Doctorial Thesis for the Degree of Doctor of Philosophy, Faculty of Medicine

The effects of stress on atherosclerosis in mice

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2010

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Printed by Geson Hulte Tryck
Göteborg, Sweden, 2010
ISBN 978-91-628-8191-7
Abstract and the summary section of this thesis are available online:
http://hdl.handle.net/2077/22934

Therefore I tell you, do not worry about your life, what you will eat or drink; or about your body, what you will wear. Is not life more important than food, and the body more important than clothes? Look at the birds of the air; they do not sow or reap or store away in barns, and yet your heavenly Father feeds them. Are you not much more valuable than they?

Who of you by worrying can add a single hour to his life?

So do not worry. Your heavenly Father knows that you need all these things. But seek first his kingdom and his righteousness, and all these things will be given to you as well. Therefore do not worry about tomorrow, for tomorrow will worry about itself. Each day has enough trouble of its own.

Matthews chapter 6 verse 25-27 and 31-34

Därför säger jag er: bekymra er inte för mat och dryck att leva av eller för kläder att sätta på kroppen. Är inte livet mer än födan och kroppen mer än kläderna? Se på himlens fåglar, de sår inte, skördar inte och samlar inte i lador, men er himmelske Fader föder dem. Är inte ni värda mycket mer än de?

Vem av er kan med sina bekymmer lägga en enda aln till sin livslängd?

Gör er därför inga bekymmer. Er himmelske Fader vet att ni behöver allt detta. Sök först hans rike och hans rättfärdighet, så skall ni få allt det andra också. Gör er därför inga bekymmer för morgondagen. Den får själv bära sina bekymmer. Var dag har nog av sin egen plåga.

Matteusevangeliet kapitel 6 vers 25-27 och 31-34

ABSTRACT

Psychosocial stress has been recognized as an independent risk factor for cardiovascular disease and atherosclerosis. However, little is known about the mechanisms converting this psychosocial load into physical disease. This thesis aims to find and evaluate a well controlled animal model for stress and use it to study the long term consequences of stress on atherosclerosis. We also aim to use this model to search for mechanisms causing stress to accelerate the progression of atherosclerosis.

We exposed atherosclerosis-prone ApoE$^{-/-}$ mice to social isolation, five physical stressors or social disruption stress (SDR-stress). A subgroup of SDR-mice and unstressed mice were treated with metoprolol. Atherosclerosis was assessed and blood samples were collected for analysis of corticosterone, lipids and cytokines.

We found that social isolation and SDR-stress increased atherosclerosis, while the five more physical stressors failed to be atherogenic. Metoprolol per se reduced atherosclerosis in unstressed mice. Plasma corticosterone levels were increased after all 5 physical stressors and SDR-stress, but not in socially isolated mice. Plasma lipid levels were increased in socially isolated mice. Serum levels of the haemotopoietic cytokine G-CSF were decreased in socially isolated mice, pro-inflammatory cytokines IL-6 and CXCL1 were increased after SDR-stress, but no effects on cytokine release was found after the five physical stressors. β-blockade with metoprolol likely reduced SDR-stress-induced increases in both IL-6 and CXCL1, and significantly reduced CXCL1 and TNF-α levels in unstressed mice.

This thesis has provided important information on how social stress accelerates atherosclerosis, and has suggested the release of pro-inflammatory cytokines as an underlying mechanism. Our hope is that our results, and further studies exploring mechanisms converting psychosocial stress into physical disease, will help to reduce the deleterious effects of psychosocial stress.

Keywords: Psychosocial stress, Social isolation, stressors, social disruption stress, atherosclerosis, cytokines, corticosterone, metoprolol

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärt-kärlsjukdomar till följd av åderförkalkning är den vanligaste dödsorsaken i världen. Vi vet att de klassiska riskfaktorerna högt blodtryck, höga kolesterolnivåer, diabetes och rökning dramatiskt ökar risken för att drabbas av hjärt-kärlsjukdomar, men vi ser också att personer som saknar dessa riskfaktorer drabbas. En förklarning till detta kan vara stress, som under senare tid har visats vara ytterligare en riskfaktor för hjärt-kärlsjukdom. Trots att mycket numera tyder på att stress ökar risken att drabbas av hjärt-kärlsjukdom, så vet vi fortfarande inte varför. Syftet med denna avhandling var att försöka förstå sambandet mellan stress och åderförkalkning, och att hitta bakomliggande mekanismer.

Vi har använt oss av genetiskt modifierade möss som spontant utvecklar åderförkalkning och fann att olika sorters stress påverkade åderförkalkning olika. Socialt betingad stress, som stör den sociala miljön som mössen normalt lever i, ökade åderförkalkningen, medan stress som är mer fysiskt betingad inte påverkade åderförkalkningen. Vi såg också att den socialt betingade stressen ökade blodnivåerna av olika inflammatoriska markörer, cytokiner, som tidigare visats påskynda utvecklingen av åderförkalkning. Vidare fann vi vissa bevis för att denna ökning av cytokiner kan vara medierad via det sympatiska nervsystemet, eftersom effekten kunde minskas av en β-blockerare, metoprolol. Samma β-blockerare minskade också åderförkalkningen och frisättningen av cytokiner i möss som inte stressats, vilket visar att det sympatiska nervsystemet spelar en viktigt roll i åderförkalkningsutvecklingen.

Sammanfattningsvis kan sägas att social stress som aktiverar immunförsvaret så att pro-inflammatoriska cytokiner frisätts, också är den sorts stress som leder till bildandet av åderförkalkning. Möjligen kan vanliga β-blockerare till viss del förhindra att denna stress leder till åderförkalkning.

LIST OF PUBLICATIONS

This thesis is based upon the following papers, referred to in the text by their roman numerals:

Paper I **Effects of social isolation and environmental enrichment on atherosclerosis in ApoE$^{-/-}$ mice**
Evelina Bernberg, Irene J Andersson, Li-ming Gan, Andrew S Naylor, Maria E Johansson, Göran Bergström
Stress **2008**. 11(5): 381–389

Paper II **Repeated exposure to stressors do not accelerate atherosclerosis in ApoE$^{-/-}$ mice**
Evelina Bernberg, Irene J Andersson, Sofia Tidstrand, Maria E Johansson, Göran Bergström
Atherosclerosis **2009**. 204: 90–95

Paper III **Social disruption stress increases IL-6 levels and accelerates atherosclerosis in ApoE$^{-/-}$ mice**
Evelina Bernberg, Maria E Johansson, Göran ML Bergström
In manuscript

Paper IV **Metoprolol reduces pro-inflammatory cytokines and atherosclerosis in ApoE$^{-/-}$ mice**
Evelina Bernberg, Maria E Johansson, Göran ML Bergström
In manuscript

LIST OF ABBREVIATIONS

Ang II	angiotensin II
$ApoE^{-/-}$ mouse	apolipoprotein E deficient mouse
BP	blood pressure
CRP	C-reactive protein
CVD	cardiovascular disease
ECG	electrocardiography
G-CSF	granulocyte-colony stimulating factor
HDL	high density lipoprotein
HPA-Axis	hypothalamus pituitary adrenal axis
HR	heart rate
ICAM-1	inter-cellular adhesion molecule
IL-6	interleukin-6
INF-γ	interferon- γ
LDL	low density lipoprotein
MAP	mean arterial pressure
MCP-1	monocyte chemotactic protein-1
MMP	matrix metalloproteinase
NF-κB	nuclear factor-κB
oxLDL	oxidized LDL
RAAS	renin-angiotensin-aldosterone system
ROS	reactive oxygen species
SAA	serum amyloid A
SBP	systolic blood pressure
SDR-stress	social disruption stress
SNS	sympathetic nervous system
Th1	T-helper cell type 1
Th2	T-helper cell type 2
TNF-α	tumor necrosis factor-α
VCAM-1	vascular cell adhesion molecule 1
VLA-4	very late antigen-4
VLDL	very low density lipoprotein
WHO	world health organization

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INTRODUCTORY REMARKS

Today we see a society with increasing expectations to succeed at work, financially, at home, among friends, indeed in every aspect of life. Our effort to succeed and please our surroundings is in many areas pushed to the limits, and beyond our capacity. On top of this, many people live alone with few people around for support and comfort in a stressful every day life. Accumulating evidence suggest that the resultant strain from all these aspects of modern day life is intimately linked to development of a range of diseases.

Cardiovascular disease (CVD) is the major cause of death in today's society [1] and a number of publications suggest that psychosocial stress is an important and often neglected risk factor for the disease [2, 3]. More specifically, social isolation has recently been suggested to be a risk factor for all cause mortality comparable with cigarette smoking and exceeding risk factors such as obesity and physical inactivity [4]. Although, it is increasingly clear that psychosocial stress plays a role in the development of CVD, little is known about the mechanisms converting a psychosocial load into physical disease. We know that a person experiencing a high level of stress is under a greater risk for myocardial infarction, stroke or any other cardiovascular event, but we do not know why.

The aim of this thesis is to explore the underlying mechanisms converting psychosocial stress into physical disease, i.e. atherosclerosis. We have studied the effects of stress on atherosclerosis in genetically engineered atherosclerosis prone mice ($ApoE^{-/-}$ mice), since it is difficult to gain mechanistic insight from studies in humans.

