or mortality was observed.[143] This apparent discrepancy might be caused by the often large standard deviation in psychological parameters, necessitating huge samples sizes to show correlations with "hard" cardiovascular end points (e.g. MI or stroke). Also, such trials are generally set up to determine the safety of the use of specific drugs in patients with cardiovascular disorders, who are at risk of depression, and in that sense the number of patients included is too limited to be able to draw any conclusions regarding their increased risk of having an acute cardiovascular event. Furthermore, dose response relationships in behavioral research are difficult to determine as therapy frequency and duration to achieve a certain psychological improved vary much between patients.[143] More information on cardiovascular risk after treatment with e.g. antidepressants may be gathered from larger clinical trials including patients suffering from psychological disorders, but without prior cardiovascular issues. Future research should also be aimed at early diagnosis of both cardiovascular disease and chronic psychological conditions in individual patients. In light of the reciprocal interactions between brain-initiated and peripheral mechanism, interventions in psychological or cardiovascular disease, or especially both combined, could thus have a major impact on cardiovascular health.

Conclusions

In conclusion, acute cardiovascular syndromes and its underlying disease atherosclerosis remain a leading cause of death and identification and understanding of all its risk factors is key in combating this disease. Psychological stress is receiving increased attention as a substantial but also modifiable risk factor for various diseases, including cardiovascular disease, however the exact mechanism underlying these effects have not been completely elucidated yet. Here, we reviewed current literature on both clinical and experimental data on the association between acute and chronic stress exposure and the incidence of CHD and myocardial infarction as well as biological pathways affecting stress-induced atherosclerosis development and progression. Increased insight in the causal processes will improve patient care and have implications beyond cardiovascular disease.

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Chapter 3

Stress-induced mast cell activation contributes to atherosclerotic plaque destabilization

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Abstract

Objective: Mast cells accumulate in the perivascular tissue during atherosclerotic plaque progression and contribute to plaque development and destabilization. However, the specific triggers for mast cell activation in atherosclerosis remain unresolved. We hypothesized that psychological stress-induced activation of mast cells may contribute to plaque destabilization.

Methods and Results: To investigate this, apoE$^{-/-}$ mice on Western-type diet were exposed to 120' restraint stress with or without treatment with the mast cell stabilizer cromolyn. A single episode of restraint caused a significant increase in mast cell activation in the heart. In addition to a rise in serum corticosterone and changes in circulating leukocyte populations, we observed a transient increase in the circulating pro-inflammatory cytokine interleukin-6 in the stressed mice. Subsequent characterization of the atherosclerotic plaques revealed a significant reduction in lesion collagen content and a higher incidence and larger size of intraplaque hemorrhages in stressed versus non-stressed mice. These effects were partially inhibited by cromolyn treatment and completely absent in mast cell-deficient mice, strongly indicating the involvement of a mast cell-dependent response to stress in atherosclerotic plaque destabilization.

Conclusions: We demonstrate that acute stress activates mast cells, which contributes to plaque destabilization in vivo, identifying acute stress as a risk factor for atherosclerotic plaque destabilization.

Introduction

Acute coronary syndromes (ACS), such as acute myocardial infarction, stroke and sudden cardiac death remain principle causes of death worldwide.[1,2] These events often occur in patients with severe (coronary) atherosclerosis and the underlying pathology is generally rupture or erosion of such advanced atherosclerotic lesions accompanied with thrombosis.[3] Besides dyslipidemia, vascular inflammation is a driving force behind atherosclerosis development and progression[4] and modulation of the immune system has been shown to contribute to the course of the disease. In addition to traditional risk factors for atherosclerosis such as dyslipidemia, hypertension, diabetes, obesity and genetic predisposition, (psychological) stress is receiving increased attention as both a contributing factor to and consequence of various diseases, including cardiovascular disease.[5] Physical and psychological stress, acting via activation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system, results in the systemic and local release of multiple hormones and neuropeptides and has been demonstrated to exhibit potent immunomodulatory effects. Although more established for its role in exacerbating allergic responses, there is accumulating evidence for a pathological role of stress in cardiovascular disease. For instance, results of the INTERHEART study (a case-control study with >24000 participants) indicate that the presence of psychosocial stressors greatly increase the risk of acute myocardial infarction in men[6] and a recent meta-analysis of prospective cohort studies evaluating job-strain and cardiovascular disease showed an increased hazard ratio for coronary heart disease of 1.2-1.3 in participants reporting job strain.[7] However, to study the cardiovascular implications of stress exposure in humans is complex due to strong differences in individual perception of stress. Furthermore, the direct effects of acute stress as a precipitating factor for ACS are difficult to establish, due to reverse causation bias. Thus, to gain mechanistic insight into the molecular pathways involved in stress-induced cardiovascular complications remains challenging.

Extensive research into the characteristics and morphology of the vulnerable plaque, which is prone to disruption and give rise to clinical complications, has demonstrated that especially the highly inflamed shoulder regions are common sites of rupture.[8] Previously, we and others have established a key role for an innate immune cell type, the mast cell, in atherosclerotic disease progression and especially in the subsequent destabilization of advanced atherosclerotic plaques.[8,9,10] In animal models of atherosclerosis, both systemic and local activation of (peri) vascular mast cells resulted in increased lesion progression, and importantly also decreased lesion stability. Mast cells mediate these effect via the release of pro-inflammatory cytokines, extracellular matrix degrading proteases and microvessel inducing growth factors.[9,10] Furthermore, these experimental animal data are in line with recent human immunohistochemical data, correlating perivascular mast

cell numbers and activation status with disease progression and acute coronary events.[11]

As mast cells are tissue-resident immune cells and shown to accumulate near atherosclerotic lesions, these cells are uniquely located to respond to acute (perivascular) triggers and subsequently release their pro-atherogenic content. Despite extensive knowledge on mast cell biology and activation routes, the specific mast cell triggers in relation to ACS remain unresolved. Being in close proximity to perivascular neurons[12] and expressing different types of neuropeptide and hormone receptors, this neuron-mast cell connection is likely to play an exacerbating role in cardiovascular diseases. Previously, plasma histamine and IL-6 levels were seen to be increased in a mouse model of acute physical and psychological stress[13], suggesting that mast cells are indeed activated after the induction of acute stress.

Combined, the epidemiological and experimental data strongly indicate a pro-atherogenic role for stress-induced mast cell activation. However, the direct implications for atherosclerotic lesion progression and composition have not been established. In this study we evaluated mast cell-dependent effects of acute stress on atherosclerotic lesion stability by applying a restraint stress model in apoE$^{-/-}$ and mast cell deficient RMB-apoE$^{-/-}$ mice.[14]

Material and Methods

Animals

All animal work was approved by the Leiden University Animal Ethics committee and performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. 10-12 weeks old male apoE$^{-/-}$ mice were obtained from the local animal breeding facility. The inducible mast cell deficient red mast cell and basophil (RMB) mice were obtained from the lab of Prof. Pierre Launay[14] and were backcrossed with apoE$^{-/-}$ mice to obtain RMB-apoE$^{-/-}$ mice.

Restraint-stress model

All stress experiments were performed between 9am and noon, a period in which individual differences in corticosterone levels in mice are relatively small.[31] 24 male apoE$^{-/-}$ mice were subjected to restrained stress by immobilization in a well-ventilated 50 mL Corning tube for 30', 60', 90' or 120' (n=6) while control mice were left undisturbed in their home cage. Directly after the indicated stress-time the mice were terminally anaesthetized and sacrificed. Total cell count, neutrophils, monocyte, lymphocyte, and eosinophil counts in blood were analyzed using an automated XT-2000iV veterinary hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany). Serum IL-6, (BD Biosciences) levels were determined by

ELISA according to the manufacturer's protocol. Heartswere excised and briefly fixated in 3.7% neutral-buffered formalin (Formal-Fixx; Shandon Scientific Ltd, UK) before further processing.

Corticosterone analysis

Blood samples for basal corticosterone analysis were drawn by tailcut between 9:00 and 12:00 AM. Levels of corticosterone were determined using a 125-I radioimmuno assay (RIA) with a lower detection limit of 5 ng/ml, according to the manufacturer's specifications (MP Biomedicals, Illkirch-Graffenstaden, France).

B-hexosaminidase assay

To measure β-hexosaminidase levels 50 µL of serum was added to 50 µL 2 mM 4-nitrophenyl N-acetyl-b-D-glucosaminide (Sigma, Germany) in 0.2 M citrate (pH 4.5) in a 96-well plate (Greiner Bio-One, The Netherlands) and incubated at 37 °C for 2 hours. After addition of 150 µL 1 M Tris (pH 9.0), absorbance was measured at 405 nm.

Atherosclerosis

Atherosclerotic lesion formation was induced by feeding male apoE mice (n=39) a Western-type diet (0.25% cholesterol and 15% cocoa butter; Special Diet Services, Witham, Essex, UK) for 6 weeks. A schematic representation of the experimental setup is depicted in Supplemental figure 1A. Blood samples were taken at the start of the experiment, at 2 and 5 weeks of WTD feeding by tail-cut and plasma was obtained by centrifugation at 8,000 rpm for 10 min. Levels of total plasma cholesterol were measured spectrophotometrically using enzymatic procedures (Roche Diagnostics, Almere, the Netherlands). At 6 weeks mice were randomly assigned to either the undisturbed control, 120' restraint stress or 120' restraint stress + cromolyn group (n=13 per group) and subjected to the stress protocol. Cromolyn-treated mice were injected 30' prior to the stressor and once daily over the next three days with the mast cell stabilizer cromolyn (50 mg/kg, i.p). Three days after stress all mice were terminally anaesthetized and sacrificed. Blood was processed for fluorescence flow cytometry (Sysmex) or ELISA/RIA as described above. Heart, skin and lung tissue were fixated in 3.7% neutral-buffered formalin (Formal-fixx; Shandon Scientific Ltd, UK) before further processing. A similar experimental setting was used to evaluate the pro-atherogenic effects of stress in mast cell deficient RMB-apoE$^{-/-}$mice (supplemental figure 2A). Three intraperitoneal injections of 1 µg of diphtheria toxin (#322-326, Calbiochem, CA, USA) in these mice resulted in complete eradication of all mast cells, including in the aortic root (supplemental figure 2B).

Plaque morphometry and immunohistochemistry

Harvested hearts were fixated in 3.7% neutral-buffered formalin solution (Formal-fixx; Shandon Scientific Ltd, UK) and embedded in O.C.T compound (Tissue-Tek, Sakura Finetek, CA, USA). Once the aortic root was identified by the appearance of aortic valve leaflets, transverse 10-µm sections were prepared on a Leica CM 3050S Cryostat (Leica Instruments, Nassloch, Germany) and mounted on gelatin-coated slides. Mean lesion area (in µm2) was calculated from six Oil-Red-O stained sections in distal direction starting at the point where all three aortic valve leaflets first appeared. Leica Qwin image analysis software was used for morphometric analysis of the atherosclerotic burden.

Mast cells were visualized by chloroacetate esterase staining according to manufacturer's protocol (CAE, Sigma, Germany) and degranulation status was assessed manually by bright-field microscopy as described previously[10]. A mast cell was considered resting when all granulae were maintained inside the cell, while mast cells were assessed as activated when granulae were deposited in the tissue surrounding the mast cell.

In addition, immunohistochemical stainings were performed to assess plaque composition and stability. Sections were stained for collagen (picrosirius red) and macrophages (MOMA-2) as described previously[32]. Necrotic core area was defined as the a-cellular, debris-rich plaque area and represented as percentage of total plaque area. Apoptosis was visualized using a terminal dUTP nick-end labelling (TUNEL) kit (Roche Diagnostics). As another measure of lesions destabilization the amount and surface area of intraplaque hemorrhages (IPH) were determined as described before.[15] Presence of masses of intimal erythrocytes, free in the plaque matrix or filling the necrotic core, was classified as intraplaque hemorrhage.[18] For detection of intraplaque hemorrhage, we analyzed all hematoxylin & eosin-stained sections (at 50 µM intervals) for each mouse having atherosclerotic plaques. Furthermore, erythrocytes were visualized by fluorescence microscopy (erythrocyte autofluorescence at 560 nm emission wavelength). All morphometric analyses were performed by blinded independent operators (IB and HML).

Statistical analysis

Data are expressed as mean ± SEM. A two-tailed student T-test was used to compare normally distributed data between individual groups. For comparison of three or more different groups, data were analyzed with a one-way ANOVA followed by Tukey's multiple comparisons test. Frequency data analysis was performed by means of the Fisher's exact test. Probability values of $P<0.05$ were considered significant.

Results

Restraint stress dose-dependently activates cardiac mast cells in male apoE$^{-/-}$ mice

To evaluate the effect of acute physical and psychological stress on cardiac and vascular mast cell activation we subjected male apoE$^{-/-}$ mice to a restraint stress protocol. A time course experiment (n=6 per timepoint) was performed to determine the optimal stress time resulting in mast cell activation. As depicted in figure 1A, restraint stress induced a quick and strong increase in circulation glucocorticoid levels (169.4 ± 50.9 ng/ml in unstressed vs 332.3 ± 35.7 ng/ml in 60' stressed mice, P<0.05), indicating HPA-axis activation. Next, we assessed cardiac mast cell numbers and activation status by immunohistochemical staining of heart cross-sections at the level of the aortic root. While absolute numbers of cardiac mast cells were similar in all groups, the percentage of activated mast cells, scored by the presence of granules deposited outside the mast cell (figure 1B), was significantly increased upon stress exposure (37.3 ± 1.8 % unstressed vs 50.7 ± 5.0% in 120' stressed mice, figure 1C, P<0.05). Furthermore, the length of stress exposure (and thus severity) seemed to correlate with the degree of mast cell activation. Serum levels of the mast cell-granule derived mediator β-hexosaminidase significantly correlated with the percentage of activated cardiac mast cells (figure 1D). As apoE$^{-/-}$ mice subjected to 2 hours of restraint stress showed the most prominent increase in cardiac mast cell activation we used this experimental setup for subsequent experiments.

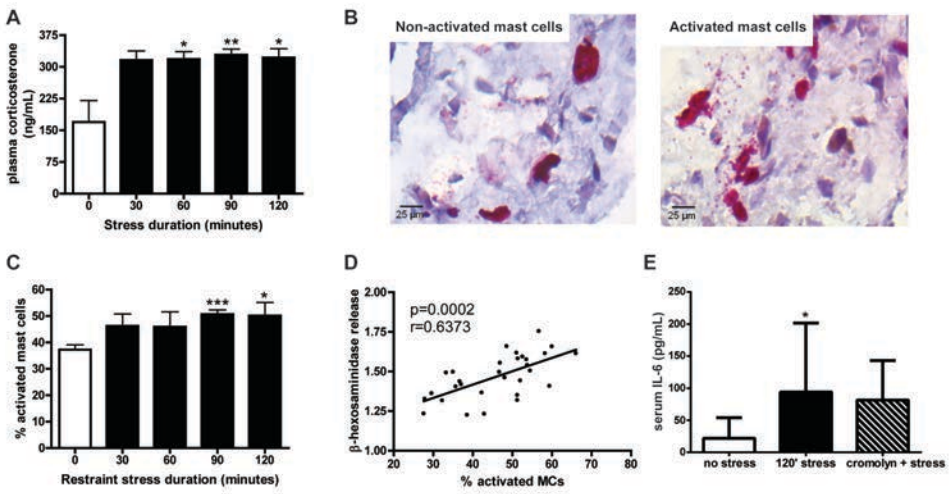


Figure 1. Restraint stress time course in apoE$^{-/-}$ mice. A) Restraint stress rapidly and significantly increased plasma corticosterone levels. B) Representative pictures of resting (non-activated) and degranulating (activated) cardiac mast cells. C) Restraint stress severity-dependently increased cardiac mast cell activation status. D) Percentage of activated cardiac mast cells significantly correlated with serum β-hexosaminidase level. E) 120' restraint stress significantly increased serum IL-6 levels. *P<0.05, **P<0.01, ***P<0.001

To further investigate the effect of mast cell-specific responses to acute stress exposure on circulating cells and blood cytokine levels an additional pilot experiment was performed. Again the control mice were left undisturbed, while treated mice were subjected to 120' restraint stress. A subset of the stressed mice was pre-treated with the mast cell stabilizer cromolyn. As before, acute stress resulted in a strong increase in serum corticosterone levels, indicating stress responsiveness. Furthermore, we observed a significant decrease in blood monocyte and lymphocyte numbers, while the amount of circulating neutrophils was not affected by stress. Therefore the relative leukocyte composition in blood shifted to an increased percentage of neutrophils and a reduction in lymphocytes and monocytes. Pre-treatment with cromolyn did not significantly affect the blood leukocyte distribution after stress. (supplemental figure 3). Also, the overall inflammatory status of the mice tended to be increased by acute stress exposure, as significantly higher serum IL-6 levels were observed in the stressed mice. (21.9 ± 8.0 pg/mL in unstressed versus 94.1 ± 26.9 pg/mL in 120' stressed mice, P=0.015) (figure 1E).

Acute stress activates cardiac mast cells in close proximity to atherosclerotic plaques

Having established that mast cells are indeed activated after a single exposure to an acute stressor, we next investigated the effects of acute stress-induced mast cell activation in an atherosclerotic setting. Mice were sacrificed three days after exposure to the stressor, a time point after which mast cell activation has previously been shown to have the most prominent effects on the composition of the atherosclerotic lesion[15] (supplemental figure 1A).

In line with our pilot experiment results, 120' restraint produced a strong stress response, indicated by a transient rise in glucocorticoid levels (supplemental figure 1B). As expected three days after a single restraint stress episode, lesion size in the aortic root was similar between all three treatment groups (figure 2A). At this time-point, we did not observe a significant increase in the percentage of activated cardiac mast cells in the stressed mice (figure 2B).

Decreased lesional collagen content and increased incidence of intraplaque hemorrhages.

As increasing evidence associates both stress and mast cell activation with cardiovascular complications such as cardiac ischemia and MI[16], we analyzed the degree of lesion stability in our animal model. Important features of the vulnerable and rupture prone lesion are a decrease in relative collagen content and the presence of large necrotic cores.[3,17,18] We did not observe significant differences in apoptotic cell numbers or relative necrotic core area between stressed and unstressed mice. However, collagen content, expressed as percentage of total

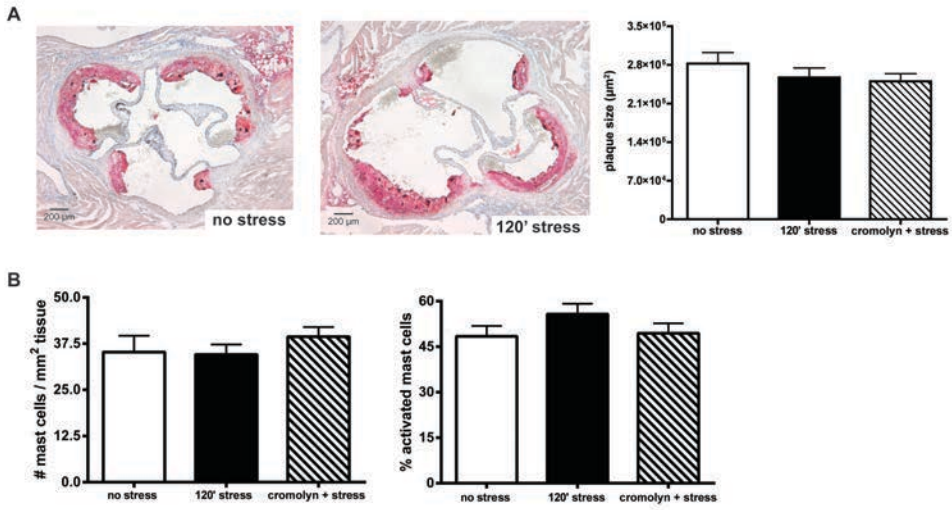
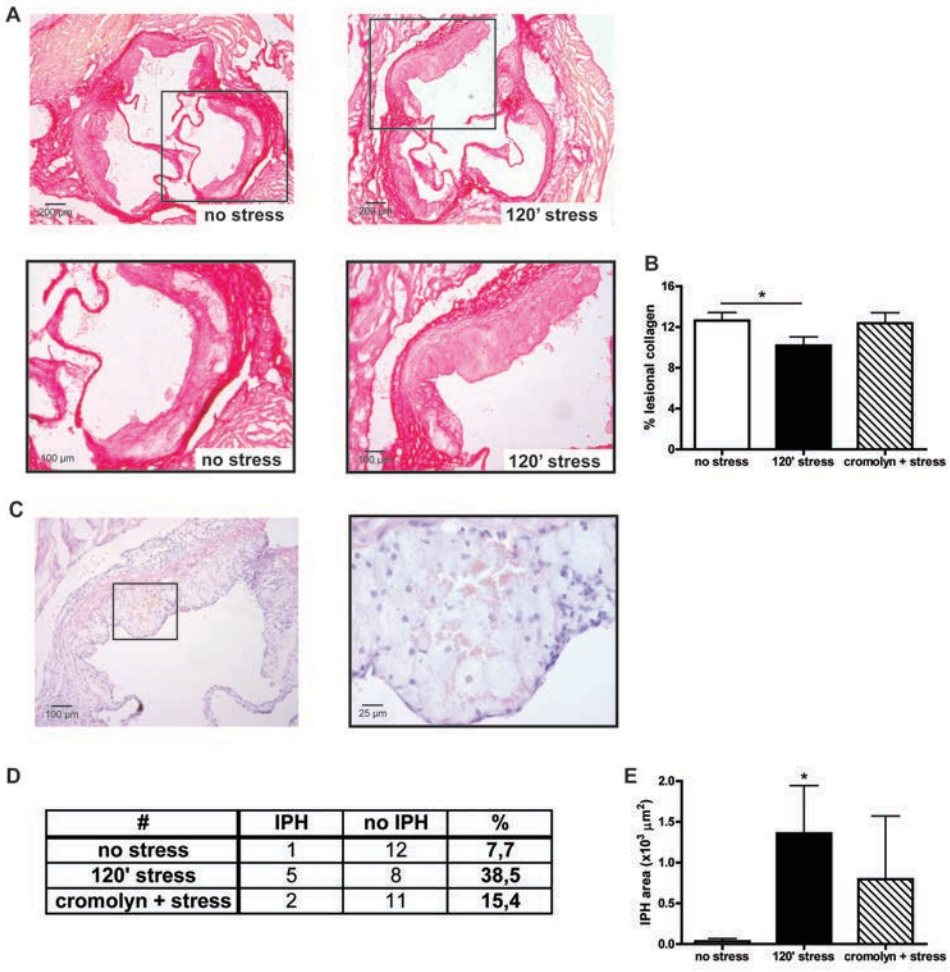


Figure 2. A) Atherosclerotic plaque size in the aortic root of apoE$^{-/-}$ mice was not significantly different between the treatment groups. B) Cardiac mast cell number and activation status three days after stress exposure.

plaque area, was significantly reduced in aortic root lesions of the stressed mice (figure 3A; B). Furthermore, this decrease was not observed in the cromolyn treated mice, supporting an, at least partial, mast cell-dependent effect on lesion stability. Another hallmark of rupture-prone lesions is the presence of intraplaque hemorrhages (IPH).[19] In 5 out of 13 mice in the stress group these IPH, characterized by the presence of intimal erythrocytes, were observed (figure 3D). In contrast, only one IPH was detected in the unstressed mice versus two in the stressed cromolyn-treated mice. In addition, when we quantified the relative area of the intimal erythrocytes, the IPH in the stressed mice were significant larger compared to control mice. These results further strengthen the previous observations that exposure to acute stress leads to a more vulnerable plaque phenotype.

Lack of intraplaque hemorrhages in stressed mast cell deficient mice.

To firmly establish the contribution of mast cells to the stress-dependent effects on plaque stability, we next evaluated the effects of acute stress on plaque stability in a mast cell deficient mouse model. As we depleted mast cells only just prior to the induction of acute stress, the number of mast cells and thus its contribution to atherosclerotic lesion development was completely comparable between the apoE$^{-/-}$ mice and the RMB mice up to the moment of mast cell depletion at 5 weeks of lesion development. This is an advantage compared to often used constitutive mast cell-deficient mice models (e.g. Kit(W^{-sh}/W^{-sh}) mice), in which mast cells are absent during lesion development and thus affect plaque stability already prior

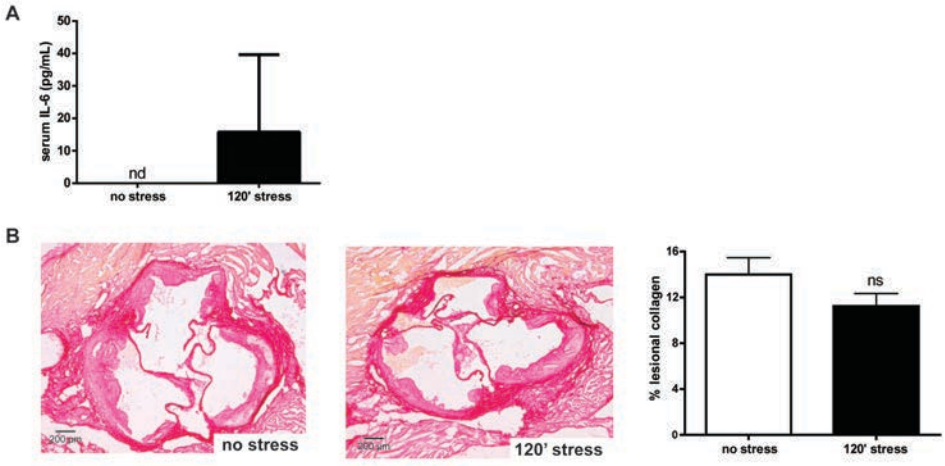


#	IPH	no IPH	%
no stress	1	12	7,7
120' stress	5	8	38,5
cromolyn + stress	2	11	15,4

Figure 3. A) Representative pictures of picrosirius red stained aortic root sections. Less dense (collagen) staining was observed in the stressed hearts compared to control. The 20x magnification insert demonstrates limited collagen and a thin fibrous cap in a plaque of a stressed mouse. B) Quantification of the lesional collagen content showed a significant reduction in the stressed mice compared to non-stressed controls. This effect was not observed in cromolyn treated stressed mice. C) Representative pictures of an intraplaque hemorrhage (IPH), displaying erythrocytes inside the atherosclerotic lesion. D) Amount and relative percentage of intraplaque hemorrhages in each treatment group. E) Quantification of the IPH area demonstrated significantly larger bleedings in the stressed mice compared to the non-stressed controls.

to the moment of stress. Interestingly, plasma IL-6 was not detectable in non-stressed RMB-apoE$^{-/-}$ mice, and only in low amounts upon stress induction (figure 4A), suggesting that the rise in plasma IL-6 upon acute stress is largely mast cell derived. The stress-response itself, based on its effects on blood composition of the different leukocyte subpopulations, was similar as compared to the apoE$^{-/-}$ mice (supplemental figure 4).

No significant differences in plaque size were observed between the stressed and non-stressed RMB-apoE$^{-/-}$mice, while we also did not observe a significant difference in collagen content of the plaques (figure 4B). Strikingly, no intraplaque hemorrhages could be detected in any of the aortic root plaques of either the non-stressed or stressed mice, suggesting that stress-induced plaque destabilization is, at least partly, mediated by a mast cell-dependent response (figure 4C).



C

#	IPH	no IPH	%
no stress	0	12	0,0
120' stress	0	11	0,0

Figure 4. A) Circulating IL-6 in non-stressed and stressed mast cell deficient RMB-apoE$^{-/-}$ mice. B) Representative pictures and quantification of the lesional collagen content demonstrating no significant difference between the stressed and non-stressed mast cell deficient RMB-apoE$^{-/-}$ mice. C) Amount and relative percentage of intraplaque hemorrhages in both treatment groups.

Discussion

In the current study we show that restraint stress, a mouse model for acute physical and psychological stress, activates cardiac mast cells which resulted in a more unstable atherosclerotic plaque phenotype. Acute cardiovascular syndromes (ACS) such as myocardial infarction and stroke remain leading causes of death and warrant further research into the physiological and molecular mechanisms contributing to these complications of the underlying vascular disease, atherosclerosis. Despite much effort and increased insight into the development and morphology of the rupture prone atherosclerotic plaque, the triggers of acute cardiovascular events remain incompletely understood. Plaque inflammation and degradation of the plaque's fibrous cap are clear prerequisites for plaque rupture and identifying risk factors fueling the inflammatory response is key in preventing ACS.[20]

The stress response and its main mediators (glucocorticoids, adrenalin and noradrenalin) are among the most potent immune modulatory agents known to men. Considerable evidence exist for a detrimental and bi-directional role for psychological stress in allergies, asthma and skin diseases.[21,22] However, such implications for atherosclerosis development and ACS are less well understood.

In line with previous results showing cardiac mast cell degranulation and histamine secretion upon acute stress exposure[23], we demonstrate that exposure of apoE$^{-/-}$ mice to a single episode of restraint stress (up to 120 minutes in duration) significantly increased cardiac mast cell activation in a stress-severity dependent manner. Furthermore, acute stress induced a significant increase in serum IL-6 levels, which was dampened in mast cell deficient mice. While such a mast cell dependent pro-inflammatory response has been described previously[13], it's implication for atherosclerotic lesion progression was not addressed. In the current study, we applied this restraint stress protocol to 6-week WTD-fed male apoE$^{-/-}$ mice, which have already established atherosclerotic lesions in the aortic root. Analysis of the plaques three days after stress exposure revealed a significant reduction in lesional collagen content and an increase in the amount of intraplaque hermorrhages in the stressed animals compared to non-stressed controls, indicative of an unstable plaque phenotype. Importantly, these stress-induced effects were reduced in cromolyn-treated mice and abolished in the mast cell deficient RMB-apoE$^{-/-}$ mice.

The immunomodulatory effects of acute and chronic stress are often considered contradictory with the acute stress response resulting in immunoprotection (e,g, increased bacterial and parasitic resistance, efficient wound healing and vaccine-induced memory) and chronic stress resulting in immunopathology (e.g. general immunosupression followed by a maladaptive stress and inflammatory state).[24] As such, stress research in relation to cardiovascular disease has focused primarily on the detrimental effects of chronic stress. In humans, significant correlations have been found between chronic stress induced by job strain or effort-reward imbalance and the incidence of coronary heart disease.[7] A number of chronic stress models in mice have shown the proatherogenic effects of chronic stress to be mediated via both the induction of chronic inflammation (IL-6 and CXCL1)[25] and by effecting lipid homeostasis.[26] Here, exposure to acute stress induced a, possibly mast cell dependent, inflammatory IL-6 response, while not affecting plasma lipid levels.

