MOLECULAR MECHANISMS OF HEME OXYGENASE-1 MEDIATED CYTOPROTECTION IN CARDIOVASCULAR DISEASE

Moleculaire mechanismen van heme oxygenase-1 gemedieerde celbeschermende effecten in cardiovasculaire ziekten

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There was a time when meadow, grove, and stream,

The earth, and every common sight,

To me did seem

Apparelled in celestial light,

The glory and the freshness of a dream.

It is not now as it hath been of yore:

Turn wheresoe'er I may,

By night or day,

The things which I have seen I now can see no more.

By William Wordsworth

For Thijmen

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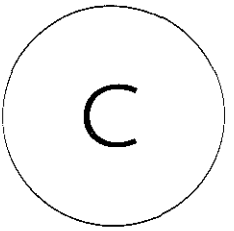
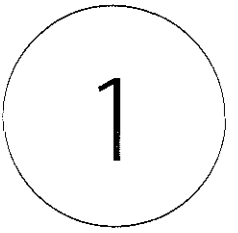
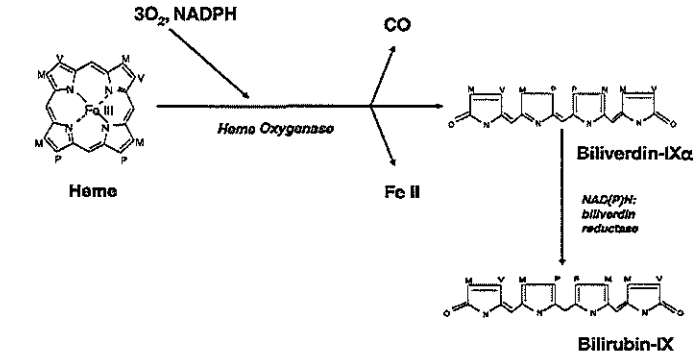


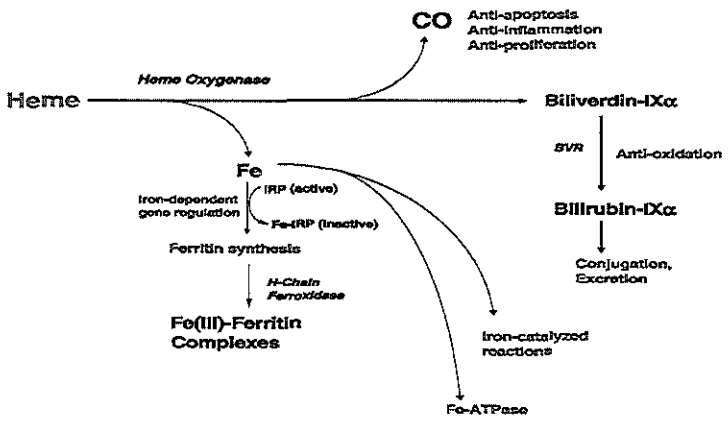


INTRODUCTION

INTRODUCTION

The scientific interest in gaseous molecules started in 1980 with the reports of endothelium-dependent vasorelaxation, which led to the unequivocal identification of EDRF as nitric oxide (NO) [1-3]. Since the discovery that NO acts as a potent regulator of many processes, including vascular tone, neurotransmission, inflammation, intensive investigation has followed on the role(s) of NO in inducible gene regulation[4]. A major mechanism for NO-mediated signaling effects involves the activation of soluble guanylate cyclase (sGC), leading to enhanced cGMP formation [5]. Strikingly however, it was known for a longer time, that cells can produce another endogenous gaseous molecule by an enzymatic reaction initially described by Tenhunen and Schmid in 1968: the catalytic breakdown of heme by the microsomal heme oxygenase (HO) enzyme system which releases carbon monoxide (CO) [6,7]. In the past decade, the interest in HO isozymes shifted from their metabolic function of heme degradation to another critical physiological function as a cytoprotective chaperone in numerous models of cellular stress [4, 8]. Heme oxygenases catalyze the rate-limiting step in the metabolic conversion of heme to the bile pigments (i.e., biliverdin and bilirubin) and generate equimolar amounts of biliverdin, iron and carbon monoxide (CO).





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The degradation products from HO-1 activity were for many years viewed as harmful waste products.

HO catalyzes the first and rate-limiting step in the oxidative degradation of heme (Fe-protoporphyrin-IX) to form the open-chain tetrapyrrole biliverdin-IXα [6].

The reaction initiates with the NADPH-dependent reduction of the ferric heme-iron in the HO-heme complex, which binds O_2 to form an oxyferrous intermediate that in turn accepts a second electron from NADPH [7]. The resulting ferric hydroperoxide (Fe III-OOH) intermediate hydroxylates the heme ring at the α-methene bridge carbon, forming α-hydroxy heme [4, 9]. Two further oxidation cycles involve the elimination of the α-methene bridge carbon as CO and the subsequent formation of a ferribiliverdin-IX complex (BV-Fe III) [10]. Iron released from HO enzymatic activity evokes pathways which activate the iron regulatory protein (IRP), leading to its sequestration. Biliverdin and bilirubin produced from HO activity potentially contribute to cellular defense against oxidative stress prior to their excretion [4].

Besides the important anti-oxidative properties of the first two degradation products of heme catabolism, CO has been shown to possess multiple signaling properties which affect cellular functions including inflammation, cellular proliferation, and apoptotic cell death [11-13]. In this thesis we sougth to further define the signaling pathway of HO-1/CO in cardiovascular disease.

Three genetically distinct isozymes of HO have been characterized:The first one is heme oxygenase-1 (HO-1), which expression is inducible by cellular stress, furthermore two more constitutively expressed forms have been identified, heme oxygenase-2 (HO-2) and heme-oxygenase-3 (HO-3) [14].HO-1 proteins (32 kDa) were first purified from the livers of heme-induced rats and from porcine spleen [15, 16].The resulting HO-1 proteins bind heme in a 1:1 complex.The inducible form of heme oxygenase, HO-1, occurs at a high level of expression in the spleen and other tissues that degrade red blood cells, including specialized reticuloendothelial cells of the liver and bone marrow. High levels of HO activity are detected in these tissues [6]. HO is present in hematopoietic stem cells of the bone marrow, where it may inhibit cellular differentiation by lowering the intracellular concentration of heme [17]. HO is also present in the liver parenchyma, which is the site of uptake and degradation of plasma heme, hemoglobin, and methemalbumin. Under conditions of hemolysis, HO activity dramatically increases in the liver parenchyma, kidney, and macrophages in response to increased levels of circulating hemoglobin [18].

Increased synthesis of the HO-1 protein occurs as a general response to cellular stress.This response depends on de novo synthesis of mRNA induced by transcriptional activation of the Hmox-1 gene. HO-1 belongs to a larger family of stress proteins whose transcriptional regulation also responds to adverse environmental and intracellular conditions. Of the known mammalian stress protein families, the expression of the heat shock proteins (HSPs) (20–30 kDa, 70–73 kDa, 90 kDa, 104–110 kDa) constitutes a global cellular response to protein denaturation associated with hyperthermia. Hence HO-1 has sometimes been classified as a heat shock protein (Hsp32) due to its ability to be induced by hyperthermia.

The substrate and catalytic cofactor of HO-1, heme (iron-protoporphyrin-IX), acts as an inducer of Hmox gene expression and activity [4, 8, 13]. Several metalloporphyrins, including SnPPIX and ZnPPIX, can induce HO-1 transcription, while paradoxically acting as competitive inhibitors of HO activity both in vitro and *in vivo*. In contrast, CoPPIX, which is a potent inhibitor in vitro, is a powerful inducer of HO activity *in vivo* [19].

Heme Oxygenase -2 (HO-2) is a constitutive expressed member of the heme oxygenase family. HO-2, has been identified in the liver, spleen, the brain, and as well in the testes [20, 21]. HO-2 was initially purified from rat testes with a molecular mass of 36 kDa whereas human HO-2 displays an 88% amino acid homology to rat HO-2 [22]. HO-1 and HO-2 represent the products of distinct genes (Hmox1, Hmox2) [23].The human, mouse, and rat Hmox-1 genes (14 kb) and the rat Hmox-2 gene (12 kb) share similar organization into five exons and four introns [24].

McCoubrey and co-workers [25] isolated a cDNA (2.4 kb) encoding a third HO isozyme (HO-3) in rat tissues. The predicted polypeptide has a high amino acid sequence homology to HO-2 (90%) and thus shares many common sequence motifs, including putative heme regulatory domains. HO-3 protein has very little heme degrading activity, and therefore, the function of this protein

still remains largely unknown.It was however speculated that this HO isotype was involved in the regulation of heme dependent cellular processes since the HO-3 protein has two heme regulatory motifs that may be involved in heme binding.

Biliverdin (BV), the first product of HO-catalyzed heme cleavage is under normal physiological conditions processed for rapid elimination. In cell culture studies the potential antioxidant capacity of the bile pigments was detected [26]. A number of studies have recently explored the potential of BV as potential therapeutic agents in ischemia / reperfusion (I/R) stress models [27].In an isolated perfused heart model, heme preconditioning provided protection against myocardial infarction following I/R injury, these effects were associated with increased HO-1 expression and bilirubin (BR) formation.In addition general anti-inflammatory effects of bilirubin due to ROS scavenging have been addressed [4].

Also CO reversibly binds to hemoglobin, to form carboxyhemoglobin (CO-Hb) [28]. The binding affinity of CO for the hemoglobin heme iron is 240 times that of O_2 and thus CO competes against oxygen,which causes the intoxicating effects if large doses are inhaled.The association of endogenous CO production with a distinct enzymatic mechanism, HO, was first described by Tenhunen and colleagues [13].

Today multiple reports suggested that CO has profound effects on intracellular signaling processes, resulting in anti-inflammatory, antiproliferative, antiapoptotic effects.The physiological signaling effects of CO known to date involve relatively few defined mechanisms; the modulation of soluble guanylyl cyclase (sGC) activity and subsequent stimulation of cGMP production are the most described [13]. Like the classical agonist NO, CO binds directly to sGC, leading to stimulation of its enzymatic activity. The importance of CO in sGC activation likely increases in cells or tissues with low endogenous NO production. In the presence of NO, however, CO may even act as a partial antagonist of NO function by competing against NO binding to sGC [29].

Recent studies have described anti-inflammatory effects of both HO-1-derived CO and exogenous CO in cell culture models and animal models.The production of the anti-inflammatory cytokine IL-10 has been shown to be augmented by the CO treatment [30]. In the presence of LPS, CO increased p38 MAPK activation without modulating ERK1/2 and JNK activation.

The cytostatic effects of CO were first examined by Morita and colleagues [31]. CO exerted a general antiproliferative effect in hypoxic smooth muscle cells that could be reversed by chemical inhibitors of sGC. CO produced endogenously and released from vascular smooth muscle cells (SMC) as a consequence of hypoxia-inducible HO-1 expression, elevated the endogenous production of cGMP of endothelial cells in co-culture and was also associated with a cGMP-dependent downregulation of the expression of endothelial-derived mitogens such as PDGF and ET-1 [32].In addition to that it was suggested that CO inhibited cell growth by influencing the expression and/or activation of cell cycle-related factors cyclin dependent kinases and cyclin complexes. Hmox-1 expression and CO treatment induced $p21^{Waf1/Cip1}$ expression in SMC [33].The antiproliferative effect of CO was inhibited in SMC derived from the $p21^{Waf1/Cip1}$ knockout mice ($p21^{-/-}$). Although $p21^{Waf1/Cip1}$ is regulated by p53, the absence of the p53 gene in SMC derived from the p53-knockout mice did not affect the antiproliferative effect of CO [13].

The importance of HO-1 in vascular biology was evident with the discovery of a patient with HO-1 deficiency. In this HO-1–deficiency subject, both intravascular hemolysis and endothelial cell injury were prominent. Importantly, oxidation of hemoglobin to methemoglobin occurred in the plasma, and Iron-induced oxidative modification of lipoproteins was cytotoxic and causes endothelial damage, leading to the development of fatty streaks and fibrous plaques in the aorta. Endothelial cells in this case were susceptible to oxidative stress due to of heme-mediated oxidation of LDL [4, 34].

Accumulating evidence suggests that HO-1 contributes to the balance of pro-oxidant and antioxidant factors in the vascular wall through multiple mechanisms. Endothelial dysfunction caused by reactive oxidant species (ROS) is a key step in the initiation of lesion formation. Previous studies have shown that increased expression of HO-1 attenuated ROS-mediated cell growth and apoptosis of endothelial cells [35].

In this thesis we evaluated the effects of HO-1 against the background of cardiovascular disease with the purpose to obtain insight in the role of HO-1 in cardiovascular pathology and the molecular mechanism by which HO-1 exerts its effects.

We assessed the effects of HO-1 transgenesis on atherogenesis, transplantation atherosclerosis and allograft rejection and angiogenesis and we studied the effects of HO-1 on SMC migration. It is now generally believed that most of the stimuli which induce HO-1 modulate intracellular signal transduction pathways via protein kinase cascades. We have made an attempt to elucidate some of these signaling cascades initiated by HO-1 activity against the background of cardiovascular pathology.

In **chapter 2 and 3** of this thesis we will focus on the effect of HO-1 on the development of atherosclerotic plaque and the transition of stable to vulnerable lesions. The expression of HO-1 is induced by a number of pro-atherogenic stimuli, including hypertension, smoking, and oxidized lipids. In murine models, induction of HO-1 impeded the development of atherosclerotic lesions, whereas inhibition of HO-1 stimulated atherogenesis. Likewise, ApoE/Hmox-1 double knockout mice developed accelerated atherosclerosis compared to ApoE knockout mice when subjected to a high cholesterol diet [36]. Taken together, these anti-atherogenenic properties of HO-1 expression suggest a prominent role for Hmox-1 in the genetic regulation in the development of atherosclerosis.

CO may also cause a general down regulation of proinflammatory cytokine production through p38 MAPK-dependent pathways leading to anti-inflammatory tissue protection [13]. The potential physiological effects of CO also include inhibition of apoptosis, cellular proliferation, and thrombosis. In chapter 2 we assessed the role of HO-1 on the development of atherosclerosis in a hyperlipidaemic mouse model, mainly focusing on the effects of the gaseous HO-1 metabolite CO.

In **chapter 3** we postulate that HO-1 may be an important regulator of advanced atherosclerotic lesion progression and eventually plaque destabilization. In this chapter, we sought to correlate HO-1 expression with phenotypes of atherosclerotic lesions in the carotid end atherectomy (CEA) material obtained from patients with documented cardiovascular disease.

Furthermore, we sought to validate the data obtained from human CEA material in a house-developed murine model for vulnerable plaque which we combined with the systemic induction of HO-1 by cobalt-protoporphyrin (CoPPIX).

Increasing survival rates in organ transplantation represent one of the successes of modern medicine. However, graft rejection limits its efficacy especially following transplantation. The frequency and severity of acute rejection episodes is a predominant risk for chronic graft rejection. Expression of the stress protein HO-1 in rodent allografts (kidney, heart, and liver) and xenografts (heart) correlated with long-term graft survival in several models of transplantation [33]. In a rodent model of renal transplantation, HO-1 expression increased in the allograft in response to immune injury. The reduced expression of HO-1 in chronic rejection compared with acute rejection may represent an inadequate response to injury or a consequence of prior injury that sensitizes further tissue response to immune attack [37]. Considering the anti-oxidative and anti-apoptotic properties of HO-1 observed in allograft protection, in **chapter 4** we sought to define the role of HO-1 in the genetic regulation of the alloimmune response directed by the dendritic cell.

The signal transduction mechanism by which HO-1 deficiency in dendritic cells regulates the alloimmune response is further elucidated. HO-1 deletion was shown to preferentially initiate CD8+T cell maturation and proliferation, furthermore it was shown that HO-1 regulated MHC class II expression in DC by an CTIIa dependent pathway.

In **chapter 5** we investigated the effects of HO-1 expression in dendritic cells (DC) in regard to their ability to initiate a T-helper 1 (Th1) vs. T-helper 2 (Th2) differentiation and subsequent immune responses of naive T cells stimulated by either HO-1 +/+ or HO-1-/- dendritic cells in a murine model of asthma, in which mice develop an airway hyperresponsiveness similar to that seen in human asthma, when challenged with aerosolized ovalbumin (OV) after initial sensitization. This study provided us with novel and valuable insigths in the genetic regulation of the immune respons evoked by DC.

Dysregulated growth and motility of vascular smooth muscle cells (VSMC) contributes to neointimal lesion development during the pathogenesis of vasculoproliferative disease, including atherosclerosis and restenosis. Previous studies have shown that overexpression of heme oxygenase-1 (HO-1) inhibits formation of neointima after vascular injury, an effect

partly explained by inhibition of VSMC proliferation. However, the effects of HO-1 on the regulation of VSMC migration remain unclear. In **chapter 6** we assessed the role of HO-1 in VSMC migration and the effect of HO-1 overexpression on focal adhesion formation and cellular polarity.

In atherosclerosis, the complex biology of intralesional angiogenesis remains to be elucidated. Intra-lesional neovascularization is observed in atherosclerotic lesions in humans and in animal models, while modulators of angiogenesis have been reported to affect atherosclerotic plaque growth. In addition, intra-lesional neovascularization is associated with advanced atherosclerotic lesions (vulnerable plaques), which sustain a characteristic morphology that renders them vulnerable to rupture, causing cardiac infarction [38]. In **chapter 7** we investigate the effects of

HO-1 overexpression and silencing in an established in vitro model for angiogenesis. The results obtained from these experiments are further evaluated in a validated murine model for vulnerable plaque in order to investigate the role of HO-1 on neovascularization and in vulnerable plaque development.

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2

HEME OXYGENASE-1/CARBON MONOXIDE SIGNALING INHIBITS ATHEROSCLEROSIS DEVELOPMENT IN APOE KNOCKOUT MICE

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ABSTRACT

Heme oxygenase-1 (HO-1) is a stress inducible enzyme that catalyzes the degradation of heme generating biliverdin, ferrous iron and carbon monoxide

(CO) in the process. HO-1 and its degradation products have been shown to exert protective effects on the formation of atherosclerotic lesions in mice and cardiovascular patients. In this study the effects of the gaseous HO-1 metabolite CO on plaque formation in the ApoE knockout (ApoE-/-) receiving a high cholesterol diet (HCD) was assessed. Carbon monoxide inhalation starting either I week prior before or simultaneously with HCD suppressed the development of atherosclerotic lesions by 32-54% in aortas and in the supra valvular area and compared to air treated controls ($P<0.05$, as determined by Students t-test). Furthermore, in ApoE / HO-1 double knockout mice (ApoE-/- HO-1 -/-) deletion of HO-1 promoted atherogenesis as compared to the ApoE-/- mice whereas no significant differences were observed in heterozygous mice ($P=0.46$ and $P=0.89$ determined by Students t-test).

Our data indicate that the heme oxygenase-1/ carbon monoxide signaling cascade attenuated atherogenesis in the ApoE-/- mouse and may function as a compensating mechanism in atherogenesis. Furthermore we showed that HO-1 deletion deteriorated lesion progression in the ApoE/HO-1 double knockout, which confirms the right genotype of this generated double knockout mice and makes them suitable for future experiments. One of these future options will be to assess whether CO administration would be successful to rescue the atherosclerotic lesion progression in ApoE/HO-1 double knockout mice. These experiments would further contribute to the possibly therapeutical applicability of CO, which holds great promise for future strategies treating vasculoproliferative disease.

INTRODUCTION

Heme oxygenase -1 (HO-1) is a stress responsive, ubiquitous enzyme that degrades heme, generating equimolar amounts of iron, biliverdin and carbon monoxide (CO)[3, 4]. Previous work has shown that induction of

HO-1 and its metabolites counter vascular remodelling associated with the development of atherosclerotic lesions in mice[5, 6]. The vasculature in a human case of a documented deletion of the HO-1 locus contained fatty streaks and fibrous plaques, accompanied by high blood level of triglycerides. In addition, deficiency of HO-1 led to increased iron deposition in liver and kidney with damage to the local vascular endothelium [7]. This endothelial cytotoxic effect was not only attributed to the direct cytotoxicity of heme, but also appeared to be caused by heme-associated LDL oxidation generating primarily lipid hydroperoxides which are toxic to endothelium. HO-1 is induced by oxidized LDL and is expressed in atherosclerotic plaques. In the LDL-receptor (LDL-R) knockout mice, HO-1 expression was detected in atherosclerotic plaques after dietary cholesterol feeding[8]. HO-1 degradation products, biliverdin and bilirubin, have been shown to contribute to the cellular antioxidant balance and ROS scavenging and thus protect vascular cells from oxidative stress injury [5, 9]

Indeed diminished expression or loss of HO-1 resulted in increased lipid uptake and foam cell formation and correlated with increased ROS generation and proinflammatory cytokine production. Also in ApoE-/-/HO-1-/- mice larger and more advanced lesions were formed as compared to ApoE knockout mice[6].Therefore, it has been suggested that HO-1 activation may represent the protective response mechanism against oxidative stress and inflammation during development of atherosclerosis. However, the molecular basis of HO-1 induced signalling pathways rendering atheroprotection remains incompletely understood. In this study, we focussed on the effects of carbon monoxide on atherosclerotic lesion development. CO is able to activate soluble guanylyl cyclase (sGC) leading to upregulation of cGMP. Further, CO is able to modulate the mitogen-activated protein kinase pathway (MAPK), including p38 MAPK, ERK, and JNK which will down regulate proinflammatory cytokine production [10].