INTRODUCTION

Atherosclerosis

As a result of reduced smoking and lower cholesterol levels in the population, and the advantages of new treatments for cardiovascular disease (CVD), the incidence of coronary artery disease and the cardiovascular mortality rate have declined in Western societies over the last few decades [5, 6]. However, CVD is still the leading cause of death worldwide today and causes an estimated 30-40 % of all deaths [6, 7]. CVD is predicted to remain the single leading cause of death globally over the next 20 years [1]. Atherosclerosis is the major underlying cause for CVD.

Atherosclerosis is a slowly progressing disease that is initiated early in life and develops further throughout life. The progression of atherosclerosis may continue for decades without symptoms, but eventually the atherosclerotic lesions may rupture and cause a clinical event such as stroke or myocardial infarction.

The traditional risk factors for atherosclerosis and CVD are hypertension, hyperlipidemia, smoking and diabetes [8]. These risk factors contribute substantially to the development of atherosclerosis.

A role for stress in atherosclerosis

The traditional risk factors do, however, not account for all cases of disease [8] and lately, psychosocial stress has been identified as an important contributor [2, 3]. In a recent meta-analysis, social isolation was suggested to be a risk factor for total mortality comparable with cigarette smoking and exceeding risk factors such as obesity and physical inactivity [4]. Moreover, depression and the lack of social support are two forms of stress that after acute myocardial infarction are

associated with increased cardiac morbidity and mortality [9-11]. However, despite accumulating evidence and increased awareness of the importance of stress in the pathogenesis of CVD, the underlying mechanisms are still largely unknown. It is believed that the prolonged, multifaceted neurohormonal activation seen during chronic exposure to stress may be harmful for the cardiovascular system [3, 12].

During stress the sympathetic nervous system (SNS) is activated, noradrenalin and epinephrine are released and bind to their receptors, adrenoceptors, resulting in increased heart rate, vasoconstriction etc. Moreover, the hypothalamus-pituitary-adrenal axis (HPA-axis) is activated by stress. When the HPA-axis is activated ACTH (adrenocorticotropic hormone) is released from the pituitary and stimulates the release of corticosterone from the adrenal glands. Cortisol is a classical "stress hormone" that is released within minutes after activation and has, among many properties, immunosuppressive effects. The role for cortisol in atherogenesis is however complex, and not fully understood [13]. Thus, in response to stress the SNS and the HPA-axis are activated and subsequently mediate their distinctive effects on the cardiovascular system and other target organs.

Clinical investigations and population studies have provided important information on the association between stress and development of atherosclerosis, but have not been able to provide information on underlying mechanisms. Animal experiments are therefore crucial to determine causality and to understand mechanisms by which stress accelerates atherosclerosis.

In landmark studies, Kaplan and coworkers showed that atherosclerosis development was accelerated in male cynomolgus monkeys living in an unstable social environment [14, 15]. The effect on atherosclerosis was blocked by non-selective β-blockade using propranolol [16]. Furthermore, selective β_1-

adrenoceptor blockade by metoprolol protected against endothelial injury induced by stress (Skantze et al., 1998). Social isolation has also been shown to increase development of atherosclerosis in rabbits living in isolation [17].

Mechanistic links between stress and atherosclerosis

The interactions between stress and atherosclerosis may occur at several levels. Although the mechanisms linking CVD and stress are not clarified, a number of hypothetical interactions exist. The following text is an outline of the most important steps in atherosclerosis development and how stress may interfere with these processes.

Sympathetic activation – a link between stress, the immune system and atherosclerosis

Blockade of the sympathetic nervous system (SNS) by β-blockers have been shown to reduce the risk of cardiovascular events after myocardial infarction, in patients with hypertension and in heart failure [18, 19]. The mechanisms behind this cardioprotective effect have been attributed to the many positive effects β-blockers have on cardiac function: anti-arrhythmic effects, improvement of myocardial function and lowering of cardiac oxygen consumption and lowering of blood pressure. In addition, a few studies have also shown that β-blockers may have a direct anti-atherosclerotic effect [20, 21]. Interestingly, a SNS mediated induction of the transcription factor nuclear factor-κB (NF-κB) by psychosocial stress has been observed in humans as well as in mouse models [22, 23]. This stress- and noradrenalin-mediated NF-κB activation induce IL-6 mRNA transcription and can be inhibited by adrenergic blockade [22]. In this way psychosocial stress, via activation of the SNS, can be linked to the immune system.

Atherosclerotic plaque formation

Atherosclerotic lesions are formed specifically in regions where blood flow is disturbed, i.e. in bifurcations and curvatures [24, 25], and very little in regions with laminar flow. Atherosclerosis is an inflammatory disease and leukocytes, mostly monocyte-derived macrophages and T cells [26], are abundant in the lesions and play very important roles for the progression of the disease [24, 27]. Hence, the activation of endothelial cells and the subsequent expression of adhesion molecules and chemokines, in regions with turbulent flow [28-30] are important for the recruitment of leukocytes to these sites.

Atherosclerosis is initiated when infiltration and accumulation of low density lipoprotein (LDL) in the arterial intima initiate an immune response in the vessel wall [31, 32]. Modified LDL particles, such as oxidized LDL (oxLDL) activate endothelial cells, which express leukocyte adhesion molecules such as vascular cell-adhesion molecule 1 (VCAM-1) [33]. Leukocytes, mainly monocytes and T-cells, then bind to VCAM-1 on endothelial cells and, by the guidance of chemokines produced by vascular cells, migrate into the intima. In the intima, monocytes differentiate into macrophages which release pro-inflammatory cytokines, chemokines etc. that further augment the inflammatory response. Macrophage uptake of oxLDL through scavenger receptors, leads to intracellular accumulation of cholesterol and the subsequent formation of foam cells (reviewed in [34]). Activated macrophages express class II major histocompatibility complex (MHC class II) that allow presentation of processed oxLDL as antigens to T cells, which are then activated and secrete cytokines [34]. Early atherosclerotic lesions, fatty streaks, are mainly composed of foam cells [24]. Fatty streaks progress into atheromas, which are complex atherosclerotic lesions with a lipid rich core and a covering fibrous cap that contains smooth muscle cells and a collagen-rich matrix [26]. Although the lesions grow in the intima the lumen diameter of the vessel remains constant, due to remodulation of the outer boundaries of the artery. This

phenomenon compensates for plaque expansion to a certain extent. However, as the plaque continues to grow narrowing of the lumen will eventually occur with stenosis as a consequence [35].

There are a few animal models in which stress has been shown to increase formation of atherosclerotic lesions in monkeys, rabbits and possibly also in mice [15, 17, 36]. It is, however, unclear at what mechanisms these actions take place. There are evidence suggesting that stress may lead to increased plasma lipid levels [37], and may thus lead to increased lipid accumulation in the vessel wall. Moreover, administration of the β-blocker metoprolol to rabbits decreased the expression of adhesion molecules VCAM-1 and ICAM-1, reduced lipid accumulation in lesions, and subsequently stabilized vulnerable atherosclerotic plaques [38].

Plaque rupture leads to clinical events

Fatty streaks are present from young age and do not cause symptoms. Fatty streaks can, however, progress into advanced atherosclerotic plaques (atheromas) and these can in some cases cause disease. When the fibrous cap covering the plaque ruptures and the pro-thrombolytic interior is exposed to the blood stream a thrombus forms blocking the artery and causing an acute event like stroke or myocardial infarction [39]. Pro-inflammatory cytokines including INF-γ and TNF-α are released by activated immune cells in the plaque. INF-γ inhibits proliferation, collagen secretion and expression of contractile proteins by smooth muscle cells [40, 41]. These cytokines also stimulate the production of proteases (MMP's etc.) that attack and degrade collagen in the fibrous cap. Both these effects reduce the stability of the plaque. Activated immune cells can also produce pro-thrombotic and pro-coagulant factors that directly enhance the formation of a thrombus [27].

Stress may, through the activation of SNS, trigger cardiovascular events such as myocardial infarction [12, 42]. It is well known that increased sympathetic activity activates platelets, and thus increases the thrombogenic properties of blood. Moreover, stress has been shown to cause endothelial dysfunction and arrhythmia (reviewed in [12]). All of these effects may as a response to stress trigger cardiovascular events.

Cytokines and chemokines in atherosclerosis

Cytokines produced by macrophages and T cells are key players during acute and chronic inflammation. Many cytokines have been assigned important roles in atherogenesis, most of them with pro-atherogenic effects (e.g. IL-1, IL-6, IL-12, IL-18, IFN-γ, TNF-α, MIF and M-CSF) while only a few anti-atherogenic cytokines has been identified (IL-10 and TGF-β) [43-45].

In the tissue, activated T cells generally differentiate into T helper cells type 1 (Th1) or T helper cells type 2 (Th2) and start producing cytokines specific for Th1 and Th2 responses, respectively [46]. Th1 cytokines are predominant in atherosclerotic lesions, while Th2 cytokines are less common [44]. Activated Th1 cells begin producing INF-γ, TNF-α and IL-1 [27]. These cytokines induce the production of IL-6, which is a potent inducer of acute phase proteins like C-reactive protein (CRP) and serum amyloid A (SAA) [47]. In fact, both IL-6 and CRP are independent risk factors for CVD [48-50]. Moreover, pro-inflammatory cytokines like IFN-γ, TNF-α and IL-6 has been shown to increase in humans after exposure to both acute and chronic stress [51-53], suggesting that psychosocial stress may initiate a Th1-like response.