Interestingly, the potential benefit or harm of acute stress-induced enhanced immune function also seems to depend largely on the effects on leukocyte distribution and the timing between stress exposure and immune activation.[27] The atherosclerotic plaque represents a continuous site of inflammation in the vessel wall and stress-induced local immune cell activation is thus likely to aggravate the disease. Mast cells are uniquely located in the perivascular tissue and shoulder

regions of the atherosclerotic plaque and are, in concert with other (recruited) inflammatory cells capable of inducing lesion progression and destabilization[28]. Previous work from our group and others has demonstrated that mast cell-derived mediators such as chemokines, specific proteases (chymase and tryptase) and cytokines (IL-6, TNFα) enhance macrophage infiltration, vascular smooth muscle cell apoptosis[29], collagen degradation[30] and overall inflammatory status of the plaque[9], thereby contributing to a more rupture-prone phenotype. Interestingly, the presence and activation status of mast cells in carotid endarterectomy specimens was recently shown to associate with the incidence of future adverse cardiovascular events.[11] Furthermore, a recent literature review indeed showed that stress can precipitate acute coronary syndromes and demonstrated an important role for coronary mast cell activation through the stress hormone corticotropin-releasing hormone as well as other neuropeptides.[16]

In conclusion, we here demonstrate that a single episode of restraint stress, is sufficient to significantly activate cardiac mast cells, resulting in an increase in serum IL-6 and β-hexosaminidase levels. Such acute stress-induced mast cell activation in mice with established atherosclerotic lesions significantly affected plaque stability by reducing plaque collagen content and by inducing intraplaque hemorrhages. Combined these results provide further evidence for the important role of mast cells in modulating atherosclerotic plaque vulnerability and highlight the acute stress response as a risk factor and therapeutic target.

Acknowledgements

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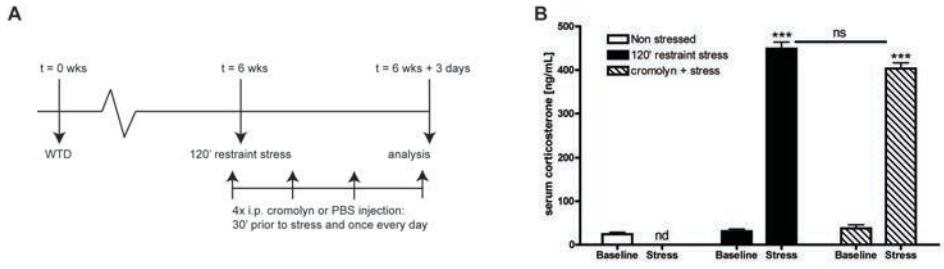
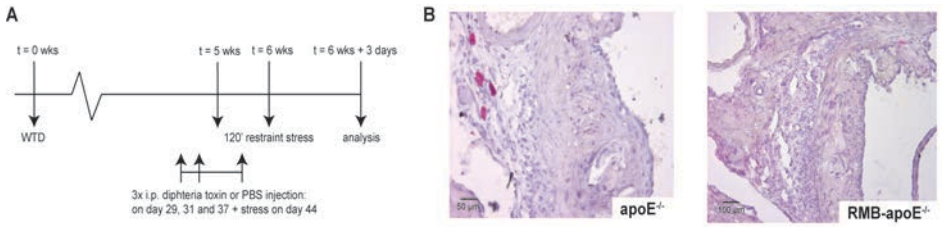
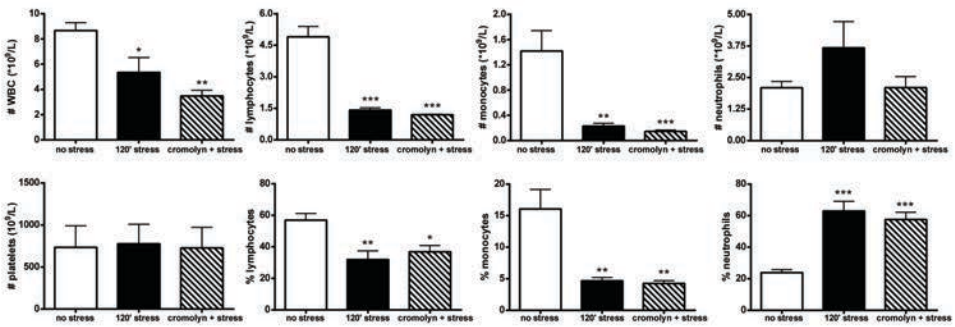
Supplemental data



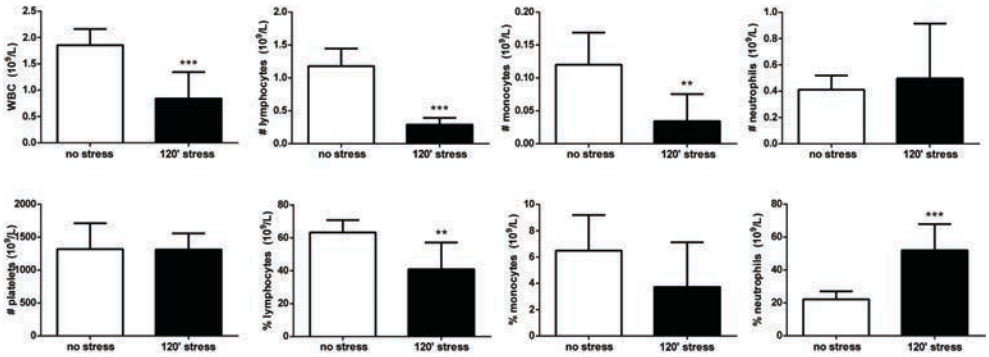
Supplemental figure 1. A) Schematic overview of the experimental setup to evaluate the mast cell contribution to acute stress-induced cardiovascular complications. B) Stress-induced increase in circulating corticosterone level was not affected by cromolyn pre-treatment.



Supplemental figure 2. A) Schematic overview of the experimental setup to evaluate the pro-atherogenic stress response in mast cell deficient RMB-apoE$^{-/-}$ mice. B) Cardiac mast cell staining of apoE$^{-/-}$ and DT-treated RMB-apoE$^{-/-}$ mice, confirming the absence of cardiac mast cells in the inducible knockout model.



Supplemental figure 3. Automated differential cell count analysis of blood from non-stressed, 120' stressed or 120' stressed mice pretreated with cromolyn apoE$^{-/-}$ mice, showed a significant decrease in circulating lymphocytes and monocytes directly after stress exposure suggesting glucocorticoid induced cell death and/or recruitment to sites of inflammation. Circulating neutrophil numbers were slightly increased in a mast cell dependent manner. *P<0.05, **P<0.01, ***P<0.001



Supplemental figure 4. Differential cell count analysis of blood from non-stressed and 120' stressed mice mast cell deficient RMB-apoE$^{-/-}$ mice, demonstrated a similar stress-response compared to the response observed in apoE$^{-/-}$ mice. Significant decreases in circulating lymphocytes and monocytes directly after stress exposure and non-affected neutrophil numbers were observed. *P<0.05, **P<0.01, ***P<0.001

Chapter 4

Acute stress exposure induces a transient decrease in bleeding time without affecting thrombus formation

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Manuscript in preparation

Abstract

Objective: Atherothrombosis is the major cause of myocardial infarction and stroke. Rupture or erosion of an advanced atherosclerotic lesion exposes the thrombogenic content of the plaque and initiates platelet activation and blood coagulation, which can result in a vessel occluding thrombus. Psychological stress has been increasingly implicated as a risk factor for atherosclerosis and atherothrombotic complications. Here we assessed the effects of acute stress exposure on blood composition, coagulation and thrombus formation.

Methods and Results; 120' restraint stress in apoE$^{-/-}$ mice resulted in a transient but significant reduction in circulating leukocytes and a shift in the relative amount of the different leukocyte subpopulations, without affecting platelet and red blood cell counts or haematocrit values. Tail bleeding time was significantly increased directly after stress and clot retraction was impaired for up to 24 hours after a single acute stressor. Acute stress did however not result in platelet activation or have a significant effect on $FeCl_3$-induced thrombus formation. In addition, we analysed the contribution of mast cell-derived pro-inflammatory and pro-thrombotic mediators herein by means of mast cell deficient (RMB-apoE$^{-/-}$) mice. A significant proportion of the observed stress-induced increase in circulating IL-6 could be attributed to mast cell dependent responses and the stress-induced increase in tail bleeding time was not observed in the mast cell deficient mice. However clot retraction was similarly affected by stress exposure in the mast cell deficient mice compared to controls. Interestingly, while acute stress did not contribute to thrombus formation in a $FeCl_3$-induced thrombosis model, mast cell deficiency by itself prolonged the time to occlusion.

Conclusion: Combined, our results demonstrate that acute stress exposure causes a transient increase in circulating inflammatory markers and a redistribution of blood leukocyte subpopulations, in part via the activation of mast cells. However, no direct contributing role for the acute stress response to thrombus formation could be observed in the current experimental setup, indicating additional factors to be involved in stress induced atherothrombic complications.

Introduction

Atherothrombosis is defined as the process in which an advanced and unstable atherosclerotic lesion ruptures and gives rise to a vessel-occluding thrombus. Such atherothrombotic complications, resulting in a myocardial infarction or a stroke, are a leading cause of death worldwide. Like atherosclerotic lesion development itself, the pathophysiological pathways and mechanisms involved in atherothrombosis are complex and multifactorial. In addition to procoagulant and thrombogenic factors such as fibrinogen and tissue factor (TF) released from the ruptured atherosclerotic plaque, blood composition and blood-borne mediators play a pivotal role in this disease process and influence the magnitude and stability of the resulting thrombus.[1]

Atherosclerosis is a lipid-driven chronic inflammatory disease and vascular inflammation is intimately linked to the risk of arterial thrombosis.[2] As elegantly reviewed by Engelmann and Massberg, thrombosis may be considered the pathological deviation of hemostasis and involves two main components, platelets and the coagulation system.[3] In addition, there is a significant bi-directional interaction with cells of the innate immune system, including neutrophils, monocytes and mast cells which promote coagulation and the formation of a stable clot or thrombus. Previously we and others have demonstrated the importance of arterial mast cells in atherosclerotic lesion formation and plaque destabilization.[4,5] Pro-inflammatory and matrix modifying cytokines and proteases released from activated (peri)vascular mast cells contribute to the ongoing inflammatory response and compromise plaque integrity.[6] Furthermore, mast cell also contain various vasoactive mediators such as histamine and heparin which affect platelet-collagen interactions and growth of the thrombus.[7] Within the vessel wall, mast cell express numerous surface receptors and may be activated by various stimuli such as IgE molecules via crosslinking of the FcεR, inflammatory mediators and complement factors, lipid mediators as well as neurogenic stimuli.[8]

With respect to acute triggers of acute cardiovascular syndromes, the psychological stress response is among the most potent endogenous immunomodulatory pathways known to men. Acute mental stress, for example outbursts of anger[9] or exposure to traumatic events[10], has been demonstrated to strongly associate with the incidence of acute coronary syndromes. Especially, stress-induced changes in hemostatic factors and inflammatory mediators have been suggested to play an important role herein.[11] Enhanced platelet activation and coagulation may be considered part of the fight-or-flight response aimed at preparing the organism for possible injury. However, chronic stress exposure or acute stress exposure in a vulnerable individual with advanced atherosclerosis, the stress-induced inflammatory response and hypercoagulabililty of the blood can result in atherothrombotic complications. The direct impact of acute stress

on platelet aggregation has been evaluated in humans and multiple animal models demonstrating diverse effects on aggregation depending on the stress conditions. Especially the duration of stress exposure may be important as a recent study demonstrated only chronic, but not acute stress exposure to result in enhanced agonist-induced aggregation of mouse platelets.[12] As vascular inflammation is intimately linked to atherosclerosis progression and the incidence of atherothrombotic complications, we focused in this study on stress-induced changes in immune cell composition of the blood and circulating pro-inflammatory cytokines levels as well as subsequent effects on coagulation and fibrinolysis. With the mast cell uniquely located in the vessel wall, packed with pro-inflammatory and thrombogenic mediators and previously shown to be activated upon acute stress exposure (Lagraauw *et al*, manuscript in preparation), we also aimed to dissect out mast cell-specific effects to acute stress-induced changes in platelet activation, coagulation and thrombosis.

To this end, acute stress was induced in apoE$^{-/-}$ mice and mast cell deficient red mast cell and basophil (RMB) apoE$^{-/-}$ mice by means of 2 hour restraint, followed by subsequent analysis of blood composition, bleeding time, clot retraction capacity and thrombus formation.

Materials and Methods

Animals and acute restraint stress model

All animal work was approved by the Leiden University Animal Ethics committee and performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. 10-12 weeks old male apoE$^{-/-}$ mice were obtained from the local animal breeding facility. The inducible mast cell deficient red mast cell and basophil (RMB) mice were obtained from the lab of Prof. Pierre Launay[13] and were backcrossed with apoE$^{-/-}$ mice to obtain RMB-apoE$^{-/-}$ mice. In all experiments mice were kept under standard laboratory conditions, and administered a Western type diet, containing 0.25% cholesterol and 0.15% cocoabutter (SDS, Sussex, UK) 2 weeks prior to stress exposure and throughout the experiment. To induce acute stress, mice were subjected to 2 hours restraint stress by immobilization in a well-ventilated 50 mL conical centrifuge tube. This setup was previously shown to result in a robust stress response as indicated by a 10-fold increase in plasma corticosterone levels (Lagraauw *et al*., manuscript in preparation). Directly or 24 hours after the acute stress protocol the mice were terminally anaesthetized and sacrificed. Blood was collected from the eye, anti-coagulated with 3.8% sodium citrate in a ratio of 1:10 and subsequently processed for fluorescence flow cytometry (Sysmex and FACS analysis), clot retraction measurement and ELISA.

Blood composition

Total white blood cell count, red blood cell count, platelet count, mean platelet volume and neutrophil, lymphocyte, monocyte and eosinophil counts in blood were analyzed using an automated XT-2000iV veterinary hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany).

Tail bleeding time

Tail bleeding time after acute stress exposure was assessed in anesthetized mice by surgical removal of the distal 3-mm segment of the tail. Bleeding time was subsequently monitored by absorbing the blood droplets with filter paper at 15-second intervals, without touching the wound, as described previously.[14]

Clot retraction

Clot retraction was assessed in anti-coagulated blood diluted 1:20 in HBS (10 mM HEPES; 150 mM NaCl; 1 mM MgSO4; 5 mM KCl; pH 7.4). After addition of 1 mM CaCl2 clot retraction was monitored by taking photographic images every 15 minutes. Clot area was subsequently quantified with ImageJ software (National Institute of Health, Washington, DC).

Cytokine analysis

Serum interleukin-6 (IL-6) and macrophage inhibitory factor (MIF) levels were determined by ELISA according to manufacturer's protocol (IL-6 ELISA from BD Biosciences, MIF ELISA from R&D Systems).

$FeCl_3$-induced arterial thrombosis model

Western-type diet fed male $apoE^{-/-}$ or RMB-$apoE^{-/-}$, which were exposed to 120' restraint stress 24 hours or just before FeCl3-induced thrombosis measurement and their respective non-stressed controls, were anaesthetized by subcutaneous injection of ketamine (60 mg/kg; Eurovet Animal Health, The Netherlands) and hypnorm (5%; VetaPharma, UK). Subsequently, the right carotid artery was dissected free and equipped with an ultrasonic 0.5 PSB Doppler flow nanoprobe and T420-PB-flowmeter (Transonic Systems Europe B.V., Maastricht, The Netherlands) and subsequently assessed for stable blood flow. After topical application of a 1x2mm filter paper saturated with 20% $FeCl_3$ for 3 minutes, flow was monitored until complete occlusion of the vessel was observed. Mice were subsequently sacrificed by exsanguination and blood was collected for serum cytokine analysis. The occluded carotid artery was harvested for morphometric and immunohistochemical analysis.

Thrombus morphometry and immunohistochemistry

The harvested thrombi were fixed in 4% formaldehyde solution (FormalFix) and embedded in O.C.T compound. 10 µm thick transverse cryosections were prepared on a Leica CM 3050S cryostat (Leica Instruments, Nassloch, Germany). Morphological analysis of the thrombi was performed on hematoxylin-eosin stained section. Thrombus collagen content was assessed by picrosirius red (SR; Direct Red 80, Sigma-Aldrich) staining and polarized light (PL) microscopy (Leica DM-RE microscope and LeicaQwin software, Leica Imaging Systems, Cambridge, UK).

Statistical analysis

Data are expressed as mean ± SEM. An unpaired two-tailed Student's t-test was used to compare normally distributed data between two groups of animals. Data of three groups were analyzed with one-way ANOVA and data of two groups with more than one variable were analyzed by two-way ANOVA, both followed by Tukey's multiple comparison test. A level of $P<0.05$ was considered significant.

Results

Acute stress-induced changes in blood composition

Exposure to acute stress has been demonstrated to strongly affect blood leukocyte distribution and to differentially affect the different leukocyte subpopulations.[15,16] In this study, 120' restraint stress induced a strong but transient decrease in white blood cell count, which was primarily due to significant reductions in the amounts of circulating lymphocytes and monocytes (figure 1A). As depicted in figure 1B, also the relative blood leukocyte content is drastically changed upon acute stress exposure. As described in literature, neutrophils are actually mobilized and indeed demonstrate an increase in relative amounts in the circulation. Recent data indicate contributing roles for neutrophils[17,18] in the induction of arterial and deep venous thrombosis, and the relative increase in circulating neutrophil numbers seen after acute stress exposure may impact thrombus formation and the stability of the developing thrombus.

Platelet count as well as mean platelet volume, red blood cell count and hematocrit levels were not significantly affected by the stress protocol. Interestingly, all stress-induced changes were completely reversed to baseline (non-stressed control) level 24 hours after acute stress exposure. Stress responsiveness, assessed by the effects on blood leukocyte distribution, was similar between apoE$^{-/-}$ and the mast cell deficient RMB-apoE$^{-/-}$ mice (supplemental figure 1A and B).

Acute stress-induced increase in circulating IL-6 and MIF partly mast cell derived

In addition to a quick rise in circulating levels of the principal stress hormones (corticosterone, adrenalin, noradrenalin) primarily released from the adrenal

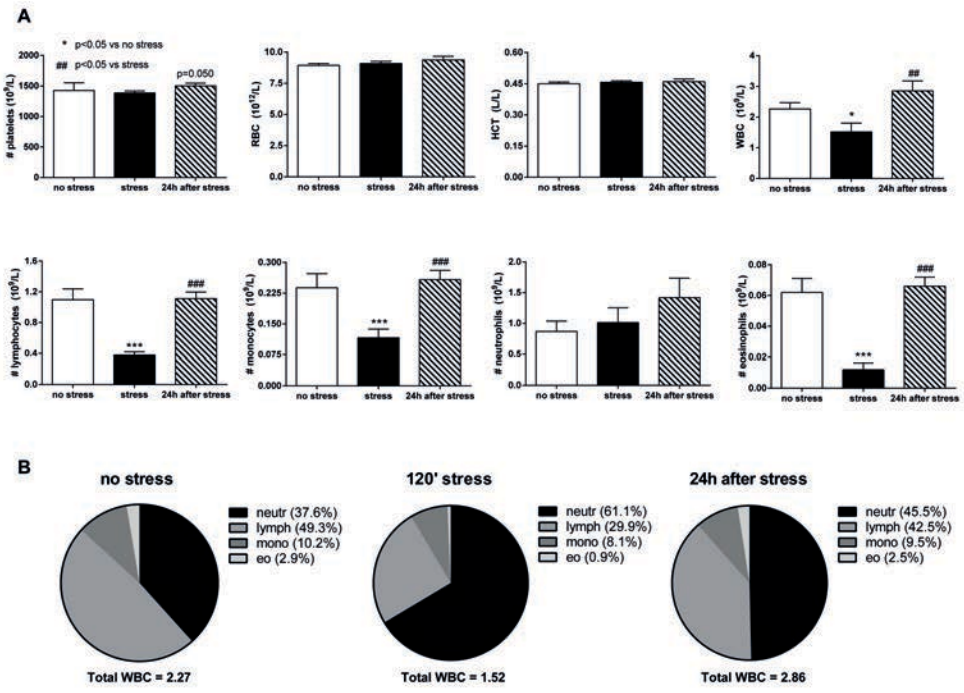

Figure 1. A) Blood platelet count, red blood cell count (RBC), hematocrit and leukocyte distribution directly and 24 hours after acute restraint stress exposure in apoE$^{-/-}$ mice. B) Relative blood leukocyte composition after acute stress, demonstrating a significant increase in the percentage of circulating neutrophils and decreases in lymphocytes and monocytes.

glands, acute stress exposure causes immune cell activation and pro-inflammatory cytokine to be released in the circulation. In the context of mast cell-derived pro-inflammatory and atherothrombotic mediators we focused on IL-6 and MIF. IL-6 has been demonstrate to promote coagulation without affecting fibrinolysis[19] and although many immune cells produce and secrete IL-6, mast cells contribute significantly to the amount of circulating IL-6 after acute stress exposure.[20] MIF is another potent cytokine with a well-established contributing role in atherosclerosis progression.[21] Furthermore, MIF seems to serve as the endogenous counterpart of glucocorticoids to dampen and regulate their potent anti-inflammatory effects and thus its circulating levels are strongly affected by the acute stress response.[22] Similar to previous results, 120' restraint stress resulted in a significant increase in serum IL-6 levels, an effect that was dampened in the RMB-apoE$^{-/-}$ mice, suggesting that part of the circulating IL-6 to be mast cell derived (figure 2). MIF demonstrated a similar secretion pattern after stress in apoE$^{-/-}$ mice. In mast cell-deficient mice however, acute stress did not significantly increase the circulating amount of this cytokine (figure 2).

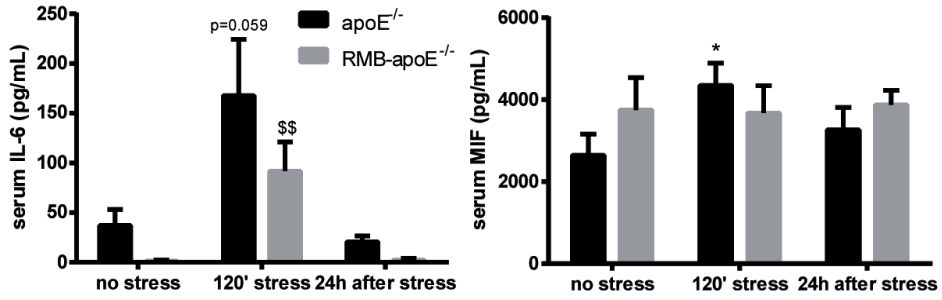

Figure 2. Serum IL-6 and MIF levels are significantly increased directly after acute stress exposure in apoE$^{-/-}$ mice. Basal circulating IL-6 and the stress-induced increase in IL-6 is less prominent in mast cell deficient mice. MIF levels were generally higher in the mast cell deficient mice and no apparent stress-induced increase in circulating MIF could be observed in those mice. *, $p<0.05$ vs no stress (apoE$^{-/-}$). $$ $p<0.01$ vs no stress (RMB-apoE$^{-/-}$).

Acute stress exposure increases in tail bleeding time in a mast cell-independent manner

To assess the effect of acute stress exposure on hemostasis, a tail bleeding assay was performed in 120' stressed apoE$^{-/-}$ mice and their non-stressed controls. Interestingly, bleeding time was significantly increased in stressed mice compared to non-stressed controls (376 ± 131 seconds in control mice compared to 718 ± 306 seconds after acute stress exposure (figure 3)). Previous results from our lab indicate the acute stress response as a potent activator of (cardiac) mast cells (Lagraauw *et al*. manuscript in preparation) and the mast cell-derived vasoactive mediators, histamine and heparin have been implicated in bleeding abnormalities. Therefore, a similar experiment was performed in the mast cell depleted RMB-apoE$^{-/-}$ mice. In contrast to the results obtained in apoE$^{-/-}$ mice, no acute stress-induced increase in tail bleeding time was observed in the mast cell deficient mice, suggesting an important role for mast cell-derived mediators (e.g. heparin) in stress-induced impaired hemostasis.

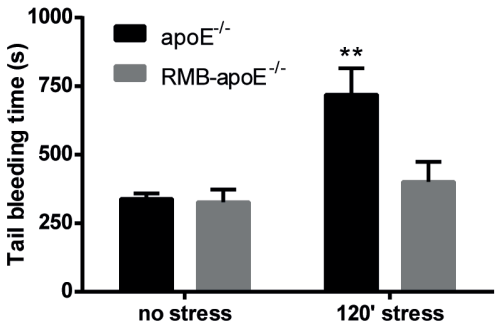

Figure 3. Acute restraint stress resulted in increased tail bleeding times in apoE$^{-/-}$ mice, but not in mast cell deficient apoE$^{-/-}$ mice. *, $p<0.05$ vs no stress.

Acute stress induces a lasting decrease in clot retraction without affecting platelet count or activation induced αIIbβ3 expression

Another measure of platelet function and blood coagulation is the clot retraction capacity, which is mediated by the interaction of fibrin with platelet integrin αIIbβ3 resulting in actin and myosin cytoskeleton rearrangements and contraction of the platelet-rich fibrin clot. Although platelet number and activation capacity, determined by basal αIIbβ3 expression level, did not differ between non-stressed control mice and mice, clot retraction upon recalcification was at least partly impaired in the stressed mice (figure 4). Interestingly, these effects disappeared in time, as clot retraction was normal 4 days after inducing acute stress (data not shown). In contrast to the effect on tail bleeding time, no clear mast cell-dependent contribution could be observed and clot retraction was similarly impaired in the stressed RMB-apoE$^{-/-}$ mice.

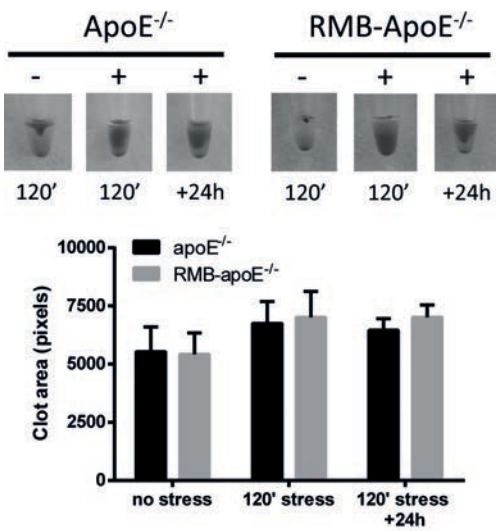

Figure 4. Acute restraint stress exposure in both apoE$^{-/-}$ and RMB-apoE$^{-/-}$ mice caused a direct and at least 24h lasting decrease in clot retraction capacity as indicated by an increased clot area. Clot retraction upon recalcification was assessed under non-stresses conditions (-) and directly or 24 hours after 120' stress exposure (+).

$FeCl_3$-induced arterial thrombosis

To determine acute stress-mediated effects on arterial thrombosis, we applied an arterial thrombosis model upon stress induction. Arterial thrombosis primarily results from rupture of a destabilized atherosclerotic plaque and exposure of the subendothelial matrix which, induces platelet adhesion to the exposed matrix, platelet activation, aggregation and blood coagulation. Animal models of arterial thrombosis mainly consist of methods to induce vascular damage, either by photochemical injury, laser injury or ferric chloride application. Topically applied ferric chloride diffuses through the vascular smooth muscle cell layer and results in

rapid removal of the endothelial layer without affecting the internal elastic lamina[23] and the resulting thrombi share a strong morphological similarity with the human situation. 120' restraint stressed mice were subjected directly or 24 hours later to this FeCl3-induced thrombosis model and compared to non-stressed control mice. No significant difference in time to occlusion of the treated right carotid artery nor the time between initial drop in blood flow and complete occlusion, the 'thrombus formation time', could be observed between stressed and non-stressed mice (figure 5). As mast cell-derived mediators, including heparin, histamine, chymase, typtase and proinflammatory cytokines (e.g. IL-6) could affect the formation of a stable clot, we assayed thrombus formation in the mast cell deficient mice. Interestingly, mast cell deficiency by itself significantly prolonged the initiation of thrombus formation. However, once initiated the thrombus formed similarly in the RMB mice. Polarized light microscopy can be used to analyze the fibrin architecture and structural integrity of thrombi.[24] However, morphological analysis of the isolated thrombi did not reveal any striking differences between the groups and mouse strains (supplemental figure 2).

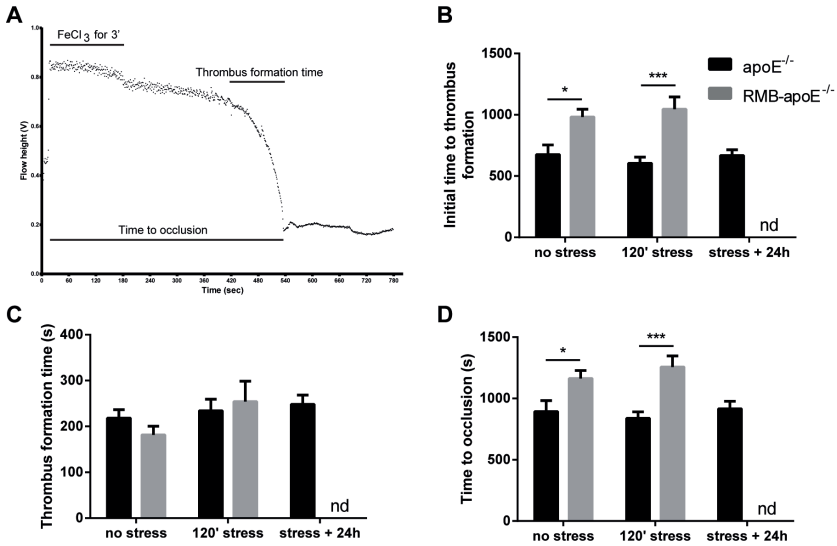

Figure 5. $FeCl_3$-induced thrombus formation in apoE$^{-/-}$ and RMB-apoE$^{-/-}$ mice. A) Representative blood flow curve trough the right carotid artery upon $FeCl_3$ application for 3 minutes. Time to initial thrombus formation represents the time at which initiation of an eventually occlusive thrombus could be observed, while time to occlusion represents total time until vessel occlusion. Thrombus formation time is calculated from initiation till complete occlusion of the vessel. B-D) While no significant effect of acute stress exposure on thrombus formation could be observed, mast cell deficiency did prolong the initiation and eventual time till occlusion.