In this study the role of heme oxygenase / carbon monoxide signaling in the development of atherosclerosis is assessed.

MATERIALS AND METHODS

Lipid mediated atherosclerosis was induced in 12 week old female Apoliploprotein deficient mice (ApoE-/-) on a C57black6 background (Jackson Labs, USA) which were fed a high cholesterol (1.25%) diet (HCD) which was continued for 8 weeks. CO inhalation (250 parts per million) was started before, during or after HCD.

HO-1 and ApoE null mice were obtained by crossbreeding ApoE-/- / C57 black6 background with HO-1-/- / C57black6 background in the Gulbenkian Institution, Oeiras, according to the guides and regulations of the Portuguese Animal Committee.

ApoE -/- mice were randomly assigned to either the control group (7 weeks HFD and 8 weeks air inhalation) or one of the three experimenal groups (group CO-I ; treated with 8 weeks CO inhalation 250ppm starting one week before and simultaneously with 7 weeks HFD, group CO-II; treated with 7 weeks CO inhalation 250ppm simultaneously with 7 weeks HFD;and group CO-III: in which mice received 7 weeks HFD and CO inhalation 250ppm during weeks 4, 5,6 and 7 of the HFD (figure 1a).

After 8 weeks of HCD mice were sacrificed and the aorta was harvested in total from the arch of the ascending aorta to the bifurcation of the ilio-femoral arteries and stained for lipid deposition with Oil-red-O.

Lipid depositions were subsequently en face imaged and quantified by a computer assisted imaging analysis system (CLEMEX).

Hearts from HO-1-/-ApoE-/- mice were harvested after 8 weeks of HCD and whole mounted in paraffin. Continuous sections were made of the entire aortic valve area (5µm each). HE staining was performed and analysed every fifth section of the supra valvular area and lesion size was quantified with Clemex system.

Adjacent sections were stained and quantified for presence of vascular smooth muscle cells (VSMC) (anti actin-Cy3, Sigma-Aldrich, The Netherlands).

The percentages of the different plaque components were calculated as the percentage of surface area positive for each specific immunostaining relative to the total intimal surface area. Statistical analysis was performed using student's t-tests. Data are presented as mean ± SEM. P values less than 0, 05 were considered to be significant.

RESULTS

CO inhalation starting either before or simultaneously with HCD suppressed the development of atherosclerotic lesions with 32 -54%. Initiation of CO exposure starting 1 week prior to HFD reduced atherosclerotic lesion formation by 53% (P=0.004) whereas CO inhalation initiated either at onset or 3 weeks after HCD initiation resulted in resp. 32% and 34% lesion reduction compared to air treated controls (p<0.003 and p<0.0013 respectively figure 1 a,b.) Lesions quantified by computer assisted image analysis of oil red-O staining in the aortic arch showed lesion reduction in the CO -treated mice specifically at the aortic arch and the area of the internal and external carotid bifurcations (figure 1c). Figure 1d shows a representative cross-section of the aortic valve area of an ApoE-/- mouse treated with CO inhalation for 8 weeks versus an air-treated ApoE -/-control mouse indicating sustained plaque/valve size reduction at the supra valvular area.

(Figure 1e. 33% P=0.026). Next ApoE -/- mice reveived either 12 weeks of HFD with CO inhalation 250ppm started at week 7 or 8 weeks with just air inhalation with subsequent 4 weeks of normal chow diet (figure 1f). Both groups displayed about 33% less atherosclerotic lesion formation compared to the air-HFD for 12 weeks control group (P< 0.0002 and P< 0.022 respectively) indicating that the effects of CO inhalation at this dosage are as potent as reduction of cholesterol levels suggesting that CO inhalation may be used therapeutically to arrest the progression of atherosclerosis.

Neither CO treatment nor biliverdin administration modified total plasma cholesterol levels.

In figure 2 data from a pilot we conducted in collaboration with the Gulbenkian Institute are shown. These data form a prerogative for a larger study in which we will assess the ability of CO to rescue the deteriorating effects on atherosclerotic lesion development in HO-1/ApoE double knockouts. Representative cross-sections are shown of the aortic valve area of ApoE -/- mice that were either wild type(+/+), heteroxygous (+/-) or nullizygous for HO-1(-/-) after 8 weeks of high fat diet.The upper panel shows that deletion of HO-1 promotes atherogenesis (a trend is observed with p< 0.099 at N=4 animals). No difference was observed in mice heterozygous for HO-1 expression compared to their wild type littermates (P< 0.46 and p<0.89 resp.) The lower pannel shows representaive cross-sections of the supra valvular area stained for α-actin. No significant difference was observed between the 3 experimental groups. These data confirm that in these home- generated HO-1/ ApoE1 double knockout mice we were able to reproduce an earlier finding [6] that HO-1 deletion promotes atherosclerotic lesion progression in the ApoE-/- mice.

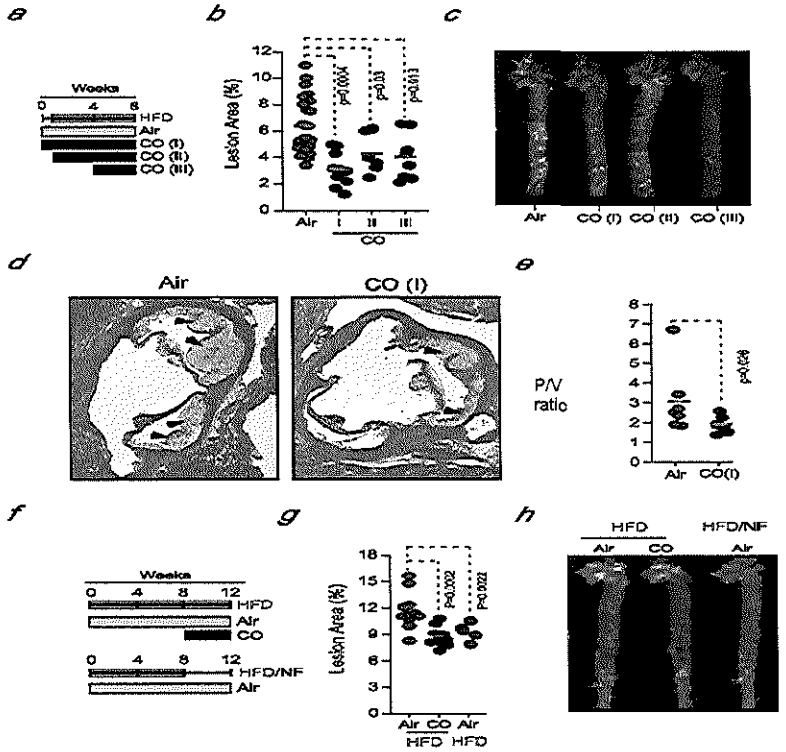

Figure 1 *(1a) Outline of experimental groups: ApoE -/- mice were randomly assigned to either the control group (these mice were fed a HFD during 7 weeks while receiving normal air inhalation over the entire time course) or one of the three CO-inhalation groups (group CO-I ;mice in this group were treated with 8 weeks CO inhalation at a concentration of 250ppm starting one week before and simultaneously with 7 weeks of HFD, and group CO-II; these mice were treated with 7 weeks CO inhalation also at 250ppm simultaneously with 7 weeks HFD;and group CO-III: in which mice received 7 weeks HFD and CO inhalation 250ppm during weeks 4, 5, 6 and 7 of the HFD, N=10 mice each group). (1b) Graph shows atherosclerotic lesion reduction in the three experimental groups in which CO was inhaled during various time points of the HFD compared to air treated control mice. CO inhalation was effective in all three setups to sustain a significant lesion size reduction (CO-I: 52% reduction,p<0.004 CO-II:32% reduction, p<0.03, COIII: 34 %reduction p<0.0013) (1c) Oil-redO staining of representative whole mounted aorta's showing reduction in atherosclerotic lesion size at aortic arch end at side branch bifurcations. (1d) HE stained representative crosssections of aortic valve area of an air treated control mice and a mice receiving CO inhalation continuously for 8 weeks during HFD. (1e) Valve area vs. Plaque area in μM was significantly reduced after Co inhalation (p< 0.026).*

(1f) Outline of second set of experiments: Mice from control group were placed on a HFD over a time course of 12 weeks and inhaled normal air during these 12 weeks. The second group switched in week 7 from air to CO while continuing the HFD and the third group inhaled normal air while being fed HFD for 8 weeks and switched to NFD in week 8. 1g: Both switching to a NFD and the inhalation of CO while continuing HFD for 4 more weeks reduced atherosclerotic lesion formation with 30% compared to mice on HFD and air inhalation for 12 weeks.

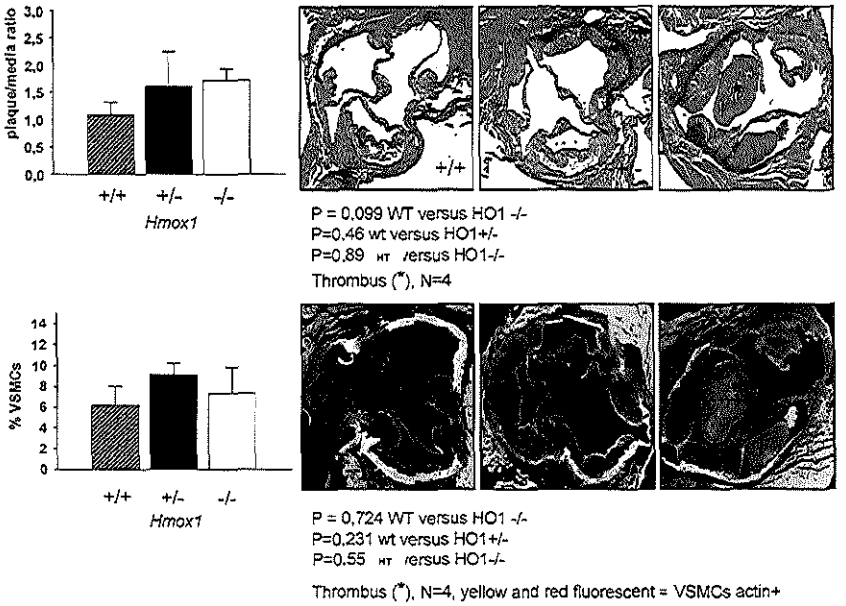

Figure 2 *Representative cross-sections are shown in the rigth panel of the supra aortic valve area of ApoE -/- mice that were either wild type (+/+) heteroxygous (+/-) or nullizygous for HO-1(-/-) after 8 weeks of high fat diet. The upper left panel depicts the effect of HO-1 deletion on lesion formation in the ApoE-/-HO-1 -/- mice (a trend is observed with p< 0.099 at N=4 animals.). No significant difference was observed for the effect in mice that were heterozygous for HO-1 expression compared to wild type littermates (P= 0.46 and P=0.89 resp.) The lower rigth panel shows representative cross-sections of the valve area stained for α actin. No significant difference was observed between the 3 groups.*

DISCUSSION

In the current study CO inhalation suppressed development of atherosclerotic lesions by 32-54% in ApoE knockout mice on a HFD when started either prior or simultaneously with compared to air treated controls. Furthermore, CO inhalation showed to be equally effective in attenuating plaque progression as reduction of cholesterol levels. In addition to that we show that the ApoE/HO-1 double knockout mice generated respond as expected to high cholesterol feeding.

HO-1 has been shown to have important antioxidant and anti-inflammatory properties that may result in vascular anti-atherogenic effects [11-15]

Enhanced production of reactive oxygen species under pathophysiological conditions is integral in the development of cardiovascular diseases (CVD). These reactive oxygen species regulate various signaling pathways involved in vascular inflammation in atherogenesis but also mediate antioxidative and anti-apoptotic responses[14]. Accumulating evidence has shown that HO-1 and its degradation products may function as adaptive molecules against oxidative insults. The proposed mechanisms by which HO-1 exerts its cytoprotective effects include its ability to degrade the pro-oxidative heme, and to release biliverdin which exerts a strong antioxidative action affecting intracellular redox signaling and reduces oxidized LDL accumulation. Carbon monoxide (CO) is a gaseous metabolite of heme oxygenase activity. Although long considered an insignificant and potentially toxic waste product of heme catabolism, CO is now recognized as a key signaling molecule that mediates several signaling cascades and has anti- proliferative and anti-inflammatory, as well as vasodilatory properties [16]

Interestingly, alterations in CO synthesis are associated with many cardiovascular disorders, including atherosclerosis, septic shock, hypertension, and ischemia-reperfusion injury [17-21] whereas restoration of physiologic CO levels exerted a beneficial effect in many of these conditions, suggesting a crucial role for CO in maintaining cardiovascular homeostasis[18,22].The observations in this study add to the belief that CO may act as the important signaling molecule that mitigates this anti-atherogenic action of HO-1. In addition we here report the finding that CO inhalation seems to be equally effective as lowering cholesterol levels in the inhibition of lesion progression in an APOE -/- model of atherosclerosis.These findings will contribute to the ongoing development of therapeutic strategies exploiting the antiatherogenic effects of HO-1/CO signaling. However there are some concerns that should be addressed before the use of CO as a therapeutic agent will become manifest. The therapeutic window of CO treatment in cardiovascular disease should be thoroughly explored. Alternatively generation of inducible or tissue specific viral vectors expressing HO-1 are a feasible option. In addition to that ongoing development of CO-releasing molecules will furthermore provide new strategies that make local or site specific delivery of CO possible while bypassing the dangers of systemic administration. We conclude with the prospect that CO therapy may hold great expectations for future treatment of atherosclerotic disease.

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3

HEME OXYGENASE-1 DETERMINES ATHEROSCLEROTIC LESION PROGRESSION INTO A VULNERABLE PLAQUE

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MATERIALS AND METHODS

Analysis of the human atherosclerotic plaques in human carotid end atherectomy specimens

Carotid end atherectomy (CEA) specimens were obtained from a biobank comprised of a collection of atherectomy-derived specimens of patients with symptomatic carotid artery disease (Athero Express biobank, Utrecht) [6]. The study was approved by an institutional review committee, and that the subjects gave informed consent. The collected specimens were routinely processed for immuno-histological analysis, as well as protein/RNA extraction, and were subsequently quantified by 2 blinded observers for presence of characteristics indicative for a vulnerable plaque morphology, as reported earlier [18]. Briefly, CEA specimens were harvested, embedded in paraffin, and immunohistochemically examined for the presence of macrophages, vascular smooth muscle cells (VSMCs), collagen and lipids. In addition, plaque thrombogenicity was assessed by the presence of intraluminal thrombus or intraplaque erythrocyte deposition, as defined by histomorphology in hematoxylin/eosin-stained sections. In addition, adjacent sections of the collected CEA atherosclerotic plaques were freshly frozen for protein analysis. Protein samples of 112 patients were randomly selected from the Athero Express biobank and analyzed.

ELISA analysis of protein expression

Proteins were extracted from atherosclerotic segments adjacent to the segments used for immunohistochemical analysis, by Tri-Pure Isolation Reagent (Roche, The Netherlands). Total protein concentration was determined by a colorimetric protein assay, followed by optical spectroscopy (Biorad, The Netherlands). HO-1, IL6, and IL8 protein expression in lysates was assessed with a commercial quantitative sandwich ELISA assay (for HO-1; EKS-800 kit, Stressgen, USA, for IL6, IL8, MMP2 and MMP9; Sanquin, The Netherlands). The association between HO-1 protein expression and patient CEA characteristics was analyzed using a Kruskal-Wallis one-way analysis of variance for ranks. Value of $P<0,05$ was considered to indicate significance.

Immunohistological analysis by confocal microscopy

Paraffin embedded sections were stained with anti-human CD68 (Hycult, The Netherlands), CD163 (clone 106D, Sanbio, The Netherlands), α-Actin Cy3 (Dako, Belgium), and HO-1 (OSA-110, Stressgen, US), and with secondary antibodies labeled with Alexa Fluor (568nm, 488nm, Molecular Probes, Invitrogen Inc., The Netherlands). Processed sections were visualized by tile analysis on the confocal microscope and quantified using LSM software (LSM510 NLO/FCS, Zeiss, The Netherlands).

Vulnerable plaque model in ApoE -/- mice

All experiments were performed in compliance with institutional (Erasmus University Medical Center, Rotterdam, The Netherlands) and national guidelines.

ApoE-/- mice on a C57BL/6J background (age 12-15 weeks) were obtained from Jackson Laboratory (Bar Harbor, USA). The protocol for this animal study is described in figure 1. (A) Two weeks before surgery, all animals were placed on a Western type diet containing 15% (w/v) cacoa butter and 0,25% (w/v) cholesterol (diet W, Hope Farms, The Netherlands). (B) Two weeks later, animals were anaesthetized by isoflurane inhalation, and the right common carotid artery was dissected from circumferential connective tissues. The cast was placed around the right common carotid artery, after which the wounds were closed with vicryl sutures and the animals were allowed to recover. (C) Six weeks after cast placement, animals were randomized to either treatment with phosphate buffered saline (Cambrex, UK) (N=10) or cobalt protoporhyrin (CoPPIX, Frontier Scientific Inc., Canada) treatment (N=10) in order to induce endogenous HO-1 (ip injection, 5mg/kg q2d, in 0,2M NaOH, ph 7,4). After 21 days of either PBS or CoPPIX treatment, animals were scarified and carotid arteries were harvested and processed for histological analysis.

Tissue preparation and histological analysis

Carotid arteries were embedded in OCT (Sakura Finetek, The Netherlands) and snap-frozen in liquid nitrogen. The abdominal aorta from each animal were excised and homogenized by sonification in NP40 lysis buffer on ice.

Quantification of histological data was performed on 7 µm cryosections, at 140 µm intervals. In addition to standard hematoxylin/eosin (H/E) staining, immunohistochemical analysis was performed, including assessment of macrophages (anti-CD68 antibody, Santa Cruz Biotechnology Inc., The Netherlands), and smooth muscle cells (anti-α-actin antibody, Sigma, Zwijndrecht, The Netherlands). Lipid and collagen deposition were visualized by Oil-red-O and picosirus red stainings, respectively. Data analysis was performed using a commercial image analysis system (Impak C, Clemex Technologies, Canada). Intima/media ratio and necrotic core/intima ratios were analyzed in sequential H/E-stained carotid sections. Necrotic area was defined as neointimal areas devoid of cellular tissue. Relative fibrous cap thickness was defined as the ratio of the average cap thickness at the shoulder and mid-plaque region divided by maximal intima cross-sectional thickness. The percentage of the different plaque components was calculated as the surface area positive for each specific indicative expressed as a percentage of the total intimal surface area. Statistical analysis was performed using student's T-test. Data are presented as mean ± SEM. P values less than 0,05 were considered to be significant.

Western blot analysis

Total protein concentration was determined using a colorimetric protein assay followed by optical spectroscopy (Biorad, The Netherlands). HO-1 protein expression was analyzed by standard Western Blot analysis (Biorad, The Netherlands) and was subsequently visualized using a fluorescent detection system (Li-cor Biosciences, The Netherlands).

RESULTS

HO-1 is specifically upregulated in human vulnerable atherosclerotic lesions

Average HO-1 expression levels in the CEA material was 0,98 ± 0,12 pg/ml. CEA patients were divided into quartiles of HO-1 expression: the first quartile contained 28 patients (HO-1 expression: 0,19 to 0,34 pg/ml), the second quartile contained 27 patients (HO-1 expression: 0,39 to 0,57 pg/ml), the third quartile contained 29 patients (HO-1-expression: 0,58 to 1,01 pg/ml), and the fourth quartile contained 28 patients (HO-1 expression: 1,02 to 9,07 pg/ml). The baseline characteristics of these patients are shown in table 1. The distribution of CAD risk factors did not differ among the HO-1 quartiles.

Increasing protein levels of HO-1 were associated with a characteristic vulnerable plaque phenotype (P=0,004; figure 2a). More specifically, increasing percentages of lipids and macrophages in the carotid lesions correlated closely with HO-1 protein expression (P=0,006, and P=0,005; figure 2b, c), whereas increasing percentages of collagen and VSMCs in the lesions correlated significantly with decreasing levels of HO-1 (P=0,04 and P<0,0005; figure 1d, e). Double labelling in imunohistlogical analysis suggested that HO-1 expression was mainly localized in the base of the intimal lesion (figure 3a), and co-localized with residing macrophages, whereas HO-1 expression in VSMCs was hardly detectable (figure 3b, c).