Chemokines are chemoattractant molecules that promote and guide leukocyte migration from the blood stream into the inflamed tissue. Several chemokines

have been associated with atherosclerosis and play critical roles in directing leukocytes into atherosclerotic-prone vessels [54]. The role for stress in the production and release of chemokines is poorly understood. However, there are some data suggesting that plasma levels of monocyte chemotactic protein-1 (MCP-1) may be increased in chronically stressed women [55, 56].

HYPOTHESIS

We hypothesize that prolonged exposure to psychosocial stress may induce the production of pro-inflammatory cytokines and chemokines and subsequently accelerate atherosclerosis. Figure 1 illustrates the specific questions we sought answers to in this thesis.

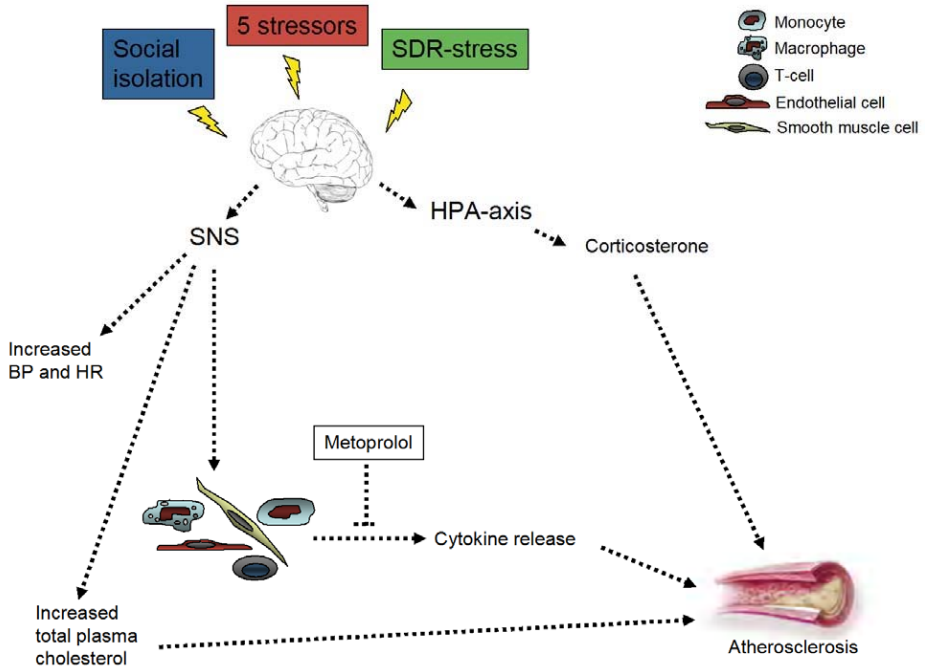

Figure 1. A summary of hypothesized mechanistic links between psychosocial stress and atherosclerosis. We hypothesized that the different forms of stress would activate the sympathetic nervous system (SNS) and the hypothalamus-pituitary-adrenal axis (HPA-axis), leading to a cascade of downstream events, eventually accelerating atherosclerosis. We also hypothesized that the β-blocker metoprolol would inhibit effects such as cytokine release mediated by the SNS. BP-blood pressure; HR-heart rate. Dashed lines represent hypothesized pathways.

AIMS OF THE THESIS

The general aim of this thesis was to find and evaluate a controllable form of social stress that in the long term leads to increased atherosclerosis in mice. Further, the aim was to search for mechanisms causing social stress to accelerate the progression of atherosclerosis.

The specific aims of this thesis were:

To investigate the effects of social isolation and environmental enrichment on atherosclerosis in ApoE$^{-/-}$ mice (**Paper I**).

To investigate the effects of five physical stressors on atherosclerosis in ApoE$^{-/-}$ mice. The aim was also to evaluate the possible synergistic effect of these stressors with a high salt intake (**Paper II**).

To investigate the effects of long-term SDR-stress on atherosclerosis in ApoE$^{-/-}$ mice. Further, the aim was to compare SDR-stress with the five physical stressors used in Paper II, regarding cytokine release (**Paper III**).

To investigate a possible anti-atherogenic effect of metoprolol, and to study the effects of this β-blocker on cytokine release in ApoE$^{-/-}$ mice (**Paper IV**).

METHODOLOGICAL CONSIDERATIONS

Mouse models for atherosclerosis

Atherosclerosis does not develop spontaneously in laboratory mice because of their lipid profile, with high HDL levels and low LDL levels, which significantly differ from humans [57-59]. However, targeted deletion of specific genes (knockout) can provide mouse strains that develop atherosclerosis. One such mouse strain is $ApoE^{-/-}$ mice, where the gene for apolipoprotein E is knocked out. These mice display severe hypercholesterolemia and spontaneously develop atherosclerosis [58]. Another genetically modified mouse strain that develop atherosclerosis is $LDL^{-/-}$ mice lacking the gene for LDL receptors. These mice preferably need to be fed a high cholesterol diet to develop atherosclerosis [60]. In this thesis $ApoE^{-/-}$ mice have been used.

The $ApoE^{-/-}$ mouse

Genetically manipulated mice, such as $ApoE^{-/-}$ mice are convenient to use in atherosclerosis research. Atherosclerosis is a disease that develops very slowly and leads to clinical events late in life. Mice have a short life span and develop atherosclerosis in a matter of weeks or month, compared to several decades in humans. Moreover, mice are small in size and are thus easy to house and can be maintained at low costs. However, with the small body size follows limitations in amount of tissue and blood samples that can be collected.

Atherosclerotic lesion development in $ApoE^{-/-}$ mice

$ApoE^{-/-}$ mice display very high cholesterol levels even on a standard diet, mostly in the VLDL and chylomicron remnant fractions [59, 61]. Importantly, although $ApoE^{-/-}$ mice display a different lipid profile than humans, atherosclerotic lesion

development appears to be similar at early stages of atherosclerosis with the initial formation of fatty streaks that further progress into advanced lesions with a fibrous cap [62].

Unexpectedly, the inter-individual variability in lesion area is very large in ApoE$^{-/-}$ mice, despite inbreeding for many generations [57]. One would expect genetically "similar" individuals to display similar lesion area after exposure to the same treatment. In the light of the theme of this thesis, it is intriguing to speculate whether the level of stress as a result of hierarchal position may contribute to this variability. This, however, remains to be investigated.

Dietary manipulations

Western diet (Paper IV)

Although ApoE$^{-/-}$ mice develop atherosclerosis on standard chow, atherosclerosis can be further accelerated by feeding mice a high cholesterol and high fat diet [61]. This diet is usually called a "western diet", and contains 0.15% cholesterol and 21% fat, in this thesis (Paper IV). However, subtle changes in plaque progression caused by other mechanism than hypercholesterolemia may be overshadowed by the atherogenic effect of the very high cholesterol levels that ApoE$^{-/-}$ mice display on western diet.

High salt diet (Paper II)

A high salt intake increases blood pressure in salt-sensitive individuals, and may also increase blood pressure in the population. However, the effect of a high salt intake on blood pressure is controversial. Although some studies suggest a positive relationship between salt intake and hypertension, intervention studies have failed to show substantial effects on blood pressure after salt restriction [63-65]. The

response to either salt loading or salt restriction is off-set by changes in salt excretion induced by changes in the activity of the sympathetic nervous system (SNS) and the renin-angiotensin aldosterone system (RAAS). If regulation of the SNS and RAAS is defect a salt load may not be excreted and thus results in a pathological rise in blood pressure. It appears as a defect in RAAS sensitivity to salt loading is responsible for high blood pressure in a subgroup of patients [66]. In accordance, our group has previously shown that fixed high Ang II levels, imitating a dysregulated RAAS, in combination with a high salt diet accelerates atherosclerosis in $ApoE^{-/-}$ mice, in a way that a high salt diet or Ang II infusion alone do not [67]. In the light of this finding we wanted to test the hypothesis that psychosocial stress may cause a dysregulation of SNS and would thus, in combination with a high salt diet accelerate atherosclerosis to a greater extent than psychosocial stress alone. In Paper II we thus administrated a high salt diet (8% NaCl) to two of four groups of mice.

Animal models for stress

In stress research several different animal models have been described in the literature. In this thesis we have used social isolation (Paper I), five physical stressors (Papers II and III), and social disruption stress (SDR-stress; Paper III) as models for psychosocial stress in mice.

Social isolation (Paper I)

Mice are social animals that prefer living in groups and develop stable social hierarchies. To deprive mice of social interaction is thus stressful. During social isolation in Paper I mice were socially deprived by individual housing during 20 weeks. We compared socially deprived mice with group housed mice with different levels of environmental enrichment.

However, there are some limitations and potential confounding factors with using social isolation as a model for psychosocial stress. Although mice are likely experiencing stress due to the lack of social support, socially isolated mice also alter their behavior compared to group-housed mice. Such behavioral changes could be decreased physical activity and changed food intake. Physical inactivity is a know risk factor for CVD and may confound our results. It is difficult and expensive to assess the level of physical activity in mice without disturbing natural behavior. Therefore, comparing individually housed mice with group housed mice has limitations that must be taken into account when analyzing data. Moreover, in the normal situation mice cuddle together to keep warm. Social isolation may thus lead to problems with maintenance of normal body temperature, and may cause a subsequent activation of the sympathetic nervous system which may also confound our results.