Discussion

Arterial thrombosis, which primarily occurs upon rupture of destabilized atherosclerotic lesions, results in exposure of plaque derived pro-thrombotic factors and vessel wall components, which subsequently initiates platelet activation and triggers blood coagulation. In line with recently increased appreciation for the contribution of psychological risk factors, including stress, to cardiovascular disease, we here aimed to demonstrate acute stress-induced changes in blood composition and coagulation.

Exposure of acute stress, by means of 120' restraint, resulted in significant redistribution of blood leukocyte subpopulations in both apoE$^{-/-}$ and mast cell-deficient RMB-apoE$^{-/-}$ mice. As previously intensively investigated by Dhabhar *et al*[15], monocyte and lymphocyte counts were strongly reduced after acute stress exposure, while circulating neutrophils amounts were increased. Recent data indicate the importance of platelets, monocytes and neutrophils in both arterial and deep venous thrombosis[17,18], suggesting the possibility of a direct contributing role for stress-induced immunomodulation in the development of atherothrombosis. In the current study, acute stress was shown to induce a significant increase in circulating IL-6 and MIF. IL-6 has demonstrated pro-coagulant effects via upregulation of various members of the coagulation cascade (e.g. fibrinogen, tissue factor, factor VIII) and to enhance platelet production.[19] In contrast, the pleiotropic inflammatory cytokine MIF, has recently been reported to delay clot retraction.[25]

A recent study, evaluating the effects of both chronic and acute stress on platelet aggregation demonstrated limited differences in platelet responsiveness after a 2 hour restraint stress protocol, while chronic stress (2h/day for 3 weeks) did significantly enhance agonist-stimulated platelet aggregation. These chronic stress-induced changes were blocked in adrenalectomized mice, suggesting the contribution of the main stress hormones, corticosterone, adrenalin and noradrenaline.[12] In our study, exposure to an acute episode of restraint stress did not alter platelet counts, mean platelet volume, or agonist-induced platelet αIIbβ3 expression. However, tail bleeding time was significantly increased and clot retraction was reduced in the stressed mice, suggestive of impaired platelet function and/or coagulation. Despite these changes, acute stress in the current experimental setting did not result in an increased prothrombotic potential in a ferric chloride-induced arterial thrombosis model. Detailed analysis of the thrombi may however provide more evidence on the composition and stability of the thrombi after acute stress.

Interestingly, severe restraint stress (20 hours) was previously shown to increase circulating levels of tissue factor (TF), the main initiator of coagulation, possibly accounting for enhanced thrombus formation in that model. This stress-induced

effect on thrombus formation could be prevented by chemical sympathectomy, suggesting the involvement of the sympathetic nervous system. In contrast to our results after 2 hours restraint stress, tail bleeding time was not affected in this study. Sympathetic innervation of the vessel wall regulates vasoconstriction and dilatation, but also modulates (resident) immune cell responses via the local release of various neuropeptides and hormones. Previous results from our lab indicate the acute stress response as a trigger of perivascular mast cell activation, resulting in increased inflammation and intraplaque hemorrhages (Lagraauw *et al*., manuscript in preparation). Furthermore, mast cell activation has been shown to take part in mediating leukocyte recruitment to sites of inflammation and vascular damage.[26] To dissect out the contribution of mast cells in the observed stress-induced increase in tail bleeding time and impaired clot retraction, we performed similar experiments in mast cell deficient RMB-apoE$^{-/-}$ mice. While blood composition was similar, mast cell deficiency limited the stress-induced increase in bleeding times, which is possibly due to a lack in mast cell derived heparin, but prolonged thrombus formation. Stress-induced IL-6 levels were diminished, identifying a significant proportion of the IL-6 to be mast cell-derived. Clot retraction capacity however, was similarly impaired up to 24 hours after acute stress exposure, while also thrombus formation time was similar in these mice.
Combined, our results indicate that acute stress significantly affects multiple components of the immune and coagulation system, which however did not result in an enhanced thrombus formation. Further research may identify more specific effects of acute stress on platelet function and aggregation, and may provide more evidence of mast cell mediated effects on the coagulation system.

Funding

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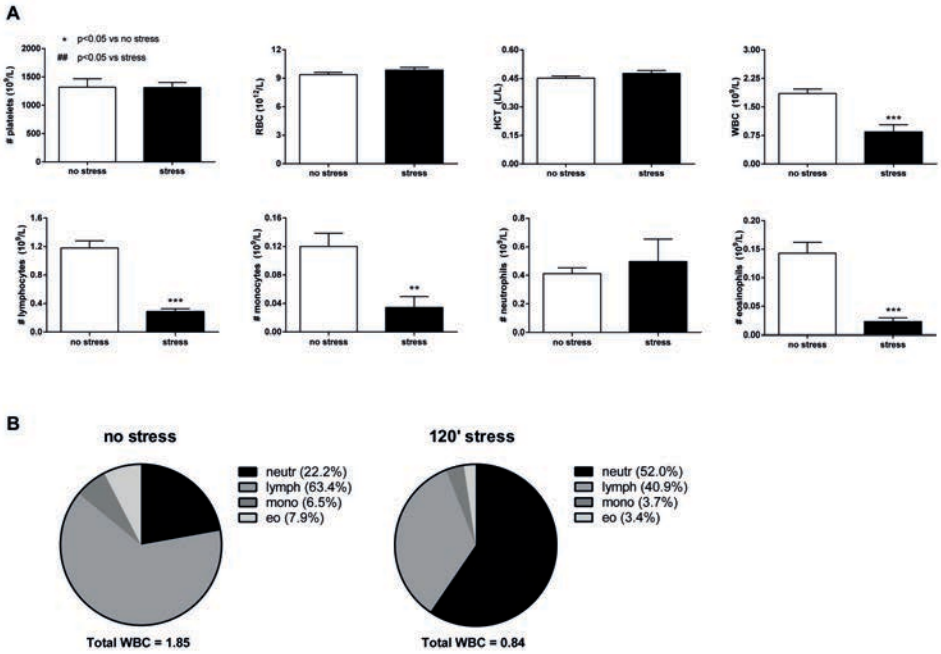
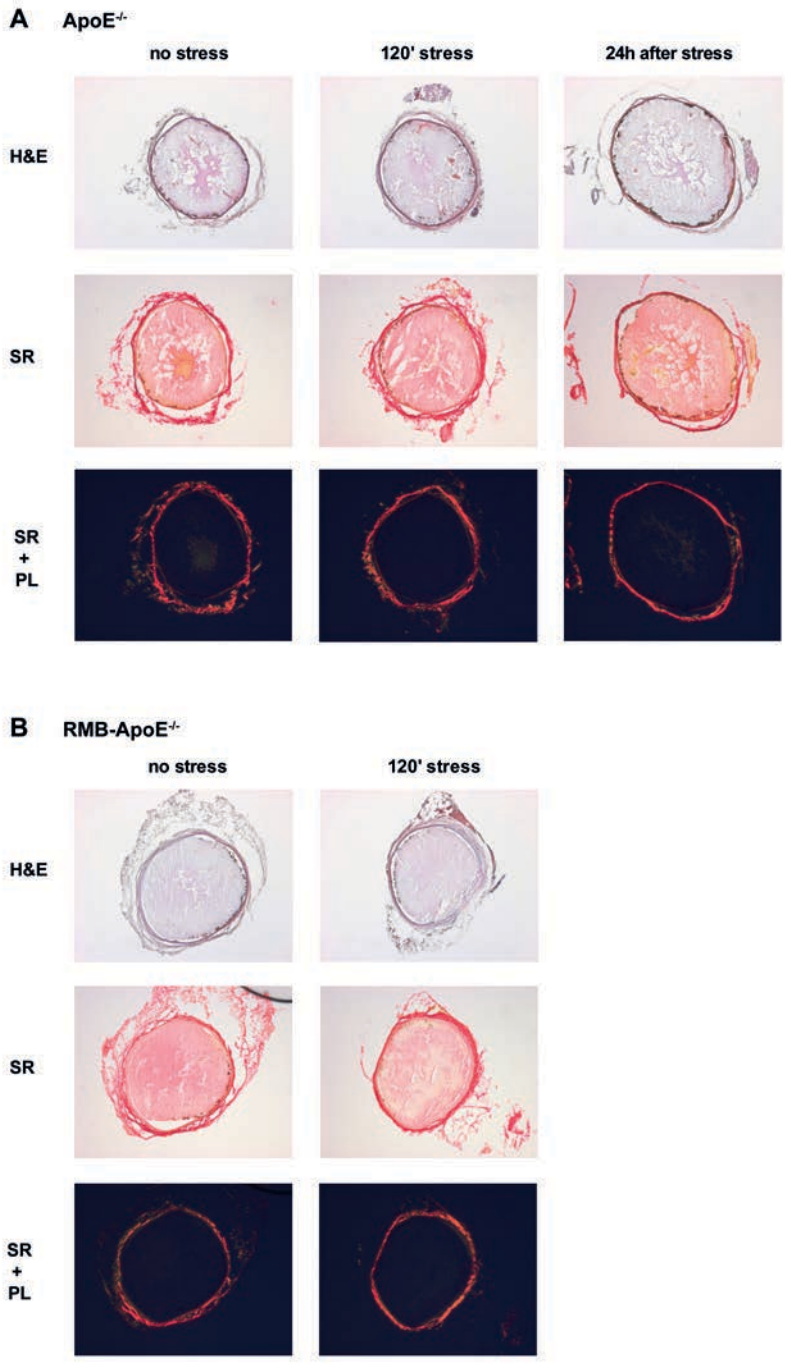
Supplemental data



Supplemental figure 1. A) Blood platelet count, red blood cell count (RBC), hematocrit and leukocyte distribution directly after acute (120') restraint stress exposure in RMB-apoE$^{-/-}$ mice. B) Relative blood leukocyte composition after acute stress, demonstrating a significant increase in the percentage of circulating neutrophils and decreases in lymphocytes and monocytes.



Supplemental figure 2. $FeCl_3$-induced thrombus morphology and collagen content was assessed in both apoE$^{-/-}$ and RMB-apoE$^{-/-}$ mice. No clear morphological differences could be observed between the groups and mouse strains.

Chapter 5

Vascular Neuropeptide Y contributes to atherosclerotic plaque progression and perivascular mast cell activation

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Abstract

Objective: Neuropeptide Y is an abundantly expressed neurotransmitter capable of modulating both immune and metabolic responses related to the development of atherosclerosis. NPY receptors are expressed by a number of vascular wall cell types, among which mast cells. However, the direct effects of NPY on atherosclerotic plaque development and progression remain to be investigated. In this study we thus aimed to determine whether NPY is expressed in atherosclerotic plaques and to establish its role in atherosclerotic plaque development.

Methods and Results: NPY expression was seen to be increased up to 2-fold in unstable human endarterectomy plaques, as compared to stable plaques, and to be significantly upregulated during lesion progression in apoE$^{-/-}$ mice. In apoE$^{-/-}$ mice focal overexpression of NPY in the carotid artery significantly increased atherosclerotic plaque size compared to controls, while plaque composition was unaffected. Interestingly, perivascular mast cell activation was significantly higher in the NPY-overexpressing mice, suggesting that NPY may impact plaque progression in part via mast cell activation. Furthermore, in vitro NPY-induced murine mast cell activation resulted in the release of pro-atherogenic mediators including IL-6 and tryptase.

Conclusion: Our data show that NPY expression is increased during atherogenesis and in particular in unstable plaques. Furthermore, perivascular overexpression of NPY promoted plaque development and perivascular mast cell activation, suggestive of a role for NPY-induced mast cell activation in lesion progression.

Introduction

Atherosclerosis is a lipid-driven inflammatory disease characterized by endothelial dysfunction, vascular inflammation and deposition of lipids, cholesterol and cellular debris within the vessel wall.[1] Being the principle cause of myocardial infarction and stroke it remains a leading cause of death in westernized society.[2] These clinical outcomes are generally caused by rupture of an advanced and unstable atherosclerotic plaque, leading to a subsequent thrombotic response and luminal obstruction. Despite the identification and management of multiple risk factors for atherosclerosis, including hyperlipidemia, genetic background, hypertension, obesity and smoking, the precise triggers that cause a plaque to rupture remain unclear.

Vessels that are affected by atherosclerotic lesion formation, such as the coronary arteries, generally are richly innervated, suggesting a role for the nervous system in disease progression and plaque stability.[3,4] The idea of neurotransmitters influencing vascular tone and remodeling as well as immune functions is generally accepted, however the precise implications for atherosclerosis progression are still unclear. Sympathetic activity, e.g. due to exposure to a stressor, mediated by its transmitters noradrenalin, ATP and neuropeptide Y (NPY) is believed to promote atherosclerosis primarily indirectly via vasoconstrictive actions.[5] However, more recent studies have demonstrated additional actions of NPY in vascular and metabolic disease models[6] in which NPY acts as a trophic factor on vascular smooth muscle cells (VSMC) and endothelial cells[7,8] and induces metabolic alterations and growth of adipose tissue.[9]

Neuropeptide Y is a 36-amino acid long peptide abundantly present in both the central and peripheral nervous system, primarily exerting its actions trough G-protein coupled Y receptors and is modified by endogenous peptidases such as dipeptidyl peptidase IV (DPPIV) into Y2 receptor-specific NPY(3-36).[10] NPY has also been implicated in modulating innate and adaptive immune responses and expression of NPY and its receptors were shown to increase following activation of macrophages, granulocytes, T- and B-cells.[11,12] The link between NPY signaling and cardiovascular complications is further strengthened by the identification of several SNPs associated with increased risk of early-onset atherosclerosis[13] and a gain-of-function polymorphism, Leu7Pro7, commonly found in Northern European populations which is now associated with hyperlipidemia, accelerated atherosclerosis and diabetic retinopathy.[14,15] The present insight into the immune modulatory actions of NPY during atherosclerotic lesion progression is currently mostly limited to descriptive studies evaluating NPY, its receptor system (Y1-Y6) and plaque characteristics.[16] While providing valuable indications for a pathogenic role of NPY in atherosclerosis the direct effects of increased perivascular NPY on atherosclerotic plaque progression remain ill-defined.

In this study we thus aimed to determine the expression levels of NPY during the progression of atherosclerosis and establish the effects of increased perivascular NPY levels on atherosclerotic plaque development and vascular inflammation.

Materials & Methods

NPY (receptor) gene expression during lesion development in apoE$^{-/-}$ mice

This study was performed in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. All animal experiments were approved by the animal welfare committee of the Leiden University Medical Center (approval reference numbers 11106 and 12102). Male apoE$^{-/-}$ mice obtained from the local animal breeding facility were fed a Western type diet, containing 0.25% cholesterol and 0.15% cacaobutter (SDS, Sussex, UK) two weeks prior to surgery and throughout the experiment. Mice were anaesthetized by subcutaneous injection of ketamine (60 mg/kg, Eurovet Animal Health, Bladel, The Netherlands), fentanyl citrate and fluanisone (1.26 mg/kg and 2 mg/kg respectively, Janssen Animal Health, Sauderton, UK). The adequacy of the anaesthesia was monitored by keeping track of the breathing frequency and the response to toe pinching of the mice. Atherosclerotic carotid artery plaque formation was induced by bilateral collar placement as described previously.[17] From week 0 to week 8 after collar placement every 2 weeks a subset of mice (n=6) was anesthetized and perfused with PBS after which both carotid arteries were harvested, snap-frozen in liquid nitrogen and stored at -80°C until RNA isolation.[18]

RNA isolation and gene expression analysis

Three arteries were pooled per sample (n=3-4), homogenized and total RNA was isolated using Trizol reagent according to manufacturer's instructions (Invitrogen, Breda, The Netherlands). RNA was reverse transcribed using M-MuLV reverse transcriptase (RevertAid, MBI Fermentas, Leon-Roth) and used for quantitative analysis of gene expression with an ABI PRISM 7700 Taqman apparatus (Applied Biosystems, Foster City, CA) as described previously[19], using the PCR primers listed in supplementary table 1.

NPY expression profiling in human stable and unstable carotid artery plaques

For NPY expression analysis in human stable (n=9) and unstable (n=12) plaques, RNA as well as corresponding paraffin embedded sections were obtained from the AtheroExpress Biobank.[20] Plaques from all patients in this study were previously phenotyped by two independent observers using the following criteria. Fibrous plaques low in inflammatory cell and fat content with strong staining for collagen and smooth muscle cells were considered stable, while lesions were categorized unstable atheromathous plaques if they had strong staining for macrophages and

no or minor staining for collagen and smooth muscle cells.[21,22,23] Gene expression was analyzed as described for mouse gene expression during plaque progression using PCR primers listed in supplementary table 1.

Immunohistochemical analysis of NPY expression in both stable and unstable carotid endarterectomy specimens was performed with a rabbit anti-NPY antibody (1:1500, Ab30914, Abcam, Cambridge, UK), a goat anti-rabbit IgG poly-HRP conjugated secondary antibody (Powervision, Leica, Rijswijk, the Netherlands) and Nova-Red (Vector labs, Peterborough, United Kingdom) as enzyme substrate resulting in a red-brown color. The negative control to determine the specificity of the NPY staining in human endarterectomy specimens was obtained by omitting the primary antibody in the staining protocol (supplemental figure 1C).

Lentivirus vector construction and production

To obtain full length cDNA for murine NPY total RNA was extracted from mouse brain tissue, obtained from male apoE$^{-/-}$ mice sacrificed by cervical dislocation. The cDNAs were amplified using the following primers: forward: 5'-CCGCCGCTCAGCGACGACTG-3' and reverse: 5'-GTTTCATTTCCCATCACCA CATGGAAGGGT-3'. Primers contained extra Xhol and HindIII restriction sites to facilitate cloning into the pRRI-cPPt-CMV-PreSIN vector. Sequencing by means of vector specific primers confirmed 100% identity of the inserted NPY gene. Virus was produced as described previously[24] using transient calcium phosphate cotransfection of 293T cells with the LV.Empty or LV.NPY vector together with pMDL/RRE, pRSV-REV and pVSV-G. Viral titers were determined as essentially described by Sastry *et al*.[25] and modified by Bot *et al*.[26]

In vitro lentiviral NPY overexpression

The overexpression capacity of the lentiviral NPY construct was determined in 293T HEK cells, the endothelial cell-line H5V and a murine vascular smooth muscle cell (VSMC) cell-line. 293T, H5V, VSMC were cultured in a humidified atmosphere (5% CO2) at 37°C in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), 2mmol/L L-glutamine, 100U/mL penicillin and 100U/mL streptomycin (all from PAA, Colbe, Germany). Cells were transduced with concentrated LV.NPY for 24h, after which the medium was refreshed and the cells incubated for another 36-48 hours. Total RNA was extracted from these cells with GTC, reverse transcribed using M-MuLV reverse transcriptase (RevertAid, MBI Fermentas, Leon-Roth, Germany) and expression of NPY was measured by qPCR on an ABI PRISM 7500 Taqman apparatus (Applied Biosystems, Foster City, CA).

Lentiviral perivascular overexpression of NPY in apoE$^{-/-}$ mice

Local perivascular overexpression of NPY was assessed in the previously described carotid artery collar model for accelerated atherosclerosis. 24 male apoE$^{-/-}$ mice,

fed a Western type diet throughout the experiment, were equipped with bilateral carotid artery collars and treated perivascularly with either an empty (pRRI-cPPt-CMV-PreSIN)[27], designated LV.Empty, n=12) or NPY expression vector (pRRI-cPPt-CMV.NPY-PreSIN, designated LV.NPY, n=12; 4*108 TU/mouse) in a pluronic F-127 gel (25% w/v, 20 µl/mouse, Sigma, Zwijndrecht, The Netherlands). Blood samples were taken biweekly from the mice by tail-cut and plasma was obtained by centrifugation at 8,000 rpm for 10 min. Levels of total plasma cholesterol were measured spectrophotometrically using enzymatic procedures (Roche Diagnostics, Almere, the Netherlands). Four weeks after collar placement the animals were anesthetized, perfused with PBS and the carotid arteries harvested for morphometric and immunohistochemical analysis.

Plaque morphometry and immunohistochemistry

Harvested carotid arteries were fixed in 4% formaldehyde solution (FormalFix) and embedded in O.C.T compound (Tissue-Tek, Sakura Finetek, CA, USA). Transverse 5µm cryosections were prepared on a Leica CM 3050S Cryostat (Leica Instruments, Nassloch, Germany). Hematoxylin-eosin stained sections of the common carotid arteries were used for morphometric analysis. Each vessel was assessed approximately 0.5 mm proximal to the collar and the site of maximal stenosis was used for morphometric assessment of lesion size and necrotic core area (defined as the a-cellular, debris-rich plaque area as percentage of total plaque area) using Leica Qwin image analysis software. Immunohistochemical analysis of NPY expression was performed with a rabbit anti-NPY (1:1500, #Abcam, Cambridge, UK) and goat anti-rabbit poly-HRP conjugated secondary antibody (Immunologic, Duiven, The Netherlands). Both positive and negative controls were used to determine the specificity of the NPY staining. Mouse brain tissue showed clear NPY positivity in the cerebral cortex and cerebellum (supplemental figure 1G;H), while no staining was observed in negative control samples of collar-induced murine lesions (supplemental figure 1E;F) or mouse brain tissue (supplemental figure 1I) in which the primary antibody was omitted. In addition, immunohistochemical stainings were performed to assess plaque composition and stability. Sections were stained for collagen (picosirius red), macrophages (MOMA-2) and vascular smooth muscle cells (α-SMA) as described previously.[28] Mast cells were visualized by chloroacetate esterase staining according to manufacturer's protocol (CAE, Sigma, Germany) and degranulation status was assessed manually by bright-field microscopy as described previously.[29] A mast cell was considered resting when all granulae were maintained inside the cell, while mast cells were assessed as activated when granulae were deposited in the tissue surrounding the mast cell. All morphometric analyses were performed by blinded independent operators (HML and IB).

NPY-induced mast cell activation

The capacity of NPY to activate murine mast cells was further studied on bone marrow derived mast cells (BMMCs) skewed towards either a mucosal-like (MMC) or connective tissue-like (CTMC) phenotype. BMMCs were cultured by culturing bone marrow cells, obtained from male apoE$^{-/-}$ mice sacrificed by cervical dislocation, at a density of 0.25*106 cells in T175 culture flasks (Greiner Bio-One, The Netherlands) in RPMI containing 10% fetal bovine serum (FBS), 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and mIL3 for 3 weeks. Cells were then stimulated towards a MMC phenotype by continued culture in mIL-3 containing medium or a CTMC phenotype in complete medium with 300 U/ml mIL-4 and 50 ng/ml SCF (PeproTech, Inc., Rocky Hill, NJ) for another week.[30] In vitro mast cell activation was assessed by 4 hour incubation with NPY-conditioned medium obtained from LV.NPY-transduced VSMC or control VSMC conditioned medium. Stimulated BMMCs (1*106 cells; n=3 per condition) were centrifuged (1500 rpm, 5 minutes) and the releasate was used for further experiments. IL-6 and Monocyte Chemoattractant Protein (MCP)-1 levels in the mast cell releasate and conditioned medium itself were determined by ELISA according to manufacturer's protocol (eBioscience). In a similar in vitro experimental setup, we evaluated skewing of bone marrow-derived macrophages (grown for 7 days in RPMI containing 20% fetal bovine serum (FBS), 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 10ng/ml M-CSF) towards either an M1 or M2 phenotype by NPY. Four hours after incubation with either NPY or control medium (n=3 per condition) cells were harvested and total RNA extracted as described previously. M1 and M2 phenotype was assessed by qPCR analysis of M1 (ao. CCL2, iNos, TNFα, IL-6) or M2 (ao. IL-10, YM-1, CD206, Arg-1) marker gene expression. Primers are listed in supplemental table 1. To further determine the NPY concentrations capable of activating murine mast cells, BMMCs (0.5*106 cells; n=3 per condition) were activated by incubation with compound 48/80 (0.5 µg/mL, Sigma) or recombinant NPY (10-5M – 10-12M, Tocris Bioscience) for 30 minutes at 37°C in HEPES-tyrode supplemented with 0.1% fatty acid free bovine serum albumin (BSA, Sigma). Stimulated BMMCs (0.5*106 cells) were centrifuged (1500 rpm, 5 minutes) and the releasate was used for further experiments. For total (100%) content measurements, mast cells were lysed with 10% Triton X-100 and untreated control cell supernatant served as 0% release controls. β-Hexosaminidase activity was determined by adding 50 µL of releasate to 50 µL 2 mM 4-nitrophenyl N-acetyl-b-D-glucosaminide (Sigma) in 0.2 M citrate (pH 4.5) and incubated at 37 °C for 2 hours. After addition of 150 µL 1 M Tris (pH 9.0), absorbance (optical density, OD) was measured at 405 nm. To measure tryptase release after degranulation, 50 µL supernatant was added to 2 mM S-2288 (tryptase substrate, Chromogenix, Lexington, USA) in PBS supplemented with 100 U/mL heparin. After 2 hours (tryptase) at 37°C, OD405 was measured.

Statistical analysis

Data are expressed as mean ± SEM. A 2-tailed Student's t-test was used to compare individual groups. Non-Gaussian distributed data were analyzed using a Mann-Whitney U test. A level of $P<0.05$ was considered significant.

Results

NPY expression is increased in unstable versus stable human atherosclerotic plaques.

As NPY is an abundantly expressed neuropeptide and has well known growth promoting and immune modulatory actions on various cells implicated in atherogenesis we first assessed the abundance of vascular NPY expression. Plaques from all patients in this study were previously phenotyped, enabling the comparison between stable and unstable plaques.[20] Fibrous plaques low in inflammatory cell and fat content with strong staining for collagen and smooth muscle cells were considered stable, while lesions were categorized unstable atheromathous plaques if they had strong staining for macrophages and no or minor staining for collagen and smooth muscle cells. NPY immunostaining in stable and unstable carotid endarterectomy specimens revealed NPY positive staining to be primarily localized to intimal vascular smooth muscle cell-rich regions in the stable lesions (brown staining in figure 1A; B and supplemental figure 1A) and both smooth muscle cell- and macrophage-rich areas in the unstable plaques (supplemental figure 1B). Interestingly, significantly higher lesional NPY expression could be detected in unstable compared to stable plaques (>2-fold, $P=0.036$, figure 1C), thus suggesting that NPY may actively participate in plaque progression.

Vascular neuropeptide Y expression correlates with plaque progression in apoE$^{-/-}$ mice.

Next, we measured expression of NPY and its receptors during atherogenesis in hyperlipidemic apoE$^{-/-}$ mice. Gene expression analysis both by microarray and qPCR showed NPY to be increasingly expressed from week 0 (non-diseased artery) to week 8 and the expression maintained a high level till at least 8 weeks after initiation of plaque development (figure 1D). We further confirmed the presence of NPY by immunohistochemistry and show that similar to the human atherosclerotic plaques, lesional NPY (figure 1E and G) colocalized with vascular smooth muscle cells (figure 1H) and to some extent with infiltrated macrophages or foam cells (figure 1I) . Interestingly, strong NPY positive staining was also observed in neurons innervating the perivascular tissue (figure 1F). In vitro analysis of NPY expression in several cell types involved in atherosclerosis indeed showed high relative gene expression in VSMC and neuronal cell lines as well as in bone marrow-derived macrophages (supplemental figure 1D).

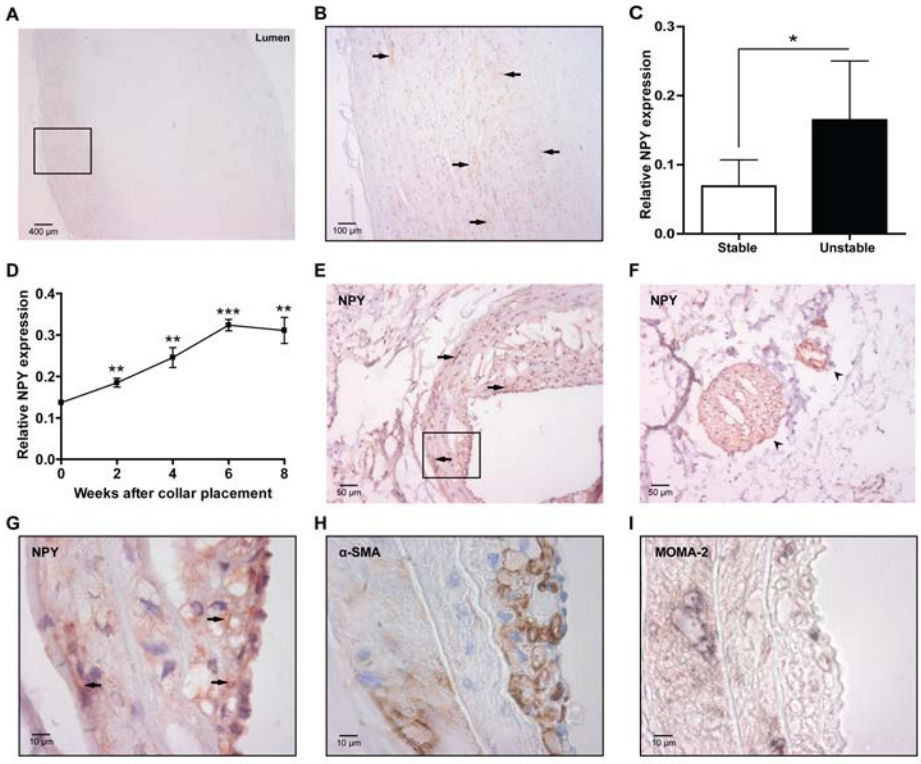

Figure 1. NPY gene expression (C) in stable (n=9) and unstable (n=12) human carotid endarterectomy specimens, demonstrating NPY expression in the plaque, especially in the medial vascular smooth muscle cells (A, B: 100x magnification; brown staining (arrows)). Murine carotid NPY expression was quantified during atherogenesis by (D) qPCR (n=3-4 per timepoint) or (E) visualized by immunohistochemical staining (arrows, 200x magnification). Perivascular NPY colocalized with neuronal structures (F, arrowheads). Higher magnification (1000x) inserts display NPY positive staining (G) to correlate with (H) vascular smooth muscle cells (α-SMA) and (I) macrophages (MOMA-2) *P<0.05, **P<0.01, ***P<0.001

Perivascular overexpression of NPY during lesion development in apoE$^{-/-}$ mice.