HO-1 expression correlates with distinct molecular markers of plaque vulnerability in human CEA material

Next, we assessed the relation between HO-1 levels, and protein expression of local matrix metalloproteinase (MMP), or various inflammatory cytokines, which previously were shown to promote plaque vulnerability. HO-1 expression levels in the carotid lesions correlated closely with MMP9 expression levels (P=0,02), whereas a trend was observed with MMP2 protein levels (P=0,06, figure 4a, b). Likewise, HO-1 strongly predicted IL-6 and IL-8 protein levels (P<0,01, figure 4c, d). In contrast, no clear relation was detected with MMP8, and IL-10 (P=0,13, and P=0,52, respectively, data not shown).

HO-1 expression levels are associated with plaque thrombogenicity in human CEA material

Luminal or intraplaque thrombus formation is an established characteristic sign of plaque vulnerability [20-22]. We therefore evaluated the relation between thrombus formation with the expression levels of HO-1. High intraplaque protein expression of HO-1 was correlated with the presence of thrombus in the assessed human carotid lesions (P=0,04; as shown in figure 5).

Table 1. *Baseline patients' characteristics*

Risk factors N=112 pts	**Ntiles Hmox-1 I 28**	**Ntiles Hmox-1 II 27**	**Ntiles Hmox-1 III 29**	**Ntiles Hmox-1 IV 28**	**P-Value**
male (N=81, 72,3%)	20/28	20/27	19/29	22/28	0,58
Age (range)	67,7 (63,5-72,0)	65,4 (62,1-68,7)	70,2 (66,8-73,6)	68,9 (65,8-71,9)	0,25
Hypertension (N=107, 95,5%)	27/28	27/27	29/29	24/28	0,20
Diabetes (N=75, 67,0%)	23/28	20/27	16/29	16/28	0,25
Hypercholes-Terolemia (N=17, 15,2%)	4/28	6/27	1/29	6/28	0,07
Current smoker (N=62, 55,4%)	14/28	18/27	16/29	14/28	0,24
Previous smoker (N=25, 22,3%)	4/28	7/27	4/29	10/28	0,13
Systolic tension mmHg (range)	160,4 (149,3-171,5)	155,0 (145,3-164,6)	163,3 (150,5-176,0)	165,3 (154,4-176,1)	0,52
Diastolic tension mmHg (range)	82,4 (77,9-86,9)	83,2 (78,5-87,9)	79,8 (74,4-85,2)	86,7 (82,3-91,1)	0,28
Total cholesterol mmol/l (range)	5,0 (4,3-5,8)	4,8 (3,6-5,8)	4,3 (3,7-4,8)	5,1 (4,5-5,6)	0,22

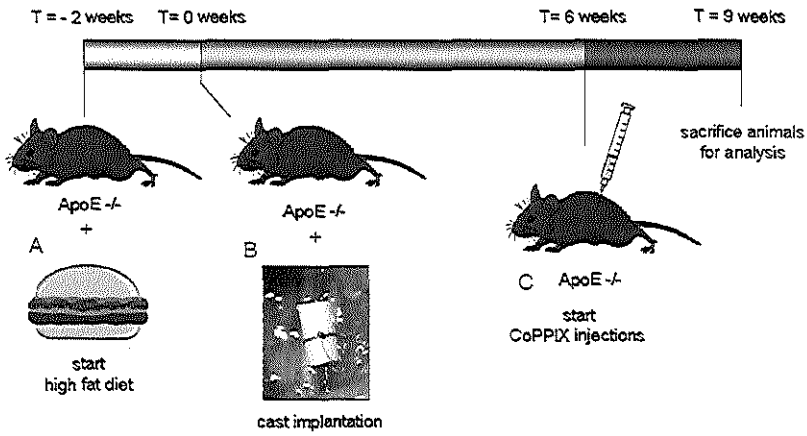

Figure 1 *Schematic overview of the experimental animal model. (A) C57bl/6 ApoE -/- mice were fed a cholesterol rich, high fat diet starting from 2 weeks before cast implantation (T=-2 weeks). (B) The cast device was implanted into the right carotid artery (T= 0 weeks). (C) CoPPIX was injected i.p. three times a week starting from week 6 (T= 6 weeks). The animals were sacrificed 9 weeks post cast-implantation, and the carotid artery segments were harvested for immunohistological analysis. Two groups were included in the study: (I) The control group was injected with saline from T= 6 weeks. (II) The Hmox-1 induced group was injected with CoPPIX from T = 6 weeks.*

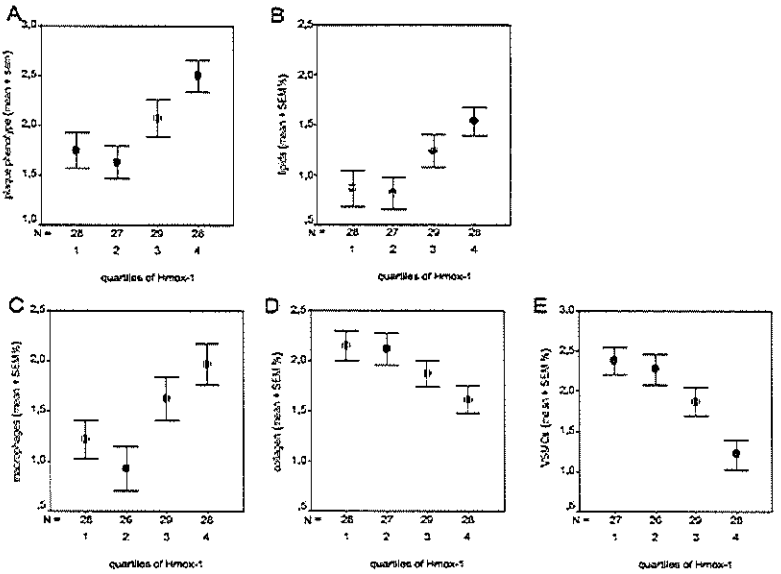

Figure 2 *Intraplaque Hmox-1 levels correlated strongly to the characteristic parameters of vulnerable plaque. (A) A correlation between Hmox-1 expression and plaque vulnerability was observed in the human CEA samples (P=0,004 based on correlations found between Hmox-1 expression and characteristic vulnerable plaque parameters: including a linear correlation of Hmox-1 with (B) intimal lipid deposition, (C) macrophages (P=0,006, and P=0,005 respectively), and an inverse correlation of Hmox-1 with (D) intimal collagen and (E) VSMCs (P=0,04 and P<0,0005, respectively), divided into four quartiles ranging from 0.5 (representing the group of patients with the lowest percentage) to 2 (representing the group of patients with the highest percentage) on the y-axes. On the x-axes, Hmox-1 protein expression levels are shown, divided into four quartiles of expression ranging from 1 (representing the group of patients with the lowest percentage) to 4 (representing the group of patients with the highest percentage).*

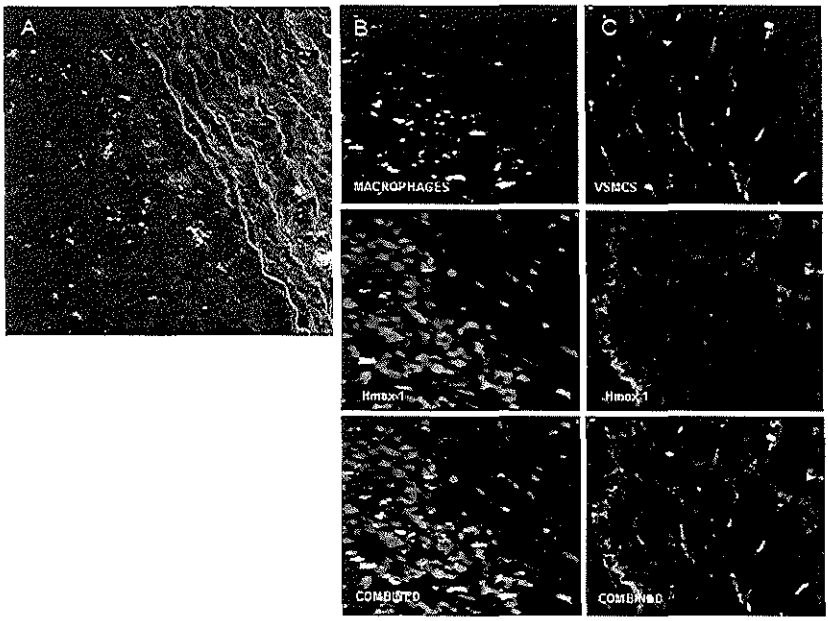

Figure 3 *Hmox-1 expression is mainly co-localized with macrophages infiltrating in human atherosclerotic lesions. Representative pictures from paraffin cross-sections of human CEA material are shown. Hmox-1 expression was mainly localized in the base of the intimal lesion, close to the media and the adventitia (A). Hmox-1 is defined by a green fluorescent signal, while the red fluorescent signal defines the elastic laminas of the media. Hmox-1 expression was co-localized with macrophages residing in the intima (B). The middle panel depicts Hmox-1 expression, the upper panel shows CD68+ cells, and the lower panel shows the combined images. Hmox-1 expression in VSMCs rich area was hardly detectable (C). The middle panel depicts Hmox-1 expression, the upper panel shows α-actin+ VSMCs, whereas the lower panel shows the integrated images. (magnification 200X).*

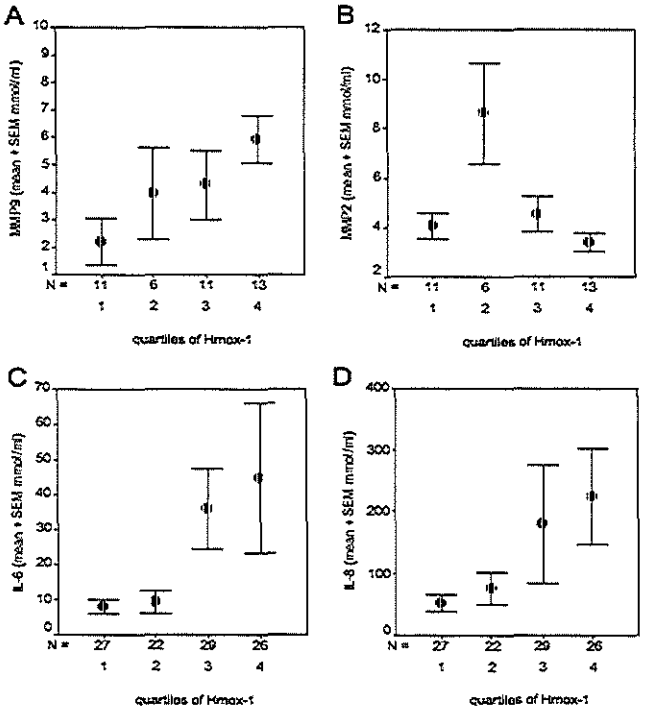

Figure 4 *Hmox-1 expression correlates with biomarkers of advanced atherosclerosis. Hmox-1 expression levels in the carotid lesions correlated with IL6, IL8, and MMP9 expression levels in the vulnerable atherosclerotic plaque. For MMP2, a trend was observed. The graphs show the expression levels in mmol/ml for (A) MMP9 (P=0,02), (B) MMP2 (P=0,06), (C) IL-6 (P<0,01), and (D) IL-8 (P<0,01) on the y-axes. On the x-axes, Hmox-1 protein expression levels are shown, divided into four quartiles ranging from 1 (representing the group of patients with the lowest intra plaque level) to 4 (representing the group of patients with the highest intraplaque level).*

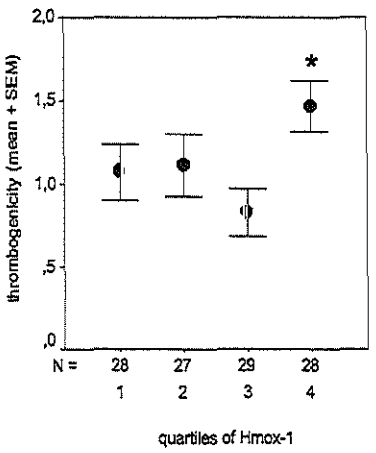

Figure 5 *Hmox-1 expression correlates with lesion thrombogenecity.*

Hmox-1 expression levels in the carotid lesions correlated with thrombus formation and erythrocyte extravasation in the human carotid vulnerable plaque lesions (P=0,04). The y-axes show the degree of accumulation of erythrocytes/blood plasma in CEA lesions divided into four quartiles ranging from 0,5 (representing the group of patients with minor deposition) to 2 (representing the group of patients with the most severe deposition). The x-axes show Hmox-1 expression levels divided into four quartiles ranging from 1 (representing low expression level) to 4 (representing high expression level).

HO-1 induction inhibits vulnerable plaque development in ApoE-/- mice without modulating lesion size.

The observations in the human carotidectomy material suggest that HO-1 expression is compensatory upregulated in advanced atherosclerotic plaques with a vulnerable phenotype. Induction of HO-1 expression could therefore aid to the stabilization of the atheromatous plaque. In order to study this, HO-1 expression was induced by cobalt protoporhyrin (CoPPIX) treatment in a mouse model for vulnerable atherosclerotic plaque formation. ApoE knockout mice were fed on a high cholesterol diet and implanted with an carotid cast as previously described. This flow-modifying device induces atherosclerotic lesions with a vulnerable phenotype in the proximal segment, whereas downstream from the device stable atherosclerotic lesions are formed. The murine proximal vulnerable lesions typically comprise a low content of plaque-stabilizing components, including collagen and VSMCs, and high percentages of plaque-destabilizing components, including lipids and macrophages, which taken together composes a lesion phenotype typically seen in human vulnerable plaques described by Virmani [23, 24].

Endogenous HO-1 mRNA expression was increased in the proximal lesions with the vulnerable phenotype, but not in the distal lesions with stable phenotype or in the contralateral naïve carotid arteries (figure 6a) at 9 weeks post implantation.

CoPPIX treatment to induce HO-1 expression was initiated at 8 weeks of Western diet (6 weeks post cast implantation), when the early lesions in the carotids do not yet show the histomorphological characteristics of vulnerable plaque development. Histomorphological analysis was performed after 3 weeks of CoPPIX treatment and 9 weeks of cast placement. HO-1 protein levels were increased in vascular segments of CoPPIX-treated mice, as compared to the saline-treated group by 20-fold as measured by western blot analysis of aorta segments. HO-1 expression levels were elevated as early as 2 days after initiation of CoPPIX injections (figure 6b).

Although HO-1 overexpression did not affect neointima/media ratio of advanced vulnerable lesions (2,97 ± 1,32 versus 3,40 ± 0,71, control versus CoPPIX, figure 7a), induction of HO-1 reversed the vulnerable plaque into a stable lesion, with an increase of relative fibrous cap thickness by 333% (0,067 ± 0,019 mm versus 0,29 ± 0,077 mm, control versus CoPPIX, $P<0,05$, figure 7b), and a significant decrease of necrotic core/intima (N/I) ratio by 49% (34,9 ± 2,3% versus 17,8 ± 2,2%, $P<0,05$, figure 7c). In addition, HO-1 overexpression was associated with a diminished lipid deposition in the vulnerable atherosclerotic lesions by 40% (19,2 ± 2,8% versus 11,5 ± 1,4%, control versus CoPPIX, $P<0,01$, figure 7d). HO-1 expression also was associated with the induction of plaques reminiscent of stable lesions, indicated by an increase in VSMCs residing in the intima by 66% (11,0 ± 0,27% versus 18,3 ± 2,6%, control versus CoPPIX, $P<0,05$, figure 7e). Interestingly, HO-1 overexpression did not affect intimal collagen formation in the atherosclerotic lesions (15,0 ± 0,6% versus 15,6 ± 0,62%, control versus CoPPIX, figure 7f). A trend towards reduction was observed in intimal CD68+ macrophage infiltration by 37% (23,7 ± 6,5% versus 14,9 ± 6,7%, control versus CoPPIX, figure 7g).

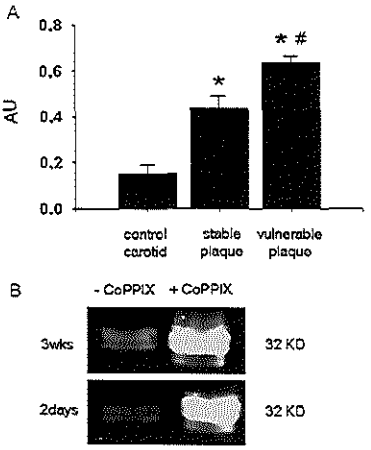

Figure 6 *Hmox-1 expression in murine vulnerable plaques and stable atherosclerotic lesions.*

*(A) QPCR analysis indicated that endogenous Hmox-1 mRNA level was increased in lesions with the vulnerable phenotype, compared to the contralateral naïve carotid arteries and the stable plaques. (B) Western blot analysis show Hmox-1 protein levels in pooled aortas of CoPPIX treated ApoE-/- mice compared to the saline-treated group after 2 days and 3 weeks of CoPPIX injections. * P<0,05 versus control. # P<0,05 versus stable lesion.*

Figure 7 *Hmox-1 induction stabilizes the progression of advanced lesions into vulnerable plaque.*

*Morphometric and immuno-histological analysis of vulnerable plaques in CoPPIX-treated and control ApoE -/- mice. In the right column, representative cross-sections are shown. In the left column, bar-graphs show the effects of Hmox-1 induction on (A) intima/media (I/M) ratio, (B) relative cap thickness (i.e. mean cap thickness at plaque shoulders and midregion / maximal intimal thickness), (C) necrotic core/intima (N/I) ratio, (D) % intimal lipids, (E) % intimal VSMCs, (F) % intimal collagen, and (G) % intimal CD68+ macrophages in vulnerable plaque lesions. * P<0,05 versus control.*

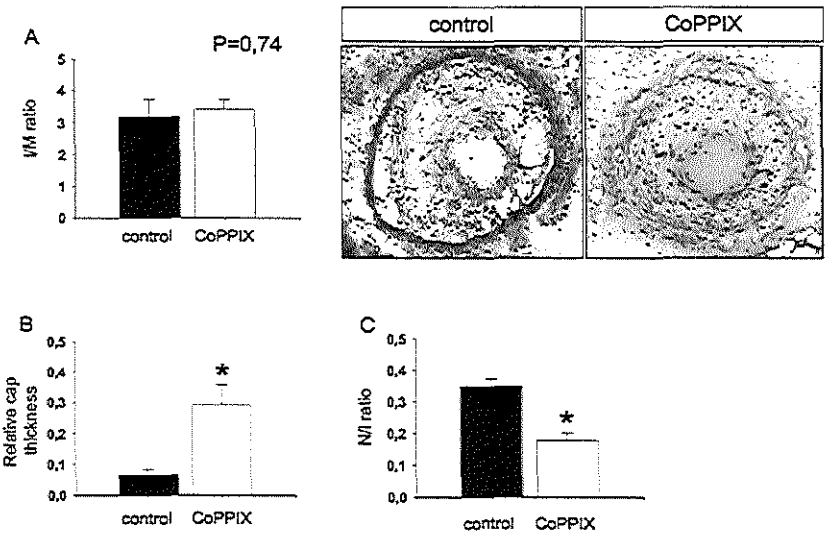

Figure 7 *Hmox-1 induction stabilizes the progression of advanced lesions into vulnerable plaque.*

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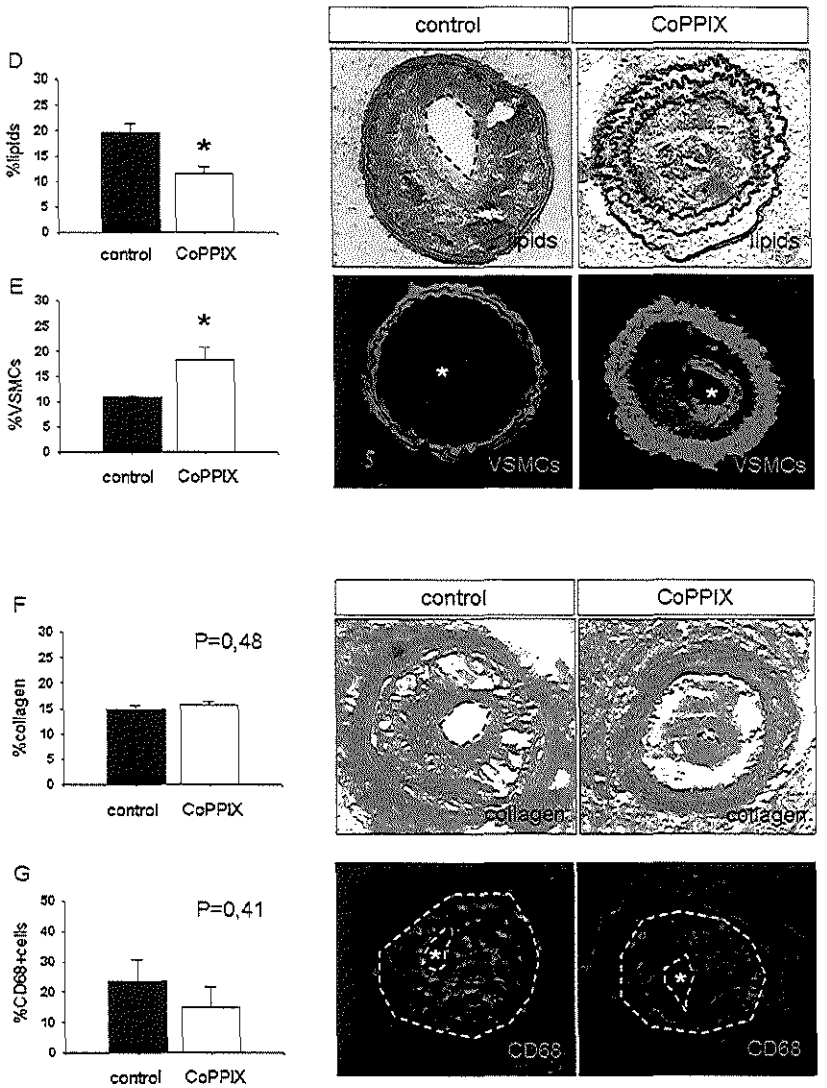

DISCUSSION

The current data in human carotid end atherecthomy atherosclerotic plaques and in murine vulnerable lesions suggest that HO-1 expression is strongly associated with a vulnerable plaque morphology: HO-1 protein expression was specifically upregulated in human vulnerable atherosclerotic lesions with lipid and macrophage accumulation and low collagen and VSMCs content. These lesions typically express high levels of proteolytic factors (MMP9 and MMP2) and pro-atherogenic cytokines (IL6, IL8), and show increased plaque thrombogenicity. HO-1 expression level correlated closely with the extend of these vulnerable plaque characteristics. Further on, in a well-validated murine vulnerable plaque model, HO-1 expression prevented vulnerable plaques formation and lead to the development of lesions with a more stable phenotype: HO-1 induction diminished the necrotic core and increased fibrous cap thickness, without affecting lesion size, whereas HO-1 induction reduced lipid and increased VSMCs accumulation in the intima.