Physical stressors (Papers II and III)

To overcome problems with confounding factors when comparing group-housed mice with individually housed mice, we wanted to find a form of stress that could be performed in a more controlled manner, in group housed mice. We exposed mice to five different physical stressors during 2 hours per day for twelve weeks. We used restraint stress, rat odor stress, the combination of restraint and rat odor stress, balance stress, and air-jet stress (Fig. 2A-E). Mice were exposed to each of the five stressors once a week in a randomized order, so that mice were exposed to all five stressors every week.

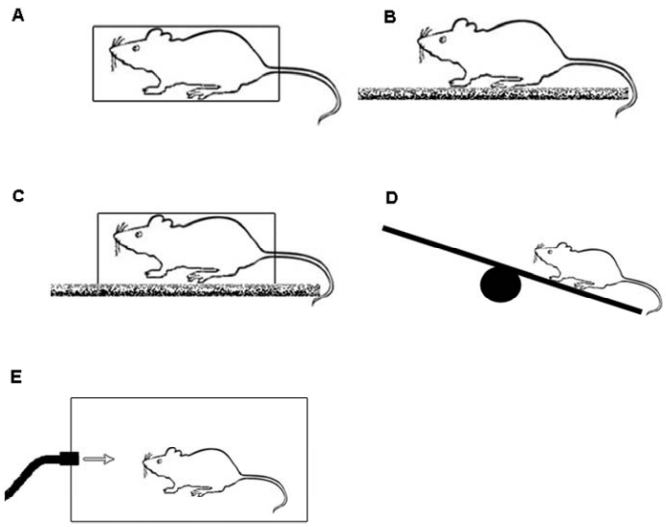

Figure 2. The five stressors used in Paper II and III. (A) Restraint stress, where mice were immobilized in a well ventilated plastic tube; (B) Rat odor stress, where mice were placed in a cage with saw dust where male rats had previously been held; (C) Rat odor combined with restraint stress; (D) Balance stress, where mice were placed in a cage with an unstable floor; and (E) Air-jet stress, where mice were placed in a specially designed cage into with a stream of compressed air was intermittently blown in periods of 2-10 minutes followed by 2-10 minutes of rest.

SDR-stress (Paper III)

In Paper III we wanted to evaluate a more social form of stress in group-housed mice. We used a social form of stress, termed social disruption stress (SDR-stress), which was controllable in the same way as the five physical stressors. SDR-stress is based on the fact that male mice housed together develop social hierarchies. Disruption of these established hierarchies is a model for social stress in rodents [68].When a resident mouse becomes subordinate to an intruding mouse this causes immune-endocrine alternations [69]. During SDR-stress a dominant intruder is introduced into a group of mice with established hierarchies (Fig. 3).

SDR-stress has been shown to increase the release of pro-inflammatory cytokines like IL-6 and TNF-α [70, 71], with known pro-atherogenic effects. We therefore hypothesized that SDR-stress triggers an immune response that in the long term would be atherogenic.

During SDR-stress sessions mice fight to defend hierarchal position. This may lead to wounds with subsequent inflammation. Previous studies have shown that cytokine levels after SDR-stress correlate to wounds caused by the fights [72]. To ensure that the potential effects of SDR-stress on cytokine release was due to increased stress, and not infected wounds, mice were carefully monitored during SDR-stress in Paper III. Mice were allowed to fight, but if biting occurred the attack was immediately interrupted by the researcher. In this way wounds were successfully avoided and could be eliminated as a confounder in this study.

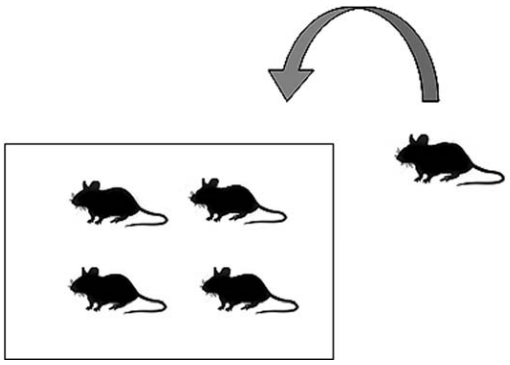

Figure 3. Social disruption stress (SDR-stress) where a dominant intruder was introduced into a cage with male mice with stable social hierarchies.

Quantification of atherosclerosis (Papers I-IV)

Atherosclerotic plaque area was quantified at two different sites, using two different methods both en face area and the cross-sectional area. En face quantification is a lipid staining of the inner (intimal) surface of the opened vessel showing how much of the intimal surface that is covered by lesions. En face

quantifications tell nothing about the developmental stage of the plaque (i.e. fatty streaks, advanced lesions) or plaque composition (collagen and immune cell content etc). Cross-sections of vessels, on the other hand, show both how much of the vessel lumen that is occluded by the lesion and can be immunohistologically stained and lesion composition can be investigated. Importantly, there is only a weak correlation between plaque size in the thoracic aorta and the innominate artery [57], possibly dependent on atherosclerotic stimuli. It is therefore important to quantify atherosclerosis at more then one site.

En face quantification (Papers I-IV)

The thoracic aorta (from the left common carotid artery to the left renal artery) was used in Papers I, II and IV (Fig. 4A), while the whole aorta (from the left common carotid artery to the aortic bifurcation) was used in Paper III.

Cross-sectional quantification (Papers I-III)

Cross-sections of the innominate artery (Papers I and III) were stained with Miller's elastin (Paper I) or Picrosirius red (Paper III; Fig. 4B). The aortic root (Paper II and III) were stained with Picrosirius red (Paper II) or Oil red O (Paper III). Lesions were measured by a blinded observer.

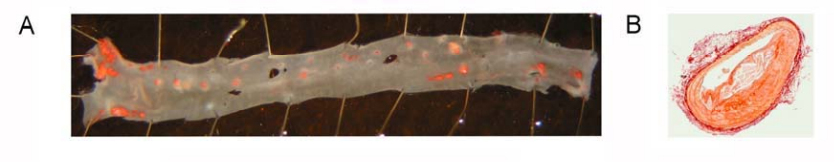

Figure 4. Quantification of atherosclerosis. (A) Thoracic aorta pinned onto a silicone-coated dish and stained with Sudan IV for lipids. (B) A cross-section of the innominate artery stained with Picrosirius red for collagen.

Immunohistochemistry (Paper II)

With the immunohistochemical technique protein expression can be quantified in cross-sections of vessels, or other tissue, by the interaction with specific antibodies. In Paper II an antibody specific for macrophages (MAC-2 primary antibody) were used in cross-sections of the aortic root to quantify the macrophage content of the lesions.

Osmotic minipump operations (Papers III and IV)

Reliable drug delivery during an experiment is very important to achieve good results in research. In animal experiments per oral administration is complicated, while injections are invasive, time consuming, and there is always a risk that the drug is not always injected to the right compartment (i.e. intraperitoneal injection can easily become a subcutaneous or intramuscular injection if the animal suddenly moves). To overcome these drug delivery problems, osmotic minipumps were used in Papers III and IV for metoprolol administration. These osmotic minipumps are implanted subcutaneously on the back of the mouse and reliably deliver drugs at a specific infusion rate during up to 6 weeks (depending on model).

During minipump implantation mice were anesthetized with isoflurane during 5-10 minutes and minipumps were implanted subcutaneously on the back of the mouse. Mice were given analgesics (Temgesic) subcutaneously before the operation.

Blood pressure measurements (Papers I and II)

Blood pressure measurements are difficult to perform in a reliable way in mice. In this thesis three different methods have been used, each with its advantages and drawbacks.

Tail-cuff technique (Paper I)

A non-invasive tail cuff system was used in Paper for measurement of systolic blood pressure (SBP). The conscious mouse was kept in a restrainer, with a standard acclimatization time of 10 min. An advantage with this method is that it is non-invasive and mice are conscious. However, although mice are allowed acclimatization to restraint, the situation is stressful and may affect blood pressure (see results in Paper II, where both heart rate and blood pressure increase for 2 hours during restraint stress; [73]).

Anesthetized mean arterial pressure measurement (Papers I and II)

On the day of termination, mice were anaesthetized and mean arterial pressure (MAP) was measured by placing a catheter in the left common carotid artery.

An advantage with this method is that mice are not exposed to stress since they are anesthetized. However, the blood pressure may vary with the level of anesthesia. Further, depending on previous treatment mice may react differently to anesthesia.

Blood pressure telemetry (Paper II)

To overcome problems with exposing mice to a stressful situation during BP measurements in conscious mice and the risk that mice react differently to anesthesia during measurements in anesthetized mice, radiotelemetry transmitters can be used. Transmitters were implanted in the abdomen of the mice (Paper II)

and a small catheter attached to the telemetry transmitter was implanted into the aorta via the left common carotid artery. During recording conscious mice are left undisturbed, freely moving in their home cages, thus avoiding both stress and problems caused by anesthesia. This method therefore provides very reliable blood pressure measurements. However, a drawback is that the apparatus and maintenance of the system for telemetry measurement is expensive and implantation demands surgical skills.

Electrocardiography (Paper II)

ECG for mice in Paper II was obtained by telemetry technique similar to BP telemetry. Mice were implanted with a transmitter in the abdomen and ECG electrodes were placed under the skin on the chest for HR measurements.