To address the atherogenic potential of increased vascular NPY expression we constructed an NPY-expressing lentivirus for perivascular application at the site of atherosclerotic lesion formation. Initially, NPY gene transduction was assessed in vitro by transducing 293T, VSMC and endothelial (H5V) cell lines with LV.NPY followed by mRNA expression analysis, resulting in overexpression of NPY in all cell lines tested (supplemental figure 2A).

In vivo, overexpression was assessed by treating apoE$^{-/-}$ mice focally at the lesion site with LV.NPY or LV.Empty at the time of collar placement. NPY expression was determined two weeks after lentiviral application by qPCR analysis and demonstrated a 1.5-fold increase in NPY expression compared to LV.Empty treated vessels (supplemental figure 2B). No systemic effects on bodyweight

gain, serum cholesterol level or white blood cell levels between the treatment groups was observed using this experimental setup (supplementary figure 2C; D). After 4 weeks the mice were sacrificed and the atherosclerotic plaques analyzed. Morphometric analysis of the carotid lesions revealed a significant increase in atherosclerotic area in the NPY-overexpressing mice compared to control (LV.NPY: 54 ± 9*103 μm^2 versus LV.Empty: 31 ± 6*103 μm^2, P=0.047, figure 2A; E; F) and a concomitant decrease in lumen size (figure 2B).

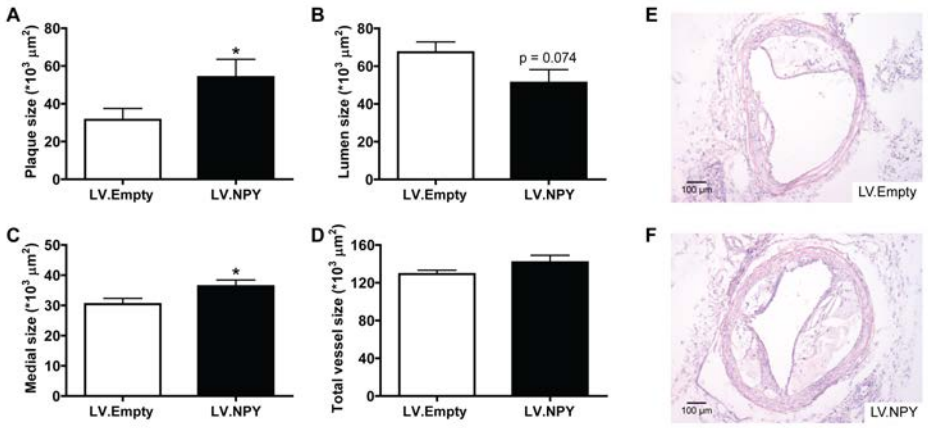

Figure 2. Advanced plaque formation was induced by semi-constrictive collar placement with addition of local perivascular NPY or empty lentivirus treatment in male apoE$^{-/-}$ mice (n=12 per group) fed a Western-type diet for 4 weeks. (A) LV.NPY treatment significantly increased lesion size and a (B) concomitant decrease in lumen area. (C) A small but significant increase in medial area was observed, while (D) the total vessel area was similar in both treatment groups. Representative cross-sections of LV.empty (E) or LV.NPY (F) carotid artery lesions stained with hematoxylin & eosin. 100x magnification, *P<0.05

Furthermore, medial size was significantly increased in the NPY-overexpressing carotids (36 ± 2*10^3 μm^2 versus 30 ± 2*10^3 μm^2, P=0.041, figure 2C) without signs of outward remodeling (total vessel area: LV.NPY 142 ± 7*10^3 μm^2 versus LV.Empty: 129 ± 4*10^3 μm^2, P=0.370, figure 2D).

Next, we assessed the composition of the plaques. While the absolute necrotic core area showed a trend towards an increase in the NPY group (LV.NPY: 25 ± 6*10^3 μm^2 versus LV.Empty: 14 ± 4*10^3 μm^2, P=0.097, Figure 3A), the relative necrotic area to intima area was similar (LV.NPY: 44.0 ± 4.4 % versus LV.Empty: 37.9 ± 5.1 %, P=0.365, figure 3A). Furthermore, picrosirius red staining of the carotid artery sections revealed no differences in collagen content (LV.NPY: 14.5 ± 2.1 % versus LV.Empty: 12.6 ± 1.3 %, P=0.460, figure 3B). As NPY was previously shown to have a chemotactic effect on monocytes[31], a MOMA-2 staining was performed to quantify the amount of infiltrated macrophages. As depicted, the foam cell rich areas stained strongly positive without clear differences between both groups (16.1 ± 2.9 % versus 17.2 ± 2.8 %, P=0.792) (supplemental figure 3A).

Despite an increase in the medial layer upon NPY-overexpression, the relative area of α-actin positive VSMCs in both treatment groups was comparable (supplementary figure 3B).

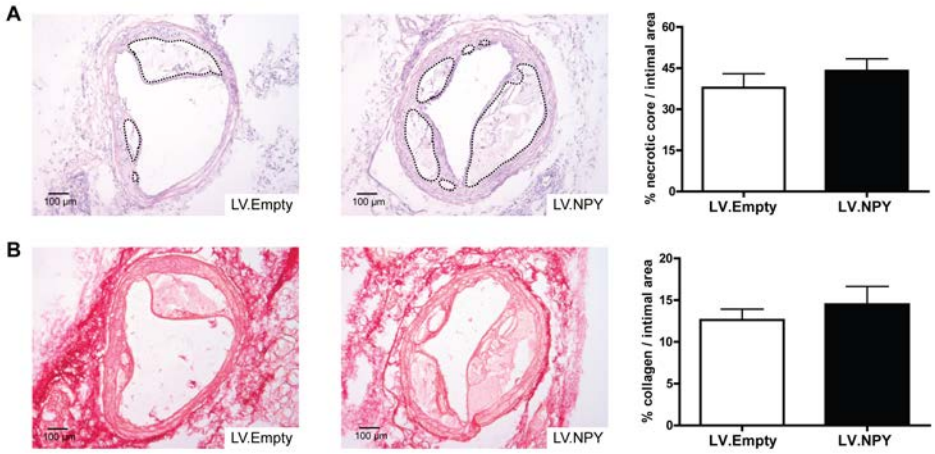

Figure 3. No difference in necrotic core area and relative collagen content of the lesions between LV.NPY or LV.Empty treated mice (n=12 per group). (A) Necrotic core area was defined as the a-cellular, debris-rich plaque area as percentage of total plaque area. (B) Collagen content of the plaque was determined by picosirius red staining and polarized light microscopy. 100x magnification

Another immune cell, which warrants attention when investigating atherosclerotic lesion progression and stability, is the mast cell. Previously, we and others have shown perivascular mast cell accumulation during atherogenesis and a destabilizing role for the mast cell protease chymase.[18,29,32] As mast cells contain receptors for NPY, the abundant vascular NPY expression and neuronal release of NPY near blood vessels may be an important endogenous trigger of mast cell activation in the context of atherosclerosis development. Interestingly, while the absolute number of perivascular mast cells did not differ between the groups (LV.NPY: 9.5 ± 1.2 MC/mm^2 versus LV.Empty: 7.3 ± 1.5 MC/mm^2, P=0.29), the amount of activated mast cell in the NPY-overexpressing mice, was significantly increased as compared to the controls (48.1 ± 4.0 % versus 30.2 ± 6.0 %, P=0.018, figure 4). These data thus suggest that NPY may contribute to lesion progression, in part, by its capacity to activate perivascular mast cells.

Increased vascular smooth muscle cell NPY expression promotes a proinflammatory and proatherogenic environment

We further established the effects of NPY on mast cell function and activation in vitro. PCR analysis of MC/9 and connective tissue-like bone marrow-derived mast cells (CTMC), the predominant mast cell type in the vessel wall, revealed expression of NPY and Y1, Y2 and Y5 receptors on murine mast cells as well (figure 5A). Furthermore, in vitro incubation of CTMC with LV.NPY-conditioned VSMC medium

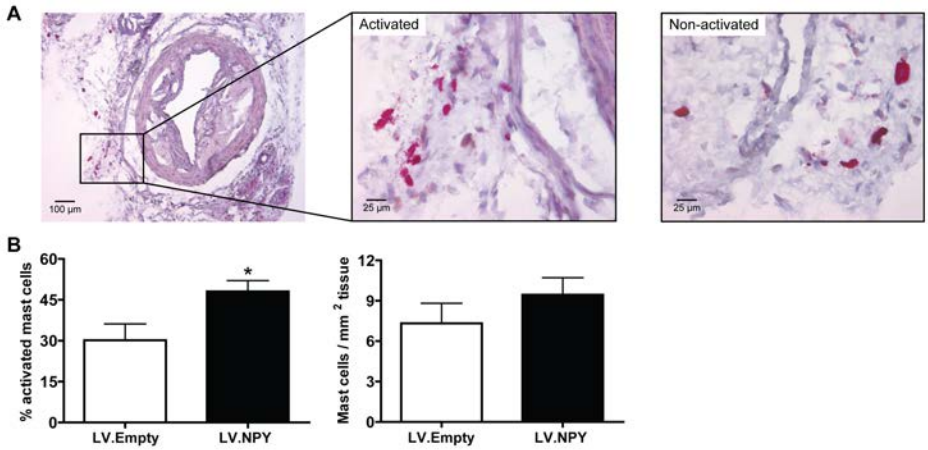

Figure 4. (A) Representative sections of perivascular tissue stained with Naphthol AS-D Chloroacetate to visualize activated and non-activated mast cells in close proximity to the atherosclerotic vessel. (B) Perivascular mast cell activation, but not mast cell number, is significantly increased in LV.NPY treated mice compared to controls (n=12 per group). 100x magnification, inserts 400x magnification. *P<0.05

induced IL-6, but not MCP-1 release into the medium (figure 5B; C). In addition, acute activation (30') with recombinant NPY caused release of β-hexosaminidase and tryptase already at picomolar concentrations with effectiveness compared to the positive control compound 48/80. Taken together, these data demonstrate that NPY can activate murine mast cells resulting in the release of pro-atherogenic mediators.

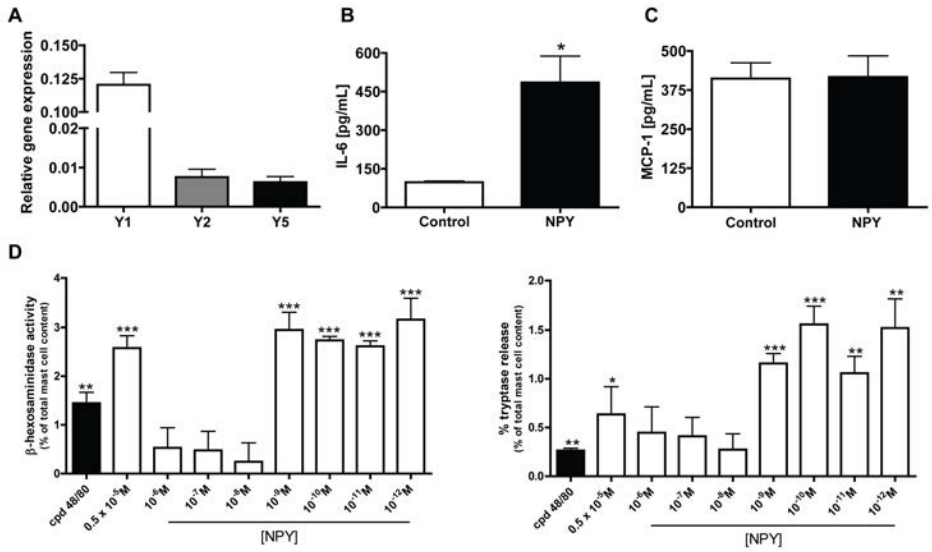

Figure 5. (A) Murine bone marrow-derived mast cells express neuropeptide Y receptors, with Y1R as the main receptor expressed in our cultures. Incubation of BMMCs with LV.NPY-conditioned VSMC medium resulted in significant IL-6 (B), but not MCP-1 release into the medium (C). Recombinant NPY activates BMMCs both at micromolar and picomolar concentrations. D) β-hexosaminidase activity and tryptase release in the releasate after 30' activation with NPY. (n=3 per condition) *P<0.05, **P<0.01, ***P<0.001

Discussion

Neuropeptide Y (NPY) is an abundant neurotransmitter peripherally co-released with noradrenaline upon stress and could therefore well be implicated in atherosclerotic plaque destabilization. Previously, several SNPs in the NPY gene and a gain-of-function polymorphism was associated with increased atherosclerosis in human.[13,14,15] Furthermore, lesional expression of NPY and its receptors (Y1-6) was assed immunohistochemically in patients with peripheral artery disease (PAD) and healthy volunteers, demonstrating a 3-fold increase in NPY-positive area in PAD.[33]

Here, we demonstrate a similar increase in NPY expression in the vessel wall during atherogenesis and importantly, that its expression is additionally higher in unstable compared to stable lesions and may thus have a contributing effect to both lesion progression and destabilization. We now confirm a pathogenic role for perivascular NPY by using a lentivirus to overexpress NPY near advanced murine carotid artery plaques, which resulted in increased plaque progression. Previously, it was established in rats that angioplasty in combination with NPY pellets near the injured vessel caused rapid occlusive restenosis, with macrophage rich lesions and thrombus formation.[6] Similar results were obtained in carotid or femoral artery endothelial denudation models in mice, demonstrating decreased restenosis upon NPY Y1R antagonism or increased restenosis upon NPY overexpression, respectively.[13,34] Although valuable, the models used in these studies are predominantly injury based and exert their effects via smooth muscle cell proliferation resulting in intimal hyperplasia and as such are less representative for the complex lesion composition observed in atherosclerotic vessels. In contrast, we are the first to show a direct pro-atherogenic effect of NPY in a mouse model of shear and flow dependent atherosclerosis, which results in heterogeneous lesions more closely resembling human atherosclerotic plaques.[17,35] As described before, the collar-induced plaques contain lipid and extracellular matrix deposits and consist of an acellular necrotic core with well-defined fibrous cap.[17]

Interestingly, local NPY overexpression in our model resulted in increased perivascular mast cell degranulation, suggesting that NPY-mediated mast cell activation may be an underappreciated mechanism by which NPY contributes to atherosclerotic plaque progression. In line with these results we and others have previously demonstrated that systemic mast cell activation contributes to atherosclerotic lesion development.[29,32] Mast cell activation was shown to affect lesion progression and destabilization in a mast cell-dependent manner at multiple locations including the aorta, aortic root[32], brachiocephalic artery[29], but also collar-induced carotid artery plaques.[36,37] The results obtained in this study indicate that NPY may be an effective endogenous mast cell activator, particularly as NPY is highly expressed in advanced stages of atherosclerosis, when also the number

of mast cells that have accumulated within the lesion and perivascular tissue is high.[4] Furthermore, immunohistochemical and gene expression analysis of NPY localization in and near the atherosclerotic lesion suggest NPY to be primarily derived from perivascular neurons and medial vascular smooth muscle cells. These results provide further evidence for a role of NPY in triggering mast cells, as they also accumulate near the intima-media interface.[38]

Current literature on NPY-mediated mast cell activation is limited. While earlier studies have demonstrated that high (μM) concentrations of NPY potently trigger histamine release from isolated human skin mast cells or rat peritoneal and dural mast cells, no further characterization of the mast cell releasate was described.[39,40,41,42] Although specific NPY receptor antagonists were not widely available at that time, the use of receptor-specific truncated forms of NPY did not conclusively point towards a specific receptor pathway and at high concentrations even suggested receptor-independent G-protein activation.[39] Various other neuropeptides are known to activate mast cells both in a receptor-independent and receptor-dependent manner, at high (μM) and low (pM - nM) neuropeptide concentrations, respectively.[42] Here we show that mouse BMMCs express the NPY receptor Y1, and to a lesser extent Y2 and Y5 and release several pro-atherogenic mediators including IL-6, tryptase and β-hexosaminidase in response to NPY. Whether activation of BMMCs at the different concentrations depends on one or a combination of specific NPY receptor types remains to be elucidated. The potent release of IL-6 from NPY-stimulated BMMCs is especially interesting, as mast cell derived IL-6 and IFN-γ, but not TNFα, were previously shown to be the prime factors necessary to restore atherogenesis in mast cell reconstitution experiments in mast cell-deficient mice.[32] As physiological levels of NPY, both in human[43] and mice[44] seem to be in the high picomolar range to low nanomolar range, activation and release of pro-inflammatory mediators at these concentrations is relevant for disease progression. Several studies have shown destabilization of atherosclerotic lesions by acute mast cell activation, illustrated by increased incidence of intraplaque hemorrhaging and vascular smooth muscle cell apoptosis.[4,45,36] Here we demonstrate that despite an increase in perivascular mast cell activation, no apparent differences in collagen content and overall stability of the plaques. This may suggest a difference in the pathological effects of acute mast cell activation compared to the prolonged presence of mast cell triggers. Furthermore, these data are in line with our previous studies, in which systemic mast cell activation also resulted in increased plaque progression, while not affecting lesion composition.[29] In contrast to sustained high or accumulating NPY concentrations, acute NPY release from sympathetic nerve terminals, for example after exposure to certain types of stressors, may induce acute mast cell activation in the vessel wall and have an additional impact on plaque stability.

Although the proinflammatory mast cell mediator release upon NPY treatment seems an attractive mechanisms by which perivascular NPY overexpression contributes to atherosclerosis development in our model, we cannot exclude additional mast cell-independent effects. In addition to well-described vasopressor effects, NPY has been shown to affect monocyte and macrophage function.[11,31] While we did not observe significant differences in relative macrophage content of the lesions, functional differences may have been underappreciated. However, in vitro incubation of bone marrow-derived macrophages with NPY, a condition which resulted in pro-inflammatory mediator release from mast cells, did not skew the expression of several macrophage phenotype markers towards a more pro-inflammatory M1 state. The current experimental setup primarily focused the effects of NPY to the adventitial side of the carotid artery, thus without systemic effects on vascular tone. Therefore, we argue that, if any, possible effects on vascular tone and endothelial cells are probably indirectly mediated.

In conclusion, we here demonstrate that NPY is increasingly expressed during human and murine atherosclerosis development with a 2-fold increase in unstable compared to stable lesions. Local overexpression of NPY at the site of plaque formation resulted in a 70% increase in neointima area, while relative composition of the plaques was similar. Importantly NPY overexpression caused a significant increase in perivascular mast cell activation, which may be, at least partly, responsible for the increased progression of atherosclerosis. In favor of this, we demonstrate that NPY induces proinflammatory mediator release from isolated mast cells. The above results highlight NPY as an important factor in plaque progression and an endogenous trigger of perivascular mast cell activation.

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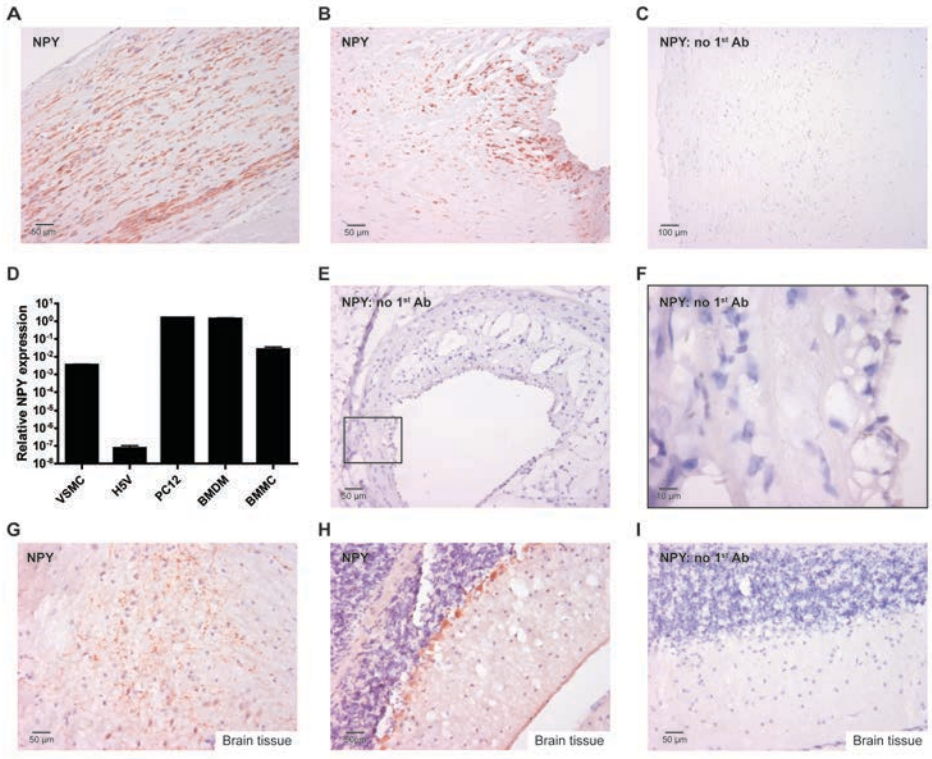
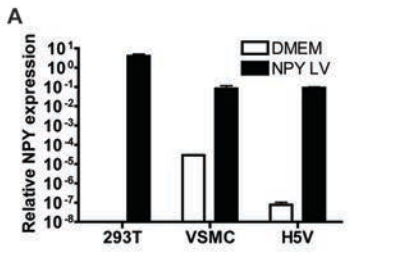
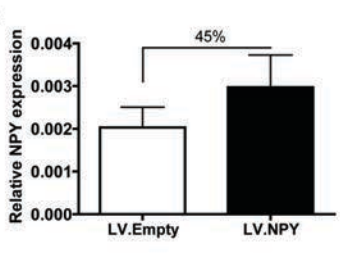
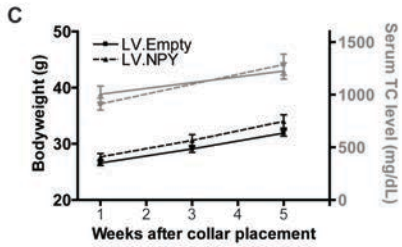
Supplemental data



Supplemental figure 1. Representative micrographs of NPY immunohistochemical staining (brown color, arrows) in stable (A) and unstable (B) human carotid endarterectomy specimens, demonstrating NPY expression in medial vascular smooth muscle cells and lesional macrophages. 200x magnification. (C) Human endarterectomy specimen negative control staining without primary antibody. 100x magnification. (D) RT-PCR analysis of in vitro NPY expression (n=3 per condition) in vascular smooth muscle cells (VSMC), endothelial cells (H5V), neuronal cells (PC12), bone marrow-derived macrophages (BMDM) and bone marrow-derived mast cells (BMMC). (E, F) Murine carotid artery plaque negative control staining without primary antibody. 200x and 1000x magnification. (G-H) NPY positive control staining of mouse brain tissue, demonstrating clear NPY-positive soma and processes. 200x magnification. (I) Negative control. nd, not detectable.



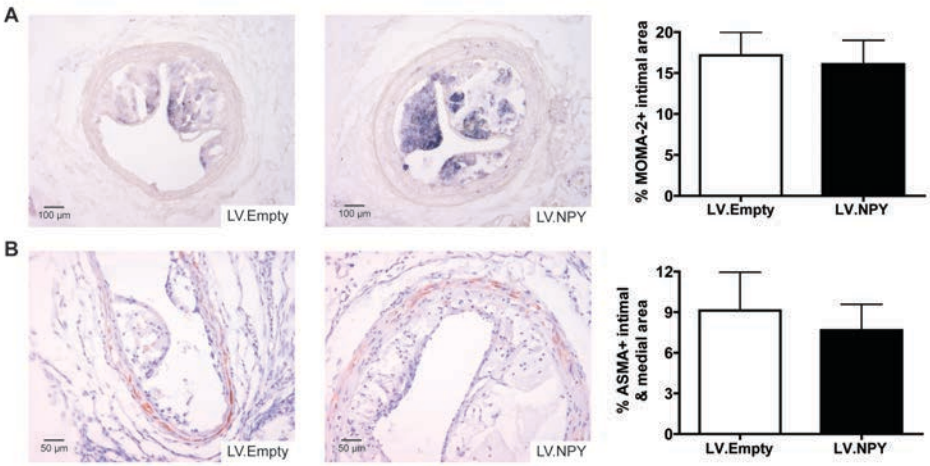




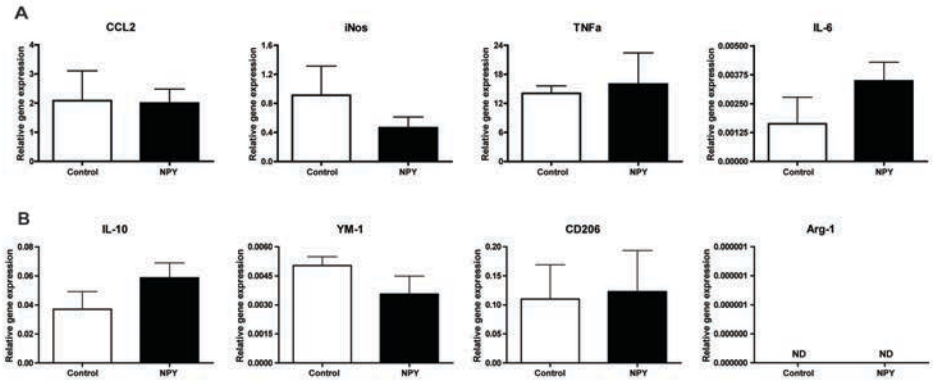
D

	LV.Empty (10^9 / L)	LV.NPY (10^9 / L)
WBC	7.44 ± 0.38	6.80 ± 0.43
lymphocytes	4.61 ± 0.31	4.48 ± 0.34
monocytes	0.93 ± 0.06	0.79 ± 0.05
neutrophils	1.60 ± 0.03	1.33 ± 0.13
eosinophils	0.31 ± 0.02	0.21 ± 0.03

Supplemental figure 2. (A) Lentiviral NPY overexpression (n=3 per condition) was determined in vitro in three cell lines, 293T cells, VSMC and the endothelial cell line H5V. (B) In vivo NPY expression in the carotid artery was determined 2 weeks after collar placement and lentivirus application, demonstrating a 45% increase in NPY expression (n=4 per group). (C) No systemic effects on bodyweight, total cholesterol or circulating white blood cell levels upon LV. NPY treatment (n=12 per group).



Supplemental figure 3. No difference in relative macrophage content and α-smooth muscle cell positive area between LV.NPY and LV.Empty treated mice (n=12 per group). (A) Macrophage content of the lesions was determined by MOMA-2 staining. 100x magnification. (B) Contractile smooth muscle cell content of the medial and intimal layer was determined by α-SMA staining. 200x magnification.



Supplemental figure 4. RT-PCR analysis of macrophage M1 (A) and M2 (B) phenotype marker gene expression in bone marrow-derived macrophages incubated with control or NPY conditioned medium. No significant differences were observed between the two treatments (n=3 per condition).

5

Chapter 6

Neuropeptide Y receptor Y1, Y2 and Y5 antagonism accelerates atherosclerotic lesion development in LDL receptor-deficient mice

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Abstract

Objective: Neuropeptide Y is an abundantly expressed stress–related hormone capable of modulating metabolic and immune responses involved in the development and progression of atherosclerosis. NPY mediates its effects via multiple G-protein coupled receptors (Y1-Y6), which are differentially expressed throughout the central nervous system and the periphery. Here we investigated the therapeutic potential of systemic NPY Y1, Y2 or Y5 receptor antagonism on atherosclerosis development in $LDLr^{-/-}$ mice.

Methods and Results: Gene expression profiles of NPY and its receptors were obtained by qPCR analysis of collar-induced atherosclerosis specimens and guide wire-induced restenotic carotid arteries. NPY was significantly increased during disease progression in both models, while Y1 receptor expression was almost completely abolished. Expression of Y2 and Y5 as well as DPPIV was generally increased, rendering intervention in Y2 and Y5 signalling of therapeutic interest. Systemic treatment of high fat diet-fed $LDLr^{-/-}$ mice with the Y1 (BIBO-3304), Y2 (BIIE-0246) or the Y5 (CGP-71683) antagonists for 6 weeks resulted in increased lesion development, especially in the Y2 receptor antagonist treated mice. Changes in triglyceride metabolism in white adipose tissue and a general increase in systemic inflammation, e.g. the pro-inflammatory and proatherogenic IL-12 family cytokines, seem to contribute to the observed increase in atherosclerosis.

Conclusions: NPY and its receptor system is highly involved in a wide array of physiological and pathological processes including atherosclerosis. Here we provide evidence for a pro-atherogenic effect of chronic systemic NPY receptor antagonism acting through both metabolic changes and inflammatory cytokine production.

Introduction

Neuropeptide Y (NPY), a 36-amino acid peptide, is a ubiquitous hormone with well-established central and peripheral functions, ranging from regulation of energy balance to modulation of immune responses. NPY is expressed in the brain and peripheral nervous system and acts through multiple G-protein coupled receptors (Y1-Y6), which are differentially expressed throughout the body.[1]

After its discovery by Tatemoto *et al*. in 1982[2], NPY-like immunoreactivity was demonstrated in specific areas of the brain, especially the hypothalamic brain structures, and peripherally throughout the sympathetic nervous system. Peripheral NPY distribution parallels that of tyrosine hydroxylase (TH) and dopamine-beta-hydroxylase, both rate limiting enzymes in the catecholamine synthesis. NPY is often co-stored and co-released with norepinephrine and dopamine. High concentrations of NPY were demonstrated in highly innervated tissues, such as the heart, spleen, kidney and around blood vessels. The connection with the stress response is further strengthened by bidirectional modulation of the expression of NPY by glucocorticoids and vice versa.[3]

The first identified biological function of NPY was its potent vasoconstrictive effect on multiple vascular beds, including the cerebral and renal arteries[4,5,6] and points towards involvement of NPY signalling in vascular homeostasis and disease. Extensive work by Zukowska et al. elucidated potent growth promoting properties of NPY on vascular smooth muscle cells, endothelial cells and adipocytes. Signalling through its Y1 or Y2 receptor was demonstrated to induce vascular smooth muscle cell and endothelial cell proliferation contributing to angiogenesis.[7,8] Furthermore, the NPY-NPY2R system was shown to be involved in stress-induced augmentation of obesity and metabolic syndrome by stimulation adipogenesis, as well as white adipose tissue inflammation and angiogenesis.[9]

While all animals express NPY and its receptors, clear species differences exist. For example, the Y4 receptor is found in rodents and has high affinity for the NPY-related peptide PP but is not found in humans. The Y6 receptor is expressed in many species except the rat and only in a truncated form in various primates, including humans.[10] In addition, the different receptors have different binding preferences for NPY-family members and truncated forms of the NPY protein. A key enzyme involved in the proteolysis of NPY is the abundantly expressed dipeptidyl peptidase IV (DPPIV) cleaving NPY into NPY3-36, which lacks Y1 receptor affinity and thus shifts the responses towards Y2- and Y5 receptor mediated effects. High vascular expression of NPY is considered to be pro-atherogenic. A gain-of-function mutation in the preproNPY gene strongly correlates with increase intima media thickness[11] and increased expression of NPY and the Y1, Y2 and Y5 receptors was demonstrated in patients with peripheral artery disease in the carotid or femoral arteries by means of immunohistochemical stainings.[12] In addition, NPY signalling

was shown to be intimately linked to stress induced accelerated atherosclerosis in various animal models.[12,13,14] Furthermore, we recently indicated plaque NPY to be associated with plaque vulnerability and NPY-induced mast cell activation as a contributing mechanism to atherosclerosis development.[15] Like mast cells, many, if not all, cells of the immune system express functional NPY receptors. For example, NPY induced polarization of T cell responses[16] and pro-inflammatory cytokine production by macrophages[17] are likely mechanisms involved in NPY-mediated accelerated atherosclerosis.