HO-1 was designated as a protein involved in heme protein degradation, but more recently was suggested to play a more versatile role, as the heme catabolic products were shown to be cytoprotective. HO-1 has been associated with early atherogenesis by previous clinical studies. Kaneda and coworkers showed in a study with 554 patients that short (GT)(n) repeats in the HO-1 gene promoter with elevated HO-1 expression was predictive of a positive outcome in coronary atherosclerotic disease (CAD) progression, on angiographic follow-up [25]. Likewise, the ability of blood-derived mononuclear cells to express HO-1 correlated with short (GT)n promoter repeats, suggested that high HO-1 expression levels protects against the initiation of atherosclerosis [26]. Previously, increased HO-1 expression was also detected in advanced atherosclerotic lesions [7, 27]. However, data regarding the genetic regulation of advanced atherosclerotic lesions and the progression into vulnerable plaque are lacking.

In the current study, we sought to define the role of HO-1 in the genetic regulation of vulnerable plaque formation. Intra-plaque HO-1 expression in CAD patients was correlated with plaque vulnerability, as assessed by intimal distribution of destabilizing components (lipids and macrophages), and plaque stabilizing components (VSMCs and collagen). These observations are corroborated by correlations with pro-atherogenic cytokines IL6 and IL8, and with the matrix metallo-proteases MMP9 and MMP2, previously shown to be involved in collagen breakdown that weakens the atherosclerotic cap and add to the vulnerability of the advanced atherosclerotic lesion.

Increased HO-1 levels in lesions with an atheromatous/vulnerable plaque phenotype suggested that HO-1 could be upregulated to modulate plaque morphology and stability. In line with this finding, HO-1 was expressed in endothelial cells (ECs) overlying advanced atherosclerotic lesions, whereas ECs derived from early lesions did not express HO-1 in a small group of patients [28]. Immuno-histological analysis of these human atherosclerotic lesions by others and in the current study showed that HO-1 was mainly expressed by the macrophages/foam cells residing or infiltrating in the neointima.

In addition, atherosclerotic vulnerable lesions are characterized by an increase in frequency and extend of intraplaque haemorrhages, due to intimal neovascularization, increased permeability of the vasa vasorum, or extravasation of hemoglobin due to small ruptures. Clearance and degradation of haemoglobin by infiltrated CD163+ macrophages again induces HO-1 expression. Schaer and coworkers described colocalization of HO-1 and macrophages that express the hemoglobin scavenger receptor, CD163, in human atherosclerotic lesions. In vitro, HO-1 expression indeed was induced by CD163 internalization after haemoglobin binding in macrophages [27]. In the current analysis of human carotid vulnerable plaque, HO-1 was specifically upregulated in vulnerable lesions with the highest thrombogenecity, suggesting that intimal haemorrhages indeed stimulate HO-1 expression in this type of lesions, presumably as a compensatory mechanism.

The induction of HO-1 that occurs in the vulnerable plaque strongly suggests a role for this enzyme in the regulation of plaque destabilization and stabilization. To study the role of HO-1 in the progression of plaque destabilization, a murine model for vulnerable plaque was used. This model permits the comparison of stable versus vulnerable plaque in a single ApoE-/- mouse. Endogenous HO-1 expression was indeed increased in the vulnerable plaque region, as compared to the downstream stable plaque region in this animal model. Previously it was shown that adenoviral gene transfer of HO-1 inhibites initiation of atherogenesis, as suggested by a reduction in intimal size of lesions located in the aortic root and aortic arch in ApoE -/- mice [29]. Induction of HO-1 by hemin injections also decreased the lesion size in LDL-receptor -/- mice, whereas HO-1 inhibition by Sn-protoporhyrin IX promoted only lesion development as compared to the saline-treated control animals [8]. In HO-1/ApoE double knockout mice, accelerated atherosclerotic lesion formation was observed, whereas the ApoE knockout control mice developed lesions with the characteristics of a fatty streak after 8 weeks of Western diet. However, the role of HO-1 in the molecular regulation of plaque progression into an advanced complex lesion with plaque vulnerability remains poorly understood.

The function of HO-1 in the progression of early atherosclerotic lesions into advanced vulnerable plaques was therefore investigated in an established vulnerable plaque model developed in the mouse using a shear stress modifying device placed around the carotid arteries of ApoE knockout mice [30]. Cast placement has been shown to induce low shear stress proximal, and oscillatory shear stress, distal to the device [30], which will induce atherosclerotic lesions that are histologically reminiscent to human vulnerable plaques in the proximal region, and stable plaques distal to the cast [23, 24]. HO-1 induction by CoPPIX injections was initiated at 6 weeks after cast placement. Previous studies have indicated that at this timepoint, in the proximal segments, lesions are already developed with an advanced phenotype with VSMCs infiltration and cap-formation, although a necrotic core is still absent. Although late HO-1 induction had no effect on lesion size, further progression of the vulnerable plaque phenotype was prevented in the HO-1 expression group, indicated by a decrease in the necrotic content by 49%, and reduced lipid deposition by 40% in the vulnerable lesions compared to control. In line with this finding, HO-1 deficiency was associated with increased oxLDL uptake by macrophages, and lipid accumulation in foam cells in vitro [31]. Accumulation and apoptosis of foam cells in the atherosclerotic lesion leads to extracellular lipid deposition and the formation of the necrotic core characteristic of a vulnerable plaque. The observed

decrease in intimal lipids in response to HO-1 expression could have aided in plaque stabilization by limiting the necrotic core area. In addition, HO-1 expression was associated with a significant increase in the percentage of intimal VSMCs, and the relative cap thickness of a more stabilized atherosclerotic lesion. HO-1 expression might prevent cap thickening by inhibition of VSMC proliferation. HO-1 expression inhibited progression of wire or balloon injury induced restenosis by VSMC cell-cycle arrest via its catabolic end-products [32, 33]. However, Yet and coworkers showed in an autologous vein graft transplantation model that neointimal VSMCs in HO-1 -/- vein grafts were severely reduced by increased VSMC death as detected by a TUNEL assay [15]. In addition, HO-1 deletion significantly decreased viability of VSMCs following oxidative stress stimulation in vitro and *in vivo* associated with hemodynamic pressure in the vein graft model [15]. Taken together, these data support the idea that HO-1 expression may protect VSMCs from oxidative stress-induced cell death. Due to the distinct morphology of the vulnerable plaque, the VSMC rich fibrous cap is indeed exposed to high vascular strain and apoptosis during the cardiac cycle. This renders the lesion prone to rupture by sheer mechanical force [1], but also weakens the cap by inducing apoptosis in the residing VSMCs. In our study, intimal VSMC accumulation was significantly preserved in the arteries with HO-1 induction. This finding suggested that HO-1 prolongs VSMC survival in the fibrous cap and in the neointima by protecting the cells against oxidative stress damage, surpassing the cytostatic effect of HO-1.

In conclusion, the current study provides first evidence that HO-1 expression defines progression of an advanced atherosclerotic lesion into a vulnerable plaque, both in human carotid atherosclerotic lesions (in 112 patients) and in a hyperlipidaemic vulnerable plaque mouse model. HO-1 expression in vulnerable plaques is enhanced as a compensatory atheroprotective response, in which HO-1 prevents plaque instability by impeding lipid deposition and necrotic core growth, and by prolonging VSMCs survival in the fibrous cap. Genetic or pharmaceutical enhancement of HO-1 levels could protect this type of lesion from rupture, thereby reducing the risks on subsequent acute coronary events.

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4

HEME OXYGENASE-1 IN DENDRITIC CELLS REGULATES THE DEVELOPMENT OF TRANSPLANTATION ATHEROSCLEROSIS

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ABSTRACT

Heme oxygenase-1 (Hmox1) is an important modulator of physiological function with vascular cytoprotective properties. Here we show that Hmox1 also plays a crucial role in the molecular regulation of the dendritic cell (DC) function that governs the alloimmune response leading to the development of transplantation associated vasculopathy.

SiRNA Hmox1-knockdown and Hmox1-knockout DCs showed increased expression of MHC class II in culture. MHC class II upregulation was induced by STAT1 phosphorylation with subsequent CIITA-driven activation of MHC II genes transcription. As a result, increased MHC class II expression and alloantigen presentation by Hmox1-/- DCs directed the primary T-cell response preferentially towards a CD4+ T-cell, rather than a CD8+ T-cell response. In a murine model for transplantation atherosclerosis, presentisization by transfer of alloantigen-primed recipient syngeneic DCs accelerated development of allograft vasculopathy. More importantly, adoptive transfer of Hmox1-/- DCs prior to allograft transplantation was indeed associated with pronounced CD4+ T-cell accumulation, with attenuated CD8+ T-cell and macrophage infiltration, compared to wildtype DCs pretreated recipients, which is suggestive of an advanced chronic phase in vasculopathy, whereas animals presensitized with wildtype DCs showed an early phenotype phase. Hence, deletion of Hmox1 in DCs promoted the alloimmune response delegated by dendritic cells and resulted in an aggravated development of transplant atherogenesis, by increased intima hyperplasia and medial thickening in the arterial allograft. These findings imply that HO-1 plays an important role in the genetic regulation of the vascular alloimmune response elicited by DCs.

INTRODUCTION

Transplant atherosclerosis subsequent to transplantation continues to impede long-term allograft survival [1]. Transplant atherosclerosis is thought to be initiated by alloimmune-mediated injury to graft endothelial cells lining the vascular bed as a response to ischemia/reperfusion injury concominant to the transplantation procedure which induces the generation of reactive oxygen species. This subsequently leads to Toll-Like Receptor (TLR)-mediated maturation of resident dendritic cells (DCs), as well as infiltration of recipient-derived DCs into the graft. Alloantigen presentation by these mature DCs initiates the development of an alloantigen-specific adaptive immune response against the allograft [1], resulting in endothelial cell activation, and dysfunction, and perivascular infiltration of lymphocytes. Intimal vascular smooth muscle cells respond through activation, consequent migration into the intima of the vessel, and cell proliferation, which results in progressive intimal hyperplasia with impediment of allograft perfusion, and eventual graft failure. Although involvement of DCs in allograft rejection is presumed, the direct function of DCs in the pathogenesis of transplantation atherosclerosis still remains to be elucidated.

Allograft vasculopathy in humans, as well as in rodent experimental models, is associated with expression of a series of cytoprotective genes in the graft including the IL10 and the anti-apoptotic gene A20, as well as Heme oxygenase-1 [2,3]. The latter has previously been associated with increased recipient adaptation to the graft and improved survival of the vascular allograft in rat models in response to induction of heme oxygenase-1 by cobalt-protoporphyrin [3, 4]. Heme oxygenase-1 is a stress-inducible enzyme that degrades heme proteins into free iron, carbon monoxide (CO), and biliverdin, which is then rapidly converted into bilirubin. These catabolic end products exert anti-oxidant, and potentially anti-apoptotic properties, rendering the overall function of Hmox1 to be cytoprotective. Indeed, HO-1 upregulation by induction or transgenic expression has been shown to protect allografts from oxidative stress mediated-ischemia/reperfusion injury [5-7], to reduce restenosis after arterial injury [8], and to attenuate development of atherosclerotic lesions in LDL-receptor-deficient mice [9]. Conversely, loss of Hmox1 aggravated atherogenesis in ApoE-KO mice, and augmented vein graft stenosis [10], which were attributed to the anti-proliferative properties of HO-1 and its metabolites. The enzyme and its catalytic products may also exert vascular protection by virtue of its immune-modulating actions. Hmox1 overexpression and CO, as its downstream effector, reduced the response of macrophages to LPS, shifting cytokine production from pro-inflammatory TNF-α, towards the anti-inflammatory cytokine, IL-10 [11], whereas Hmox1-deletion enhanced the pro-inflammatory response in atherosclerosis by stimulating MCP1 and IL-6 production in macrophages [12].

Considering the anti-oxidative and anti-apoptotic properties of HO-1 observed in vascular disease and allograft protection, and the potential role of DCs in allograft rejection, we sought to define the role of HO-1 in the genetic regulation of the alloimmune response directed by DCs in transplantation atherosclerosis. The current study demonstrates that HO-1 in DCs, beside its catabolic and anti-oxidative properties, may also function as a regulator of the alloimmune response

targeted against the vascular grafts. Here, we found that Hmox1 deletion in DCs accelerated transplantation atherosclerosis by eliciting an alloimmune CD4+ T-cell driven response against the allograft, eventually leading to allograft failure. This process is mediated through induction of MHC class II expression by MHC class II transactivator (CIITA)-driven transcriptional initiation, which was associated with increased STAT1 phosphorylation. Taken together, these data indicate that Hmox1 plays a crucial role in DC activation of the CD4+ T cell alloimmune response during development of transplantation atherosclerosis.

MATERIALS AND METHODS

Dendritic cell culture

Bone marrow was isolated by crushing femur bones from 12-week old, wild type or Hmox1-/- C57BL/6 mice. Myeloid dendritic cells (mDC) were subsequently cultured according to the Lutz protocol(13) in RPMI medium (Cambrex, The Netherlands) containing 10% FBS, gentamycin and -mercaptoethanol after stimulation with GMCSF for 9 days (10ng/ml). At day 9, mDC cultures were pulsed for 24 hours with allogeneic splenocyte lysate derived from BALB/c mice in a ratio of 1:10. Lysates were obtained by 5 repetitive freeze-thaw cycles of homogenized spleen tissue. After 24 hours, mDC were collected and washed three times in fresh RPMI medium.

Mixed lymphocyte response assay and flow cytometry analysis

In the mixed lymphocyte response assay (MLR), naïve T-cells obtained from C57BL/6 spleens were co-cultured with mDCs, previously stimulated with alloantigens. The number of CD8+ T-cells and CD4+ T-cells were assessed at day 4 (MLR4) and day 7 (MLR7). Cell suspensions were stained for cell surface markers (0,2 µg antibody/300.000 cells) and were analyzed by flow cytometry (FACSCanto, BD Bioscience, USA). Fluorescence-labeled antibodies directed against CD8, CD4, MHC class II, CD80, CD40, CD86 and CD11c antibodies were all obtained from eBioscience (San Diego, CA, USA).

RNA and protein expression analysis

CIITA expression in cultured mDCs was assessed by quantitative PCR (QPCR). Total RNA was isolated using the RNAeasy kit (Qiagen, Germany) and reverse transcribed into cDNA using random hexamers. QPCR reactions were performed using cybergreen incorporation and real time detection in the iCycler iQ Detection system (Biorad, The Netherlands). Target gene mRNA levels were assessed relative to the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT). Statistical analysis was performed by use of Student's t-test analysis. Data are presented as mean ±SEM unless stated otherwise. (14)P values <0,05 were considered statistically significant.

Western blot analysis was performed using antibodies against STAT1 and phosphoTyr701 STAT1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti rabbit-IgG or goat-IgG antibodies (IRdye 680 CW, IRdye 800 CW, Licor Bioscience, USA) using an Odyssey infrared imaging system (Licor Bioscience, USA).

siRNA knockdown of Hmox1 in bone marrow derived mDCs

Selective silencing of Hmox1 was achieved by transfection of C57BL/6 bone marrow derived mDCs at day 7 of the Lutz protocol [13]. Transfection was carried out in Dharmafect transfection agent, using a pool of 4 different HO-1 mRNA targeting siRNAs, whereas control cultures were transfected with a pool of 4 different scrambled siRNAs, according to the protocol provided by the manufacturer (Dharmacon, USA). For silencing of CIITA, a similar combination of 4 CIITA mRNA targeting siRNA sequences was used (Dharmacon, USA)

Mouse vascular allograft model

All animal experiments were performed according to protocols approved by the national and local committees for use and care of laboratory animals.

For a schematic overview of the experimental setup, see figure 1. Ten days before arterial allografting, 12-week old C57BL/6 mice were injected intraperitoneally with either saline (n=6, group 1), or $2x10^6$ C57BL/6-derived, wild type (n=6, group 2) or Hmox1-/- mDC (n=6, group 3) (figure 1c). The mDCs cultured from C57BL/6 bone marrow (figure 1a) were presensitized with BALB/c alloantigens derived from freeze-thawed, BALB/c spleen lysate (in a ratio of 1:10, between DCs and spleen cells) at day 9, 24 hours prior to injection (figure 1b).

Aortic allograft transplantation of 12-week old BALB/c mice into the right carotid artery position of age-matched C57BL/6 mice, was performed as described previously [15]. Briefly, isoflurane-anesthetized, C57BL/6 mice were intubated and the right common carotid artery was ligated and cut. The proximal and distal portions of the carotid artery were passed through nylon cuffs (Portex ltd. Smiths medical international, UK) and fixed using aneurysm clips (Yasargil gold, B-braun medical, The Netherlands). The extending carotid artery segment was inverted over the cuff followed by ligation. The arterial segments derived from BALB/c donor mice were implanted by placing the arterial segment over the cuff followed by ligation with sutures (figure 1d). At 14 days

post transplantation, mice were sacrificed and perfused with 0,9% saline via a cardiac puncture. Vascular grafts were embedded in OTC medium (Sakura, The Netherlands), frozen in liquid nitrogen and subsequently processed for immunohistological analysis.

Immuno-histological and morphometric analysis

Contineous serial sections (6 µm) were cut with a cryotome from one cuff end to the other. Cryosections were stained for every 5th section with Hematoxilin/Eosin (HE) in order to gain an overall histological overview. Evaluation started in each graft, 80 µm after one cuff end, towards 80 µm before the opposite cuff end. For quantification of cellular infiltration in the media, the media was divided into two areas; the luminal side of the media was defined as the area confined by the three inner medial elastic lamina adjacent to the neointima. The adventitial side of the media was defined as the area confined to the outer medial elastic laminae adjacent to the adventitia. For immuno-histological evaluation, every 10th cross-sections was stained for immuno-histological analysis. Sections were analyzed by (confocal) microscopy using primary antibodies against IL-6 (clone 20F3, Genzyme, USA; 1:100), CD8, (YTS169, Bioceros, USA; 1:150), CD11c (APC conjugated, Ebioscience, USA; 1:50) CD4 (Ebiosciences, USA; 1:100), and α-actin antibody (Cy3-conjugated; Sigma, The Netherlands; 1:200) followed by DAP-PO or AEC incubation according to the manufacturer's protocol. Apotosis was analyzed using a TUNEL assay (Roche Diagnostics, The Netherlands). Blind data analysis was performed, using a commercial, quantitative image analysis system and an adjusted color threshold routine (Clemex Vision Image Analysis System, Clemex Technologies). Areas of specific cell staining were expressed as the percentage of medial or intimal surface area. Statistical analysis was performed by Student's t-test analysis. Data are presented as mean ±SEM. P values <0,05 were considered statistically significant.