Sample collection and biochemical analysis

Corticosterone (Papers I-III)

When analyzing corticosterone levels correct sampling methods are crucial to obtain reliable results. Corticosterone, the rodent homolog of cortisol, is rapidly released into the blood stream upon arousal and stress and can be detected after about 2 minutes [74]. It is therefore of great importance to collect blood samples within a maximum time of 2 minutes from removing the mouse from its home cage.

Furthermore, besides collecting blood samples rapidly it is also important to collect all samples during the same time of the day since corticosterone is a hormone with a circadian rhythm. Corticosterone levels are reasonably stable between 08.00 and 12.00 am [75], a few hours into the light period when mice

sleep. In all experiments in this thesis samples for corticosterone analysis were taken during this time period.

A way to overcome the problems with rapid release of corticosterone into the blood stream upon handling the mice, urinary corticosterone can be measured. Upon activation of the HPA-axis increases in urinary corticosterone levels can be detected after 1 hour, instead of within 2 minutes as is the case for plasma samples [74]. Further, collection of urine is less invasive than blood sampling.

It is difficult to obtain good baseline or control levels of corticosterone in plasma samples, because of the rapid release of the hormone during sample collection. As a consequence, there is a risk that subtle changes in corticosterone levels can be missed when plasma samples are used. Therefore, in this thesis plasma samples were only used to measure acute effects of different forms of stress on corticosterone release (Papers II and III). To investigate more subtle chronic changes urine samples were used (Papers I-III).

Cytokines (Papers I, III and IV)

Blood for analysis of cytokines was collected at termination from the right ventricle of the heart. Cytokine measurements are not as sensitive to handling stress during sample collection as corticosterone measurements are. Most cytokines are not stored within the cells but are produced de novo upon activation, a process that usually takes a couple of hours [76].

IL-6 (Paper III)

Plasma IL-6 levels were analyzed using a Quantikine Mouse IL-6 ELISA kit (R&D Systems, Inc. Minneapolis, USA), according to the manufacturer's

protocol. IL-6 is synthesized mainly by macrophages and T cells upon activation. However, during baseline conditions IL-6 levels are very low, and can be difficult to detect in plasma. Many samples in control mice usually fall below the detection limit of the ELISA. These samples were therefore assigned a value corresponding to the sensitivity of the assay (1.6 pg/mL). However, this raises statistical problems when analyzing IL-6 data (commented in the "Statistics" section below). Another problem with very low baseline levels of IL-6 it that is not possible to detect decreases in IL-6 compared to a control situation.

Th1/Th2 cytokines (Papers III and IV)

Th1 cytokines (IL-1β, IL-2, IL-12 total, IFN-γ, TNF-α and CXCL1) and Th2 cytokines (IL-4, IL-5 and IL-10) were measured using a Mouse Th1/Th2 Multiplex ELISA (Meso Scale Discovery, Gaithersburg, Maryland, USA) according to the manufacturer's protocol. This multiplex ELISA measures 9 cytokines in a MULTI-SPOT 96-well plate where the capture antibodies are coated on the bottom of the wells on specific spots for each cytokine. During incubation each cytokine binds to its corresponding capture antibody spot, and cytokine levels are quantified using a labeled cytokine-specific detection antibody.

This method makes it possible to specifically measure several different cytokines in one assay using only a small amount of sample, and still get a reliable result. When using mouse models for research, there are always limitations in sample size, because mice are small animals. Therefore, a multiplex ELISA opens up the possibility to measure many parameters although sample supply is limited.

CXCL1 (Paper III)

Serum levels of CXCL1 were analyzed using a Quantikine Mouse CXCL1 ELISA kit (R&D Systems, Inc. Minneapolis, USA), according to the manufacturer's protocol.

G-CSF (Paper I)

G-CSF was measured in Paper I using a premixed Bio-Plex Mouse Cytokine panel (Bio-Rad Laboratories, Hercules, CA, USA).

However, there are some limitations to this method. This method has an inter-/intra-assay variability of <30 %CV and <20, respectively. Such high inter-/intra-assay variability, decreases the reliability of the assay. This method was still used, because it was the only method available at that time. Moreover, samples had been stored at approximately -20°C more than six month, which may have affected the quality of the samples. Due to space restrictions we could only perform this analysis on samples from two of the four groups in Paper I (socially deprived and environmentally enriched mice). Still, the result of this assay may contribute with important information.

Lipids (Papers I-IV)

In Papers I and II, total cholesterol and triglycerides in plasma were analyzed enzymatically and the concentrations were subsequently determined spectrophotometrically (Roche/Hitachi analyzer, Roche Diagnostics, Indianapolis, IN, USA). Total serum cholesterol in Papers III and IV were determined colorimetrically after enzymatic hydrolysis and oxidation using a cholesterol kit (Cholesterol enzymatic endpoint method, RANDOX Laboratories Ltd., United Kingdom), according to the manufacturer's protocol.

Triglycerides levels are affected by food intake and should be measured in the fasting state. However, fasting per se may potentially be stressful to the mice and the mice were hence not fasted in this thesis. In Paper III triglycerides were not measured because, as a consequence of the experimental design, control mice but not SDR-mice had access to food immediately before termination. Triglycerides were not measured in Paper IV, either, because mice in this study were fed a high fat diet.

Isoprostanes (Paper I)

Isoprostanes are prostaglandin-like compounds produced during lipid peroxidation and have been suggested to be a marker for in vivo lipid oxidation possible to measure in a urine sample [77] However, there are limitations when urinary isoprostanes are measured. Urinary isoprostane only reveals changes in systemic oxidative stress and does not assess local changes of oxidative stress possibly present in the blood vessel wall.

Behavioral studies (Paper I)

Exploratory behavior

We analyzed exploratory behavior in Paper I to find out if social isolation changed natural behavior of mice. Exploratory behavior was measured in a novel environment, using activity boxes. Interpretation of data obtained from this analysis is, however, very complex. It has been suggested that corner time is a measure of anxiety, fear or inactivation, and that rearing activity is a measure of exploratory activity, while locomotor activity is merely a measure of the mouse's physical activity [78]

Salt appetite

Psychosocial stress has previously been shown to induce an increased appetite for salt in rats [79, 80]. We measured salt appetite, in Paper I, as a mean to assess the level of stress in mice. Preference for salt in drinking water was letting mice choose between tap water and a 1% NaCl solution. Although salt appetite is not an absolute measure of psychosocial stress, it still provides important information in that mice are indeed affected by the treatment, in this case social isolation.

Statistics

When the number of observations is low (4-20 individuals per group in this thesis), it is difficult to know if data is normally distributed. Skewness and kurtosis values close to zero suggest that data is normally distributed, but with small sample size this is not always true. Therefore, mainly non-parametric statistics, Kruskal-Wallis followed by Mann-Whitney U test (SPSS Statistics version 17.0, Chicago, IL, USA), have been used to analyze data in this thesis. Blood pressure, heart rate and body weight are known to be normally distributed (and displayed skewness and kurtosis values close to zero in Paper I) and was thus analyzed with parametric one-way ANOVA followed by post hoc testing using Tukey's HSD (SPSS). Atherosclerotic plaque area in aortic root was analyzed with a repeated measurement two-way ANOVA (SPSS).

In the IL-6 analysis in Paper III many samples fell below the detection limit of the assay and were therefore assigned a value corresponding to the sensitivity of the assay (1.6 pg/mL). Median values are compared when using non-parametric statistics, and in this case when many values equal 1.6, the median value may actually equal 1.6 for several study groups, and thus group median values appear similar. However, this is a limitation of the method rather then a biological phenomenon. A way to overcome this problem is to logarithmically transform IL-

6 data and subsequently analyze with one-way ANOVA followed by post hoc testing using Tukey's HSD (SPSS). This method allows the comparison of mean values, although data is non-parametric, and statistical significance can be declared.

A p-value <0.05 is considered statistically significant in this thesis. However, when many measurements are performed there is always a risk for mass-significance and subsequent type 1 errors. There are different approaches to reduce the risk for mass-significance. One approach is to use Bonferroni correction [81] where the p-value is divided by the number of measurements. However, Bonferroni is a conservative method, with a risk to miss significant changes (type 2 error) when the number of measurements are high. In the Th1/Th2 analysis in Papers III and IV 9 cytokines were analyzed. Using Bonferroni correction would result in statistical significance if $p<0.006$ ($\alpha/9$). We considered this too conservative. We therefore chose to use a 99 % significance level ($p<0.01$) to determine statistical significance.

SUMMARY OF RESULTS AND DISCUSSION

Social but not physical stress increase atherosclerosis (Papers I-III)

In this thesis we have found that different forms of stress affect atherogenesis differently. In Paper I, social isolation accelerated atherosclerosis in the innominate artery, while the five physical stressors used in Paper II failed to be atherogenic [73, 82]. In Paper III we used SDR-stress, and found a significant correlation between stress level and atherosclerosis in the aorta. Moreover, we found a numerical increased plaque area in aortic root, but this increase was not statistically significant ($p= 0.096$). Nevertheless, taken together plaque data from aorta and aortic root indicate that SDR-stress indeed may accelerate atherosclerosis. Thus, we suggest that social stress, which intervenes with the social environment, accelerates atherosclerosis in $ApoE^{-/-}$ mice. The five more physical stressors, on the other hand, appear easier to cope with.