With respect to NPY receptor antagonism in atherosclerosis, the main focus has been on the Y1 receptor-mediated vasoconstrictive and growth promoting properties, mostly in concert with Y5. Accelerated angioplasty-induced restenosis by local NPY treatment was shown to be completely blocked continuous infusion with H409/22 or CGP71683, which are Y1 or Y5 receptor antagonists, respectively.[13] Similar beneficial results were obtained in a restenosis model in apoE$^{-/-}$ mice by BIBP-3226 treatment, another Y1 receptor specific antagonist.[18] In contrast, a recent study evaluating systemic Y1 receptor antagonism in apoE$^{-/-}$ mice fed a high fat diet, a model of atherosclerosis rather than restenosis, surprisingly demonstrated accelerated atherosclerosis in NPY and Y1 receptor antagonist treated mice.[19] Mainly pro-atherogenic inflammatory IL-12 production and possibly increased leptin secretion was thought to result in increased atherosclerosis under hyperlipidemic conditions.

Considering the proatherogenic effects of NPY, and the vascular expression of the different NPY receptors and differential expression at specific stages of human disease progression[20], we aimed to determine NPY receptor expression during the development and progression of atherosclerosis and subsequently evaluate the therapeutic potential of systemic Y1, Y2 or Y5 receptor antagonism in the prevention of atherosclerosis.

Materials and Methods

Neuropeptide Y and receptor expression during atherosclerosis development and restenosis

All animal studies were performed in compliance with Dutch government guidelines, the Directive 2010/63/EU of the European Parliament and were approved by the animal welfare committee of the Leiden University Medical Center (approval reference number 12102). Diet and water were provided ad libitum.

To determine the gene expression profiles of NPY, Y1R, Y1R, Y1R and DPPIV during atherosclerotic lesion progression apoE$^{-/-}$ mice were put on Western type diet containing 0.25% cholesterol and 15% cacao butter (SDS, Sussex, UK) and equipped with bilateral semi-constrictive collars (2mm long, 0,3mm in diameter) around the left and right common carotid artery as described by von der Thusen *et*

al.[21] Before and at 2 week intervals after collar placement, 6 mice were sacrificed and the carotids isolated for gene expression analysis. Total RNA was extracted from 3 pooled carotids (n=4 per timepoint) with the guanidium thiocyanate-phenol-bromochloropropane extraction method[22] and RNA concentration, purity and integrity were examined by nanodrop (Nanodrop® Technologies). RNA was reverse transcribed by M-MuLV reverse transcriptase (RevertAid, MBI Fermentas, Landsmeer, The Netherlands) and used for quantitative PCR analysis with an ABI PRISM 7700 Taqman apparatus (Applied Biosystems). Murine HPRT, RPL27, Gusb and 36B4 were used as standard housekeeping genes. qPCR primer pairs are given in supplemental table 1. A similar setup was used to determine the gene expression of NPY and its receptors during progression of restenosis using a wire-induced denudation model as described previously.[23,24] In short, the right carotid artery was dissected free and denudated using a 0.36-mm guide wire, which results in a proliferative VSMC-rich lesions within 4 weeks. Before and every week after denudation 6 mice were sacrificed and the carotids isolated for gene expression analysis.

Systemic NPY receptor (Y1, Y2 or Y5) antagonism during atherosclerosis development

To study the effect of NPY receptor antagonism on atherosclerosis development we fed low-density lipoprotein receptor deficient ($LDLr^{-/-}$) mice a Western-type diet (WTD) for eight weeks. Mice were obtained from the local animal breeding facility (Gorlaeus Laboratories, Leiden, The Netherlands) and treatment groups were randomized based on age and weight at the start of the experiment. After 2 weeks on WTD and onwards mice were injected i.p. three times a week with 100ul PBS (1% DMSO), Y1 receptor antagonist BIBO-3304 trifluoroacetate (400μM; #2412; Tocris Bioscience, Ellisville, MO, USA), Y2 receptor antagonist BIIE-0246 (350μM; #1700; Tocris Bioscience) or Y5 receptor antagonist CGP 71683 hydrochloride (750μM; #2199; Tocris Bioscience). Bodyweight, serum cholesterol and triglyceride levels were checked regularly throughout the study. Hematological parameters (including white blood cell count and leukocyte subpopulations) were analyzed using an automated XT-2000iV veterinary hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany). After 6 weeks of treatment, mice were anaesthetized by subcutaneous injection of ketamine (60 mg/kg, Eurovet Animal Health, Bladel, The Netherlands), fentanyl citrate and fluanisone (1.26 and 2 mg/kg, respectively, Janssen Animal Health, Sauderton, UK). Adequacy of anaesthesia was monitored by regular visual inspection and toe pinch reflex. Mice were exsanguinated via orbital bleeding and in situ perfused with PBS after which the hearts were excised and stored in 3.7% neutral-buffered formalin (Formal-Fixx, Shandon Scientific Ltd., Runcorn, UK) for further analysis.

Histological analysis and morphometry

To determine plaque size, serial 10 µm cryosections of the aortic root were cut using a Leica CM3050S cryostat and stained with Oil-Red-O and hematoxylin (Sigma-Aldrich, Zwijndrecht, The Netherlands). Plaque size was analyzed in 5 consecutive sections, starting at the point where all three aortic valve leaflets first appeared, with a Leica DM-RE microscope and LeicaQwin software and represented as mean plaque area and total plaque volume (AUC). Corresponding sections on separate slides were stained with a macrophage specific antigen (MOMA-2, monoclonal rat IgG2b, Serotec, Raleigh, NC, US) and alkaline phosphatase conjugated secondary antibody (Sigma-Aldrich). A Masson's trichrome (Sigma-Aldrich) staining was performed to analyze plaque collagen content and necrotic core area, which was defined as the a-cellular, debris-rich plaque area. Macrophage, collagen and necrotic core area were measured as percentage of total plaque area. Medial and intimal vascular smooth muscle cell content was determined by α-smooth muscle cell actin staining (Sigma-Aldrich). Mast cells were visualized with a naphthol AS-D chloroacetate esterase staining kit (Sigma-Aldrich) and counted manually. A mast cell was considered resting when all granula were maintained inside the cell, while mast cells were assessed as activated when granula were deposited in the tissue surrounding the mast cell. All morphometric analyses were performed by blinded independent operators.

Spleen and liver RNA isolation and gene expression

At sacrifice spleen and liver tissue were isolated, immediately snap frozen in liquid nitrogen and stored at -80°C until RNA isolation. After tissue homogenization with a Potter tissue homogenizer Total RNA was extracted, reverse transcribed and used for gene expression analysis as described above.

Serum cholesterol and triglyceride levels

Serum concentrations of total cholesterol and triglyceride were determined by enzymatic colorimetric assays (Roche Diagnostics, Mannheim, Germany) in 96-well plates (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). Precipath (standardized serum, Roche Diagnostics) was used as internal standard in the cholesterol and triglyceride assay.

Statistical analysis

Data are expressed as mean ± SEM. An unpaired two-tailed Student's t-test was used to compare normally distributed data between two groups of animals. Data of three groups were analyzed with one-way ANOVA and data of two groups with more than one variable were analyzed by two-way ANOVA, both followed by Tukey's multiple comparison test. A level of $P<0.05$ was considered significant.

Results

Differential expression of NPY and its receptors during atherosclerosis and restenosis

To investigate the involvement of NPY signaling through its G-protein coupled receptors Y1, Y2 or Y5, in atherosclerotic lesion development we first determined gene expression levels in two different vascular disease models (i.e. a collar-induced atherosclerosis model and a wire-induced denudation model of restenosis). Atherosclerotic lesions were induced in apoE$^{-/-}$ mice fed a Western-type diet by means of bilateral collar placement. Similar to previously published microarray results[15], expression of NPY was significantly increased during atherosclerotic lesion development (figure 1A). Interestingly, while Npy1r was highly expressed in healthy carotid arteries, its expression was almost completely abolished upon disease initiation and remained low during the whole experiment. Expression of Npy2r and Npy5r fluctuated but was generally higher in advanced atherosclerosis. Interestingly, DPPIV expression was highly induced and mirrored the effect on expression of the Y1 receptor, possibly further inducing NPY signaling via the Y2 and Y5 receptors. As NPY is known to have potent vasoconstrictive and angiogenic properties, we analyzed the expression NPY and its receptors in restenotic vessels, which is a primarily VSMC proliferation-driven process, in contrast to atherosclerosis, which is known to mainly be macrophage- and lipid-driven. Strikingly, the expression profiles were very similar to the atherosclerotic lesions, with increased NPY expression especially at later stages of the disease and a severe reduction in Npy1r expression (figure 1B). In contrast to the atherosclerotic lesions, Npy2r and Npy5r expression was initially reduced after endothelial denudation, but recovered gradually to baseline expression levels at 4 weeks.

Effects of systemic NPY receptor antagonism on bodyweight, cholesterol and triglyceride homeostasis and glucocorticoid levels.

The differential expression of the NPY receptors on various tissues and cell types involved in atherosclerosis combined with the expression profiles during atherosclerosis development prompted us to investigate the effects of Y1, Y2 and Y5 receptor antagonists in an atherosclerotic mouse model. To induce atherosclerosis we fed male LDL$^{r-/-}$ mice a high cholesterol diet for 8 weeks combined with systemic antagonist treatment for the Y1, Y2 or Y5 receptor. As central NPY is a potent orexigenic hormone, mediating appetite, hepatic triglyceride (TG) secretion and TG storage in white adipose tissue[25,26], we determined bodyweight, total cholesterol and triglyceride levels throughout the study. No apparent effect of the antagonist on bodyweight or serum cholesterol could be observed (figure 2A; B), however plasma triglyceride levels were significantly elevated at 4 weeks

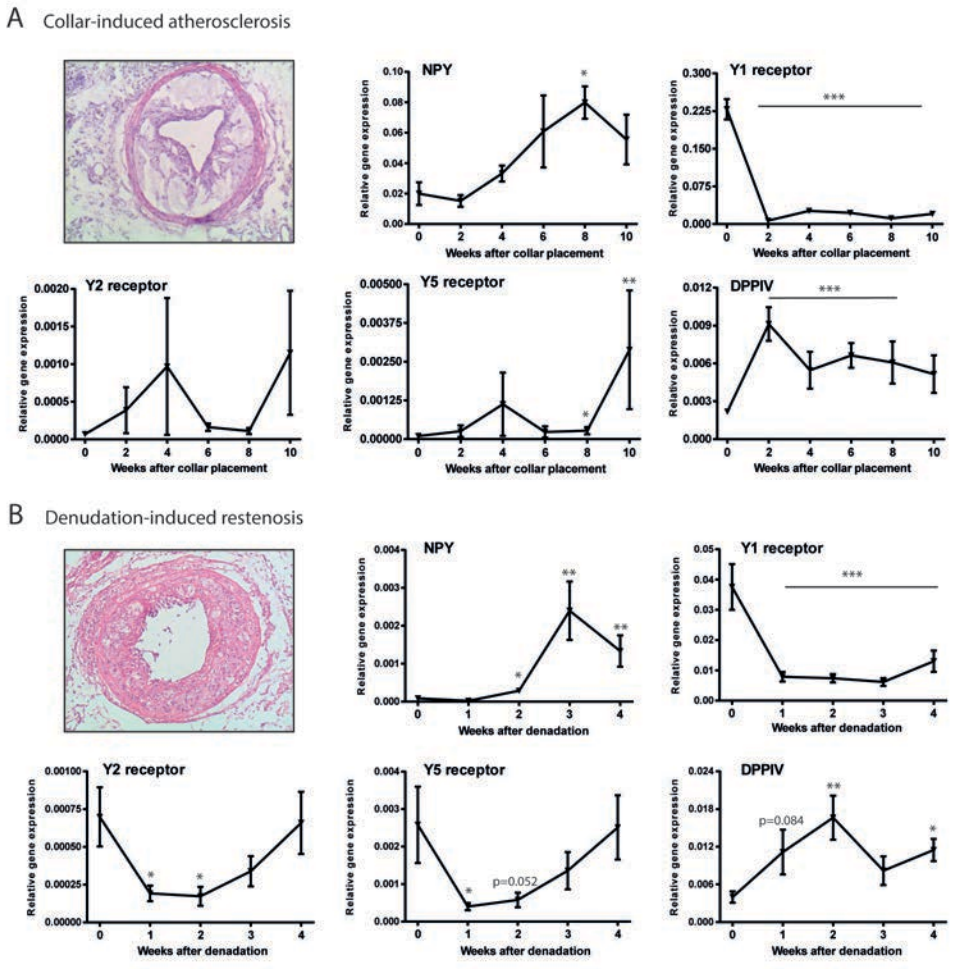

Figure 1. Gene expression levels of NPY, its receptors Y1, Y2, Y5 and peptidase DPPIV the during (A) carotid artery atherosclerosis progression induced by semi-constrictive collar placement and (B) carotid guide wire-induced restenosis.

of Y1 treatment (figure 2C). As previous research indicated the effect of central NPY infusion on TG levels to act within 2 hours, we determined plasma TG levels 1,5 hour after injection with the antagonists. A significant increase in plasma TG levels upon Y1 and Y5, but not Y2 receptor antagonism could be observed (figure 2D). NPY is co-released with catecholamine and glucocorticoids during stress and NPY receptor expression is abundant on the adrenal glands.[27] To account for the possible contribution of changes in circadian levels of glucocorticoids, we measured morning and late afternoon plasma corticosterone levels after 5 weeks of receptor antagonist treatment. As depicted in figure 2E, no significant differences in circulating hormone levels could be observed.

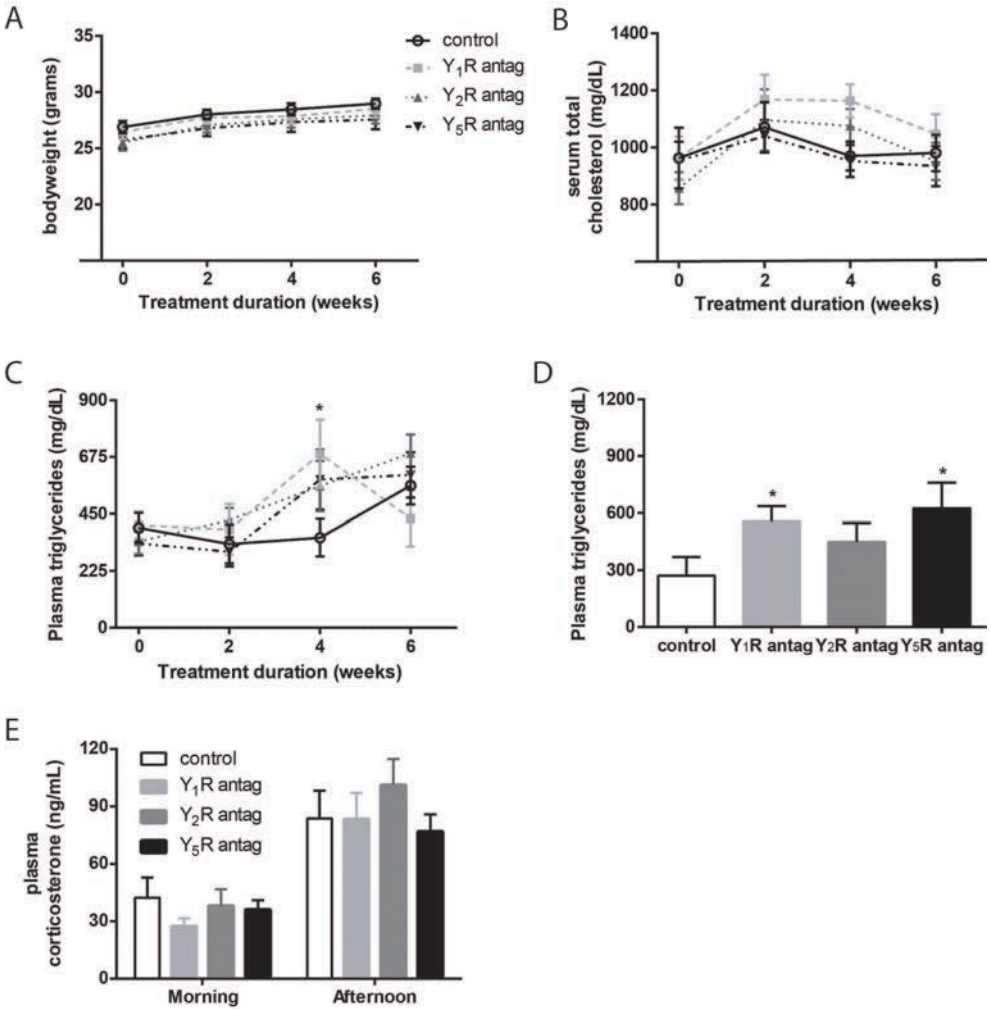

Figure 2. A) Bodyweight throughout the experiment. Mice were put on Western type diet t = -2 weeks before the start of NPYr antagonist treatment at t = 0. B) Total serum cholesterol levels at 2 week intervals during the treatment period. C) Plasma triglyceride levels at two week intervals during the antagonist treatment period. Significant increase in TG levels at 4 weeks of Y1 receptor antagonist treatment compared to control. D) Plasma triglyceride levels 1,5 hour after antagonist injection at 5 weeks of treatment. E) Plasma corticosterone levels in the resting (early morning) and active phase (late afternoon). P <0.05 vs control treatment is considered significant.

Chronic NPY receptor antagonism during atherosclerotic lesion progression.

In our study we monitored circulation levels of the main leukocyte subpopulations (i.e. neutrophils, lymphocytes, monocytes and basophils) during systemic antagonist treatment. We observed limited effects on these cell populations (supplemental figure 1), only Y5 antagonism reduced neutrophil monocyte numbers at two weeks of treatment and Y2 antagonism significantly increase the neutrophil count at sacrifice, after 6 weeks of treatment.

Next we analyzed the atherosclerotic plaque burden in the aortic root by means

of Oil-red-O staining of histological sections. While all antagonist-treated mice appeared to have a higher plaque burden compared to control (1% DMSO) mice, only the Y2 treated mice had statistically significant larger plaques (figure 3A; 1.4-fold increase). In addition to plaque burden, lesion stability or vulnerability to erosion or rupture is an important measure of disease progression and strongly correlates with clinical outcome. We determined lesion stability by quantifying the macrophage and collagen content of the plaque, as well as and the necrotic core area. The amount of intimal macrophages (as percentage of total plaque area) was significantly reduced in Y1 and Y2 antagonist treated mice (Figure 3B).

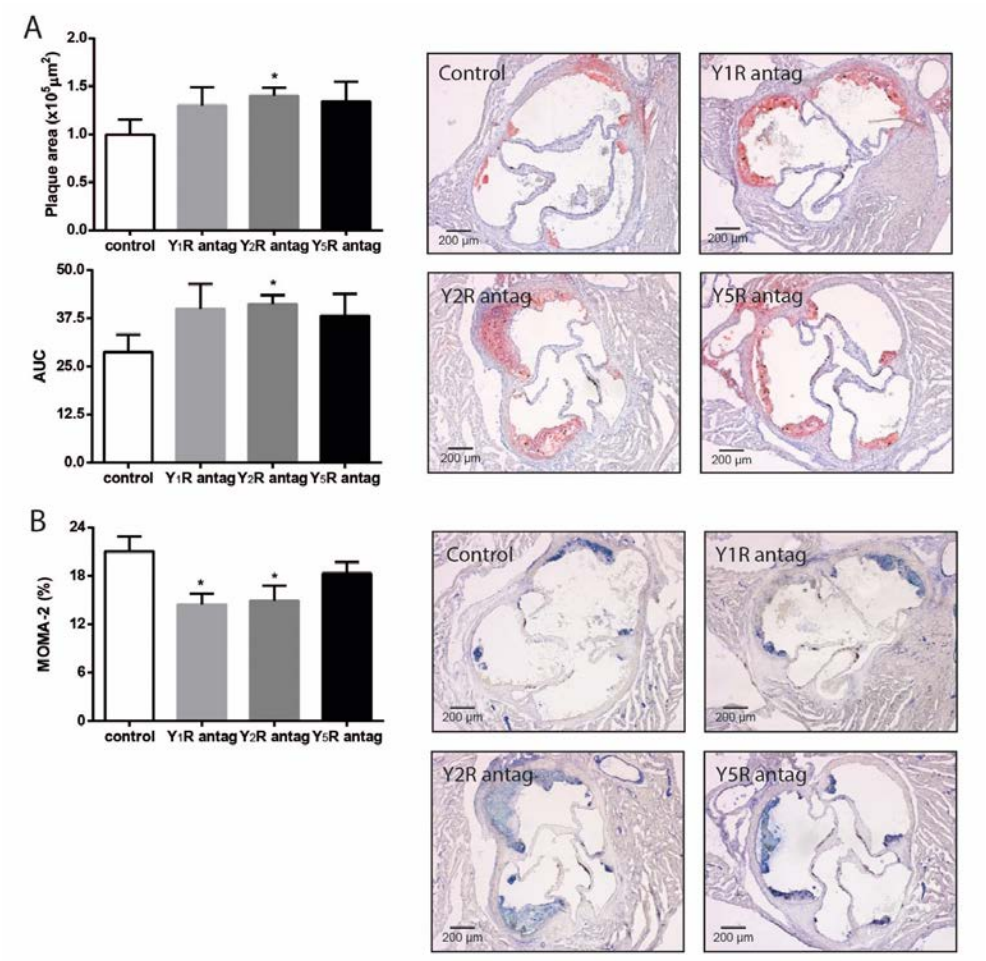

Figure 3. A) Atherosclerotic lesion formation in the three valve area of the aortic root was assessed by Oil-red-O staining. B) Macrophage content of the lesions as determined by MOMA-2 staining and depicted as percentage of lesion area.

As most lesions were early lesions, intimal collagen deposition was limited and not significantly different between treatment groups (figure 4A). Also the amount of necrosis was not significantly different, although appeared somewhat more

prominent in the larger lesions of Y2 antagonist treated mice. Intimal smooth muscle cells, measured by α-smooth muscle staining, were decreased by Y2 antagonist treatment (2.41 ± 0.006% vs 4.43 ± 0.007% in control mice) and demonstrated a trend towards a decrease in the media of Y2 and Y5 antagonist treated mice (figure 4B). Combined, the morphological analysis at least partly confirm the pro-atherogenic effect of systemic Y1 receptor antagonism, but provides additional evidence for an even more pronounced pro-atherogenic role for Y2 receptor antagonist treatment.

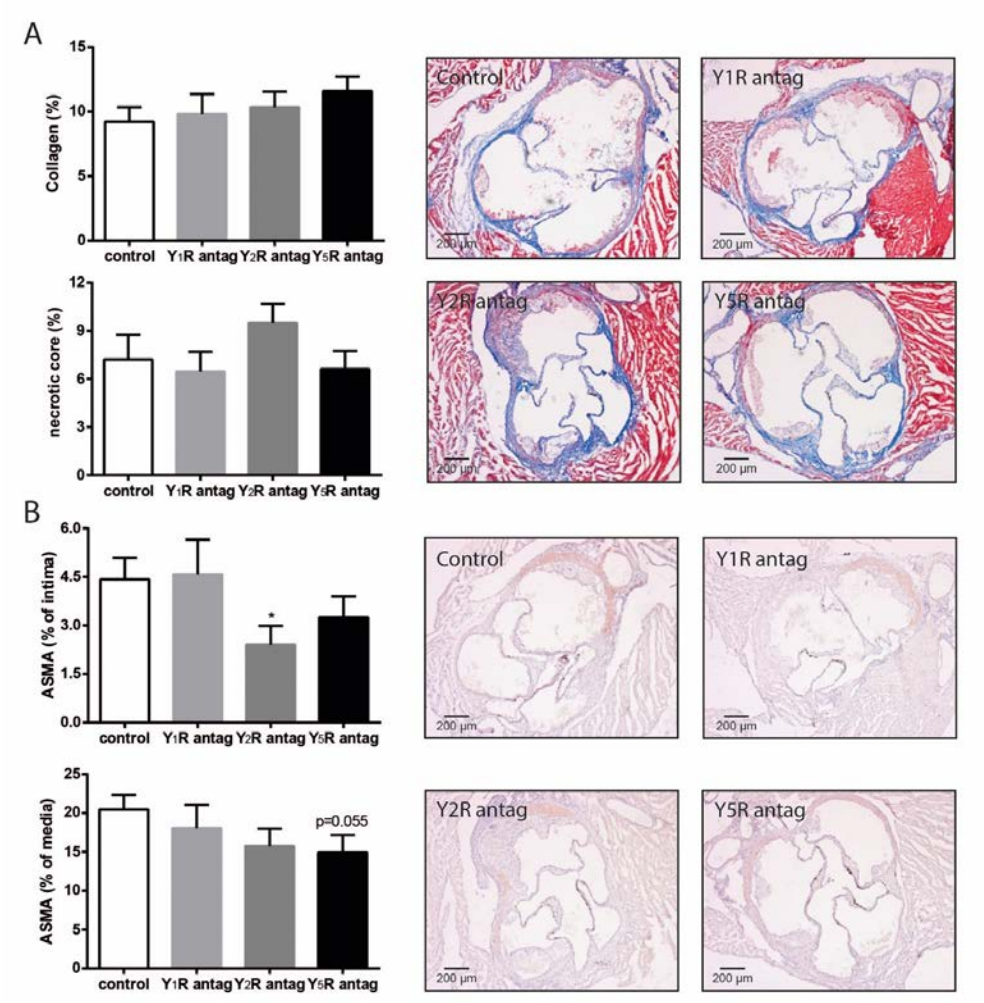

Figure 4. A) Collagen content of the atherosclerotic lesions in the three valve area of the aortic root was assessed by Masson's trichrome staining as percentage of total lesion area. B) Intima and media vascular smooth muscle was determined by alpha smooth muscle actin staining and represented as percentage of the intima or media area.

Mast cell accumulation upon systemic Y2 receptor antagonist treatment.

Previous results from our lab have implicated NPY-induced perivascular mast cell activation as a potential contributing factor to atherosclerotic lesion development.[10] To evaluate the effect of systemic NPY receptor antagonist treatment on perivascular mast cell responses we quantified the amount of cardiac mast cells in the aortic root sections and determined their activation status. A trend towards more mast cells in the Y2 antagonist treated mice (p=0.055) could be observed (figure 5A). However, the percentage of activated mast cells was similar between treatment groups (figure 5B), suggesting the increased number to primarily reflect the more advanced and inflamed plaque in the Y2 antagonist mice.

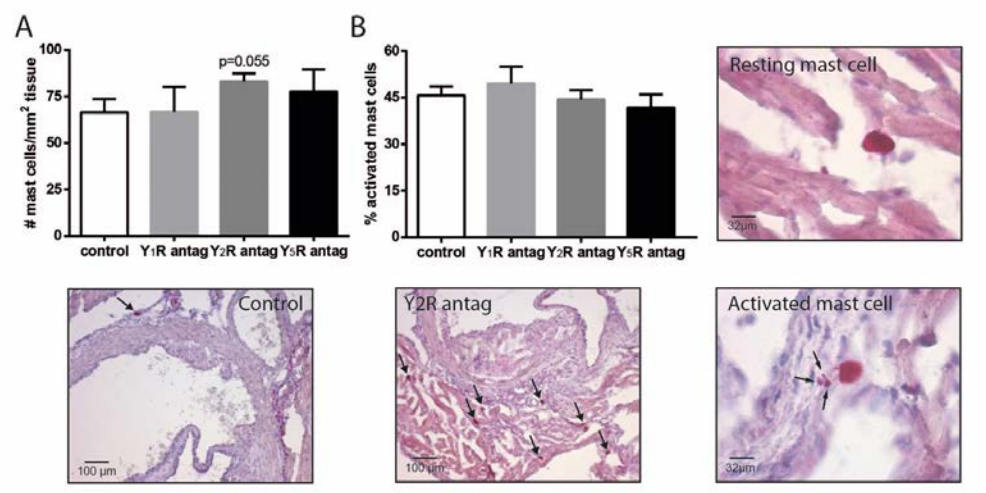

Figure 5. A) Mast cells were visualized and scored by naphtol AS-chloroacetate staining. B) Activation status is illustrated by the presence of granules in the surrounding tissue.

NPY receptor antagonist induced changes in the pro-atherogenic IL-12 cytokine family.