RESULTS

Pretreatment with alloantigen stimulated mDCs accelerates transplant atherosclerosis in the vascular allograft.

In order to asses the effect of Hmox1 expression specifically in mDCs in adoptive transfer in allograft vasculopathy, three groups of C57BL/6 mice received aortic allografts from BALB/c donor mice (N=6 in all groups). The first group of age-matched control animals underwent vascular allograft transplantation without any prior treatment. In the two other groups, C57BL/6 recipient mice received an intraperitoneal injection of bone-marrow derived mDCs cultivated from Hmox1+/+ or Hmox1-/- C57BL/6 mice, 10 days prior to allograft transplantation. These mDCs were pulsed with BALB/c mice-derived spleen lysate (alloantigen) and LPS prior to adoptive transfer into the C57BL/6 recipients (See figure 1 for overview of the protocol).

Treatment with mDC cells prior to implantation of the vascular graft led to a pronounced increase of transplant atherosclerosis by 915,4% at day 14 post transplantation, as compared to control mice that received PBS injections (Intima area; 38428 ± 26546 μm^2 versus 4198 ± 2963 μm^2 respectively, $P<0,05$; figure 2b). Presensitization of BALB/c alloantigen stimulated mDCs therefore appears to accelerate the alloimmune response and substantiates the involvement of mDCs in the initiation of allotransplant-associated atherosclerosis.

Deletion of Hmox1 in mDCs significantly promotes the development of transplantation atherosclerosis in vascular allografts following adaptive transfer.

Loss of Hmox1 in alloantigen-stimulated DCs used for adoptive transfer prior to allografting increased transplant atherosclerosis function by 2686,9%, as compared to control animals (116995 ± 2704 μm^2 versus 4198 ± 2963 μm^2 respectively, $P<0,05$; figure 2b).

Pretreatment with Hmox1-/- mDC significantly stimulated neointimal hyperplasia in vascular allografts, as compared to pretreatment with wild type mDC by 204,7% (116995 ± 2704 μm^2 versus 38428 ± 26546 μm^2 respectively, $P<0,05$; figure 2b). The upper panel shows representative cross-sections of the allografts derived from the various groups (figure 2a).

Loss of Hmox1 in mDCs used for presensitization prior to allografting facilitates vascular infiltration.

The medial surface area of grafts in mice pretreated with Hmox1-/- mDC was increased by 32,4%, as compared to WT mDC-pretreated recipients (15936 ± 1060 μm^2 versus 12034 ± 698 μm^2, $P<0,05$; figure 3a). Furthermore, the grafts in Hmox1-/- mDC-treated recipients contained 66,1% less VSMCs, as compared to the wild type mDC-pretreated group (46,0 ± 11,1% versus 76,4 ± 5,3% of total graft area, $P<0,05$; figure 3b and c). Therefore the observed increase in medial thickness did not appear to be associated with increased VSMCs proliferation, but could potentially be explained by pronounced vascular graft infiltration from the luminal or adventitial sides. Administration of Hmox1-/- mDC

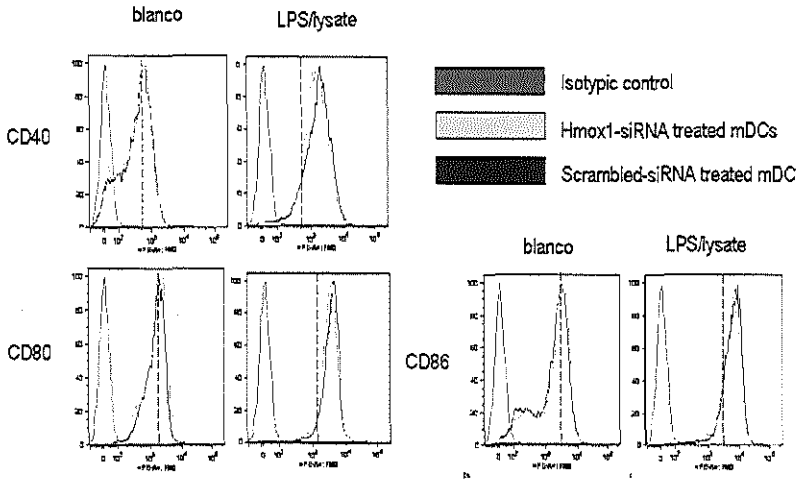


Figure 1 *Flow cytometry analysis of mDC maturation markers. Hmox1 deletion did not affect dendritic cell maturation. Expression of CD40, CD80, and CD86 dendritic cell maturation markers were present on unstimulated (blanco) cultured dendritic cells, and were increased in response to alloantigen stimulation (LPS/BALB/c spleen lysate) as shown by flow cytometry analysis. However, no difference was observed in the expression of these maturation markers between the Hmox1-deficient and WT mDCs. Red histographs represent the isotypic control, green histographs represent the Hmox1-silenced mDCs and blue represent the mDCs treated with scrambled siRNA.*

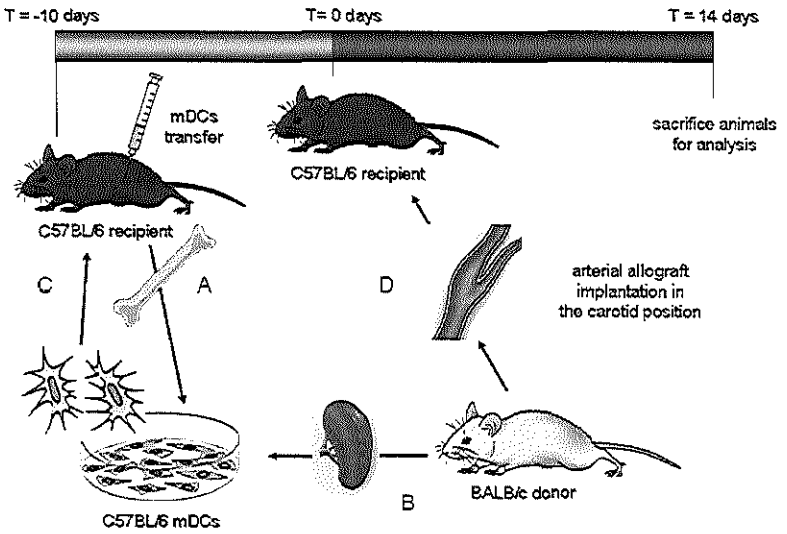


Figure 1 *Schematic overview of the experimental allograft model. (A) Dendritic cells were cultured from bone marrow derived from Hmox1 -/- and WT C57BL/6 mice. (B) Dendritic cells were presensitized with alloantigen derived from BALB/c spleen lysate. (C) The presensitized WT (Hmox1 +/+) or Hmox1-deficient (Hmox1 -/-) DCs were injected intraperitoneally in C57BL/6 mice, 10 days before aortic allograft transplantation. (D) Aortic allografts derived from BALB/c mice were transplanted into the right carotid artery position of these pretreated C57BL/6 recipients. Mice were sacrificed 14 days post transplantation, and the allografts were harvested for immunohistological analysis. Three groups were included in the study: (I) The control group without adoptive transfer of dendritic cells. (II) A second group received adoptive transfer of alloantigen stimulated C57BL/6 mDCs at day -10 prior to aortic allograft transplantation from BALB/c mice. (III) A third group received adoptive transfer of Hmox1-deficient C57BL/6 mDCs at day -10, prior to implantation of the aortic allograft.*

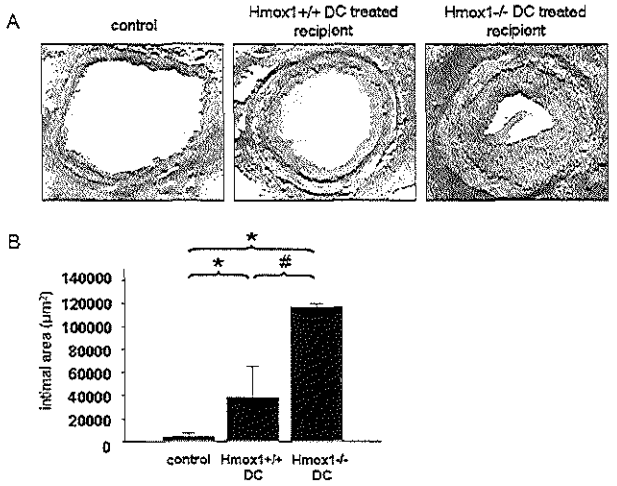


Figure 2 *Histological analysis of arterial allografts at day 14 after transplantation. Representative images from regions in the middle section of the graft are shown. (A) Images of control (no additional treatment), and Hmox1+/+ mDC or Hmox1-/- mDC presensitized recipients are shown. (B) The bar-graph shows the mean surface area of the neointima, as measured by computer-assisted image analysis of the intimal and medial surface area of the grafts. (data shown in µm², # P<0.05 Hmox1-/- versus Hmox1 +/+ mDC-treated group. *P<0,05 Hmox1+/+ mDC and Hmox1 -/- mDC-treated group versus the control group as defined by the Student's t-tests, mean ± sem.)*

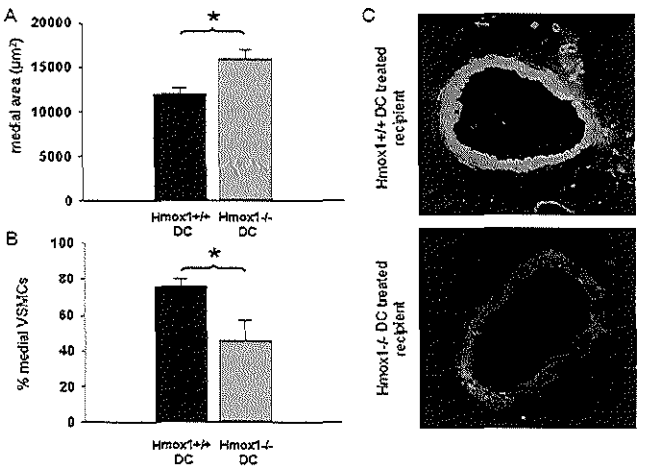


Figure 3 *(A) Mean medial surface area in allografts. Cross-sections were analyzed throughout the allografts at equal distance of both cuffed ends by confocal microscopy after staining with -actin-Cy3. Medial surface area was defined as the difference in total surface area between the inner and outer lamina elastica in µm². (B) Percentage of medial VSMC content per µm² medial surface area. (*P<0,05 Hmox1-/- versus Hmox1 +/+ mDC-treated, mean ± sem.) (C) Representative confocal images of allografts from Hmox1+/+ and Hmox1 -/- mDC-pretreated recipients (magnification: 100X).*

indeed resulted in accumulation of cells at the luminal side of the media by 98,7% (461 ± 58 cells versus 232 ± 81 cells, Hmox1-/- mDCs versus WT mDCs pretreated group respectively, P<0,05, figure 4a). Since a difference in total medial surface area between both groups was observed, the number of infiltrating cells on the luminal side was corrected for differences in surface area in this particular region, by calculating the number of cells per μm^2 medial surface area. A trend towards an increase in the number of cells per μm^2 medial surface area by 51,2% was observed (19,5 ± 1,5 cells/ μm^2 for the Hmox1-/- mDC versus 12,9 ± 4,0 cells/ μm^2 in the WT mDC pretreated group, P=0,08, figure 4b).

In contrast, at the adventitial side of the media, the absolute number of infiltrating cells and relative cell accumulation did not differ between groups (489 ± 74 cells in Hmox1-/- mDC versus 514 ± 144 cells in the WT mDC group, P=0,44, and percentage of infiltrating cells; 20,8 ± 3,9 cells/ μm^2 in the Hmox1-/- mDC versus 20,9 ± 5,2 cells/ μm^2 in the WT mDC-pretreated group, respectively). Figure 4c depicts examples of HE-stained cross-sections of arterial allografts derived from the WT mDC and the Hmox1-/- mDC-pretreated group, 2 weeks after allograft transplantation. Perivascular infiltration of blood-derived cells is characteristic of the early vascular allograft response and precedes neotimal formation [15]. In both groups, accumulation of infiltrating cells at the luminal side of the media was mostly observed in the areas with no or limited neo-intima formation (indicated in the figure by black arrows).

Hmox1 deletion in mDCs used for adoptive transfer prior to allografting is associated with pronounced CD4+ T-cell infiltration and IgG accumulation, whereas CD8+ T-cell and CD68+ macrophage infiltration is attenuated.

In both WT and Hmox1-/- mDC-treated mice, mDCs were detected in the vascular allografts. No significant differences between mDC accumulation were detected in the groups (2,3 ± 0,3% versus 1,9 ± 0,7%, in the Hmox1-/- mDC versus WT mDC-treated group, respectively). However, treatment with Hmox1-/- mDC prior to the transplantation procedure was associated with extensive CD4+ T-cell infiltration in the intima, media and the adventitia of the allograft by 4650% (9,5 ± 4,5% in the Hmox1-/- mDC-pretreated group versus 0,2 ± 0,1% in the WT mDC-pretreated group, P<0,05; figure 5a). This specific CD4+ T-cell infiltration was accompanied by a decrease in CD8+ T-cell infiltration of 76,5% (8,1 ± 1,0% versus 14,3 ± 1,6% in the Hmox1-/- mDC versus WT mDC-pretreated group, P<0,05; figure 5b).

However, Hmox1 deletion in mDC did change local IL-6 levels (17756 ± 12647 μm^2 versus 11854 ± 4824 μm^2 surface of IL-6+ staining area in Hmox1-/- mDC versus WT mDC-treated mice, P=0,34; 5,2 ± 2,4% versus 2,3 ± 0,7% Hmox1-/- mDC versus WT mDC-pretreated mice, P=0,26). A TUNEL assay indicated that the CD8+ T-cell response in the allograft was not associated with increased apoptosis of graft ECs and VSMCs. Apoptosis was rarely detected in vascular allografts (figure 6), whereas no difference was observed between the WT and Hmox1-/- mDC-pretreated mice.

The acute phase of the vascular alloimmune response is characterized by infiltration of CD68+ macrophages and CD8+ T cells into the allograft, as early as 2 weeks post transplantation. In contrast, the chronic phase of the alloimmune response is defined by IgG deposition, and perivascular CD4+ T cell infiltration [15]. Concomitant to the significant CD4+ T cell accumulation, Hmox1 deletion in

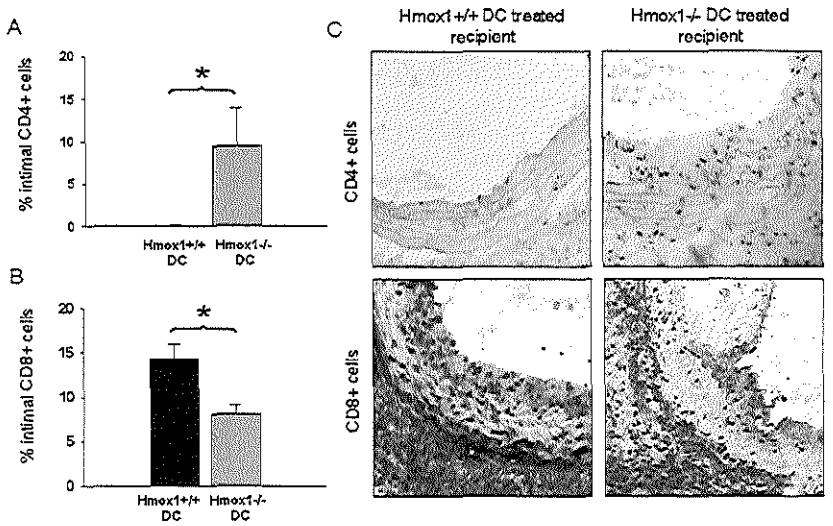


Figure 4 *Number of cells infiltrating the media. (A) The number of infiltrating cells into the luminal side of the media was increased. (*P<0,05 Hmox1-/- versus Hmox1 +/+ mDC-treated, mean ± sem.) (B) Number of infiltrating cells per μm² medial surface area at the luminal side (P<0,08: Hmox1-/- versus Hmox1 +/+ mDC-treated group as defined by the Student's t-tests). (C) Representative sections of arterial allografts show cell infiltration at the luminal side of the media (magnification: 100X).*

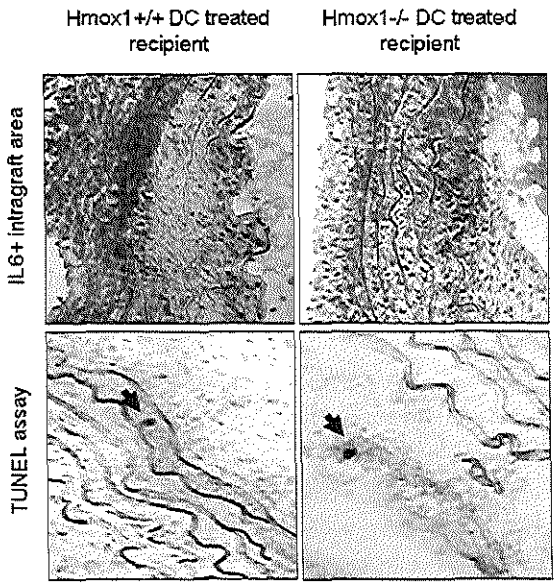


Figure 5 *(A) CD4+ T-cell graft infiltration (B) and CD8+ T cell infiltration into the allograft. Number of CD4+ or CD8+ cells was corrected for the total allograft surface area (mm²), and is presented as a percentage of total surface area. (C) Representative sections of arterial allografts obtained from WT and Hmox1-/- mDCs-pretreated groups are shown (magnification: 100X, * P<0,05 Hmox1-/- versus Hmox1+/+ mDC-treated group, Student's t-tests, mean± sem).*

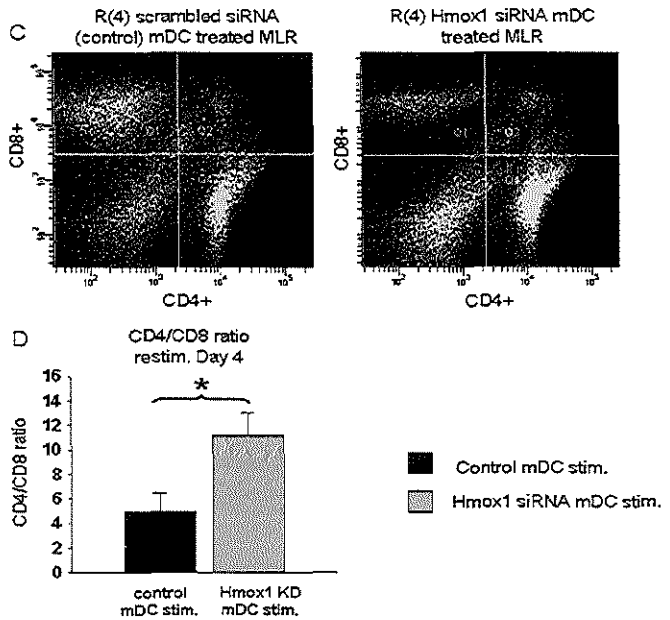


Figure 8 *Flow cytometry analysis of CD8+ and CD4+ T-cell populations in alloantigen-primed mixed leukocyte reaction (MLR) cultures. (A) Knockdown of Hmox1 RNA expression by Hmox1-targeting siRNA decreased Hmox1 mRNA expression by 80% as compared to DCs transfected with non-targeting siRNA. (B) Flow cytometry analysis of CD8+ T-cells and CD4+ T-cells, and the CD4+/CD8+ T-cell ratio in MLR cultures at 4 (MLR4), and 7 days (MLR7) and 4 days after restimulation with alloantigen (R(4)) (C) In Hmox1-silenced mDC MLR cultures, the %CD8+ T-cells were decreased (Quarter 1), while the %CD4+ T-cells simultaneously increased after MLR restimulation with alloantigen (Quarter 4). (D) This resulted in a significant increase in CD4+/CD8+ T-cell ratio in Hmox1 silenced mDC co-cultures. (* P<0,05 scrambled versus Hmox1-siRNA knockdown MLR, paired t-tests per culture, N=4, mean ± sem.)*

mDCs cultures, no difference in these maturation markers was observed between the two groups (see supplemental data). This indicated that Hmox1 knockdown did not interfere with mDC maturation in vitro in response to alloantigen.