The lack of social support is an important source of psychosocial stress in humans [12] as well as in animal models [83, 84]. Social isolation is also known to increase cortisol levels in monkeys and to increase atherosclerosis in rabbits [17, 85]. However, although restraint and rat odor stress earlier in a brief report have been suggested to be associated with increased atherosclerosis [36], we found no such association in Paper II [73]. The lack of atherogenicity of the five physical stressors may be due to the fact that mice, after 2 hours of stress exposure, were returned to their home cages and group mates. Thus, mice had social support and time to recover during 22 hour per day. In fact, studies have shown that social support protects against CVD [4, 12]. In Paper III mice, were exposed to SDR-stress during 2 hours, but the stress caused by disturbed hierarchies and the following struggles to re-establish hierarchies continued after the SDR-session

was ended. In this way, mice were exposed to a socially stressful environment during a larger part of the day, compared to the five stressors in Paper II. Furthermore, four of the five physical stressors did not trigger the release of pro-inflammatory cytokines that occurred after SDR-stress, which may also explain why these stressors were not associated with increased atherosclerosis.

Possible mechanistic links between stress and atherosclerosis

In this thesis we have studied the effect of three different forms of stress on atherosclerosis. We found that social stress accelerated atherosclerosis while more physical forms of stress did not. Possible mechanisms converting this psychosocial load into physical disease, i.e. atherosclerosis, are discussed below.

Changed cytokine release

In line with the effects on atherosclerosis, social but not physical stress changed the release of specific cytokines (Table 1). SDR-stress resulted in increased plasma/serum levels of IL-6 and CXCL1, and social isolation decreased serum levels of G-CSF, while after exposure to four of the five physical stressors cytokine levels remained unchanged [73, 82]. We therefore hypothesize that the five more physical stressors fail to be atherogenic because these stressors did not activate the immune system, and did not trigger the release of pro-inflammatory cytokines. SDR-stress, and social isolation to some extent, did affect the release of cytokines, and subsequently also accelerated atherosclerosis. Social stress has been associated with immune disorders in rodents [86], and more specifically with the increase of pro-inflammatory cytokines [71, 87].

One of the five physical stressors, restraint combined with rat odor stress, triggered the release of pro-inflammatory cytokines, although mice exposed rat

odor or restraint alone did not [73]. A speculative explanation could be that the smell of a predator, a rat, only becomes a real threat and psychologically stressful when the mouse cannot move or explore the surroundings. If the mouse can move freely in a cage with rat odor it only takes a little while before the mouse has explored the surroundings and found that there is no threat, no predator. In fact, when we observed these mice we saw that they ran around in the cage for approximately 20-30 min and then lay down in a corner for the rest of the session.

Table 1. The effect of different forms of stress and metoprolol treatment on cytokine release.

	Social isolation	5 physical stressors	SDR-stress	SDR-stress + Metoprolol	Metoprolol
IL-6	↔	↔	↑	⇣	na
TNF-α	↔	↔	↔	na	↓
CXCL1	↔	↔	↑	⇣	↓
G-CSF	↓	na	na	na	na

Black arrows represent data reported in Papers I-IV. Dashed arrows represent numerical but not statistically significant decreases. na = not assessed.

Social stress specifically changed the release of a few cytokines (IL-6, G-CSF and the chemokine CXCL1) that all play a role in the development of atherosclerosis. The role of these cytokines in atherosclerosis is discussed below.

IL-6

SDR-stress increased plasma levels of IL-6 in all four studies in Paper III. In fact, we have seen this effect in seven different experiments, in two different mouse strains and in mice from two different breeders (Paper III and unpublished data),

indicating that this is a very robust finding. Activated immune cells in the plaques produce the pro-inflammatory cytokines INF-γ, IL-1 and TNF-α which induce the production of large amounts of interleukin-6 (IL-6). IL-6 is the major mediator for liver production of acute phase proteins like C-reactive protein (CRP) and serum amyloid A (reviewed in [47]). An increased level of CRP is a well known risk factor for CVD [49], but IL-6 has also been identified as an independent risk factor for carotid atherosclerosis [48, 50]. Moreover, IL-6 administration has been show to increase atherosclerotic lesion size in $ApoE^{-/-}$ and C57BL/6 mice [88]. However, there are conflicting results showing that total depletion of IL-6 in ApoE/IL-6 double knock-out mice also leads to increased atherosclerosis [89]. The role for IL-6 in atherogenesis is thus not fully understood.

In line with our results, IL-6 has been shown to increase in supernatants of cultured splenocytes [70, 71] and in plasma/serum samples in C57BL/6 mice after exposure to SDR-stress [71, 87], as well as in humans exposed to both chronic and acute stress [51-53]. IL-6 transcription is mediated via different transcription factors such as nuclear factor κB (NF-κB) and STAT3. NF-κB is rapidly induced by psychosocial stress in humans as well as in mouse models [22, 23] and induce IL-6 mRNA transcription [22]. Some of the pro-atherosclerotic effects of stress may thus be mediated by IL-6. Moreover, in Paper III we found that the increase in IL-6 likely could be reduced by metoprolol, suggesting that IL-6 production is mediated via the sympathetic nervous system as a response to SDR-stress. However, this reduction was only near statistical significance ($p=0.059$ for SDR vs. SDR-Met 3 days). Nevertheless, there was no significant difference in IL-6 levels between control and mice exposed to SDR-stress and metoprolol for three days, supporting an inhibitory effect of metoprolol.

In a small experiment with 11 healthy volunteers and 7 unstressed controls we wanted to see if we could repeat the previous findings where IL-6 increased in

humans after acute stress [51, 90]. Subjects in the stress group were stressed with Stroop Colour Word Test (CWT), a mirror tracing task (MTT), and a modified version of Trier Social Stress Test (TSST) [91, 92] , for a period of 25 minutes. Blood samples were drawn before stress (baseline) and 45 min after stress (post-stress). Interestingly, in accordance with previous findings plasma IL-6 levels (post-stress/baseline ratio) were significantly higher in the stress group compared to controls (Fig. 5).

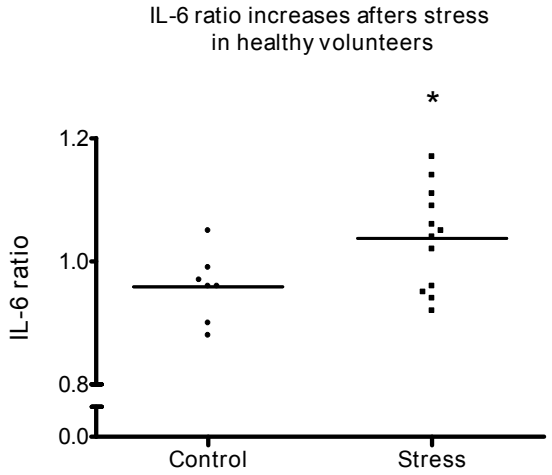

Figure 5. The effect of acute psychosocial stress on plasma IL-6 levels in healthy volunteers. Because baseline levels differ between groups a ratio for IL-6 was calculated (post-stress/baseline values). Data was logarithmically transformed and subsequently analyzed with Students T-test (SPSS). *$p<0.05$.

G-CSF

Social isolation did not stimulate the release of IL-6 and CXCL1, like SDR-stress. Nonetheless, serum levels Granulocyte-colony stimulating factor (G-CSF) was significantly decreased ($p=0.018$) in socially isolated mice compared to environmentally enriched mice. This decrease was accompanied by increased

atherosclerosis, in accordance with previous findings [93]. G-CSF is a haemotopoietic cytokine known to induce the release of haematopoietic stem cells and endothelial progenitor cells (EPCs) from the bone marrow, which is associated with an enhanced reendothelialization of injured vessels [94]. Furthermore, inflammation in the vessel wall and neointimal hyperplasia was reduced after G-CSF treatment [95]. It is possible that G-CSF reduces atherosclerosis via increased stem cell recruitment and increased regenerative capacity of the endothelium. However, the role of G-CSF in atherosclerosis is complex; a recent study shows that exogenous treatment with G-CSF actually accelerates atherosclerosis in $ApoE^{-/-}$ mice [96]. Clearly, more studies in this area are needed.

CXCL1

We found that serum levels of the chemokine CXCL1, also called KC (keratinocyte-derived chemokine), were dramatically increased by SDR-stress in Paper III. Interestingly, in Paper III, we also found that metoprolol treatment for three days numerically reduced the increased CXCL1 levels seen after SDR-stress, in a similar pattern as for IL-6. Although this reduction did not reach statistical significance compared to mice exposed to SDR-stress alone ($p=0.102$ for SDR vs. SDR-Met 3 days), CXCL1 levels in mice treated with metoprolol for three days were not significantly higher than control mice ($p=0.987$ for C vs. SDR-Met 3 days). Taken together, the fact that metoprolol numerically inhibits SDR-stress-induced increases of both IL-6 and CXCL1, the fact that these cytokines follow the same pattern both regarding SDR-stress-induced elevations and in metoprolol-induced reductions, and finally that metoprolol significantly decreased CXCL1 in unstressed mice in Paper IV, suggest that metoprolol indeed have inhibitory effects on these cytokines during exposure to SDR-stress. Moreover, we found that serum levels of CXCL1 still increased acutely after 12 weeks of SDR-stress in Paper III, suggesting that habituation did not occur.