Earlier data on systemic Y1 receptor treatment indicated an important role for the pro-inflammatory cytokine IL-12 in mediating the increase in atherosclerosis.[19] To evaluate the contribution of the IL-12 cytokine family, we determined the expression levels of p40, p35, p19, p28 and Ebi3 in the liver and spleen. The IL-12 subunits p40 and p35 were not significantly altered by antagonist treatment, however we did observe a significant reduction in p35 expression in the liver after Y5 antagonist treatment. The observed changes in gene expression in correlated to some extent with the circulating levels of IL-12 at sacrifice, demonstrating higher levels in the Y1 and Y2 treated mice and a reduction upon Y5 antagonist treatment (Suppl fig. 2A; top left panel). Interestingly, expression of Ebi3, which in complex with p28 forms the proatherogenic cytokine IL-27[29], was significantly higher in the Y1 treated mice and trended towards higher expression in the Y5

mice. Liver expression levels of Ebi3 were similarly increased in Y1 antagonist treated mice, but reduced in Y5 treated mice. (supplemental figure. 2B)

Discussion

Atherosclerosis and its clinical manifestations remain a leading cause of death and an enormous burden on society. Both metabolic- and immune-dysregulation are key pathological processes involved in atherosclerotic lesion development and progression. In addition, increased recognition is given to the brain-immune and brain-gut axis in contributing to various diseases, including cardiovascular disease. Neuropeptide Y is an abundant (stress-related) hormone with variety of known functions both in health and disease. Being co-released with and potentiating the effects of norepinephrine and ATP released from sympathetic neurons innervating for instance the vasculature, NPY has been shown to be pro-atherogenic and responsible for mediating stress-induced accelerated restenosis and atherosclerosis.[30]

In the current study we investigated the therapeutic potential of systemic NPY receptor antagonism of each of its three main receptors, Y1, Y2 and Y5 in atherosclerosis. First, expression of NPY and its receptors was assessed in atherosclerotic and restenotic carotid artery specimens. As previously reported by us and others, NPY expression was significantly increased in atherosclerotic arteries. Similar to expression levels observed in human carotid endarterectomy specimens[20], Npy1r receptor expression was significantly reduced upon disease initiation, which possibly questions the usefulness of Y1 receptor antagonism. However, contrasting data demonstrating increased expression of all three NPY receptors in the carotids and femoral arteries of patients with peripheral artery disease compared to healthy iliac arteries also exist.[12] In our study, Npy2r and Npy5r expression was generally increased and combined with the strong induction of DPPIV may suggest a shift towards Y2 and Y5-mediated effects.

Morphological analysis of the plaque burden in the aortic root after 6 weeks of NPY receptor antagonist treatment revealed a significant increase in plaque size in the Y2 receptor antagonist treated mice. In fact, also Y1 and Y5 receptor antagonism showed a trend towards more advanced lesions. Although the amount of circulating monocytes, or any of the other main white blood cell subpopulations, was not significantly altered during the treatment period, plaque macrophage content was significantly decreased by Y1 and Y2 antagonist treatment. Whether this is a direct effect on macrophage function or on monocyte recruitment via changes in endothelial adhesion molecule expression requires addition research. In vitro neutrophil and monocyte adhesion to human umbilical vein endothelial cells was previously shown to be significantly increased upon incubation with NPY[31] and NPY has been shown to have a variety of stimulatory and inhibitory functions on

macrophages and T cells via its different receptors.[16, 17]

We and others previously observed increased NPY expression in vulnerable atherosclerotic lesions compared with stable lesions, suggesting the involvement of NPY in lesion destabilization.[15,20] In line with previous results obtained with local Y1 receptor antagonism in an endothelial denudation model[18], systemic administration of NPY receptor antagonists did not result in a significant difference in collagen content nor a difference in necrotic core area of the plaques between the treatment groups. However, lesions were relatively small and primarily foam cell-rich lesions, which generally lack a clear collagen rich cap or intimal necrosis. Furthermore, no extensive differences in VSMC content of the lesions was observed except for a significant decrease in intimal α-SMA staining in the Y2 antagonist treated mice and a trend towards a decrease in the Y5 receptor antagonist treated mice. Previous results from restenosis models actually observed reduced intimal hyperplasia upon antagonism of the Y1 or Y5 receptor or both receptors combined[13,18], resulting of reduced smooth muscle cell proliferation. Lack of such effects in our atherosclerosis model may be reflect the strongly reduced Y1 receptor expression and relative initial, and thus smooth muscle cell poor, lesions. Perivascular mast cell activation and IL-6 secretion was previously shown to contribute to NPY-induced accelerated lesion development in apoE$^{-/-}$ mice[15], suggesting the potential anti-inflammatory benefit of NPY receptor antagonism. However, in line with an overall increase in atherosclerosis progression and inflammation in the Y2 receptor antagonist treated mice, mast cell numbers were actually increased in the cardiac tissue surrounding the aortic root without significant differences in activation status. Interestingly, plasma triglyceride levels were transiently increased at 4 weeks of Y1 receptor antagonist treatment and especially elevated shortly (1,5h) after both Y1 and Y5 antagonist injection. Previous results on acute central NPY infusion demonstrated increased food intake and triglyceride secretion in rats mediated via the Y1 receptor.[32] In mice however, NPY administration increased food intake without affecting hepatic VLDL-TG production. Whether the observed increase in TG secretion in our model reflects central modifications or peripheral effects deserves further investigation.

In agreement with previous results demonstrating an increased systemic inflammatory response upon Y1 receptor antagonism, splenic and liver expression of subunits of the pro-inflammatory cytokine IL-12 family were increased in the Y1 and Y5 treated mice and somewhat reflected in increased circulating level of IL-12 p70. Interestingly, especially Ebi3, which in complex with p28 form the atherogenic cytokine IL-27, was significantly increased by systemic Y1 receptor antagonism, suggesting the potential proatherogenic effect of Y1 antagonism to be mediated by multiple IL-12 family members. Of note, Y2 receptor antagonism did not affect the IL-12 cytokine family similarly, suggesting other mechanisms to account for accelerated atherosclerosis in those mice. The limited data on the

effects of Y2 signaling points toward its involvement in monocyte migration[33] and angiogenesis[34], which do not necessarily explain the increase in atherosclerosis observed here. Interestingly, recent clinical trial data of DPPIV antagonist use for glycemic control in T2DM patients indicated increased heart failure upon long-term use.[35] While NPY signaling was not evaluated, reduced signaling via the Y2 receptor may be a contributing factor.

In conclusion, our data provides further insight in the intricate role of NPY and its widely expressed receptor system in atherosclerosis development and progression. In contrast to the beneficial effects local application of Y1 receptor antagonists at sites or restenosis, systemic Y1, Y2 and Y5 receptor antagonism in a hyperlipidemic atherosclerosis model resulted in increased lesion development. Here, especially Y2 receptor antagonism increased lesion progression resulting in a more advanced state of the lesions in those mice. In light of recent detrimental cardiovascular side effects of long term DPPIV inhibitor treatment in T2DM patients[35,36], systemic modulation of the NPY-receptor system should be approached with caution and further research especially into pathological mechanisms of Y2 receptor signaling is warranted.

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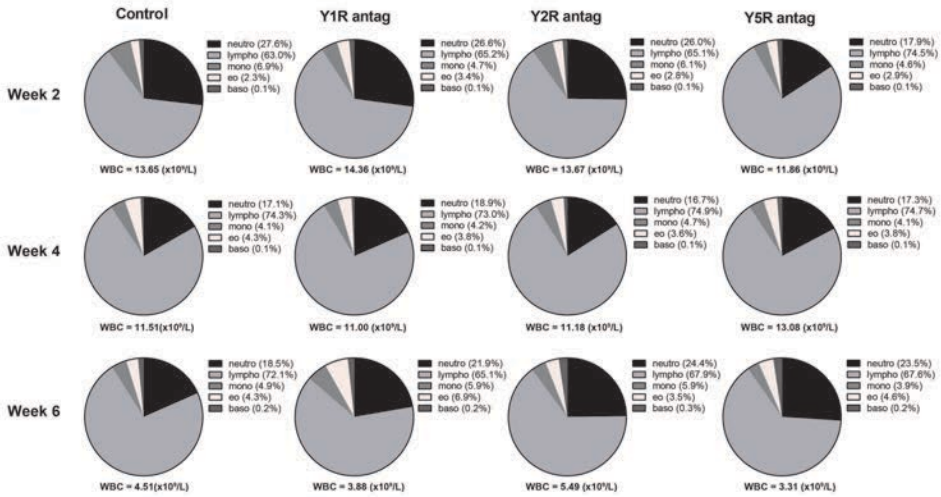
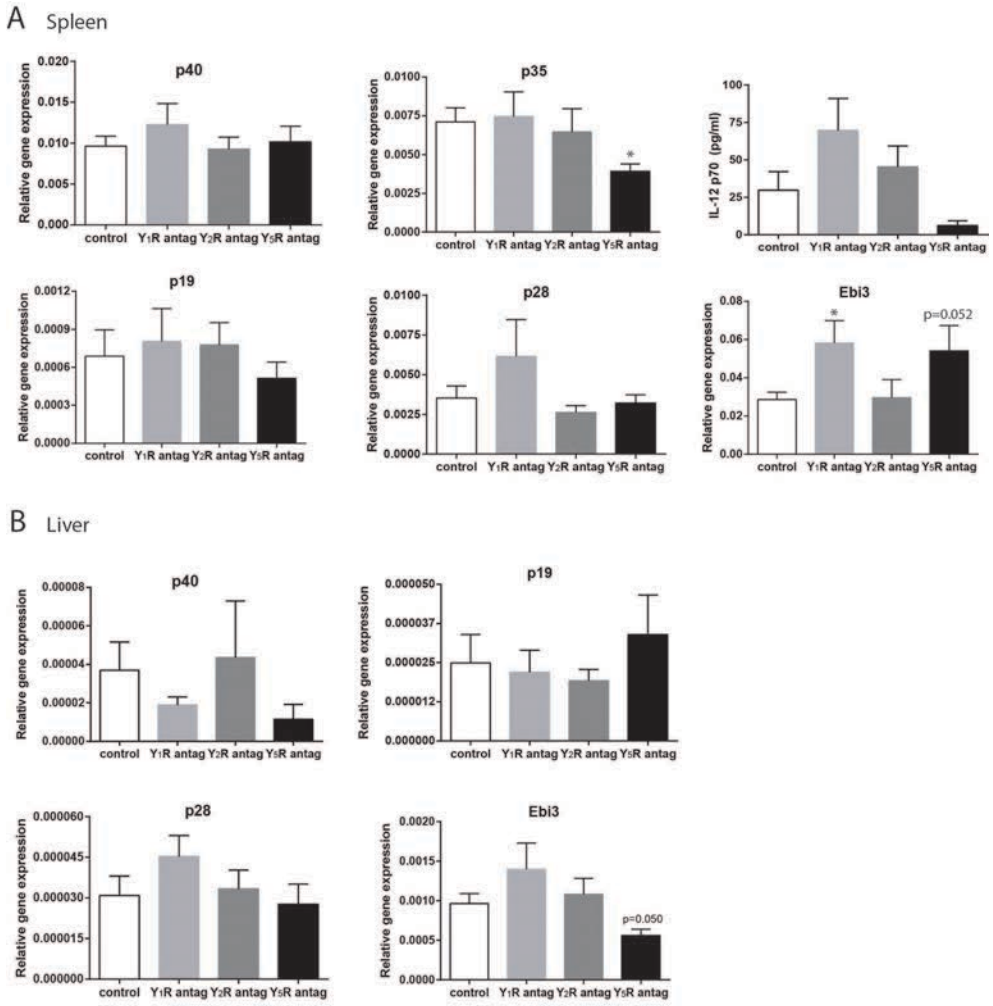
Supplemental data

Supplemental table 1: qPCR primers

Gene	Forward (5'-3')	Reverse (5'-3')
Rpl27	CGCCAAGCGATCCAAGATCAAGTCC	AGCTGGGTCCCTGAACACATCCTTG
HPRT	TACAGCCCCAAAATGGTTAAGG	AGTCAAGGGCATATCCAACAAC
Gusb	TATGGAGCAGACGCAATCCCAG	AGCTCTCCGACCACGTATTCTTTAC
36B4	CTGAGTACACCTTCCCACTTACTGA	CGACTCTTCCTTTGCTTCAGCTTT
NPY	ACATCAATCTCATCACCAGACAG	AGTTTCATTTCCCATCACCACA
NPY1R	ACACTCGTCCCGCTTCAACA	TCTTCAAAACGGATCAAATCTTCAGCA
NPY2R	GAAGGAACGCGCAAGAGTCAATAC	CCCATAGGGCTCCACTTTCACTT
NPY5R	GATGCTCAGGAGATGAGAGTCAA	TCCAGCTAACAGCGAACACTAA
p40	GATTCAGACTCCAGGGGACA	GGAGACACCAGCAAAACGAT
p35	CCAAACCAGCACATTGAAGA	CTACCAAGGCACAGGGTCAT
p19	GCCTGCTCTACTCCCTGATAGC	TGGGCATCTGTTGGGTCT
p28	CACAGGCACCTCCGCTTT	TTGGGATGACACCTGATTGG
Ebi3	CCCGGACATCTTCTCTCTCA	CAATACTTGGCATGGGGTTT



Supplemental figure 1. Relative blood leukocyte composition at two week intervals after the start of antagonist treatment, demonstrating no clear systemic effects on leukocyte subpopulations in blood.



Supplemental figure 2. Gene expression of IL-12 family subunits in A) spleen and B) liver after 6 weeks of systemic NPY receptor antagonist treatment.

6

Chapter 7

Mast cells mediate neutrophil recruitment during atherosclerotic plaque progression

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Abstract

Objective: Activated mast cells have been identified in the intima and perivascular tissue of human atherosclerotic plaques. As mast cells have been described to release a number of chemokines that mediate leukocyte fluxes, we propose that activated mast cells may play a pivotal role in leukocyte recruitment during atherosclerotic plaque progression.

Methods and Results: Systemic IgE-mediated mast cell activation in apoE$^{-/-}$µMT mice resulted in an increase in atherosclerotic lesion size as compared to control mice, and interestingly, the number of neutrophils was highly increased in these lesions. In addition, peritoneal mast cell activation led to a massive neutrophil influx into the peritoneal cavity in C57Bl6 mice, whereas neutrophil numbers in mast cell deficient Kit(W^{-sh}/W^{-sh}) mice were not affected. Within the newly recruited neutrophil population, increased levels of CXCR2^{+} and CXCR4^{+} neutrophils were observed after mast cell activation. Indeed, mast cells were seen to contain and release CXCL1 and CXCL12, the ligands for CXCR2 and CXCR4. Intriguingly, peritoneal mast cell activation in combination with anti-CXCR2 receptor antagonist resulted in decreased neutrophil recruitment, thus establishing a prominent role for the CXCL1/CXCR2 axis in mast cell-mediated neutrophil recruitment.

Conclusions: Our data suggest that chemokines, and in particular CXCL1, released from activated mast cells induce neutrophil recruitment to the site of inflammation, thereby aggravating the ongoing inflammatory response and thus affecting plaque progression and destabilization.

Introduction

Acute cardiovascular syndromes such as myocardial infarction and stroke remain the principal cause of death in western society despite increasing insight in the mechanisms of atherosclerosis, which is the main underlying cause of disease.[1] Atherosclerosis has been identified as a lipid-driven inflammatory disorder, in which various immune cells such as monocytes, macrophages but also mast cells and neutrophils have been implicated.[2,3] Although statin treatment has reduced the risk of acute cardiovascular events by its lipid-lowering and anti-inflammatory effects[4], this treatment is still insufficient for 70% of the patients, thus establishing the need for further research to obtain new therapeutic leads.

The mast cell, a potent inflammatory innate immune cell, is mainly known for its role in allergy and asthma. Nowadays, the mast cell is regarded detrimental in the development of atherosclerosis and acute cardiovascular syndromes as well. For example, we and others have previously established that mast cells induce atherosclerotic plaque growth and destabilization in a number of different mouse models of atherosclerosis.[5,6] In human atherosclerotic plaques, mast cell presence has been established[7,8] and more importantly, mast cell numbers have recently been shown to correlate with plaque progression and to associate with future cardiovascular events[9], thereby emphasizing the potential crucial contribution of mast cells to plaque destabilization. Currently, the identification of endogenous mast cell activators in atherosclerosis is of major interest. Immunoglobulin E (IgE) is commonly known for its acute effects on mast cell activation in allergy. In men with hyperlipidemia and in patients with acute cardiovascular disorders[10], plasma IgE levels were shown to be increased as well. Additional in vitro and in vivo evidence has also established a role for complement factors[11,12], neuropeptides[13,14], immune complexes[15] and lipid mediators[16] in mast cell activation during the development and progression of atherosclerosis.

Mast cells exert their detrimental effects on plaque stability by the release of a number of mediators, such as the mast cell specific proteases chymase and tryptase, histamine, and a number of cytokines, such as TNFα, IL-6, and IFNγ.[6,17] We have previously established that chymase released from mast cells can induce plaque progression.[18] Additionally, it has been shown that mast cells can promote apoptosis of various cell types present in the plaque, such as vascular smooth muscle cells[19], endothelial cells[20] and macrophages[5], thereby contributing to plaque necrosis and destabilization. However, as mast cells also secrete chemokines such as MCP-1 and IL-8, in this study we aimed to establish to which extent mast cells are involved in inducing leukocyte recruitment towards the plaque, thereby fuelling the ongoing inflammatory response and possibly aggravating plaque progression.

Materials and Methods

Systemic mast cell activation

This study was performed in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. All animal experiments were approved by the animal welfare committee of the Leiden University Medical Center (approval reference number 08014). Mice were obtained from the local animal breeding facility (Gorlaeus Laboratories, Leiden, The Netherlands). In all animal experiments, treatment groups were randomized based on age and weight. We used 10-12 weeks old male B-cell deficient apoE$^{-/-}$µMT mice, kindly provided by Prof. BH Toh (Monash University, Melbourne, Australia) for our observational study. These mice lack endogenous IgE, due to a selective deficiency in B cells.[21,22] Previous observations in our lab indicated that Western-type diet feeding results in increased circulating levels of IgE, which in turn leads to a higher basal mast cell activation level in WTD-fed apoE$^{-/-}$ mice. To control for this effect, we performed the systemic IgE-mediated mast cell activation in apoE$^{-/-}$µMT mice. All mice were fed a western-type diet containing 0.25% cholesterol and 15% cacao butter (SDS, Sussex, UK) for eight weeks. During these eight weeks, the mice were challenged with IgE (n=13) or PBS (n=10) control for 6 times (~every 1.5 weeks). In order to do so, mice were given 1 µg of anti-DNP-IgE (monoclonal mouse IgE anti-dinitrophenyl antibody, clone SPE-7, #D8406, Sigma-Aldrich, Zwijndrecht, The Netherlands) by intraperitoneal injection, and 24 hours later the mice received an intravenous injection containing 0.5 mg DNP-HSA (#A6661, Sigma-Aldrich). This treatment regimen did not affect body weight or general health status of the mice. At sacrifice, mice were anaesthetized by subcutaneous injection of ketamine (60 mg/kg, Eurovet Animal Health, Bladel, The Netherlands), fentanyl citrate and fluanisone (1.26 and 2 mg/kg, respectively, Janssen Animal Health, Sauderton, UK). Adequacy of anaesthesia was monitored by regular visual inspection and toe pinch reflex. Mice were exsanguinated via orbital bleeding and in situ fixation through the left cardiac chamber was performed, after which the hearts were excised for further analysis. The hearts were dissected just below the atria and sectioned perpendicular to the axis of the aorta, starting within the heart and working in the direction of the aortic arch. Once the aortic root was identified by the appearance of aortic valve leaflets, 10 µm sections were taken and mounted on gelatin-coated slides. Mean lesion area (in µm^2) was calculated from six Oil-Red-O stained sections in distal direction starting at the point where all three aortic valve leaflets first appeared. Collagen content in the lesion was determined with a Sirius Red staining, while macrophages were visualized with a Moma-2 antibody (1:1000, #MCA519G, Serotec, Puchheim, Germany). The necrotic core size was defined as the a-cellular, debris-rich plaque area as percentage of the total plaque area. The aortic roots were quantified by the Leica image analysis

system (Leica Ltd, Cambridge, UK). T cell numbers in the intima and adventitia were determined by staining for CD3 (1:50, Neomarkers, Fremont, CA, USA) and were counted manually. Mast cells and neutrophils were visualized by staining of 10 µm cryosections with a naphthol AS-D chloroacetate esterase staining kit (#91C, Sigma-Aldrich) and counted manually. A mast cell was considered resting when all granula were maintained inside the cell, while mast cells were assessed as activated when granula were deposited in the tissue surrounding the mast cell. Neutrophils were identified as round cells with a characteristic lobular nucleus and pink granular cytoplasm. All morphometric analyses were performed by blinded independent operators.

Leukocyte influx

Mice were obtained from the local animal breeding facility (Gorlaeus Laboratories, Leiden, The Netherlands). Peritoneal mast cells of either male C57BL/6 or mast cell deficient male Kit(W^{-sh}/W^{-sh}) mice were activated by intraperitoneal injection of compound 48/80 (1.2 mg/kg). After 30 minutes and 3 hours (n=4 per group), mice were anaesthetized as described above, after which peritoneal cells were collected by flushing the peritoneal cavity with 10 ml PBS. After collection of the peritoneal fluid, mice were sacrificed via cervical dislocation. Total cell count and neutrophil, lymphocyte, monocyte and eosinophil counts in blood were analyzed using an automated XT-2000iV veterinary hematology analyzer (Sysmex Europe GMBH, Norderstedt, Germany). After centrifugation of the cells (1500 rpm for 5 minutes), supernatant was collected for protease activity as described below and for chemokine quantification by ELISA according to manufacturer's protocol. Subsequently, leukocyte suspensions were incubated with 1% mouse serum in PBS and stained for surface markers (0.25 µg/0.2*106 cells, eBioscience, San Diego, CA, USA), after which surface marker expression was determined by FACS analysis (FACS Canto, BD Biosciences, Breda, The Netherlands).

An additional influx study was performed in order to investigate CXCR2 mediated neutrophil influx after mast cell activation. Male $apoE^{-/-}$ mice (n=8) were injected intraperitoneally with anti-CXCR2 (5 µg/mouse, #MAB2164, R&D systems, Minneapolis, MN, USA) or PBS. One day later the mice received a second injection with anti-CXCR2 an hour prior to intraperitoneal mast cell activation with compound 48/80 (1.2 mg/kg) or PBS. 3 Hours later mice were anaesthetized and peritoneal fluid was collected as described above, after which mice were sacrificed via cervical dislocation. Subsequently, cells were stained for CD11b, Ly6C, Ly6G ,CXCR2 and CXCR4 after which they were analyzed by FACS.

β-Hexosaminidase activity was determined by adding 50 µL of peritoneal fluid to 50 µL 2 mM 4-nitrophenyl N-acetyl-b-D-glucosaminide (Sigma) in 0.2 M citrate (pH 4.5) and incubated at 37 °C for 2 hours. After addition of 150 µL 1 M Tris (pH 9.0), absorbance (optical density, OD) was measured at 405 nm. To measure chymase

release after degranulation, 50 µL peritoneal fluid was added to 2 mM S-2586 (chymase substrate, #820894, Chromogenix, Llanelli, UK) in PBS supplemented with 100 U/mL heparin. After 24 hours at 37 °C, OD405 was measured. Values are expressed as percentage of total content. The CXCL1 ELISA was performed according to manufacturer's protocol (#DY453, R&D systems).

Neutrophil isolation

Neutrophils were isolated by negative selection as described earlier.[23] To obtain large quantities of functionally competent neutrophils for in vitro analysis of mast cell-mediated neutrophil migration, we isolated neutrophils from bone marrow. Previous results have indicated the bone marrow to be an excellent source of functional neutrophils with longer survival rates in culture compared to blood-derived neutrophils.[24] These functional bone marrow-derived neutrophils have been suggested replace peripheral neutrophils and to be recruited at times of increased demand during infection. In short, C57BL/6 mice were anaesthetized as described above and sacrificed via cervical dislocation after which bone marrow was isolated by flushing the femurs and tibias. Cell suspensions were incubated with an antibody cocktail containing α-CD5, α-CD45R, α-CD49b, α-CD117, α-F4/80 and α-TER119 (eBioscience) (4°C, 10 minutes under constant shaking). After washing, cells were incubated with α-biotin microbeads (4°C, 10 minutes under constant shaking, Miltenyi, Leiden, the Netherlands). Subsequently neutrophils were isolated by magnetic bead isolation (magnetic-activated cell sorting LS column, Miltenyi). We obtained neutrophils at ~90% purity, as validated by flow cytometry and histology[25], which were used for further experiments.

Cell culture

C57BL/6 mice were anaesthetized as described above and sacrificed via cervical dislocation after which bone marrow was isolated by flushing the femurs and tibias. Bone marrow derived mast cells (BMMCs) were grown by culturing bone marrow cells at a density of 0.25*106 cells in RPMI containing 10% fetal bovine serum (FBS), 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (PAA, Cölbe, Germany) and 10% mIL3 supernatant (supernatant from WEHI cells overexpressing murine Interleukin (IL)-3) for 4 weeks in T175 tissue culture flasks (Greiner Bio-one, Alphen aan den Rijn, Netherlands). BMMCs (5*105) were activated by incubation with compound 48/80 (0.5 µg/mL, Sigma, Zwijndrecht, the Netherlands (n=4 per condition) for 15-30 minutes at 37°C in HEPES-tyrode supplemented with 0.1% fatty acid free bovine serum albumin (BSA, Sigma-Aldrich). For total (100%) content measurements, mast cells were lysed with 10% Triton X-100 and untreated control cell supernatant served as 0% release controls. CXCL1 and CXCL12 ELISAs were performed according to manufacturer's protocol (#DY453, #DY350, R&D Systems)

Migration assay

BMMCs were degranulated as described above and the supernatant was collected. Neutrophils (105 per well) were applied to the upper chamber of a transwell system (24 wells, 8 µm pore size, PAA) in RPMI containing 10% fetal bovine serum (FBS), 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin. Mast cell releasate was added to the basolateral chamber. To establish the role of neutrophil derived CXCR2 and CXCR4, anti-CXCR2 or AMD3100 (500 ng/mL, #A5602, Sigma-Aldrich) were added to the system. After 4 hours incubation, the number of migrated neutrophils was counted manually.

RNA isolation, cDNA synthesis and qPCR

Total RNA was extracted from BMMCs with the guanidium thiocyanate-phenol-bromochloropropane extraction method.[26] RNA concentration, purity and integrity were examined by nanodrop (Nanodrop® Technologies). RNA was reverse transcribed by M-MuLV reverse transcriptase (RevertAid, MBI Fermentas, Landsmeer, The Netherlands) and used for quantitative analysis of the mouse genes CXCL1 and CXCL12 with an ABI PRISM 7700 Taqman apparatus (Applied Biosystems). Murine HPRT and RPL27 were used as standard housekeeping genes. For qPCR primer pairs refer to supplemental table 1.

Statistical analysis

Data are expressed as mean ± SD. An unpaired two-tailed Student's t-test was used to compare normally distributed data between two groups of animals. Data of three groups were analyzed with one-way ANOVA and data of two groups with more than one variable were analyzed by two-way ANOVA, both followed by Tukey's multiple comparison test. A level of $P<0.05$ was considered significant.

Results

Mast cell activation correlates with increased neutrophil influx to the plaque

In this study, we used apoE$^{-/-}$µMT mice, which lack endogenous IgE due to its B cell deficiency, and in these mice, mast cells were systemically activated by IgE administration during the development of atherosclerosis. Repeated treatment of apoE$^{-/-}$µMT mice with anti-DNP IgE and subsequent DNP-HSA challenge did not significantly affect total mast cell numbers in the aortic root (controls: 15.8 ± 2.2 mast cells/section versus IgE: 20.5 ± 3.1 mast cells/section), but did result in a significant increase in mast cell activation (controls: 35.2 ± 3.9% versus IgE: 48.2 ± 3.4%, $P<0.05$, Figure 1A). Concomitantly, plaque size in the aortic root increased with 40% from 2.0 ± 0.2*10^5 µm^2 in control mice to 2.8 ± 0.3*10^5 µm^2 in IgE treated mice ($P=0.05$, Figure 1B).

Collagen content of the plaques tended to be decreased by systemic mast cell activation, although this did not reach statistical significance (controls: 12.0 ± 1.7% versus IgE: 9.6 ± 1.0%, P=0.10, Figure 1C).

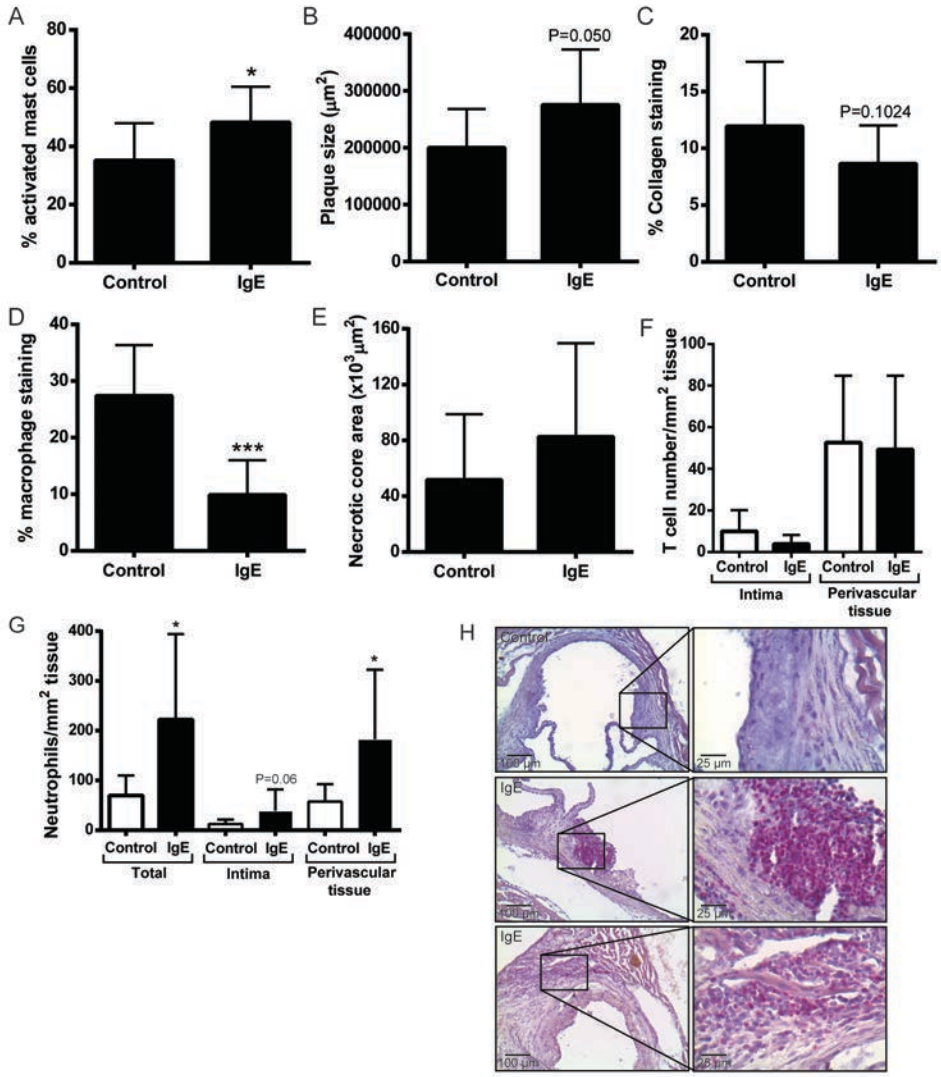

Figure 1. IgE induced mast cell activation in apoE$^{-/-}$µMT mice. During eight weeks, male apoE$^{-/-}$µMT mice treated with anti-DNP IgE followed by DNP-HSA challenge (n=13) or PBS (n=10) control for 6 times (every 10 days). IgE-DNP treatment resulted in enhanced mast cell activation within the aortic root (A) and concomitant lesion progression (B). (C) Collagen content tended to be reduced by the IgE induced mast cell activation (P=0.10), while macrophage content was significantly reduced (D). (E) Necrotic core area was somewhat, but not significantly increased in the IgE treated group. (F) T cell numbers in the intima or the perivascular tissue did not differ between the groups (G) Total neutrophil numbers were increased after IgE treatment, resulting from an increase in both intimal neutrophils, and even more pronounced, in neutrophil number in the perivascular tissue. (F) Representative images of aortic root sections stained with a naphtol chloroactetate esterase staining, illustrating large neutrophil accumulations within the lesion (middle panel) and the perivascular tissue (lower panel) after IgE treatment, but not in control mice (upper panel). Magnifications: left panels 100x, right panels 400x. *P<0.05, ***P<0.001.