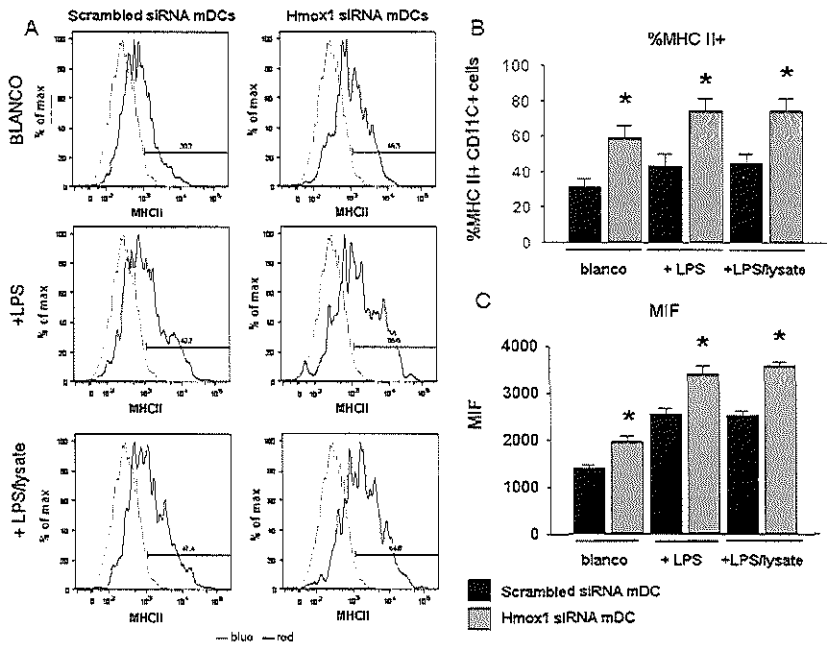
However, in previous microarray analysis, Hmox1 deletion in DC cells was associated with a predominant increase in MHC class II expression (crucial for CD4+ T-cell priming), while MHC class I (crucial for CD8+ T-cell priming) remained unaffected (data not shown). SiRNA-mediated Hmox1 silencing in cultured mDCs indeed upregulated MHC class II but not MHC class I expression levels, as verified by flow cytometry. In the CD11c+ mDC subset, the percentage of MHC class II + cells was significantly increased following Hmox1 silencing either in non-stimulated, LPS-stimulated, or alloantigen-stimulated culture conditions (figure 9a and b). In addition, the average MHC class II expression level in the CD11c+/MHC class II+ population was significantly increased upon Hmox1 siRNA knockdown in mDCs, as indicated by the two-fold induction in the mean intensity of fluorescence (figure 9c). In contrast, no effect was observed in MHC class I expression levels in the CD11c+/MHC class II+ Hmox1 silenced mDCs under all tested conditions (data not shown).

MHC class II transactivator (CIITA) is a co-transcription factor that is involved the regulation of MHC II expression in antigen presenting cells [16]. Knockdown of Hmox1 was associated with a significantly increase of CIITA expression in non-stimulated, or alloantigen-stimulated mDCs (figure 9d). CIITA gene expression is regulated by STAT1, which is activated by phosphorylation at Tyr701. SiRNA knockdown of Hmox1 led to STAT1(Tyr701) phosphorylation, without affecting STAT1 total protein levels (figure 9e en f). Subsequent siRNA-mediated silencing of the CIITA expression upon Hmox1 knockdown rescued the MHC class II upregulation, suggesting that loss of HO-1 induced MHC class II cell surface expression via CIITA transcriptional regulation (figure 9g-i).

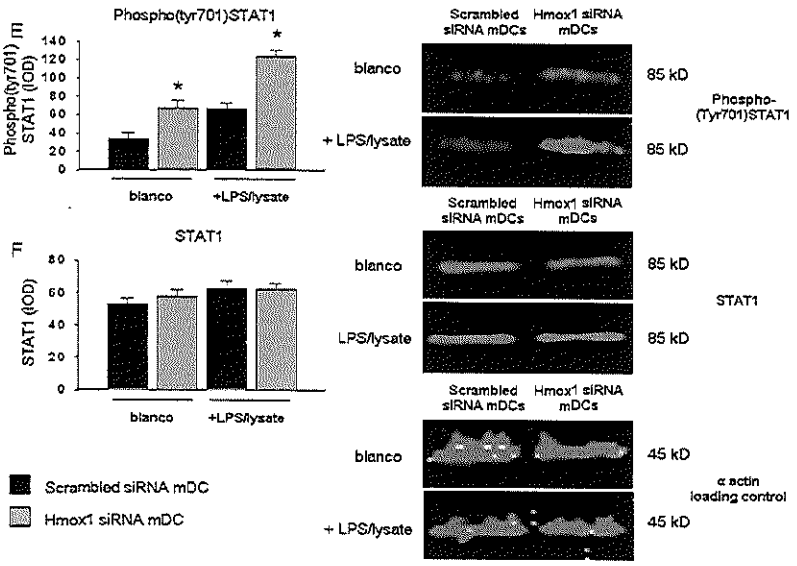
Taken together, these data indicate that Hmox1 silencing in mDCs promotes STAT1 Tyr701 phosphorylation, and subsequent gene expression of the co-transcription factor CIITA, thereby increasing the level of MHC class II on mDCs. Increased expression of MHC class II in Hmox1 knockdown mDCs facilitated the preferential priming of CD4+ T-cells (as opposed to CD8+ T-cells), thereby accelerating transplantation atherosclerosis formation.

Figure 9 *(A) MHC class II levels on mDCs cultured in non-stimulated, LPS-stimulated and alloantigen-stimulated conditions, and analyzed by flow cytometry. Red depicts the isotypic control, while blue depicts the MHC class II signal. (B) Hmox1-silencing increased the percentage of MHC class II+ cells in the CD11c+ mDC population (C) and augmented the mean intensity of fluoresence (MIF) of the CD11c+/MHC class II+ cells, in all three different conditions compared to non-targeting-siRNA transfected mDCs. (D) CIITA mRNA expression levels in mDCs cultured in non-stimulated, LPS-stimulated, and alloantigen/LPS-stimulated conditions, analyzed by QPCR. Hmox1 deletion augmented the expression levels of CIITA in mDCs in all three conditions. (E) Western blot analysis shows increased phosphorylated (Tyr701)-STAT1 protein levels in Hmox1 knockdown mDCs, (F) whereas no difference in total STAT1 protein levels was observed. Right panel shows western blot analysis of phospho(Tyr701)-STAT1, total STAT1, and β actin loading control. (G) MHC class II levels on alloantigen-activated mDCs, analyzed by flow cytometry. Red depicts the isotypic control, while blue depicts the MHC class II signal. (H) Hmox1-silencing increased the percentage of MHC class II+ cells in the mDC population. This effect was rescued by subsequent knockdown of CIITA. (I) Hmox1-silencing augmented the MIF of the CD11c+/MHC class II+ population, whereas transfection with Hmox1/CIITA targeting siRNA downregulated the average cell surface expression of MHC class II on mDCs, as indicated by a decrease in MIF. (* P<0,05 Hmox1 siRNA knockdown mDCs versus scrambled-siRNA treated (control) mDCs, † P<0,05 Hmox1/CIITA knockdown mDCs versus Hmox1 siRNA knockdown mDCs, paired t-tests per culture, N=4, mean ± sem.)*

(I)



(II)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Phospho(tyr701) STAT1 (fOD)
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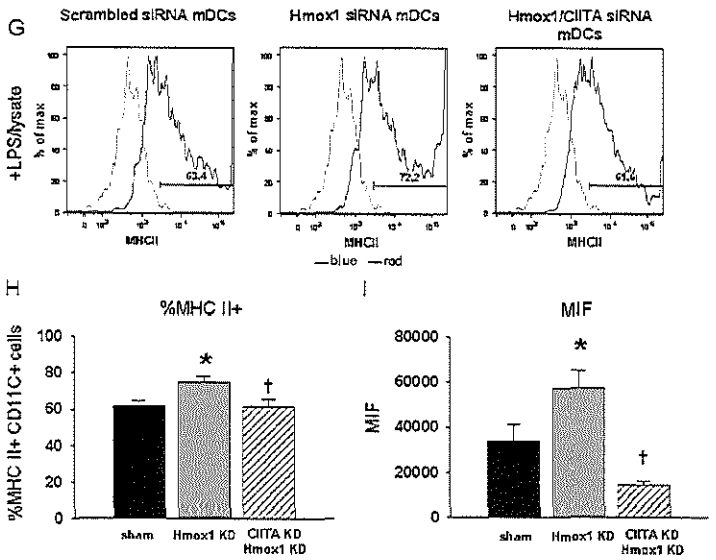
(III)


Scrambled siRNA mDCs
Hmox1 siRNA mDCs
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% of max
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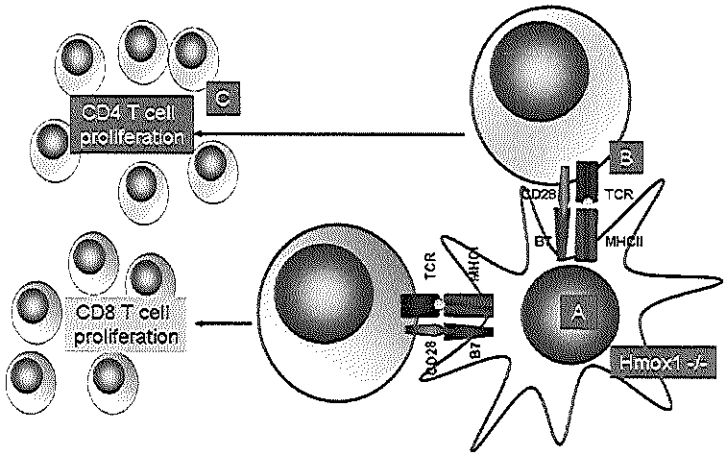
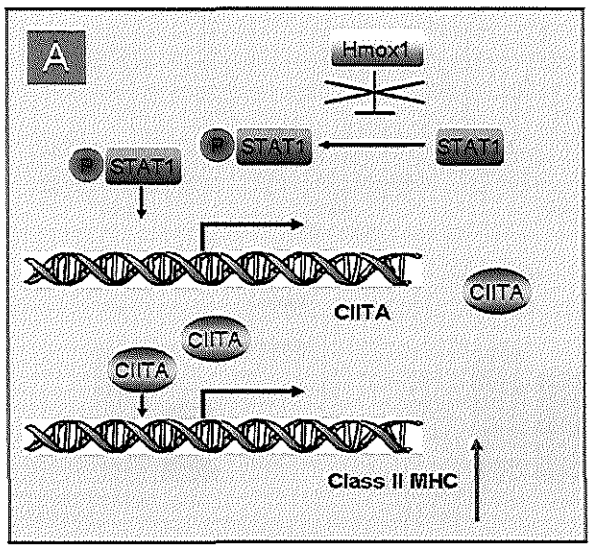
MIF
sham
Hmox1 KD
CIITA KD Hmox1 KD

Figure 10 *Proposed mechanism by which Hmox1 deletion in mDCs promotes the CD4+ T-cell response. (A) Hmox1 deletion in mDCs promotes phosphorylation of STAT1. Activation of STAT1 induces CIITA upregulation, which functions as a co-transcription factor driving the expression of MHC class II genes in the mDCs. (B) Increased MHC class II cell surface availability on Hmox1 -/- mDCs induces preferential CD4+ T-cell activation. (C) Increase in CD4+ T-cell proliferation shifts the alloimmune response towards a CD4+ T-cell mediated reaction, and promotes neointimal hyperplasia in transplantation atherosclerosis with a phenotype consistent with the chronic phase of alloimmune response.*





DISCUSSION

We were able to show that: (I) Previous adoptive transfer of alloantigen-presensitized myeloid dendritic cells accelerates transplantation atherosclerosis. (II) Loss of Hmox1 function induces STAT1 phosphorylation and subsequent CIITA expression, which leads to a rise in MHC class II in DCs without modulation in MHC class I expression. (III) Hmox1 deletion in mDCs is associated with a MHC class II-mediated, preferential CD4+ T-cell alloimmune response in DC/T-cell co-cultures (VI) loss of Hmox1 in DCs *in vivo* promotes transplant associated vasculopathy with increased perivascular infiltration of CD4+ T-cell and IgG deposition, suggestive of a chronic phase in the disease. (V) As a result, alloreactive Hmox1 -/- mDCs stimulates increased intimal hyperplasia formation in vascular allografts compared to WT mDCs-pretreatment. Taken together, these data indicate that mDCs play a key regulatory role in the initiation and progression of transplantation atherosclerosis, and provide evidence that Hmox1 is an important genetic regulator of dendritic cell function.

Progressive transplant atherosclerosis is the leading cause of late graft failure in heart transplantation, responsible for 23% to 36% of deaths among patients that survive >1 year after surgery (17), whereas substantial clinical manifestations of transplant atherosclerosis is observed in 50% of heart transplant recipients within five years after transplantation [18, 19]. The vascular lesion pattern is diffuse, and involves the entire vascular bed of the transplanted organ, which limits the options for further revascularization based on conventional surgical or percutaneous medical intervention.

In the early phase of transplantation atherosclerosis, the endothelial layer is disrupted and activated by invading blood-cells, and loss of medial VSMCs is induced by the host-derived inflammatory reaction. In the sequence of events, intimal hyperplasia is observed predominantly in the late phase of the disease, with intimal inflammatory cell infiltration, followed by intimal VSMCs migration and proliferation [20]. In the rat abdominal aortic allograft model, arterial transplants show limited hyperplasia up to 2-3 weeks [20, 21]. Our results in the mouse transplantation model adapted from Dietrich et al [15]. confirmed these findings, as transplantation of the BALB/c aortic allograft into naïve recipients initially induced modest intimal lesions within the first 14 days post transplantation. The crucial event in the priming mechanism is the interaction between (allo) antigen-presenting cells (APCs) and naïve recipient T-cells, which results in the activation of various T-cell subsets and subsequent production of antibodies directed against the allograft (the allogenic humoral response). Pre-activated T-cells and memory B-cells that react specifically to donor-derived alloantigens are responsible for accelerated graft rejection at the time of recurrent exposure (i.e. the transplantation).

DCs are professional APCs, which have been suggested to initiate the allogenic humoral reaction directed against vascular allografts: Alloantigen injection to prime autogenous DCs in rat recipients prior allografting promoted intimal lesion size [20], and adoptive transfer of alloantigen-presensitized DCs in rats with renal allografts induced hyper acute rejection, characterized by acute kidney failure and tissue damage of the grafts [22]. In contrast, inhibition of recipient DC function by Sanglifehrin A suppressed acute and chronic cardiac allograft rejection [23]. Our study provide supportive data

that mDCs play a crucial role in transplant atherosclerosis, as adoptive transfer of alloantigen-primed mDCs severely accelerated the disease progression, and subsequently promoted lesion size in the mouse aortic allograft model. The data also suggest that Hmox1 is a key regulator of mDC function during the alloimmune response in transplantation atherosclerosis. Deletion of Hmox1 in alloantigen-activated mDCs used for adoptive transfer further promoted lesion growth, as well as changed the phenotype of the transplant lesions compared to wildtype alloantigen-activated mDCs pretreated recipients. Progression of transplantation atherosclerosis can generally be divided into an acute "cell-mediated phase" and a chronic "humoral response phase" [20]. The acute phase is characterized by activation and infiltration of predominantly macrophages and CD8+ T-cells into the media of the allograft, which contribute to the early assault of the graft ECs and VSMCs(24). Immunohistological evaluation of the lesions in the wildtype alloantigen-activated mDCs-pretreated group depicted limited transplantation atherosclerosis with CD8+ T-cell infiltration and CD68+ macrophage accumulation, reminiscent of an early cell-mediated alloimmune response. In contrast, Hmox1 deletion in alloantigen-activated DCs stimulated neointimal hyperplasia with predominant CD4+ T-cell infiltration, with limited CD8+ T-cell and macrophages, consistent with a humoral chronic alloimmune response. This hypothesis is corroborated by the accumulation of IgG antibodies in the allografts of the Hmox1-/- mDC-treated group. Indeed, the chronic phase of transplant atherosclerosis is associated with IgG antibodies sequestered by the residing cells in the allograft before C1 complex formation and activation of the complement cascade [25]. Therefore, the general pathological phenotype of the lesions suggests that Hmox1-deletion in alloantigen-activated mDCs expedites progression of transplantation atherosclerosis towards the chronic, humoral response.

During progression of allograft vasculopathy, the medial area becomes devoid of VSMCs [26] and infiltrated by inflammatory cells [15]. In alloreactive Hmox1 -/- mDC primed animals, a predominant luminal infiltration of the media was observed, while VSMC content of this segment was indeed decreased. Previously, it was postulated that VSMC cell death may be induced by antibody- and complement-dependent cytotoxicity directed towards medial cells, as deposition of IgG antibodies and complement were observed in the media of arterial allografts [20, 25], and administration of alloantibodies initiated cytoxicity of graft vascular cells in vitro [25, 27]. Consistent with these observations, in our study, Hmox1 deletion in mDCs aggravated IgG accumulation in the media of the allografts of the Hmox1 -/- mDC primed group and was associated with a decrease of medial VSMCs. However, apoptosis was not prevalent at 14 days post transplantation. Complement-dependent cytotoxity may still play a role in graft VSMCs survival in a later phase in chronic vasculopathy, as apoptotic VSMCs were predominantly identified as late as 2 months post-transplantation in unprimed recipients [20, 25].

Hmox1 deletion did not impede DC maturation, as expression of maturation markers CD40, CD80, CD86 did not differ between wild type and Hmox1 knockout mDCs. Conform to our findings, CD40, CD80, CD86 cell surface expression in CD11c+ DCs was unaffected by CoPPIX induction of Hmox1 [28]. More importantly, the level of MHC class II, but not class I, was increased on Hmox1-deficient mDCs under basal and stimulated conditions. Preferential CD4+ T-cell activation by Hmox1-

deficient mDCs in vitro and *in vivo* could therefore be the result of this constitutive induction of MHC class II. MHC class II transactivator (CIITA) regulates the expression of MHC II in APCs. Macrophages or dendritic cells derived from CIITA -/- mice were unable to induce MHC II I-A and I-E expression (16), whereas Chora and co-workers found downregulation of MHC class II expression in CD11c+ DCs located in lymphoid tissues after Hmox1 induction in an EAE mouse model(28). Our results demonstrate that Hmox1-targeting siRNA increased MHC class II expression in DCs by STAT1 phoshorylation, which is involved in the transcriptional regulation of CIITA expression. More importantly, subsequent silencing of CIITA in Hmox1-knockdown mDCs impeded Hmox1 silencing induced upregulation of MHC class II.This demonstrates that cell surface availability of MHC class II for CD4+T-cell activation was affected by HO-1 levels specifically via CIITA transcriptional regulation. The increase in CIITA transcription by Hmox1 silencing could be mediated by CO, as CO stimulation of CD11c+ DCs downregulated CIITA mRNA expression in response to IFN-γ stimulation in vitro [28].These findings suggest that increase in MHC class II cell surface availability in Hmox1-deficient mDCs is mediated by activation of the STAT1/CIITA pathway (figure 10).

In conclusion, we here demonstrate the central role of Hmox1 as a genetic regulator of mDC function and subsequent T-cell priming in the allo-immune response associated with transplant atherosclerosis. Adoptive transfer of myeloid DCs accelerated the allo-immune response that amounted into an increase in neointimal hyperplasia. Hmox1 deletion in mDCs accelerated T-cell priming and progression into a CD4+ T cell mediated response, giving rise to a more chronic phenotype of transplant atherosclerosis with CD4+ T-cell infiltration and IgG deposition, and attenuation of CD8+ T-cell and CD68+ macrophage accumulation. Dendritic cell function in the alloimmune response is regulated by Hmox1 via a P(tyr701)-STAT1/CIITA/MHC II mediated pathway. Therefore, we speculate that Hmox1 could play a key role in the design of therapies to prolong allograft function, based on the potent alloimmunity modulating capacity of this enzyme.

Study limitations

Ischemia/reperfusion injury of the allograft provides the initial trigger for pathogenesis of transplantation vasculopathy, and could determine the phenotypical outcome of the lesion. Therefore, great care was taken during the procedure to keep the ischemic time to which each transplant was exposed constant (10 minutes) and to a bare minimum.

Donor DCs residing in the allograft could have contributed to the observed alloimmune response via the direct pathway of alloantigen recognition (i.e. the direct interaction of recipient T-cells with complexes of intact allogenic MHC molecules bound to the surface of donor APCs) [1]. However, since the three different experimental groups received the same type of aortic transplants derived from BALB/c mice, this process could not be accounted for the detected effects between the Hmox1-deficient mDC and the Hmox1 wildtype mDCs presensitized group.