CXCL1 is the murine homologue of human GROα (growth-regulated-oncogene-α) and is a chemoattractant for leukocytes. CXCL1 is one of the ligands for the chemokine receptor CXCR2. This chemokine and its receptor CXCR2 are present in atherosclerotic lesions and play an important role in accumulation of macrophages in established fatty streak lesions [97, 98]. CXCL1 is expressed on atherosclerotic endothelium and promotes monocyte arrest via VCAM-1 and the β-2 integrin VLA-4 in ex vivo experiments in $ApoE^{-/-}$ mice [99]. Furthermore, GROα may play a role in lipid accumulation and promote foam cell formation [100]. The pro-inflammatory cytokine TNF-α, as well as oxLDL have been found to induce GROα expression in endothelial cells [101, 102].

In line with these findings, LDL receptor-KC/GROα double knock-out mice display a reduction in atherosclerotic lesion formation compared to LDL receptor deficient mice [98]. There is also evidence for a role of GROα in atherosclerotic disease in the human situation. Patients with angina display raised plasma levels GROα compared to healthy controls. Furthermore, patients with unstable angina display particularly high levels of GROα, suggesting that GROα may also play a role in destabilization of atherosclerotic lesions [100].

Increased corticosterone levels

Plasma corticosterone levels increased acutely after all the five physical stressors and after SDR-stress, but there was no correlation between plasma corticosterone levels and atherosclerotic plaque area (neither in the aorta, the innominate artery or the aortic root). Urinary corticosterone levels were only increased chronically after exposure to the five physical stressors, and there was no correlation between urinary corticosterone levels and atherosclerosis when looking at each study separately. However, there was a weak correlation between urinary corticosterone

levels and aortic plaque area when data from Papers I-III where taken together (Fig. 6). This indicates that corticosterone per se may play a role in atherogenesis, but a large number of observations (n=116 in this case) is needed to find this correlation. Although some studies suggest a positive correlation between cortisol/corticosterone and CVD, the role for corticosterone in atherogenesis is unclear [13].

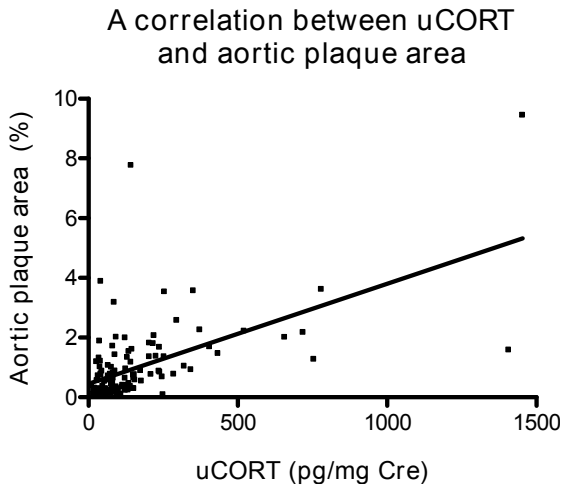

Figure 6. A correlation between aortic plaque area and urinary corticosterone levels (uCORT). Data is a summary of Papers I-III, including 116 data points. Data was analyzed with Spearman's test for nonparametric correlations (SPSS). R^2=0.323, $p<0.001$.

Sympathetic activation during stress (Papers II and III)

During stress the sympathetic nervous system is activated. In Paper II both heart rate and blood pressure increased dramatically after exposure to all five stressors [73], showing that these stressors indeed activated the sympathetic nervous system. Data in Paper III suggest that the sympathetic nervous system was

activated during SDR-stress in this setting as well. Plasma IL-6 levels were dramatically increased after SDR-stress and this increased could likely be inhibited by β-blockade with metoprolol.

Increase plasma lipid levels (Paper I)

Both total cholesterol and triglyceride levels were increased in socially deprived mice in Paper I [82]. Indeed, the increased cholesterol levels may explain the increased atherosclerosis observed in these mice.

No association between stress and oxidative stress (Paper I)

Oxidative stress plays a significant role in atherogenesis [103]. We analyzed isoprostane levels in urine samples in Paper I, as an indication of systemic oxidative stress [77]. However, neither social isolation nor different levels of environmental enrichment affected isoprostane levels [82], suggesting that this form of stress does not increase systemic oxidative stress and is thus not responsible for increased atherosclerosis in Paper I. Although, social and emotional stress has been suggested to increase markers for oxidative stress in mice [104] as well as in humans [105], we could not corroborate these findings in Paper I.

β-blockade reduces atherosclerosis and pro-inflammatory cytokines (Paper IV)

The SNS is activated during stress and in Paper IV we treated $ApoE^{-/-}$ mice with metoprolol, intervening with the SNS. Indeed, we found that intervention with metoprolol reduced atherosclerosis in $ApoE^{-/-}$ mice. The mechanisms are, however, still not clearly understood. Interestingly, in Paper IV, we found a possible mechanistic link between metoprolol and reduced atherosclerosis.

Metoprolol decreased serum levels of pro-inflammatory cytokines TNF-α (see below) and CXCL (see above), which both have known pro-atherogenic effects.

TNF-α

TNF-α is mainly produced by monocytes and macrophages and has been shown to play a role in the development of atherosclerosis. Among the pro-atherogenic effects of TNF-α are the stimulatory effect on the expression of adhesion molecules such as vascular cell adhesion molecule (VCAM), intracellular adhesion molecule (ICAM) and E-selectin, the expression of matrix metalloproteinase (MMP), the production of reactive oxygen species (ROS) through NADPH oxidase, and liver production of C-reactive protein (CRP) (as reviewed in [106]). Inhibition of TNF-α or depletion of the TNF-α gene reduces the progression of atherosclerosis in $ApoE^{-/-}$ mice [107-109]. The fact that TNF-α is present in human atherosclerotic lesions, in both diabetic and non-diabetic patients, further supports the involvement of the cytokine in atherogenesis [110].

No synergistic effect of psychosocial stress and high salt intake on atherosclerosis (Paper II)

Neither a high salt diet per se, nor the combination of a high salt diet and exposure to five stressors were associated with increased atherosclerosis in Paper II. Thus, we found no support for our hypothesis that these stressors would impair RAAS in a way that a salt load would be deleterious for atherosclerosis. However, these stressors may not have been the best form of stress to impair RAAS, since these stressors also failed to be atherogenic.

Blood pressure and heart rate (Paper I and II)

In Paper I, conscious blood pressure measurements with the tail-cuff technique showed an increased systolic blood pressure in environmentally enriched mice compared to socially deprived and environmentally deprived mice [82]. However, due to the drawbacks of this method (discussed in "Methodological considerations") it is likely that environmentally enriched mice that were rarely handled experienced a higher level of stress during blood pressure measurements. Moreover, blood pressure did not differ between groups in anaesthetized mice [82]. In Paper II, telemetry was used to study the effects of the five different stressors on blood pressure and heart rate. When using this method we could see marked increases in both blood pressure and heart rate during stress, for each of the stressors. However, in the long term atherosclerosis study in Paper II, anaesthetized blood pressure and heart rate were measured and did not differ between groups [73].

Summary

In this thesis we have evaluated three different stress models in ApoE $^{-/-}$ mice, on the effect on atherosclerosis. Figure 7 on the following page shows a summary of the results and suggests pathways through which the different stressors act on atherosclerosis.