These data are in line with previous studies, establishing that mast cell activation can result in collagen degradation, primarily via chymase release.[18,26] Macrophage staining revealed a significant decrease in relative MOMA-2$^+$ area (controls: 27.4 ± 2.7% versus IgE: 9.9 ± 1.7%, P<0.001, Figure 1D). Necrotic core area was increased from 51.7 ± 14.2*10^3 µm^2 in the control mice to 82.6 ± 18.6*10^3 µm^2 in the IgE treated group (Figure 1E), which may be caused by increased macrophage apoptosis upon mast cell activation as established previously.[5] Furthermore, intimal and adventitial T cell numbers did not differ between the groups (Figure 1F). Interestingly, IgE mediated mast cell activation resulted in a 3.2-fold increase in the number of neutrophils in the intima (controls: 12 ± 3 versus IgE: 39 ± 12 neutrophils/mm2 tissue, P=0.06). In the perivascular tissue, we observed a striking 3-fold increase in the number of neutrophils (controls: 58 ± 11 versus IgE: 183 ± 39 neutrophils/mm^2 tissue, P<0.05, Figure 1G,H). Taken intima and perivascular tissue together, we observed 70 ± 12 neutrophils/mm2 in control mice, versus 222 ± 48 neutrophils/mm^2 in mice treated with IgE (P<0.05, Figure 1G,H). Furthermore, mast cell activation status was seen to positively correlate with the number of perivascular neutrophils (P<0.05, R2=0.261). Separate analysis of each treatment group revealed that the correlation is primarily caused by effect in the IgE-DNP treated mice, i.e. controls: P=0.891 (R2=0.003), IgE: P=0.064 (R2=0.278).

In vivo mast cell activation results in neutrophil recruitment

To further investigate whether neutrophil recruitment is indeed mast cell mediated, we activated peritoneal mast cell in mast cell competent C57BL/6 mice and mast cell deficient Kit(W^{-sh}/W^{-sh}) mice, by intraperitoneal injection of the commonly used mast cell activator compound 48/80. This resulted in acute mast cell activation as indicated by β-hexosaminidase (Figure 2A) and chymase (Figure 2B) activity in the peritoneal cavity of C57BL/6 mice, but not in that of Kit(W^{-sh}/W^{-sh}) mice, at 30 minutes and up to 3 hours after injection. Leukocyte differentiation analysis using Sysmex revealed a striking influx of predominantly neutrophils in response to mast cell activation. Mast cell activation did not lead to recruitment of monocytes and lymphocytes, and its numbers were even slightly decreased after mast cell activation with compound 48/80 and these numbers remained identical between C57BL/6 and Kit(W^{-sh}/W^{-sh}) mice (Figure 2C-E). By means of flow cytometry we confirmed the influx of CD11b$^+$Ly6G^{high}CD71$^-$ neutrophils in C57BL/6 mice as displayed in Figure 3A. Interestingly, the recruited neutrophils were CXCR2 and/or CXCR4 positive (Figure 3B,C), suggesting that the ligands of these specific receptors, i.e. CXCL1 (or KC, the murine analogue of IL-8) and CXCL12, are involved in mast cell mediated neutrophil recruitment.

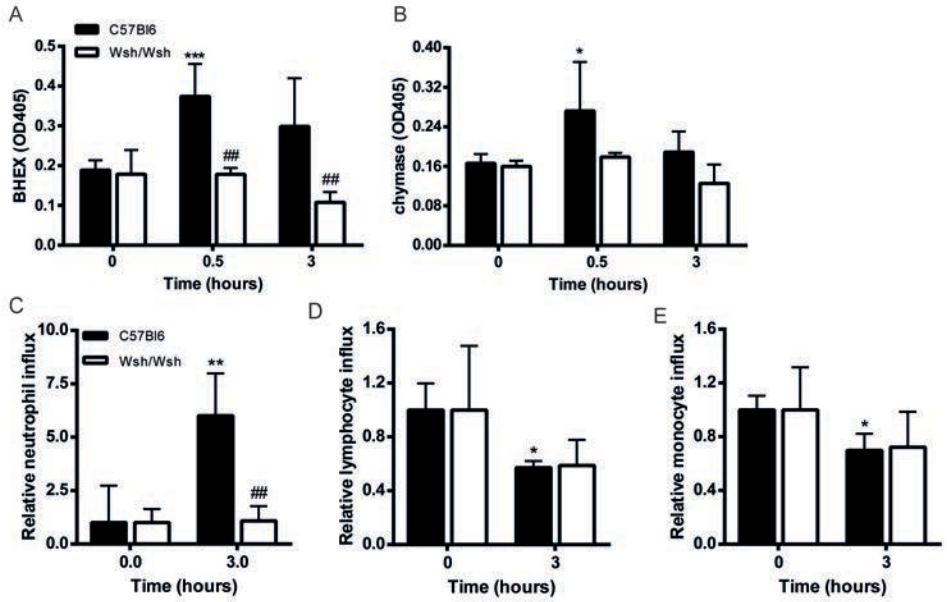

Figure 2. Mast cell induced neutrophil recruitment in vivo. C57BL/6 or mast cell deficient Kit(W^{-sh}/W^{-sh}) mice (n=4) were intraperitoneal injected with compound 48/80, which resulted in acute mast cell activation as indicated by increased β-hexosaminidase (A) and chymase (B) activity in the peritoneal cavity of C57BL/6 mice (black bars) at 30 minutes and still at 3 hours after injection, which did not occur in mast cell deficient Kit(W^{-sh}/W^{-sh}) mice (white bars). Acute peritoneal mast cell activation by compound 48/80 induced recruitment of neutrophils (C), but not of lymphocytes (D) or monocytes (E) in C57BL/6 mice as measured by Sysmex cell differentiation analysis. Injection of compound 48/80 did not significantly affect the composition of these peritoneal cell populations. *P<0.05 compared to T=0, **P<0.01 compared to T=0, ***P<0.001 compared to T=0, ##P<0.01 compared to C57BL/6.

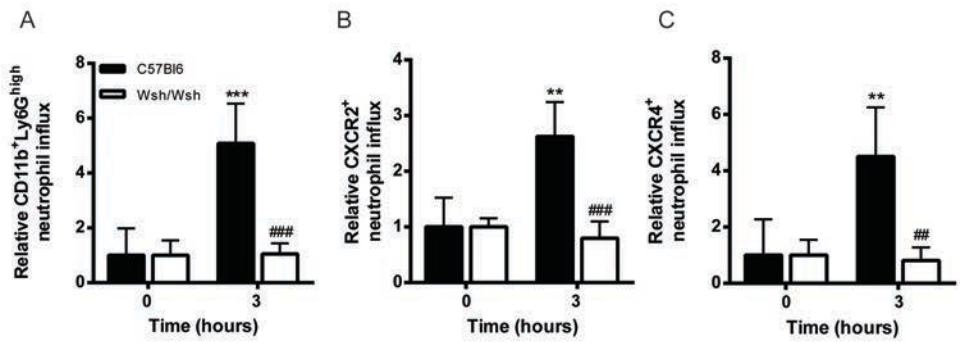

Figure 3. Mast cells recruit $CXCR2^+$ and $CXCR4^+$ neutrophils. C57BL/6 or mast cell deficient Kit(W^{-sh}/W^{-sh}) mice (n=4) were intraperitoneal injected with compound 48/80 (A). FACS analysis confirmed the recruitment of $CD11b^+Ly6G^{high}CD71^-$ neutrophils in response to compound 48/80 induced mast cell activation in C57BL/6 mice but not in Kit(W^{-sh}/W^{-sh}) mice. The mast cell dependent neutrophil influx appeared to be CXCR2 (B) and CXCR4 (C) dependent. **P<0.01 compared to T=0, ***P<0.001 compared to T=0, ##P<0.01 compared to C57BL/6, ###P<0.001 compared to C57BL/6.

Mast cells express and secrete CXCL1 and CXCL12

Next, we aimed to establish whether mast cell induced neutrophil recruitment was mediated via CXCL1 or CXCL12, the ligands for CXCR2 and CXCR4. MRNA expression of these chemokines was measured in cultured bone marrow derived mast cells (BMMCs). Indeed, we observed that both CXCL1 (relative mRNA expression: 0.005 ± 0.004) and CXCL12 (0.002 ± 0.001) were expressed by BMMCs. In the lysate of 5*105 unstimulated BMMCs, we measured 1.5 ± 0.3 ng of CXCL1. After activation with compound 48/80, CXCL1 was released by BMMCs (102 ± 15 pg/mL compared to 6 ± 12 pg/mL in the releasate of unstimulated control cells, P<0.05). Similarly, we observed an increase in CXCL12 release after stimulation with compound 48/80 (0.82 ± 0.08 ng/mL versus 0.12 ± 0.04 ng/mL in the releasate of unstimulated control BMMCs, P<0.01). These data indicate that mast cells, in accordance with previous literature, express and secrete chemokines such as CXCL1 and CXCL12 that can recruit neutrophils to the site of mast cell activation.

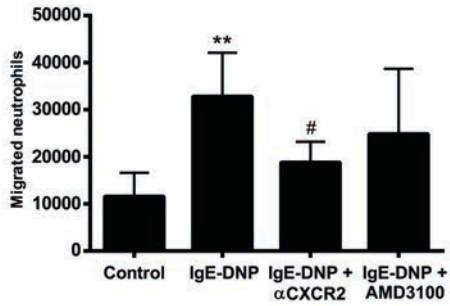

Figure 4. Mast cell induced neutrophil recruitment in vitro. Supernatant from IgE stimulated BMMCs resulted in enhanced migration of freshly isolated neutrophils, which could be inhibited by a CXCR2 blocking antibody. **P<0.01 compared to supernatant of unstimulated control BMMCs. #P<0.05 compared to IgE stimulated BMMCs.

Neutrophil recruitment in vitro

To substantiate our in vivo findings, we isolated neutrophils from bone marrow by negative selection as described previously[23] and allowed these cells to migrate towards supernatant of BMMCs stimulated with IgE. As expected, neutrophil migration towards the basolateral side of the migration chamber was significantly increased upon mast cell stimulation as compared to supernatant of unstimulated BMMCs (32.3 ± 4.7*10^3 cells versus 11.6 ± 2.5*10^3 neutrophils, P<0.01, Figure 4). Blocking neutrophil CXCR2 using a specific mouse α-CXCR2 blocking antibody inhibited the mast cell induced neutrophil migration (18.8 ± 2.2*10^3 neutrophils, P<0.05 compared to IgE stimulated mast cells), while the addition of the CXCR4 receptor antagonist AMD3100 did not affect neutrophil migration (24.8 ± 6.9*10^3 neutrophils, P=NS). These data illustrate that mast cells, when activated, can indeed directly induce neutrophil recruitment.

αCXCR2 inhibits mast cell-mediated neutrophil recruitment in vivo

We aimed to validate our in vitro findings in an in vivo setting by activating peritoneal mast cells while blocking CXCR2 with αCXCR2 (Figure 5A). Similar to the previous results in C57BL/6 mice, compound 48/80-mediated mast cell activation in apoE$^{-/-}$ mice induced a 4-fold increase in CD11b^{+}Ly6G^{high} neutrophil influx to the peritoneum compared to control mice as measured by flow cytometry (Figure 5B; P<0.05). The recruited neutrophils were primarily CXCR2+ (47%), while the amount of CXCR4^{+} (4%) and CXCR2/CXCR4 double positive (3%) neutrophils was limited. Mast cell activation, measured by CD63 expression, was similar in the compound 48/80 and αCXCR2 + compound 48/80 treated mice (Figure 5C). In line with these results, the peritoneal CXCL1 concentration was indeed increased after mast cell activation (11.6 ± 1.4 pg/mL and 13.8 ± 4.8 pg/mL compared to 1.8 ± 0.4 pg/mL in PBS treated mice, P<0.001, Figure 5D). As observed in the previous influx study, total number of monocytes in the peritoneum was reduced after mast cell activation (Figure 5E). Mast cell activation in combination

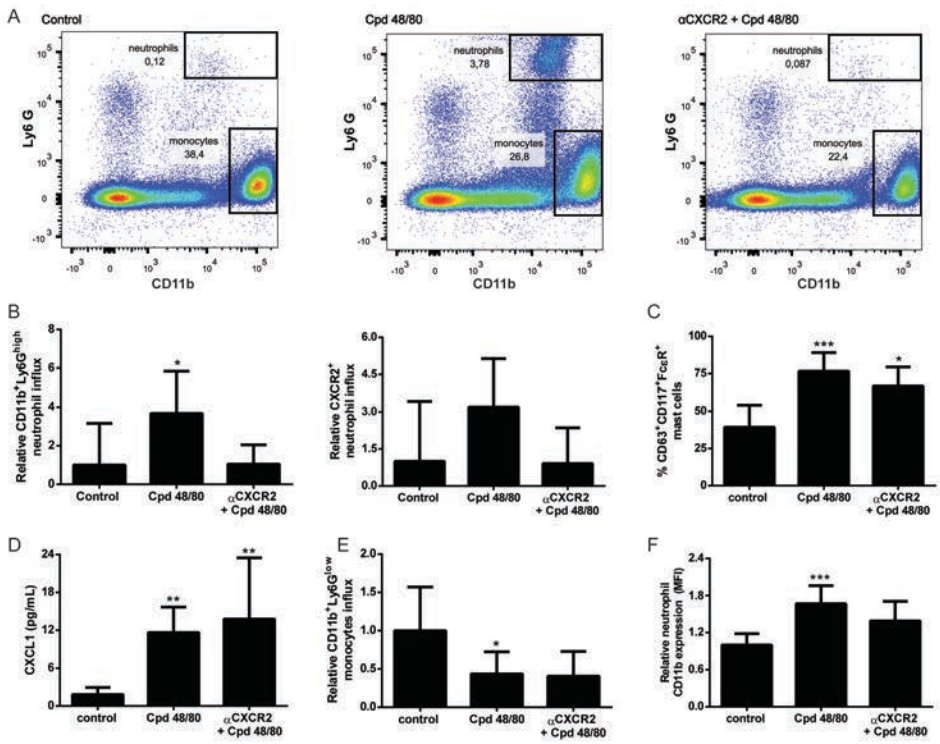

Figure 5. In vivo mast cell mediated neutrophil influx is mediated by CXCR2. Intraperitoneal mast cell activation of apoE$^{-/-}$ mice (n=8) with compound 48/80 resulted in a significant influx of CXCR2^{+} neutrophils and this effect was strongly diminished by blocking CXCR2 (A, B). Mast cell activation, measured by CD63 expression (C), caused a local increase in CXCL1 (D). Monocyte influx was significantly decreased after mast cell activation in a CXCR2-independent manner (E). Peritoneal neutrophil activation was significantly increased after mast cell activation, which was unaffected by αCXCR2 treatment (F). *P<0.05, **P<0.01, ***P<0.001 compared to control group.

with αCXCR2 resulted in a strong reduction in neutrophil chemotaxis (Figure 5B; $P<0.05$), underlining the importance of mast cell derived CXCL1 in neutrophil recruitment. Previously, mast cell derived mediators have been described to influence neutrophil effector functions. Interestingly, in our study we observed a similar increase in activation of recruited neutrophils after peritoneal mast cell stimulation, which was not affected by αCXCR2 treatment (Figure 5F).

Discussion

The current study is the first to demonstrate a correlation between systemic IgE mediated mast cell activation and neutrophil influx into the atherosclerotic plaque. Moreover, mast cell activation directly induced neutrophil migration in vitro and recruitment in vivo, in particular via the secretion of the chemokine CXCL1.

The mast cell is currently accepted as a potent contributing cell in the process of atherosclerosis. Mast cells can induce plaque destabilization by the release of a number of mediators such as chymase and tryptase, which can degrade matrix molecules that give rise to plaque stability and by the induction of plaque cell apoptosis.[5,19,20] Mast cells are also known for their capacity to store and produce a variety of cytokines and chemokines. Previously, we have provided evidence showing that MCP-1 release after either C5a[12] or lysophosphatidic acid-mediated[16] mast cell activation may results in the recruitment of monocytes towards the plaque. Acute mast cell activation can also induce upregulation of adhesion molecules on endothelial cells, thereby enabling the influx of inflammatory cells into the subendothelial space.[27] Furthermore, it was previously shown that mast cell-derived IL-6 and IFNγ are crucial for the induction of mast cell dependent atherosclerotic lesion development[6], suggesting that the contribution of mast cell derived cytokines and chemokines to atherosclerotic lesion development as such is more important than previously thought. In this study, we aimed to determine by which mechanism IgE mediated mast cell activation affects leukocyte recruitment to the atherosclerotic lesion. Unpublished data from our group have shown that in apoE$^{-/-}$ mice, plasma IgE levels rise upon Western-type diet feeding. This phenomenon renders the investigation of specific IgE mediated mast cell activation difficult, as occupation of the FcεRs on mast cells by endogenous IgE antibodies may dampen the specific activation by IgE-DNP injection. To prevent this we used apoE$^{-/-}$μMT mice that lack B cells and thus not produce endogenous IgE, and systemically injected these mice with IgE. Besides increased mast cell activation, which is thus completely induced by the injected IgE, and increased lesion size, we observed a striking increase in neutrophil numbers in the intima, and even more pronounced in the perivascular tissue. We then aimed to determine whether these effects are mast cell specific by comparing leukocyte influx to the peritoneum in mast cell deficient and control mice. Indeed, mast cell activation resulted in

exaggerated neutrophil influx to the peritoneum, which was absent in mast cell deficient mice. Neutrophils were seen to be primarily CXCR2 and CXCR4 positive, suggestive of involvement of the CXCR2 ligand CXCL1 and the CXCR4 ligand CXCL12. We then confirmed that mast cells produce and secrete both CXCL1, as previously established[15,28], and CXCL12, and investigated the contribution of these chemokines to neutrophil migration in vitro. Blockage of CXCR2, but not of CXCR4, in vitro significantly reduced mast cell induced neutrophil migration, indicating that mast cell derived CXCL1 may be more important in neutrophil recruitment than mast cell derived CXCL12. Previously, it has been described that systemic disruption of the CXCL12/CXCR4 axis aggravates atherosclerosis by expansion of neutrophils in the blood and in the plaque.[29] Also, functional blockade of CXCR4 in later stages of atherosclerosis was seen to exacerbate plaque progression, accompanied by hyperactivation of circulating neutrophils.[30] Taking into account these previous findings, and the lack of effects of CXCR4 blockade on neutrophil migration in vitro, we consider it unlikely that mast cell derived CXCL12 is a major contributor in neutrophil influx to the atherosclerotic plaque. Since blockage of CXCR2 did significantly reduce mast cell mediated neutrophil migration in vitro, we aimed to confirm this observation in vivo. Again, after peritoneal mast cell activation, a massive neutrophil influx was observed, which could be partially inhibited by αCXCR2 pretreatment. Based on these data we postulate that mast cell derived CXCL1 is a major contributor to neutrophil migration to the site of inflammation, which is in line with a previous report demonstrating that mast cell and macrophage derived CXCL1 and CXCL2 induce neutrophil recruitment in an LPS-induced peritonitis model.[31] Furthermore, in the current study, we made use of compound 48/80 and IgE mediated mast cell activation, which are well-known to cause mast cell degranulation. We showed that both these general mast cell activators are capable of inducing neutrophil recruitment.

In advanced atherosclerosis, we have previously established that mast cells can activate endothelial cells, thereby inducing the adhesion of leukocytes to the endothelial layer in a CXCR2 and VCAM-1 dependent fashion.[5] Direct mast cell dependent neutrophil recruitment has been previously associated with diseases such as EAE[32], skin diseases[33] and rheumatoid arthritis[34], either via CXCL1 or other mechanisms such as the tryptase/heparin complex. We now postulate that also in atherosclerosis mast cells may directly induce neutrophil recruitment via the CXCL1/CXCR2 axis, thus providing another mechanism by which mast cells can fuel the ongoing inflammatory response. This mechanism may also explain the massive increase in perivascular neutrophils, which can be caused by activation of perivascular mast cells and subsequent neutrophil recruitment via perivascular microvessels instead of influx through the endothelium.

In the in vivo study, both increased mast cell activation as well as increased neutrophil numbers were observed. It is therefore difficult to distinguish between

effects on plaque formation caused by either the mast cell itself or indirectly by the infiltrating neutrophils. However, a detrimental role for neutrophils in both early and late stage atherosclerosis has been previously described. Hypercholesterolemia in apoE$^{-/-}$ mice increases the amount of circulating neutrophils, which correlates with early atherosclerotic lesion size.[35] The direct presence of neutrophils has been readily detected in early fatty streaks and in advanced atherosclerosis they accumulate especially in shoulder regions of the plaque.[36] In human atherosclerotic plaques neutrophils are detected as well, and moreover, increased numbers of neutrophils correlated with rupture-prone lesions.[37] Markers of so-called neutrophil extra-cellular traps are even associated with adverse cardiac events.[38] Furthermore, neutrophils release granules containing large amounts of matrix-degrading proteases, they produce vast amounts of reactive oxygen species and go rapidly into apoptosis.[39] Thus, there are a number of mechanisms via which neutrophils can contribute to atherosclerotic plaque growth and destabilization. In our study, we have provided evidence that stimulation of mast cells resulted in increased activation of recruited neutrophils, which may cause additional detrimental effects on local inflammation.

In conclusion, systemic mast cell activation results in neutrophil accumulation within the vessel wall, and enhanced atherosclerotic lesion development. Recruitment studies revealed a direct role for mast cell derived CXCL1, which attracts CXCR2^{+} neutrophils. These data may thus provide a novel mechanism by which mast cells can aggravate the ongoing inflammatory response in atherosclerotic lesion development and progression.

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Supplemental data

Supplemental table 1: Primer pairs used for qPCR analysis. The relative gene expression of CXCL1 and CXCL12 was determined to the average expression of the two housekeeping genes: hypoxanthine phosphoribosyltransferase (HPRT) and 60S ribosomal protein L27 (RPL27).

Gene	Forward (5'-3')	Reverse (5'-3')
CXCL1	TTGACCCTGAAGCTCCCTTG	AGGTGCCATCAGAGCAGTC
CXCL12	CTGTGCCCTTCAGATTGTTG	TAATTTCGGGTCAATGCACA
HPRT	TACAGCCCCAAAATGGTTAAGG	AGTCAAGGGCATATCCAACAAC
RPL27	CGCCAAGCGATCCAAGATCAAGTCC	AGCTGGGTCCCTGAACACATCCTTG

7

Chapter 8

General Discussion and Perspectives

8.1 Introduction

Cardiovascular disease (CVD), which encompasses a group of disorders of the heart and vasculature, remains a leading cause of death world-wide. Currently, about 17.3 million people die annually as the consequence of CVD and with the growing population and increase in life expectancy this number has been estimated to grow to more than 23.2 million a year by 2030.[1,2] In the majority of these cases death is caused by a myocardial infarction or a stroke due to thrombotic blood vessel occlusion as a consequence of atherosclerotic plaque rupture or erosion. Atherosclerosis is the underlying lipid-induced chronic inflammatory disease, which already starts early in life and progresses until clinical symptoms may become apparent, often from the age of 60 and onwards. In addition to the individual grief, the economic burden on society is enormous, totaling 320 billion a year (in the US) due to health expenditures and lost productivity.[3]

The main therapeutic strategy is based on lipid lowering by means of statin treatment, which are HMG-CoA reductase inhibitors, a rate limiting enzyme in cholesterol synthesis. Under current guidelines, patient's cholesterol levels are aimed to be lowered to a considered healthy <70 mg/dL (<1.8 mmol/L), however resent trials seem to indicate that even further lowering (for example by combination therapy with Ezetimibe) results in additional risk reduction.[4] In addition to its lipid lowering potential, statins have been shown to exhibit anti-inflammatory properties, which combined have reduced the relative risk of CVD-related deaths in patient by 25-30%. Nonetheless, there remains a considerable residual risk and the absolute risk reduction, especially in patients with less extreme cholesterol levels, is limited. This clearly emphasizes the need for new therapeutic modalities based on a more thorough understanding of the disease and all its risk factors.

Besides dyslipidemia and traditional risk factors for cardiovascular disease such as smoking, untreated hypertension, physical inactivity and diabetes mellitus, a contributing role for psychosocial risk factors, including psychological stress, has become evident and inspired research into the biological pathways involved.

8.2 Psychological stress: an underappreciated risk factor for cardiovascular disease

Early work by Rosenman *et al*. indicated a clear correlation between certain behavior and personality types and the risk of developing coronary heart disease.[5] These studies provided the first link adverse between emotional states, which can be considered in general as stressful, and disease. Further evidence for a direct role for psychological stress in the development of atherosclerosis was provided by landmark studies in cynomolgus monkeys by Kaplan *et al*. demonstrating extensive coronary atherosclerosis in stressed animals compared to their unstressed controls, even under normocholesterolemic conditions.[6] More recently,

the association between multiple psychosocial risk factors and the incidence of acute myocardial infarction (AMI) was assessed in the large case-control study INTERHEART. Especially chronic exposure to for example work-related stress correlated with a more than 2-fold greater risk of AMI.

Chapter 2 of this thesis provides an overview of the major biological systems known to be involved in stress-induced exacerbation of cardiovascular disease, including the inflammatory response, the autonomic nervous system, neuroendocrine and oxidative systems. Both human epidemiological and clinical data as well as mechanistic insights obtained from various animal models are discussed. A clear distinction is made between chronic and acute stress exposure. While chronic stress results in the wear-down and maladaption of the various systems, including the immune system, acute stress boosts various responses via the central and local release of stress-related hormones and neuropeptides. Additional attention is given to the role of early life stress as atherosclerosis development initiates already in childhood and adolescence, necessitating earlier intervention strategies.

8.3 Stress-induced plaque vulnerability and atherothrombosis: a key role for mast cells?

In **Chapter 3** and **4**, the immunomodulatory potential of the acute stress response in relation to atherosclerotic lesion progression, vulnerability and atherothrombotic complications were investigated. Previous research had uncovered a pro-atherogenic effect of the sympathetic nerve-derived factor substance P, acting in part via mast cell activation[7], and a strong colocalization between nerve fibers and mast cells in the perivascular tissue. This inspired us to investigate the more general acute stress response in modulating mast cell activity with regard to atherosclerotic plaque vulnerability and atherothrombotic complications in **Chapter 3**. To establish whether acute stress, in this case a 30-120 minute period of restraint stress, was capable of activating perivascular mast cells, apoE$^{-/-}$ mice were subjected to the stress protocol and their stress response monitored. As expected, acute restraint caused a strong rise in plasma corticosteroid levels, but also resulted in a significant increase in circulating amount of the pro-inflammatory cytokine IL-6. Subsequent morphological characterization of various (vascular) tissues, demonstrated especially a significant increase in cardiac mast cell activation after 120' of restraint stress exposure. In addition to increased atherosclerosis development upon chronic mast cell activation[8,9], pro-inflammatory cytokine and protease release from mast cells at later stages of the disease was shown to decrease atherosclerotic lesion stability. In humans, acute exposure to severe stressors, such as the terror of a natural disaster or terrorist attack and the intense grief after losing a loved one, is highly linked to increased risk of myocardial infarction or stroke. To test the hypothesis, that acute stress-induced mast cell activation contributes to this we evaluated the effect of acute stress-induced mast

cell activation on atherosclerotic lesion stability. ApoE$^{-/-}$ mice were put on a high fat diet for 6 weeks to induce the development of advanced atherosclerotic lesions in the aortic root. Next, the mice were subjected to 120' restraint stress with or without prior administration of the mast cell stabilizer cromolyn. Subsequent analysis of the collagen content and the incidence of intraplaque hemorrhages (IPH) in the atherosclerotic lesions, both hallmarks of a vulnerable plaque in mice, demonstrated a significant reduction in collagen in the stressed mice compared with non-stressed controls. Furthermore, the incidence and size of the IPH were higher in the stressed mice, possibly due to increased leakiness of intraplaque neovessels as previously shown for other routes of mast cell activation.[7,9] The contribution of the mast cell herein was confirmed by the inhibition the stress-induced effects on systemic inflammation and locally on plaque vulnerability by pretreatment with cromolyn. However, the inhibition was only partial, which might be explained by the relative poor effectiveness of cromolyn in mice.[10] To further confirm the mast cell-dependency of the observed pro-atherogenic effects of acute stress, a similar experiment was performed in mast cell depleted apoE$^{-/-}$ mice. In line with the results obtained by cromolyn pretreatment, mast cell deficiency significantly reduced the stress induced increase in circulating levels of IL-6 and completely abolished the effects of stress on plaque vulnerability parameters.