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5

HEME OXYGENASE-1 INVOLVEMENT IN THE DENDRITIC CELL INDUCED SKEWING TOWARDS TH2 IMMUNE RESPONSE

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ABSTRACT

Heme oxygenase-1 (HO-1) has been shown to be a mediator in various immune responses, including the graft-versus-host alloresponse, in which HO-1 seems to play a protective role. Here, we report that HO-1 gene expression in dendritic cells (DC) is crucial in the induction of naïve CD4+ T cells towards Th2 differentiation. Ovalbumin presentation by HO-1 null myeloid DCs (mDCs) to Ova Transgenic (OT2) CD4+ T cells impeded the Th2 response, characterized by an attenuated production of the Th2-specific cytokines, including IL4, IL5, IL10, and IL13, without affecting the production of typical Th1 cytokines, including INF- . This effect was independent of DC maturation, as expression levels of CD40, CD80, and CD86 remain unaffected. In a murine model for asthma, governed by a Th2 dominant response, adoptive transfer of pulsed HO-1 null mDCs failed to elicit the typical TH2 response *in vivo* as indicated by the absence of peribronchial and perivascular infiltration of eosinophilic inflammatory cells, diminished globlet cell hyperplasia, and a significant reduction in total cell number and eosinophils in the bronchoalveolar lavage fluid (BAL). T-cells harvested from mediastinal lymph nodes (MLNs) in this model showed an alike decrease in production of Th2 associated cytokines, whereas the level of INF-γ was not affected. Taken together, the current study suggests a novel function of HO-1 signaling in the genetic regulation of dendritic cell induced T-cell priming towards a Th2 response.

INTRODUCTION

HO-1 is the key enzyme in the degradation of hemoproteins. Degradation products of this catabolic reaction including ferritin, biliverdin and carbon monoxide (CO) have been shown to play a key role in cardiovascular signaling cascades. Three distinct HO-1 isozymes have thus far been identified; HO-1,-2 and -3. HO-1 is inducible by various inflammatory stimuli, as well as by oxidative stress. HO-1 -/- mice were shown to be susceptible to chronic inflammation, manifested by enlarged lymph nodes and spleen, leukocytosis, and heme depositions in the kidney and liver [1, 2]. Interestingly, primary splenocytes derived from HO-1 -/- mice produce a Th1-associated cytokine profile following LPS stimulation, indicative of a possible correlation between HO-1 deficiency and altered T cell function [2]. Recent studies have also identified a role for HO-1 in allograft survival and organ transplantation associated vasculopathy, in which T cell activation plays a crucial role [3-5]. In an allograft model for transplantation atherosclerosis HO-1 deletion increased the capacity of myeloid dendritic cells to elicit a specific CD4+ T cell response. This CD4+ T-cell allograft response was mediated through induction of MHC class II expression by MHC class II transactivator (CIITA)-driven transcription and could be rescued by CIITA knockdown. Myeloid dendritic cells (mDCs) are potent professional antigen-presenting cells that direct the adaptive immune immunity in response to antigen stimulation. Dendritic cells are able to initiate the activation of naïve CD4+ T cells, and are able to direct T-cell polarization into distinct lineages (e.g. Th1, Th2, Th17 and regulatory T cells) [6]. The mechanism by which mDCs initiate this lineage differentiation remains poorly understood. It has been implied that differential expression of co-stimulatory surface molecules could affect the polarization process. Costimulation via CD86, CD40, and OX40L favors the development of a Th2 response [7-10]. In addition, cytokine stimulation during naïve CD4+ T cell priming could also determine the CD4+ T cell polarization. Th1 induction via mDC depends largely on IL12 stimulation, whereas IL4 and IL6 promotes a Th2 driven reaction [10-15]. However, the precise mechanisms of action of HO-1 in the regulation of DC function in CD4+ T cell polarization remains to be elucidated.

In the current study, the effects of HO-1 on mDC function are further explored, to elicit a dominant CD4+ T cell response. Here, we were able to demonstrate in both HO-1 nullizygous and HO-1 silenced mDCs that maturation of mDCs is largely independent of HO-1, as cell membrane expression levels of CD40, CD80, and CD86 are unaffected. Loss of HO-1 function however impeded the Th2 response elicited by mDC-CD4+ T cell interaction as characterized by *in vitro* T-cell and Th2 cytokine responses and *in vivo* in an adoptive transfer model of bronchial inflammation elicited by OVA-primed HO-1 null DC in OTII transgenic mice. Therefore, these studies suggest that HO-1 is involved in the genetic regulation of DC-governed Th2 response, and may provide a molecular basis for the immune-modulatory effects of HO-1 expression in immune-mediated cardiovascular diseases, including transplant atherosclerosis and vulnerable plaque formation.

MATERIALS AND METHODS

Animals

HO-1 knockout mice (HO-1-/- / C57black6) were obtained from Arthur M.E. Lee (Harvard Medical school, Boston, MA, US) and C57bl/6 OVA-TCR transgenic mice (DO11.10) were cross bred at the Erasmus Medical Center. All experiments were performed according to the institutional guidelines of the animal ethics committee at the Erasmus MC.

Generation and antigen pulsing of bone marrow DCs

DCs were cultured as previously described [16]. Briefly, bone marrow cells were obtained from femurs and tibiae of wildtype or HO-1 -/- C57bl/6 mice, and cultured at a density of $2x10^6$ per 10 cm diameter petri dish in 10 ml cell-culture medium (TCM) consisting of RPMI 1640 (Invitrogen, UK) supplemented with gentamicin (60µg/ml), 2-mercaptoethanol (5×10^{-5} mol/L), 5% fetal calf serum (Biocell Laboratories, UK) and GM-CSF (200 IU/ml) (kindly provided by Prof. B.Lambrecht). At day 3, 10 ml TCM was added to the cultures. At days 6 and 8, half of the medium was collected, centrifuged, and the pellet was resuspended in TCM and added back to the cultures.

At day 9, DCs were pulsed overnight with 100 µg/ml of OVA/LPS (LPS contamination of 2.9 ng/mg protein; Worthington) per $1x\ 10^6$ cells/ml. The viability of the DCs after treatment with OVA/LPS was >99% as assessed by trypan blue exclusion.

The DC phenotype was validated by staining for 30 minutes at RT with anti-CD11c-APC, anti-MHCII-FITC, in combination with anti-CD40-PE, anti-CD80-PE, and anti-CD86-PE (BD Biosciences, The Netherlands) dissolved in PBS containing 0.5% bovine serum albumin and 0.01% sodium azide. DCs were subsequently washed and analyzed by flow cytometry on a FACS canto (BD Bioscience, The Netherlands).

Murine asthma model

At day 0, C57bl/65 mice were anesthetized with avertin (2% v/v in PBS) and received 80 µl of the cell suspension (1×10^6 OVA/LPS pulsed wild type or HO-1 -/- DCs) via an intratracheal injection through the opening of the vocal cords.

From days 10 to 13, mice were exposed to OVA aerosols (Grade III, Sigma, The Netherlands) delivered from a jet nebulizer delivering 1% OVA in PBS for 30 min. Mice were sacrificed 24 hours after the last aerosol challenge. Bronchoalveolar lavage (BAL) was performed with 1 ml of Ca^{2+}- and Mg^{2+}-free HBSS (Invitrogen) supplemented with 0.1 mmol/L of sodium ethylenediaminetetraacetic acid. The BAL fluid was centrifuged; the cells were resuspended in HBSS, and enumerated in a hemocytometer. After washing, cells were stained for 30 minutes with anti-I-Ad/I-Ed FITC (macrophages), anti-CCR3 PE (eosinophils), anti-CD3 cy-chrome, anti-B220 cy-chrome (T and B cells, respectively), and anti-CD11c APC (macrophages) (BD Biosciences, The Netherlands), in PBS containing 0.5% bovine serum albumin and 0.01% sodium azide.

After BAL was performed, 1 ml of fixative (4% PFA/PBS) was gently infused through the catheter. The lungs were dissected and embedded in paraffin. Five-µm sections were stained with hematoxylin-eosin and assessed by brightfield microscopy.

Cytokine measurement in MLNs in a secondary response

MLNs were removed, homogenized, and resuspended in RPMI 1640 containing 5% fetal calf serum before enumeration. Ex vivo production of cytokines by T cells collected from the MLNs was measured after restimulation of 2 x 10^6 total cells/ml with 10 µg/ml of OVA/LPS for 4 days.

T cell proliferation assay

For fluorescent cell labeling, T cells were washed twice with serum-free medium, labeled with 5 µM CFSE (Molecular Probes, Oss, The Netherlands) in serum-free medium for 10 min at 37°C. For quantification of cell division based on serial halving of CFSE intensity, algorithms provided by FlowJo software (Treestar, San Carlos, US) were used as earlier described [17].

Real-time quantitative RT-PCR

HO-1 and Foxp3 expression in cultured mDCs was assessed by quantitative PCR (QPCR). Total RNA was isolated using the RNAeasy kit (Qiagen, Germany) and reversed transcribed into cDNA using random hexamers. QPCR reactions were performed using cyber green incorporation and real-time detection in the iCycler iQ Detection system (Biorad, The Netherlands). Target gene mRNA levels were assessed relative to the household gene hypoxanthine-guanine phosphoribosyltransferase (HPRT).

Elisa analysis

Cytokines levels (murine IL4, IL5, IL10, IL6, IL13, IL12P70, and IFN) were determined using commercial quantitative ELISA assays according to the instructions of the manufacturer (Bender Medsystems, The Netherlands).

siRNA transfection of DC

siRNA transfection with HO-1-/- targeting siRNA or scrambled sham siRNA was performed using Dharmacon smartpool siRNA (a commercial mix of four different sequences per target gene) according to the instructions of the manufacturer (Dharmacon, Thermoscientific, The Netherlands). siRNA transfection was initiated at day 7 of the DC culture protocol, and efficient knock down of HO-1 was validated by qPCR analysis at day 10 (1 day after stimulation with OVA/LPS) (Figure 1a).

RESULTS

HO-1 deficiency partially influences dendritic cell maturation

Bone marrow (BM) derived mDCs of HO-1 +/+ and -/- mice (C57bl/6 background) were cultured according to the Lutz-protocol and used in the various assays (figure 1a). In addition, knockdown of HO-1mRNA by gene targeting siRNA substantially decreased HO-1 mRNA expression in BM derived mDCs compared to non-targeting siRNA (sham) transfected mDCs (figure 1b). HO-1 deletion or knockdown in mDCs did not alter cell surface expression of the mDC maturation markers CD40, CD80, and CD86 on the CD11c+ mDC population compared to the controls (BM derived mDCs from wildtype littermates). However, HO-1 deficiency in mDCs was associated with significantly increase in MHC class II surface expression (Figure 2d).

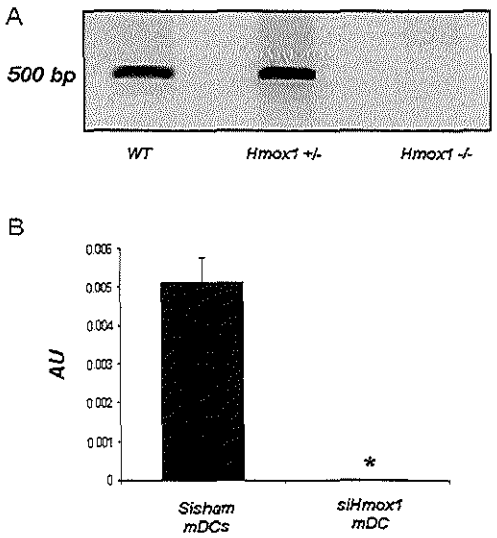


Figure 1 *HO-1 expression in transgenic and transduced mDCs.*

*(A) HO-1 mRNA expression is diminished in mDCs derived from bone marrow of HO-1 -/- C57bl/6 mice compared to wild type and heterozygous littermates. Representative result of 3 independent experiments is shown. (B) Transfection of wildtype bone marrow derived mDCs with HO-1-targeting siRNA significantly decreased HO-1 mRNA expression compared to non-targeting siRNA (si-sham) treated mDCs. N=5, *P<0.05 wildtype versus HO-1 -/- mDC as defined by the Student's t-test, mean ± sem.*

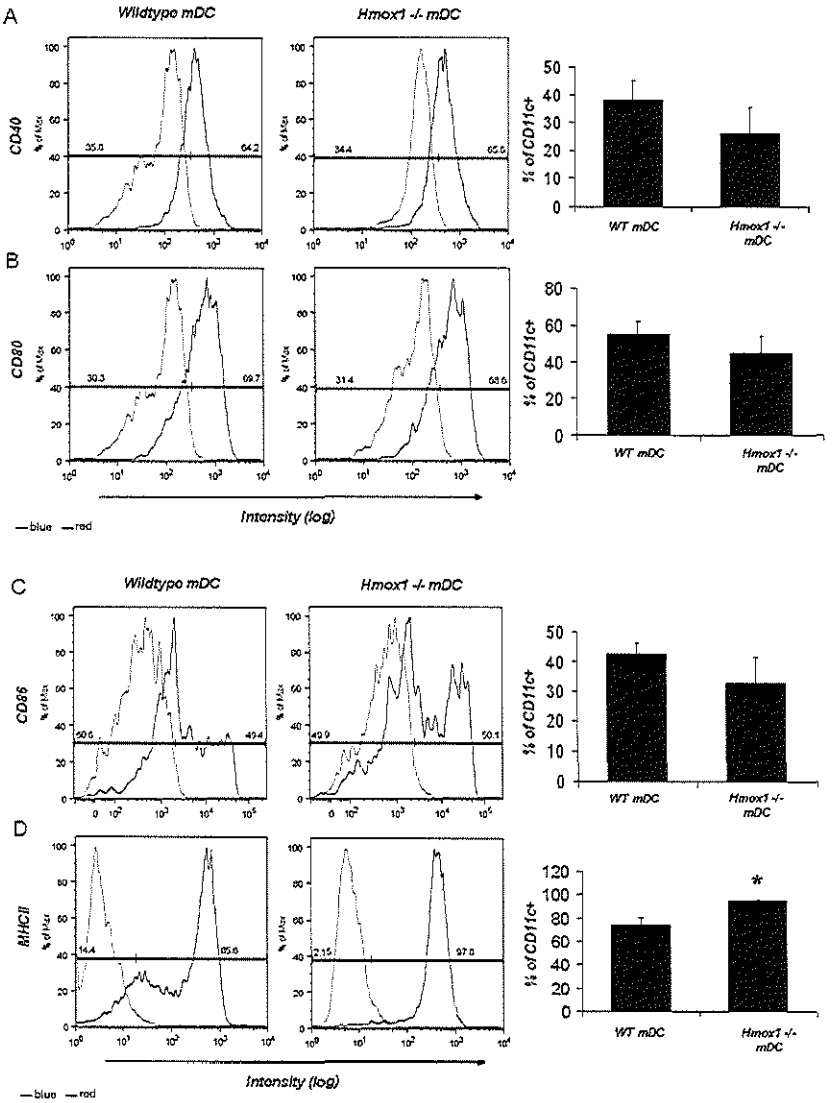


Figure 2 *Phenotype of WT and HO-1 -/- DC.*

*DCs cultured from bone marrow of wildtype and HO-1 -/- C57bl/6 mice were stimulated with OVA/LPS for 24 hours. Cell surface expression of DC specific maturation markers were analyzed by flow cytometry. Representative FACS profiles of 5 independent experiments are shown, red profile indicates the isotypic-control, blue profile indicates the CD40, CD80, CD86, and MHCII signal (left panel). The percentages of CD40+, CD80+, CD86+, and MHCII+ cells in the CD11c+ cell population of each group are shown (right panel)(A-D). N=5, *P<0.05 wildtype versus HO-1 -/- mDC as defined by the Student's t-test, mean ± sem.*

HO-1 deletion in dendritic cells impedes Th2-polarization without affecting the T cell proliferation capacity

Next, the effect of HO-1 deletion on the capacity of mDCs to activate naïve CD4+ T cells in an *in vitro* mixed lymphocyte response (MLR) assay was assessed. Murine wild type and HO-1-/- mDCs were pulsed for 24h with Ovalbumin (OVA) with trace amounts of LPS. CD4+ T cell priming capacity of these OVA pulsed mDCs was tested by co-culture with CFSE labeled OVA specific T cells purified from the spleens of OT2 C57bl/6 mice (ratio 1:10, mDCs versus CD4+ T cells respectively). Loss of HO-1 in mDCs did not affect CD4+ T cell proliferation in response to mDC priming (figure 3).

However, HO-1 deficiency in mDCs extensively affected the Th1/Th2-cytokine profile of the effector CD4+ T cells. Cytokine levels were assessed in the supernatant of wild-type and HO-1 mDC MLR cultures after 4 days of stimulation. Secretion of Th2-cytokines IL4, IL5, IL10, and IL13 by HO-1 -/-

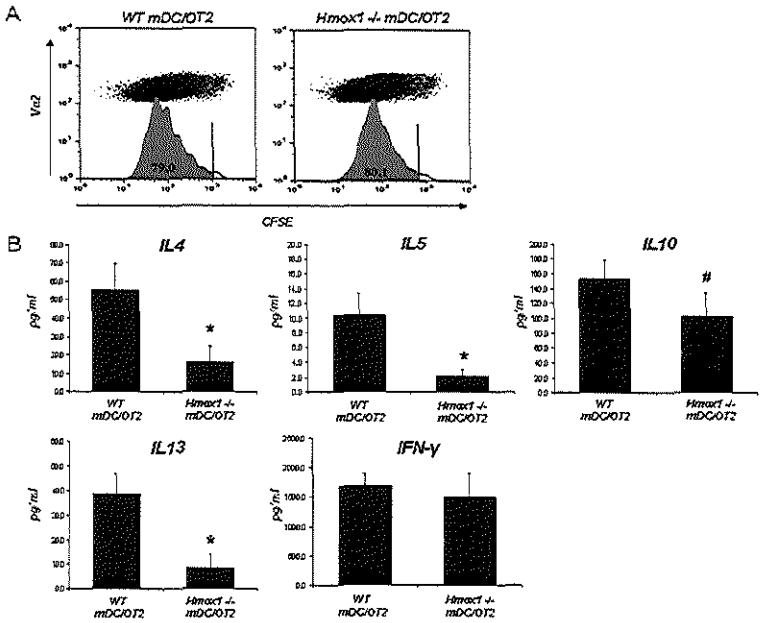


Figure 3 *Tcell response to WT and HO-1-/- DC.*

*HO-1 deficiency in mDCs decreased DC function to induce cytokine secretion by responsive CD4+ T cells without effecting DC capacity to induce CD4+ T cell proliferation. (A) CD4+ T cells isolated from the spleen of OT2 C57bl/6 mice were labeled with CFSE and co-cultured with OVA/LPS pulsed HO-1 -/- mDCs and wildtype mDCs (MLR ratio 10:1) for 4 days. Shown are representative FACS profiles of 3 independent experiments. (B) ELISA analysis of cytokine production in stimulated T cells showing the levels IL4, IL5, IL10, IL13, and IFN-γ in the supernatant derived from the MLR co-cultures of OT2 CD4+ T cells with OVA/LPS pulsed HO-1 -/- or wildtype mDCs. N=5, *P<0.05 wildtype versus HO-1 -/- mDC as defined by the Student's t-test, mean ± sem.*

mDC primed CD4+ T cells was markedly diminished compared to the CD4+ T cells primed by wild type mDCs (figure 3b). In contrast, the production of the Th1-cytokine, IFN-γ, remained unaffected (figure 3b). These *in vitro* data suggest that HO-1 deficiency in mDCs impedes Th2 polarization of naïve CD4+ T cells without affecting T cell proliferation.

HO-1-/- mDCs are incapable of induction of a Th2 response in a mouse model for asthma.

The inability of HO-1-/- mDCs to induce a Th2 serological response was further assessed in a well established Th2 driven murine model of asthma. C57bl/6 OVA-TCR transgenic mice received OVA pulsed mDCs by intratracheal injection and were subsequently challenged with OVA aerosols 10 day later, for 3 consecutive days. This treatment typically leads to production of Th2 cytokines in the draining mediastinal lymph nodes of the lung and goblet cell hyperplasia, peribronchial inflammation, and eosinophilia in the broncho-alveolar lavage (BAL) fluid, all characteristic of asthma. Histological analysis revealed that OVA-pulsed wild type mDCs induced the accumulation of inflammatory cells, which consisted mainly of granulocytes that were located around the brionchioles and lung vasculature (figure 4a). In addition, the number of goblet cells in the airway epithelium increased and mucus thickening was observed (B). In contrast, in mice that received OVA pulsed HO-1-/- mDCs, peribrionchial and perivascular infiltration was virtually absent, whereas no goblet cell hyperplasia or hyperaccumulation of mucus were observed (figure 4a, b).

Likewise, C57bl/6 mice treated with HO-1 -/- mDCs showed a significant decrease in the total cell number in the BAL fluid compared to the wild type mDC treated animals. Eosinophilia was also diminished in HO-1 -/- mDC presensitized mice, including a decrease in percentage and absolute number of eosinophils in the bronchoalveolar compartment. In addition, lymphocyte accumulation in the BAL fluid was also attenuated (figure 6). Assessment of the Th2 cytokine profile of the T cells obtained from the draining mediastinal lymph nodes (MLNs) indicated that the Th2 dominant serological response was impeded by HO-1 deletion in mDCs. The *in vivo* production of Th2 cytokines IL4, IL5, IL10, and IL13 were all diminished in the HO-1-/- mDCs treated C57bl/6 mice compared to the with wildtype mDCs treated animals. In contrast, INF-γ levels by MLN derived cells were not significantly affected (figure 5). Recent studies have implied that HO-1 suppresses the pathophysiology of asthma by inducing high levels of CD4+ CD25+ Treg cells. The mRNA level of Foxp3, a specific marker of CD4+ CD25+ T reg cells, was therefore assessed in the lungs by QPCR. Animals treated with HO-1 -/- mDCs showed reduced pulmonary mRNA levels of Foxp3 compared to animals treated with wildtype mDCs (figure 1, supplemental data).