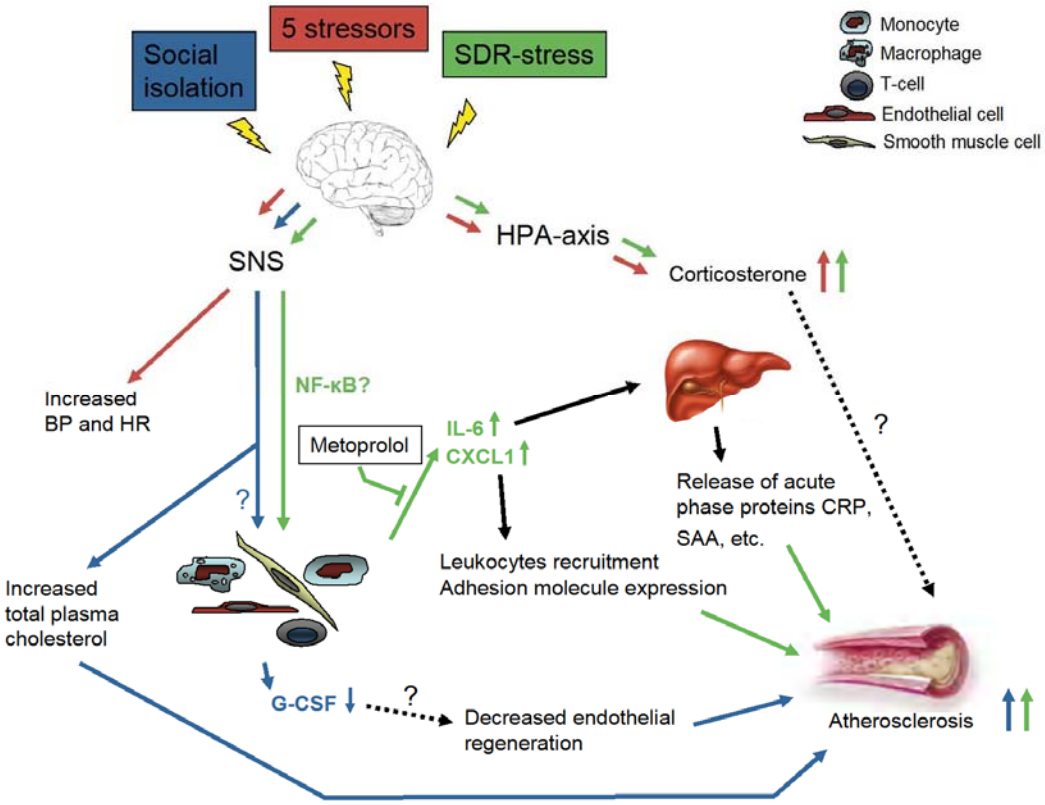

Figure 7. An overview of suggested effects of stress on atherosclerosis. Blue arrows represent possible pathways for social isolation, red arrows represent possible pathways for the 5 stressors, green arrows represent possible pathways for social disruption stress (SDR-stress), black arrows represent information from the literature, and dashed arrows represent hypothesized pathways. Social isolation increased total plasma cholesterol levels and decreased serum G-CSF levels, possibly via the sympathetic nervous system (SNS) leading to increased atherosclerosis [82]. The 5 physical stressors activated the SNS, as could be seen by increased blood pressure (BP) and heart rate (HR), but did not affect atherosclerosis [73]. SDR-stress induced the release of pro-inflammatory cytokines IL-6 and CXCL1, likely via the SNS since the effect could be reduced by metoprolol. The effect may be mediated via the transcription factor nuclear factor-κB (NF-κB) [22]. IL-6 is a powerful inducer of liver production of acute phase proteins that are known risk factors for atherosclerosis [48, 50]. Both the 5 stressors and SDR-stress activated the hypothalamus-pituitary-adrenal axis (HPA-axis) as could be seen by elevated plasma corticosterone levels. However, the role of corticosterone in atherosclerosis is unclear.

CONCLUSIONS AND FUTURE PERSPECTIVES

From the results in this thesis we can conclude that different forms of stress affect atherogenesis differently. Social forms of stress (social isolation and SDR-stress, in Paper I and III, respectively) that intervene with the social environment accelerate atherosclerosis, while more physical forms of stress (5 physical stressors, Paper II) do not. Thus, it appears that social interactions are very powerful modulators of atherosclerosis development in mice.

As a possible explanation of the difference in atherogenicity for the different forms of stress, we also found that social, but not physical forms of stress change cytokine release. Social isolation leads to decreased levels of G-CSF, SDR-stress leads to increased levels of IL-6 and CXCL1, while the physical stressors do not change cytokine release. We suggest that the increased release of pro-inflammatory cytokines is mediated via the sympathetic nervous system, since the effect likely can be reduced by metoprolol. Moreover, metoprolol per se reduces atherosclerosis and serum levels of TNF-α and CXCL1 in unstressed mice. Thus, we suggest a possible pathway in which social stress, via the sympathetic nervous system and the immune system may accelerate atherosclerosis (Fig. 8).

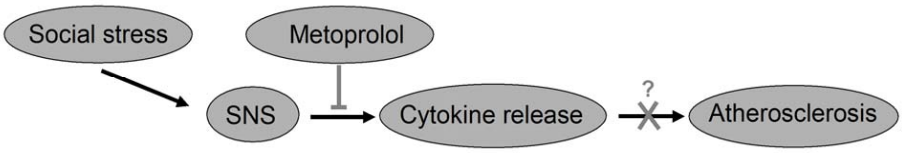

Figure 8. Suggested pathway in which social stress can accelerate atherosclerosis. Social stress activates the sympathetic nervous system (SNS) which leads to the release of pro-inflammatory cytokines with pro-atherogenic effects. Metoprolol inhibit cytokine release and may thus reduce atherosclerosis.

However, further studies are needed to confirm this hypothesis. The effects observed are subtle and no long term studies have been preformed evaluating the effect of metoprolol on atherosclerosis in mice exposed to SDR-stress. It would be of great interest to test this hypothesis and also further validate the effects of metoprolol on cytokine release after SDR-stress.

The effects of metoprolol on atherogenesis are not fully understood. In this thesis we have provided some new information on the anti-atherogenic effect of metoprolol. We found that metoprolol reduced serum levels of TNF-α and CXCL1, which both have known pro-atherosclerotic effects. Earlier studies have suggested that metoprolol can stabilize vulnerable atherosclerotic lesions by reducing expression of adhesion molecules ICAM-1 and VCAM-1 and by reducing lipid accumulation within lesions [38]. It would therefore be interesting to further study the effects of metoprolol on plaque stability.

To implement the findings of this thesis in the clinical setting could bring valuable knowledge to health care. There are already studies showing that IL-6 increases as a response to both acute and chronic stress in humans [51-53], but does GRO-α (the human homolog for CXCL1) increase in the same manner? Can metoprolol reduce this stress-induced IL-6 increase in humans? Moreover, metoprolol per se reduced serum levels of TNF-α and CXCL in unstressed mice, is this the case in humans as well? Can decreased levels of these cytokines explain some of the anti-atherogenic effects of metoprolol earlier reported in humans? [21]

This thesis has provided important information on how social stress accelerates atherosclerosis, and has suggested the release of pro-inflammatory cytokines as an underlying mechanism. Our hope is that our results, and further studies exploring mechanisms converting psychosocial stress into physical disease, will help to reduce the deleterious effects of psychosocial stress.

ACKNOWLEDGEMENTS

During my work with this thesis a number of people has helped and supported me. This thesis had not existed without these people. I especially would like to express my sincere gratitude to:

Min handledare **Göran Bergström** för att du gett mig den här möjligheten. Tack för det förtroende du gett mig (kanske lite väl mycket ibland!) och för att du alltid varit öppen för mina förslag (”Kör!”) och uppmuntrat mig. Att få ha en handledare och chef som du, som alltid tagit mitt parti och försvarat mig oavsett vad som hänt och oavsett om felet varit mitt eller inte, är få förunnat. Tack.

Mina bihandledare **Li-ming Gan**, för värdefulla råd genom åren och **Maria Johansson** som kommit med värdefulla idéer från ”The Noble Institute”! **Maria**, jag har inte bara fått ovärdelig hjälp av dig under åren, utan också funnit en vän för livet, det ska du veta att jag uppskattar! Tack också för din aldrig sinande glädje för forskningen, du är en inspiration.

Irene Andersson för all din hjälp och för att du räddade mig på EBM när jag för första gången råkade ut för möss i ”popcorn-åldern”. **Gunnel Andersson** för din ”excelens” när det gäller telemetrioperationer och för många trevliga stunder. **Julia Grönros**, **Johannes Wikström**, **Ulrika Hägg Samuelsson**, och **Henrik Nyström** för många trevliga stunder med en nygjord latte! Redan under exjobbet fick jag lära mig det viktigaste, att vispa mjölken till ett perfekt skum!

Marie-Louise Ekholm, **Josefin Kjelldahl**, **Birgitta Jannemark**, **Fredrik Olsson**, **Carl-Johan Behre**, **Eva Angwald**, **Caroline Schmidt, Dimitris Kellis, Lisbet Birke** och **Björn Fagerberg** i fysiologgruppen på Wallenberglaboratoriet för att ni välkomnade mig in i gruppen och tog hand om mig det sista året. Och för

att ni visade mig att det finns en forskningsvärld även utanför EBM och djurexperimentell forskning! Tack speciellt till **Ulrica Prahl**, min vän och ”partner”, som tillsammans med mig skrattat, gråtit och kämpat i vårt doktorandarbete! Du kommer att klara det!

Gänget på Lab 10, **Åsa Tivesten**, **Johan Bourghardt**, **Sara Sjöberg**, **Anna Wilhelmsson**, och **Camilla Alexandersson**, för att jag fått husera hos er och för all gemenskap. Speciellt tack till **Annelie Carlsson** som med sina magiska händer hjälpt mig med mina möss. Hur gör du?

Alla vänner och kollegor på **Wallenberglaboratoriet** och på **Fysiologen** som bidragit med både gemenskap och kunskap genom åren.

Examensarbetarna **Sofia Tidstrand** och **Asal Gahjavand** för alla timmar ni tillbringat på EBM när ni stressat mina möss! **Anders Hermansson, Marcus Norrby** och **Marcus Ulleryd** för trevligt sällskap och för roliga data att ha med i avhandlingen.

Susanne Exing som med outtröttlig entusiasm vårdat mina möss! ”Det ordnar vi!” har varit ditt ständiga svar, hur omöjliga och alldeles för sent påtänkta planer jag än har haft.

Mamma och **pappa** för att ni gett mig ett självförtroende och vetskapen att jag kan. **Pappa** för att du lärt mig kritiskt tänkande och att aldrig bli nöjd förrän jag förstår (men jag förstår fortfarande inte hur en luftvärmepump fungerar). Du kanske inte vet om det, men du är en liten smygforksre i allt du gör! **Mamma** för alla dagar (veckor!) som du hjälpt oss praktiskt när jag jobbat alldeles för mycket.

Storebror Magnus för att du är en föreblid, som medmänniska, förälder och yrkesman. Ditt tålamod och din flit är i en klass för sig!

Min älskade **Samuel**, för att din aldrig sinande glädje och för att du aldrig tvekat en sekund att anpassa din tid efter hur det gått för mig under en intensiv skrivar-höst. **Underbara Elna** för att du ständigt påminner mig om att jag redan har fått det bästa när jag fick dig. Inget annat är egentligen viktigt.

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