The majority of cardiovascular disease-related deaths are caused by the thrombotic occlusion of coronary or cerebral arteries, resulting in a myocardial infarction or stroke. Rupture or erosion of the fibrous cap, of an advanced and vulnerable atherosclerotic lesion exposes the thrombogenic content of the plaque and initiates platelet activation and the blood coagulation system. In **Chapter 4** the direct contribution of the acute stress response on platelet activation, blot clotting and thrombus formation was assessed. Although 120' restraint stress in apoE$^{-/-}$ mice resulted in a robust decrease in circulating white blood cells and a redistribution of the different leukocyte subpopulations, red blood cell and platelet numbers were not changed. Furthermore, platelet activation status, assessed by agonist induced expression of the glycoprotein αIIbβ3, was not affected. However, acute stress did increase tail bleeding time and somewhat reduced clot retraction capacity, both indicative of impaired coagulation. Next, we investigated the direct effect on thrombus formation, by means of a FeCl3-induced carotid artery thrombosis model. Topical application of FeCl3 in this model results in rapid endothelial damage, platelet activation and adherence to the vessel wall and subsequent formation of a vessel occluding clot. Interestingly, exposure to acute stress, either directly or 24 hours upfront did not affect the time to occlusion of results in any obvious differences in the composition of the thrombi. Similar experiments in the mast cell deficient apoE$^{-/-}$ mice did not reveal mast cell-dependent effects, except an inhibition of the acute-stress induced increase in tail bleeding time. Further research is necessary to dissect out the potential mast cell-derived mediators

implicated in this effect.

Combined, the results described in **chapter 3** and **4**, indicate the acute stress response as a potent mast cell activator, resulting in increased inflammation plaque destabilization. With regard to atherothrombotic complications and the direct effect of acute stress on thrombus formation, no clear results were obtained necessitating additional research. Possibly targeting specific mast cell-derived mediators, such as heparin could shed light on the observed difference in tail bleeding time.

8.4 Neuropeptide Y signaling in atherosclerosis

One of the most abundantly expressed stress-related neuropeptides is neuropeptide Y (NPY). After its identification in porcine brain extracts in 1982[11], further research determined the widespread distribution of this 36-amino acid peptide both in the central and peripheral nervous system. Peripherally NPY is co-stored and co-release with norepinephrine and ATP and potentiates the effects of these neurotransmitters. The first biological function identified was its potent vasoconstrictive property on cerebral and renal arteries, making NPY a possible drug target for the prevention of hypertension. However, additional research elucidated strong mitogenic effects of NPY on vascular smooth muscle cells, endothelial cells and adipocytes[12], and provided associations between chronic stress-induced increases in NPY levels and obesity and metabolic syndrome.[13] The direct involvement of NPY in atherosclerosis was inferred from increased disease progression in patients with a gain-of-function mutation in the preproNPY gene.[14] In **Chapter 5** the expression of NPY in stable versus unstable human and murine atherosclerotic lesions was investigated. NPY expression in carotid endarterectoctomy specimens obtained from the AtheroExpress biobank[15] was significantly higher in unstable compared with stable lesions. In line with these results, increased expression was also observed during atherosclerosis progression in apoE$^{-/-}$ mice. To establish a direct pro-atherogenic effect of NPY and investigate the local effect increased perivascular concentrations of NPY, for instance after local release from adventitial nerve fibers, we constructed a NPY-expressing lentivirus for perivascular application at the site of atherosclerotic lesion formation. Rapid atherosclerosis development at an accessible site was induced by collar-placement around the carotid arteries as described by von der Thusen *et al*.[16] Next the NPY-lentivirus was applied in pluronic gel to ensure the local overexpression. In line with previous results, overexpression of NPY resulted in a significant increase in lesion size. Interestingly, perivascular mast cell activation was also significantly increased, leading us to evaluate NPY-induced mast cell activation. In vitro incubation of bone marrow-derived mast cells with recombinant NPY indicated a bimodal response, resulting in the release of the pro-inflammatory cytokine IL-6 and mast cell-specific protease tryptase by NPY

concentrations in the high μM and low nM range. NPY acts both centrally and peripherally through its G-protein couple receptors (Y1-Y6). Of these receptors, Y1, Y2 and Y5 are most ubiquitously expressed and best characterized. In **Chapter 6** we obtained the expression profiles of these different receptors during atherosclerosis and restenosis in apoE$^{-/-}$ mice. In contrast to NPY itself, which was increasingly expressed in both disease models, expression of the Y1 receptor was almost completely abolished upon disease initiation. In contrast expression of the Y2 and Y5 receptors as well as the peptidase DPPIV, which cleaves NPY in NPY3-36 lacking Y1 receptor affinity, was generally higher during atherosclerosis progression. Next we assessed the therapeutic potential of systemic Y1, Y2 or Y5 antagonist treatment in atherosclerosis. LDLr$^{-/-}$ mice put on WTD were injected 3 times a week with a specific Y1 (BIBO-3304), Y2 (BIIE-0246) or Y5 (CGP-71683) receptor antagonist for a 6 week period. In contrast to previous results obtained in restenosis models in rats and mice in which NPY- or stress-induced neointima formation could be inhibited by both local and systemic Y1 receptor antagonism, atherosclerotic lesion formation was increased. Especially, Y2 receptor antagonism led to a faster disease progression resulting in significantly bigger and more advanced lesions, with lower macrophage and vascular smooth muscle cell content and increased perivascular mast cell accumulation. Interestingly, pro-atherogenic changes, upon altered Y1 signaling, including changes in food intake and triglyceride metabolism[17,18] and increased levels of the pro-inflammatory cytokine IL-12[19] could be observed in the Y1 and Y5, but not the Y2 receptor antagonist treated mice.

The results from **Chapter 3** and **5** more firmly established the important contribution of mast cells in atherosclerosis development and progression of plaques towards a vulnerable state. Packed with pro-inflammatory mediators and uniquely localized to respond quickly to pathogens, but also endogenous danger-associated signals and neurogenic stimuli, these innate immune cells contribute to the ongoing vascular inflammation and can reduce the integrity of the plaque.[20] Besides these pro-inflammatory cytokines and proteases, mast cells secrete chemokines such as MCP-1 and IL-8, which can modulate the influx of other immune cells. In **Chapter 7**, we investigated the recruitment of leukocytes towards the atherosclerotic lesion upon systemic mast cell activation. To control for a previously observed increase in circulating levels of IgE in Western type diet (WTD) fed apoE$^{-/-}$ mice, we performed the mast cell activation in apoE$^{-/-}$ μMT mice, which lack endogenous IgE. Interestingly, besides a faster lesion development, systemic mast cell activation, by means of antigen-induced FcεR crosslinking, during the 8 weeks on WTD resulted in a striking increase in intimal en perivascular neutrophil recruitment. To dissect out the mast cell-specificity of this effect, peritoneal influx studies in C57Bl/6 and mast cell deficient KitW^{-sh}/W^{-sh} were performed. Peritoneal injection of the mast cell activator compound 48/80

resulted in a significant increase in, especially the chemokine receptor CXCR2 and CXCR4 positive, neutrophils in the control apoE$^{-/-}$ mice but not in the mast cell deficient mice. In vitro migration assays, using a transwell system in which isolated neutrophils were shown to migrate towards activated mast cell supernatant, demonstrated the primary involvement of the CXCR2 receptor on the neutrophil, as CXCR2 blockade, but not CXCR4 receptor antagonism could inhibit the migration. This was further confirmed in vivo were pretreatment with an anti-CXCR2 receptor antibody similarly blocked compound 48/80 induced peritoneal recruitment of neutrophils. These data provide an additional mechanism by which these two innate immune cells reinforce each other's responses and thereby aggravate the ongoing vascular inflammation leading to accelerated atherosclerosis.

Considerations and perspectives

Cardiovascular diseases in humans develop over decades and clinical symptoms generally manifest from the age of 60 onwards. However, the vascular damage and inflammation associated with atherosclerosis initiation already occurs during early adolescence, resulting in a complex disease course modulated by many different risk factors throughout the lifetime. Psychosocial risk factors, including psychological stress, have gained well-deserved attention as important (modifiable) risk factors contributing to the metabolic, endocrine and inflammatory processes involved in atherosclerosis.

In this thesis, the immunomodulatory properties of the acute stress response and the neuronal hormone NPY in the context of atherosclerosis development and progression have been investigated. Special attention was given to the contribution of the mast cell herein, and modulation of its activity by the stress response. Our data provide evidence for a direct contribution of acute stress-induced mast cell activation in atherosclerotic plaque destabilization. Also, increased vascular expression of the stress-related hormone NPY was shown to correlate with disease severity and contribute to atherosclerosis development, at least partly, via the induction of perivascular mast cell activation. Combined with the novel insights in mast cell-mediated modulation of other immune responses involved in atherosclerosis, such as neutrophil recruitment to the plaque, therapeutic strategies aimed at mast cell inhibition or stabilization seems a valuable approach for the prevention of cardiovascular disease-related deaths. Up to date, few studies evaluating the cardiovascular benefits of mast cell stabilization, for example by means of anti-allergic drugs, have been performed. The Prevention of RESstenosis with Tranilast and its Outcomes (PRESTO) trail, assessed the anti-inflammatory and anti-proliferative properties of the anti-allergic drug tranilast for the prevention of restenosis after percutaneous coronary intervention. Unfortunately, in contrast to two smaller previous trials[21], no improvement in angiographic or clinical restenosis was observed in this multicenter randomized clinical trial.[22]

While tranilast has been shown to inhibit pro-inflammatory mediator release from mast cells it also effects proliferation and migration of smooth muscle cells and fibroblasts complicating the evaluation of mast cell specific effects. More recently, several patents regarding the use of mast cell stabilizers for the prevention and treatment of cerebral ischemia[23], cardiovascular disease in general[24], obesity[25] and in combination with statins for inflammatory disorders[26] were filed. These patents highlight the potential of mast cell stabilization in a variety of disease. However, systemic mast cell inhibition might compromise the protective responses of the mast cell as part of the immune system, in for example the skin, lung and intestine, against bacterial and parasitic infections. Intervention strategies, aimed at modulating mast cell function and activation via disease-specific ligands might circumvent this while retaining the desired beneficial effects. One such example might be inhibition of mast cell activation by specific complement factors highly expressed in vein graft disease.[27] With regard to mast cell triggers in the context of atherosclerosis development and progression, we identified increased vascular NPY expression as a potential mast cell activator, leading to increased disease progression. However, our results also indicate the difficulty of modulating NPY signaling, as its receptors are expressed both in the central nervous system and the periphery acting on various endocrine, metabolic and immunologic responses. Also systemic application of for example Y1 receptor antagonists did not influence exercise-induced ischemic parameters in patients with coronary artery disease[28] and Y5 receptor antagonism did not augment the weight loss efficacy of two anorexiants, orlistat and sibutramine.[29] Animal studies, especially with regard to restenosis, demonstrated clear beneficial effects of Y1 receptor antagonism and one might envision local application of such antagonists by means of drug eluting stents. Furthermore, the Y2 receptor was demonstrated to be indispensable in mediating stress-induced obesity and metabolic syndrome. Up to date no conclusive results demonstrating a beneficial effect of Y2 receptor antagonism in humans has been obtained and our data (in mice) indicate that systemic Y2 receptor antagonism, like previously shown for Y1 receptor antagonism[19], may actually promote atherosclerotic plaque development via a currently unresolved mechanism. Furthermore, improved glycemic control in type 2 diabetes mellitus patients treated with the DPPIV inhibitory sitagliptin, which besides the degradation of the gastrointestinal hormones GLP-1 and GIP, also modulates NPY signaling and has been associated with increased heart failure.[30] However, a recent large, multi-center trial evaluating such detrimental side-effects of sitagliptin in addition to usual care, did not show significant differences in hospitalization rates for heart failure, acute pancreatitis or pancreatic cancer[31], warranting further mechanistic insight into NPY signaling and ways of harnessing the therapeutic potential of these specific NPY receptor antagonist for the prevention of cardiovascular disease.

As mentioned before, the psychological stress response contributes to

cardiovascular and metabolic disease a.o. via the upregulation of neuropeptide Y. Furthermore, other major stress hormones, including glucocorticoids, adrenalin and noradrenaline have all been implicated in the correlation between risk of cardiovascular complications and chronic exposure to psychosocial risk factors, such as work-related stress and depression. Several trials have addressed the cardiovascular benefit of psychological treatment of depression, including the SADHART, ENRICHED and MIND-IT trials[32], but unfortunately without clear effects on cardiovascular disease. In addition to atherosclerosis progression, plaque vulnerability remains a key determinant of atherothrombotic complications. Here, we identified a direct link between acute stress exposure and plaque destabilization through increased mast cell activation. Combined with acute stress induced changes in coagulation, these results warrant further investigation into the specific mediators involved and therapeutic potential of modulating both the stress response and immune cell activation. Such therapeutic interventions might best focus on the prevention of secondary events in patients with confirmed vulnerable lesions.

In conclusion, the stress response, a.o. acting via the synthesis and release of various neuropeptides, significantly contributes to atherosclerosis development and constitutes a relatively underappreciated risk factor for acute cardiovascular syndromes. The results obtained in this thesis provide further evidence for the direct influence of the stress response in modulating innate immune responses which contribute to atherosclerotic plaque development and destabilization. Future (clinical) investigations evaluating and treating mental health combined with lipid-lowering and anti-inflammatory strategies may be the necessary next step in the prevention of cardiovascular disease.

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8

Nederlandse samenvatting

Vandaag de dag zijn hart- en vaatziekten de voornaamste doodsoorzaak in de Westerse wereld. Onder de algemene term “hart- en vaatziekten” vallen aandoeningen als een myocard infarct (hartaanval), perifeer vaatlijden en cerebrovasculaire aandoeningen (beroerte). Momenteel sterven jaarlijks wereldwijd ruim 17 miljoen mensen aan de gevolgen van hart- en vaatziekten en men schat dat met het toenemen van de wereldbevolking en de levensverwachting dit getal omstreeks 2030 gegroeid zal zijn naar ruim 23 miljoen doden per jaar. Naast groot persoonlijk leed is dit ook een enorme economische last voor de samenleving. Een groot gedeelte van deze sterfgevallen is het gevolg van een hartaanval of beroerte veroorzaakt door een bloedstolsel, welke ontstaan door een ruptuur van een instabiele atherosclerotische plaque. Atherosclerose, ook wel aderverkalking genoemd, is het proces dat leidt tot de vorming van atherosclerotische plaques. Het is een chronisch ziekteproces waarin na veranderingen in de endotheellaag van slagaders, lipiden, cholesterol en cellen van het immuunsysteem zich ophopen in de vaatwand. Deze cellen van het aangeboren immuunsysteem, de macrofagen, nemen steeds meer gemodificeerde (geoxideerde) lipiden en cholesterol op, waardoor ze veranderen in zogenoemde schuimcellen en de initiële atherosclerotische plaques, ‘fatty streaks’, vormen. Hoewel deze fatty streaks al in jongvolwassenen aangetoond kunnen worden zijn deze meestal asymptomatisch. De klinische complicaties zoals een hartinfarct of beroerte vinden meestal pas 40 à 50 jaar later plaats, een periode waarin de initiële plaques zich verder ontwikkeld hebben tot vergevorderde en soms instabiele plaques. Mede door de enorme opname van cholesterol en de daardoor geïnduceerde ontstekingsreacties gaan macrofagen en andere geïnfiltreerde cellen in de atherosclerotische plaque dood, die samen met lipiden een zogeheten necrotische kern vormen. Deze thrombogene necrotische kern wordt van het bloed gescheiden door een kapsel van spiercellen en fibrotisch materiaal. Pas wanneer dit fibrotisch kapsel scheurt en de inhoud van de plaque in contact komt met het bloed, wordt het bloedstollingssysteem geactiveerd, wat kan leiden tot een bloedvat-afsluitend stolsel met levensbedreigende complicaties.

Op dit moment is de belangrijkste therapeutische strategie om het risico op hart- en vaatziekten te verminderen het verlagen van bloed cholesterol niveaus door middel van statines. Hoewel dit medicijn het relatieve risico met 25-30% verlaagd heeft, blijft er een aanzienlijk risico over. Dit geeft aan dat voor een betere behandeling nieuwe strategieën nodig zijn, gebaseerd op een meer volledige kennis van de ziekte atherosclerose en al haar risicofactoren. Naast hyperlipidemie en traditionele risicofactoren voor hart- en vaatziekten als roken, hoge bloeddruk, diabetes en geringe lichamelijke beweging, lijken psychosociale risicofactoren, waaronder psychische stress, ook een belangrijke bijdrage te leveren aan zowel het chronische ziekteproces als de acute complicaties. **Hoofdstuk 2** van dit proefschrift geeft een overzicht van de biologische systemen die betrokken zijn bij de bijdrage van psychische stress op het verhoogde risico op hart- en vaatziekten. Naast het immuunsysteem zijn dit het autonome zenuwstelsel, het neuro-endocriene en het oxidatieve systeem. Zowel epidemiologische en klinische data alsmede inzichten in de biologische mechanismen verkregen uit proefdierstudies worden besproken, waarin een duidelijk onderscheid gemaakt wordt tussen de effecten van acute en chronische stress. Terwijl chronische stress vooral bijdraagt aan het cardiovasculaire risico door middel van foutieve aanpassingen aan de hiervoor genoemde systemen, kan acute stress een directe aanleiding zijn voor het ruptureren van een instabiele atherosclerotische plaque. Omdat atherosclerose al in een vroeg stadium aantoonbaar is, wordt hier ook gekeken naar de rol van stress in de eerste levensjaren en de gevolgen hiervan met betrekking tot het risico op hart- en vaatziekten later in het leven. Het immuunsysteem speelt een belangrijke rol bij het ontstaan en de verdere ontwikkeling van atherosclerotische plaques. Dit systeem kan ruwweg opgedeeld worden in het aangeboren en verworven (adaptieve) immuunsysteem. Cellen van het aangeboren immuunsysteem, waaronder de hiervoor genoemde macrofagen, maar ook neutrofielen en mestcellen vormen een belangrijke eerste verdediging tegen infecties en zorgen voor het inschakelen van het adaptieve immuunsysteem. In **hoofdstuk 3** zijn de effecten van acute stress op met name het aangeboren immuunsysteem in relatie tot atherosclerotische plaque ontwikkeling en destabilisatie bestudeerd. Eerder onderzoek heeft aangetoond dat mestcellen, aanwezig in het weefsel dat de bloedvaten omringt, in nauw contact staan met het perifere zenuwstelsel en dat activatie van de mestcellen door neuropeptiden, uitgescheiden door deze zenuwen, kan leiden tot versnelde atherosclerose. Dit was voor ons de aanleiding om de effecten van de acute stress reactie op mestcelactivatie en atherosclerotische plaquestabiliteit te onderzoeken. Om acute stress te induceren bij muizen werd gebruik gemaakt van een acuut stress model, waarbij de muizen 120 minuten geïmmobiliseerd werden. Naast de verwachte verhoging van stress hormonen zoals corticosteron, bleek ook het pro-inflammatoire cytokine Interleukine 6 (IL-6) in het bloed en de hoeveelheid

geactiveerde mestcellen in het hart significant hoger te zijn in de stress groep ten opzichte van de controle muizen. Omdat in mensen blootstelling aan acute stress, zoals tijdens een natuurramp, een terroristische aanval of na het overlijden van een partner, sterk geassocieerd is met een verhoogd risico op een hartinfarct of beroerte, hebben wij in deze studie gekeken naar de stabiliteit van de atherosclerotische plaques in de gestreste en controle muizen. Om ook specifiek naar de bijdrage van mestcellen te kijken werd een extra groep met de mestcel stabilisator cromolyn behandeld voor de acute stressor. Analyse van de gevormde atherosclerotische plaques toonde een verhoogd aantal intraplaque bloedingen en verminderde collageen hoeveelheden, beide kenmerken van een instabiele plaques, in de stress groep. Voorbehandeling met cromolyn kon deze veranderingen deels voorkomen. Om zeker te zijn van complete mestcel remming, is eenzelfde experiment uitgevoerd met mestcel deficiënte muizen. Ook hier zagen wij na stress geen intraplaque bloedingen, wat duidt op een belangrijke bijdrage van de mestcel in acute stress gemedieerde plaquedestabilisatie. Naast de stabiliteit van de plaque is ook de thrombogeniciteit van het bloed van belang bij het induceren van een infarct na ruptuur van een atherosclerotische plaque. In **hoofdstuk 4** is gekeken naar het effect van de acute stress respons op bloedplaatjes activatie, stollingstijd en bloedstolsel vorming. Hoewel 120 minuten stress geen significant effect op plaatjes activatie had, was de bloedstollingstijd na een staartsnede langer en stolsel retractie verminderd. De invloed van acute stress op trombose vorming werd onderzocht door middel van een ijzerchloride-geïnduceerd trombose model, waarbij plaatselijk ijzerchloride ($FeCl_3$) aangebracht wordt op de halsslagader en de bloedcirculatie gemeten wordt, totdat het bloedvat afgesloten is. Het was interessant dat wij geen duidelijke verschillen zagen in stollingstijd tussen de gestreste en de controle muizen. Ook de bijdrage van mestcellen in de effecten van acute stress op plaatjesactivatie, stolling en trombosevorming, getest door middel van mestcel deficiënte muizen, lijkt gering. Gecombineerd laten de resultaten in **hoofdstuk 3** en **4** zien dat de acute stress leidt tot mestcel activatie in het hartweefsel en dat acute stress bijdraagt aan destabilisatie van vergevorderde atherosclerotische plaques. Vervolgonderzoek is nodig om uit te wijzen welke factoren uitgescheiden door de mestcel hiervoor verantwoordelijk zijn, om deze vervolgens specifiek te kunnen remmen. Een mogelijk voorbeeld hiervan zijn de mestcel-specifieke enzymen chymase en tryptase, waarvan bekend is dat zij de extracellulaire matrix kunnen afbreken, wat bijdraagt voor plaque destabilisatie. Het is van therapeutisch belang om te bepalen hoe mestcellen in de nabijheid van de atherosclerotische plaque geactiveerd worden. Dit zou dan als drug target kunnen dienen om de ontstekingsreactie en het verder ontwikkelen van de plaque te remmen. Naast de klassieke activatieroute, die wordt gekarakteriseerd door crosslinking van IgE-antilichamen gebonden aan FcεR receptoren op het oppervlak van de mestcel, zijn andere activatoren ontdekt. Verschillende liganden van de

Toll-like receptor familie, factoren van het complement systeem (C5a, C3a) en neuropeptiden (substance P) zijn in staat perivasculaire mestcellen te activeren en zo bij te dragen aan de ontstekingsreactie in de vaatwand. Een van de meest voorkomende stress-gerelateerde neuropeptiden in het lichaam is neuropeptide Y (NPY). Buiten het centrale zenuwstelsel komt dit peptide voornamelijk samen met het hormoon noradrenaline en het signaal molecuul ATP voor en heeft vaatverwijdende effecten. Een rol voor NPY in atherosclerose bleek onder andere uit een versneld ziekteverloop in personen met een verhoogde NPY activiteit door een gain-of-function mutatie in dit gen. In **hoofdstuk 5** hebben wij de expressie van NPY vergeleken in verschillende stadia van zowel humane als muizen atherosclerotische plaques. Er werd een verhoogde expressie van NPY gemeten in instabiele humane plaques en vergevorderde muizen laesies. Om het directe effect van verhoogde NPY niveaus op de vorming van atherosclerotische plaques te onderzoeken is een lentiviraal construct gemaakt, dat na lokale toediening tot een overexpressie van NPY leidde nabij de plaats van plaque vorming. In overeenstemming met eerdere resultaten zorgde de verhoogde expressie tot versnelde plaque vorming ten opzichte van muizen behandeld met een controle virus. Opvallend was de verhoogde hoeveelheid geactiveerde mestcellen na NPY overexpressie. Dit werd hierna in vitro (in celcultuur) onderzocht door geïsoleerde mestcellen te activeren met verschillende concentraties NPY. Zowel bij nanomolaire als micromolaire concentraties NPY was er een verhoogde concentratie IL-6 en tryptase waarneembaar in het medium, wat duidt op directe mestcel activatie door NPY. NPY signalering vindt plaats via verschillende G-eiwit gekoppelde receptoren (Y1-Y6), waarvan Y1, Y2, en Y5 het meest tot expressie komen en het best gekarakteriseerd zijn. In **hoofdstuk 6** is de rol van elk van deze receptoren in atherosclerotische plaque vorming onderzocht. Genexpressie analyse van deze receptoren gedurende plaque vorming wees op verminderde expressie van Y1 en verhoogde of gelijke expressie van Y2, Y5 en het enzym DPPIV, dat NPY1-36 knipt tot NPY3-36, zodat het niet meer bindt aan Y1. Om therapeutisch de pro-atherogene effecten van NPY te remmen zou dus een Y2 of Y5 antagonist mogelijk een groter effect hebben. Deze hypothese werd getest door middel van toediening van Y1, Y2 or Y5 receptor antagonisten in muizen die atherosclerotische plaques ontwikkelen. Na 6 weken behandeling werden de muizen en de plaques geanalyseerd op plaque grootte en compositie. Anders dan verwacht waren de plaques groter in de receptor antagonist behandelde muizen. Met name de Y2 receptor antagonist behandeling resulteerde in significant grotere en meer instabiele plaques met minder macrofagen en gladde spiercellen en verhoogde mestcel hoeveelheden. Y1 en Y5 receptor antagonisme leidde daarentegen tot veranderd triglyceride metabolisme en verhoogde productie van de pro-inflammatoire IL-12 cytokine familie, zonder een duidelijk effect op atherosclerose. Op basis van deze data lijkt het toepassen van NPY receptor antagonisten ter

bescherming tegen de ontwikkeling van atherosclerose therapeutisch niet interessant, en dient er rekening te worden gehouden met dergelijke bijwerkingen door het gebruik van deze stoffen tegen het ontstaan van restenose. Naast pro-inflammatoire cytokines en proteasen scheiden mestcellen na activatie ook groeifactoren en chemokines uit. Veranderende concentraties van deze chemokines in het bloed en in weefsels vormen vervolgens gradiënten waardoor immuuncellen naar bepaalde locaties, bijvoorbeeld de plaque, kunnen migreren. In **hoofdstuk 7** is daarom onderzocht in hoeverre langdurige mestcelactivatie leidt tot actieve rekrutering en accumulatie van witte bloedcellen. Na herhaalde IgE-gemediëerde mestcelactivatie gedurende de ontwikkeling van atherosclerose in muizen vonden we sterk verhoogde hoeveelheden neutrofielen, zowel in de plaque als in het perivasculaire weefsel. Om vervolgens te bepalen in hoeverre dit effect mestcelafhankelijk was, werden zowel mestcel deficiënte als controle muizen in de buikholte geïnjecteerd met een mestcelactivator. Ook hier was voornamelijk de rekrutering van neutrofielen zichtbaar, die met name positief waren voor de chemokine receptoren CXCR2 of CXCR4. Blokkade van CXCR2 middels een anti-CXCR2 receptor antilichaam bevestigde de rol van deze receptor en het mestcel uitgescheiden ligand CXCL1 in neutrofiel rekrutering na mestcelactivatie. Deze experimenten laten een additioneel mechanisme zien waarmee deze twee celtypen van het aangeboren immuun systeem elkaars respons versterken en bijdragen aan vasculaire ontstekingen en atherosclerose ontwikkeling. Zoals eerder gezegd, zijn hart- en vaatziekten momenteel een van de belangrijkste doodsoorzaken en omvatten een ziekteproces wat al in jongvolwassenen begint maar pas op latere leeftijd voor levensbedreigende complicaties zorgt. Naast risicofactoren zoals hyperlipidemie, roken en geringe lichaamsbeweging worden psychosociale risicofactoren, als psychische stress, steeds meer erkend als belangrijk, maar ook als verander- en behandelbaar. De resultaten beschreven in dit proefschrift onderschrijven de bijdrage van stress in atherosclerotische plaqueontwikkeling en als risicofactor voor acute cardiovasculaire syndromen zoals een hartinfarct of beroerte. Met name de pro-atherogene rol van mestcellen is verder belicht, welke geactiveerd door de acute stress respons en door het stress-gerelateerde neuropeptide NPY, kan leiden tot atherosclerotische plaquedestabilisatie en versnelde plaquevorming. Daarnaast werd een interactie tussen geactiveerde mestcellen en neutrofielen ontdekt, welke sterk bijdraagt aan de ontstekingsreactie in de door atherosclerose aangetaste vaatwand. Deze resultaten identificeren verschillende potentiële therapeutische aangrijpingspunten, welke mogelijk in de toekomst toegepast kunnen worden om het residuele risico op hart- en vaatziekten, dat overblijft na cholesterol verlaging, te verminderen.

Curriculum Vitae

Max Lagraauw werd geboren op 19 december 1987 te Delft. In mei 2006 behaalde hij zijn atheneum diploma aan het Vlietland College in Leiden. In datzelfde jaar begon hij met de studie Life Science & Technology, een combinatie studie van de Universiteit Leiden en de Technische Universiteit Delft. De bachelor fase werd in 2009 afgerond met een wetenschapsstage bij de afdeling dermatologie van het LUMC. Tijdens de master fase werd de focus verder gelegd op het doen van wetenschappelijk onderzoek met twee 7-maandse stages bij de afdeling Medische Farmacologie van het Leiden Academic Centre for Drug research (LACDR) en Inflammation Research Center van de Universiteit Gent. In juli 2011 behaalde hij het doctoraal examen aan de Universiteit Leiden met een 8,3 gemiddeld. Van juli 2011 tot juni 2015 heeft hij zijn promotieonderzoek, dat beschreven staat in dit proefschrift, verricht op de afdeling Biofarmacie van het LACDR onder leiding van dr. Ilze Bot en prof. dr. Johan Kuiper. Dit onderzoek maakte deel uit van het door de Nederlandse Hartstichting gefinancierde project "Stress and Acute Cardiovascular Syndromes: A Key Role for Mast Cells?" (2010B244). Tijdens zijn periode als promovendus ontving hij in maart 2015 een travel grant van de European Atherosclerosis Society om naar het EAS congres in Glasgow te gaan. Daarnaast ontving hij in april 2015 een Young Investigator Award voor zijn posterpresentatie op de Scandinavian Society for Atherosclerosis Research conferentie in Humlebaek, Denemarken. Om zijn wetenschappelijke kennis te verbreden heeft hij van juli tot en met september 2015 deelgenomen aan de 'Treeway Summer Challenge 2015', een project opgezet door het biotechnologie bedrijf Treeway. Gedurende dit project, heeft hij samen met 8 andere PhD en Master studenten literatuur en Big Data analyses uitgevoerd, om zo tot nieuwe inzichten te komen voor de behandeling van de spier- en zenuwziekte amyotrophische laterale sclerosis (ALS).

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