Figure 4 *Murine model of asthma using WT and HO-1-/- mDC.*

*Wildtype C57bl/6 mice received intratracheal injections of OVA pulsed wildtype or HO-1 -/- mDCs at day 0, followed by intratracheal challenges with OVA aerosoles at day 10 and 13. Animals were sacrificed at day 14 for analysis. Respresentative photomicrogaphs of haematoxylin-eosin stained cross-sections of the lungs of wildtype and HO-1 -/- mDC treated mice. (A) OVA challenge in wildtype mDC treated mice induced a marked peribronchial and perivascular infiltration of inflammatory cells (indicated by black arrows), whereas HO-1 mDC presensitized mice failed to induce a marked peribronchial inflammation. (B) hyperplasia of globlet cells in the airway epithelium and mucus accumulation (indicated by black arrows) was clearly present in wildtype mDC treated mice, but was absent in HO-1 mDC treated animals. (C) FACS analysis of BAL fluids showing the total number of cells, the percentage of eosinophils, the number of eosinophils, and the number of macrophages, neurophils, and lymphocytes, in BAL fluid of wildtype mDC compared to HO-1 -/- mDC treated animals. N=10 per group, *P<0.05 wildtype versus HO-1 -/- mDC as defined by the Student's t-test, mean ± sem.*

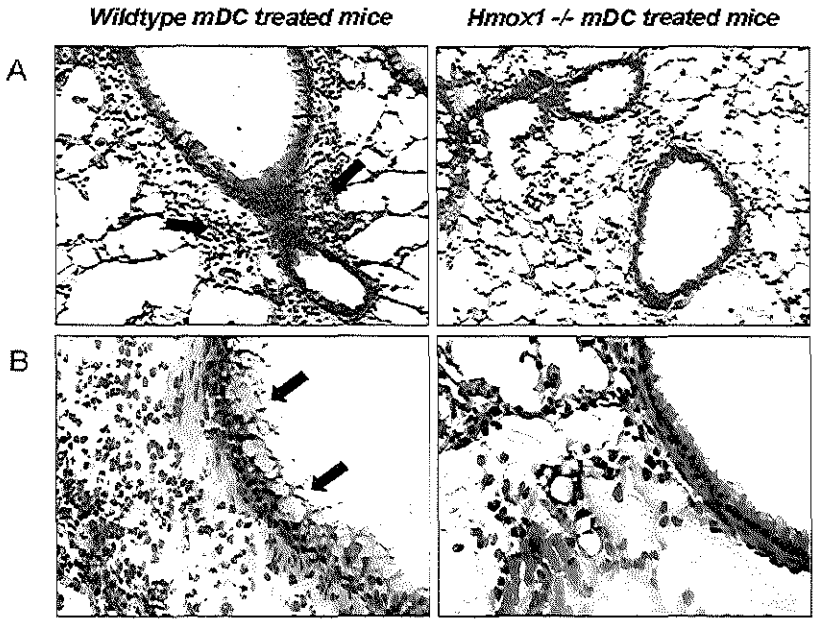


Figure 4B

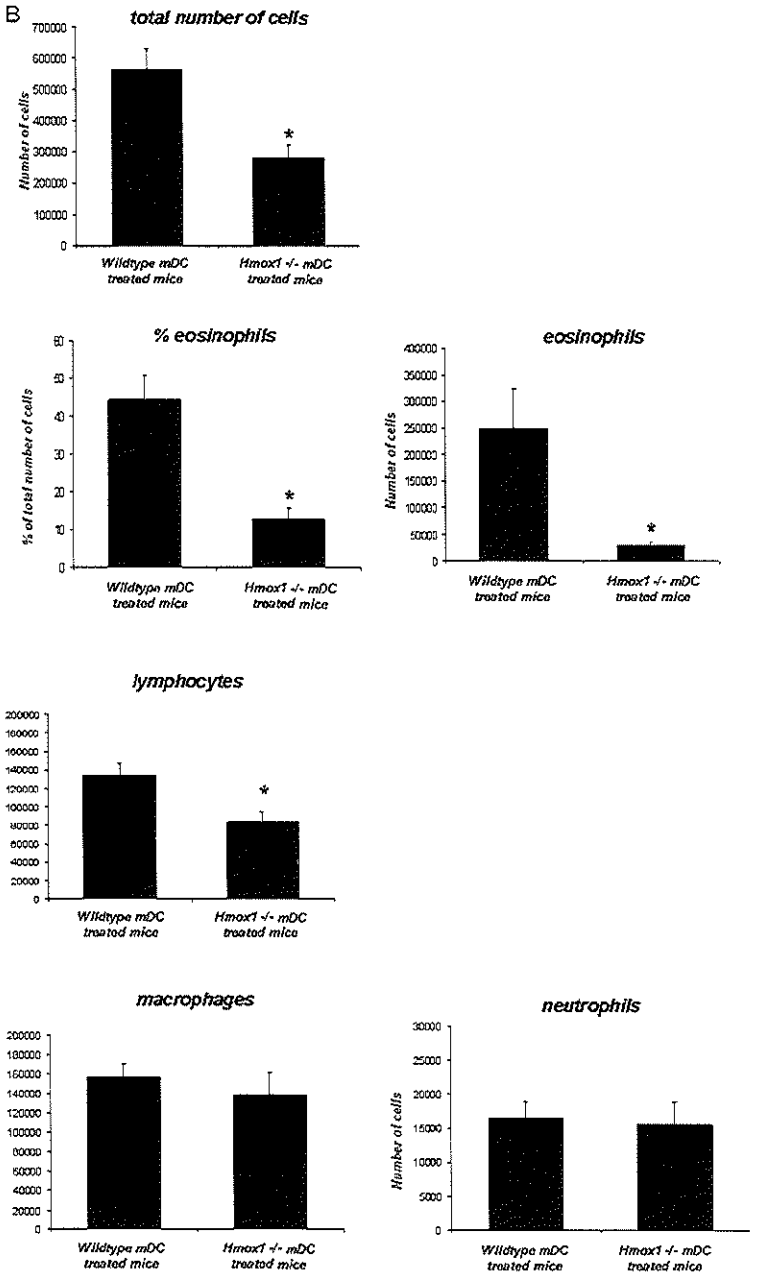

B
total number of cells
Number of cells
700000
600000
500000
400000
300000
200000
100000
0
*
Wildtype mDC treated mice
Hmox1 -/- mDC treated mice
% eosinophils
% of total number of cells
60
50
40
30
20
10
0
*
Wildtype mDC treated mice
Hmox1 -/- mDC treated mice
eosinophils
Number of cells
400000
350000
300000
250000
200000
150000
100000
50000
0
*
Wildtype mDC treated mice
Hmox1 -/- mDC treated mice
lymphocytes
Number of cells
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
*
Wildtype mDC treated mice
Hmox1 -/- mDC treated mice
macrophages
Number of cells
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Wildtype mDC treated mice
Hmox1 -/- mDC treated mice
neutrophils
Number of cells
30000
25000
20000
15000
10000
5000
0
Wildtype mDC treated mice
Hmox1 -/- mDC treated mice

Figure 5 *Cytokine profile of MLN derived T cells obtained from wildtype or HO-1 -/- mDC treated C57bl/6 mice. Cytokine levels of IL4, IL5, IL10, IL13, and INF-γ are shown. N=10 per group, *P<0.05 wildtype versus HO-1 -/- mDC as defined by the Student's t-test, mean ± sem.*

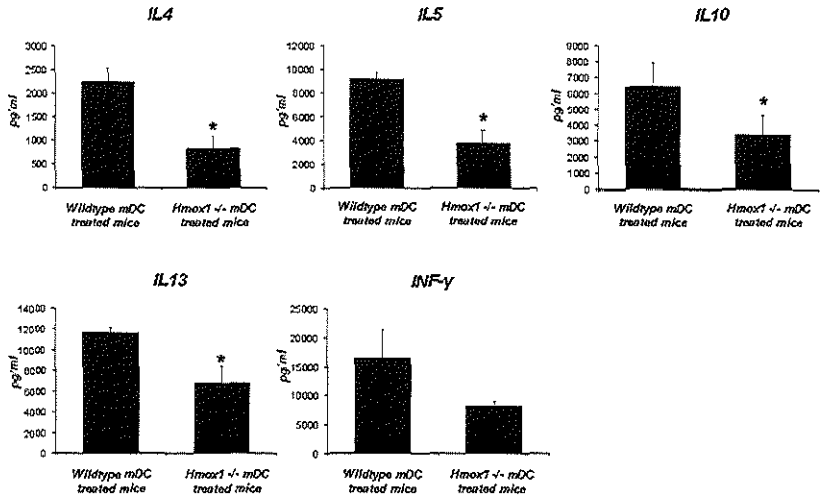
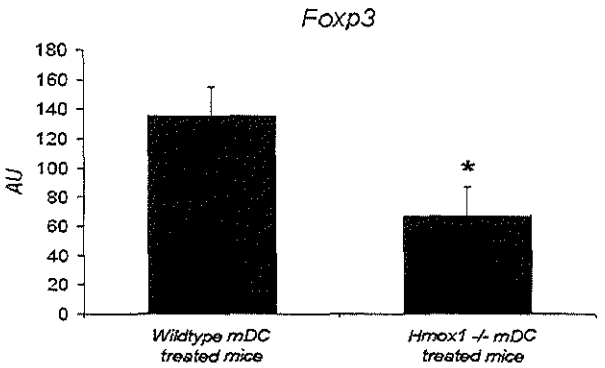


Figure 6 *Supplemented data Figure 1: Foxp3 mRNA expression in the lungs of wild type or HO-1.*

*-/- mDC treated C57bl/6 mice. N=10 per group, *P<0.05 wildtype versus HO-1 -/- mDC as defined by the Student's t-test, mean ± sem.*



DISCUSSION

The current study demonstrated that loss of HO-1 in mDC resulted in the inability of mDCs to elicit a CD4+ predominant T-cell response, and polarization towards a Th2 serological response was inhibited, as shown by MLR assays *in vitro* and in a passive transfer model of bronchial hyper reactivity *in vivo*.

HO-1 has been identified as a cytoprotective enzyme which function relies on the degradation of hemoproteins into its bioactive metabolites carbon monoxide, iron and biliverdin. Studies of graft-versus-host disease, and tolerance induction have suggested that HO-1 may impede the T-cell mediated adaptive immune response [18, 19, 20]. HO-1 deficient mice show a proinflammatory phenotype, characterized by splenomegaly, related to an increase in the total number of lymphocytes [21]. Furthermore, HO-1 was induced after activation of CD4+ T-cells by CD3 and CD28 signaling [22]. Also, HO-1 upregulation in activated CD4+ effector T-cells protected against Fas-mediated apoptosis [22, 23], but inhibited cell division by carbon monoxide mediated downregulation of the production of the mitogenic cytokine IL2 [24]. In addition, HO-1 has been implicated to play a role in the immuno suppressive function of CD25+/CD4+ regulatory T-cells (Treg cells). However, the exact contribution of HO-1 in the regulation of DC function and the subsequent differentiation and activity of T lymphocyte subsets is still debated. Systemic HO-1 induction by cobalt protoporphyrin (CoPP) suppressed the characteristic features of allergic airway inflammation in a murine model for asthma mediated by a Th2-type response, and correlated with an increased number of CD4+/CD25+ Treg cells [25]. *In vitro*, isolated Treg cells overexpressing HO-1 displayed increased expression of the specific Treg marker, Foxp3, and in addition, enhanced secretion of the Th2 cytokine IL10 [26]. In contrast, *in vivo* studies in wildtype and HO-1 deficient mice showed similar numbers of CD4+/CD25+ Treg cells with equal capacities to induce effector CD4+ T-cell proliferation *in vitro* [21]. Recently, George and coworkers have shown that loss of HO-1 in myeloid derived DCs abolished the suppressive capacity of Treg cells on effector CD4+ T cells [27], indicating that HO-1 function in antigen presenting dendritic cells may play an important role in the polarization of the general T-cell response towards Th1, Th2 or Treg differentiation.

Although initial HO-1 levels in immature DCs are high, HO-1 is downregulated during the maturation process. CoPP induction of HO-1 *in vitro* inhibited DC maturation characterized by attenuated cell surface expression of the maturation markers MHCII, CD80, and CD86 [28]. However, a more recent study suggested that CoPP may induce this refractory effect on DC maturation is dependent on STAT3 phosphorylation and independent of HO-1 activity [29]. In this study, mDCs derived from the BM of HO-1-/- mice enhanced MHCII cell surface expression in response to OVA/LPS stimulation compared to wildtype mDCs, but no significant difference in the expression levels of CD40, CD80, or CD86 could be observed. Knockdown of HO-1 by targeting siRNA in mDCs, also significantly upregulated MHCII levels in response to LPS stimulation compared to scrambled siRNA transfected control mDCs. Consistent to these findings, recent studies from Soares and co-workers have demonstrated a marked decrease in MHCII expression in mDCs exposed to CO, whereas the progression of experimental allergic encephalomyelitis was shown to be attenuated

by HO-1 expression and CO inhalation, both associated with dampened expression of MHC class II expression in DC. [30, 31] Both expression of MHCII and co-stimulatory molecules, as well as the cytokine environment define CD4+ T cell activation and differentiation. Here, we have been able to demonstrate that HO-1 deficient DCs had an impaired capacity to induce Th2-polarization in mixed lymphocyte reaction (MLR) assays *in vitro*, indicated by the reduction in the secretion of IL4, IL5, IL10, and IL13 characteristic for the cytokine profile of Th2 effector cells. Conversely, INF-γ production by effector T cells in response to priming by HO-1 deficient DCs seemed to be unaffected, indicative that the capacity to induce Th1 differentiation was sustained. This effect was validated *in vivo* in a well-defined murine model for asthma. Presensitization with HO-1 deficient mDCs dampened the Th2-dominant allergic response to the allergen ovalbumin (OVA), with failure to induce the histomorphological manifestation and immune activation characteristic to the model. The cytokine profile derived from mediastinal lymphnodes derived T-cells of HO-1-deficient mDC-treated animals also failed to express the associated cytokines involving IL4, IL5, IL10, and IL13 compared to wildtype mDC-treated animals. Together, these *in vitro* and *in vivo* data suggest that HO-1 in DCs is involved in the regulation of Th2-response, essential to various pathogenesis including asthma, transplantation atherosclerosis (chapter 4) and vulnerable plaque development (Chapter 3).

Previously systemic HO-1 induction by CoPP attenuated the induction of Th2 driven asthma in this murine model, and this was associated with increased levels of CD4+/CD25+ T-reg cells and FoxP3 [26]. Consistent with these findings, our current study showed that animals treated with HO-1 -/- mDCs had decreased levels of FoxP3 in the lungs, suggesting that CD4+/CD25+ T-reg cells presence was impeded.

In addition, Choi and co-workers demonstrated that Foxp3 is able to induce HO-1 expression whereas the expression of Foxp3 and HO-1 in peripheral $CD4^+CD25^+$ Treg and suppressive function of these Treg cells are attenuated by inhibition of HO-1 activity. Therefore, it seems that HO-1 deficiency in mDCs does not counteract the Th2 response in the current model of asthma via induction of T-reg cell subtype, but rather inhibits the disease process by directly regulating the onset of the Th2 dominant response.

In conclusion, this study provides evidence that HO-1 is a crucial factor in mDC priming of priming of naïve CD4+ T cells towards a Th2 response. Dysregulation of the adaptive immune response which results in the imbalance of the Th1/Th2 response is strongly associated with a wide range of immune disorders, including allergic asthma and EAE. The regulation of the Th1/Th2 cell response by mDCs is crucial in this process. The current new observations in our study could aid in the understanding of the pathophysiology of these Th2 dominant diseases including allergic asthma.

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6

HEME OXYGENASE-1 INHIBITS DIRECTIONAL MIGRATION OF VASCULAR SMOOTH MUSCLE CELLS

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ABSTRACT

Dysregulated growth and motility of vascular smooth muscle cells (VSMC) contribute to neointimal lesion development during the pathogenesis of vascular disease, including atherosclerosis and restenosis. Inhibition of this process is associated with reduced neointimal thickening in the setting of balloon angioplasty and chronic graft vessel disease. Previous studies have shown that overexpression of heme oxygenase-1 (HO-1) inhibits formation of neointima after vascular injury, an effect partly explained by inhibition of VSMC proliferation. However, the effects of HO-1 on the regulation of directed VSMC migration remain to be further elucidated. Directional cell migration requires the assembly of focal adhesions, a process which is orchestrated by focal adhesion kinase (FAK). In addition, directional cell migration is determined by cell polarity, regulated by both assembly and disassembly of microtubules (MT), and reorientation of the Microtubule Organization Centre (MTOC).

Previously, studies from our group showed that HO-1 overexpression inhibited cell cycle progression by $p21^{cip}$ upregulation. This $p21^{cip}$ was associated with inhibition of VSMC migration in a modified Boyden's chamber assay (Duckers, Tashiro, unpublished data). Here we explored the effect of HO-1 expression on VSMC migration and studied the effect of HO-1 on focal adhesion assembly and cell polarization as a possible molecular basis for these effects.

In the current studies migration of porcine VSMC was significantly impeded by adenoviral HO-1 overexpression in vitro in a standard scratch assay, in a modified Boyden chamber assay, and a barrier migration assay. Inhibition of cyclic GMP or the guanylate cyclase pathway led to the reversal of HO-1 induced anti-migratory effects. Overexpression of HO-1 in VSMC resulted in decreased FAK protein expression which was associated with increased Erk1 phosphorylation, but not with Erk2 phosphorylation. These effects could be reversed upon co-incubation with the HO-1 inhibitor ZnPPIX, but not with a NOS inhibitor, suggesting that the observed antimigratory effects were independent of nitric oxide synthase signaling. Immunofluoresence showed increased expression of focal contact sites concomitant to FAK downregulation in cells overexpressing HO-1, as compared to control VSMC. 24 hour time lapse imaging in a barrier assay showed a reduction of the absolute migration distance of HO-1 overexpressing VSMC vs. sham controls (absolute distance; 12.27μm ± 3.22μm vs. 13.60 μm ±3.01μm $p<0.05$; effective distance; 7.5 ±2.9μm vs 5.6 μm± 2.5μm. $p=0.11$).

Immunofluorescent staining of both acetylated and -tubulin showed an increase in stable microtubule formation and significant reduction in MTOC orientation and cell polarization in VSMC overexpressing HO-1 (38% vs. 65% $p< 0.05$).

This difference in cell polarity was accompanied by a significant increase in glycogen synthase kinase 3 beta (GSK3β) phosphorylation, which previously was associated with FAK assembly and MT-stabilization.

This study hypothesizes that HO-1 overexpression inhibits directional migration of VSMC by interfering with turnover of focal adhesions and MTOC reorientation by modulation of FAK protein expression.

INTRODUCTION

In cardiovascular disease, pathological neointima formation which occurs after vascular injury requires directional vascular smooth muscle cell (VSMC) migration from the media to the intima[1, 2]. Dysregulated growth and motility of VSMCs contributes to neointimal lesion development during the pathogenesis of vascular obstructive disease, including atherosclerosis, restenosis, and bypass graft stenosis [1,2]. Inhibition of VSMC migration was associated with reduced neointimal thickening in animal models of balloon angioplasty and chronic graft vessel disease[3, 4]. Although intracellular changes underlying directional cell migration are studied extensively [5-8], the exact molecular signaling pathways in VSMC migration still remain to be elucidated. Previous studies have shown that directed cellular migration requires co-localization of the golgi apparatus with the microtubule organizing center (MTOC) [9]. The exact mechanism and cellular signaling cascade ultimately leading to cellular polarization appears to be complex, and multiple signaling cascades were proposed to contribute to this process.

A study from Gomes and co-workers suggested that MTOC reorientation occurred by a major rearward movement of the nucleus, while the MTOC remained immobile. Nuclear movement was in this study was found to be driven by actin retrograde flow and was myosin II dependent. Cdc42 was found to mediate both this nuclear movement and myosin phosphorylation. Myotonic dystrophy kinase-related Cdc42 binding kinase (MRCK) was identified as a downstream Cdc42 effector [10] that stimulates myosin phosphorylation and activation of rearward nuclear movement. In addition a previous report suggested that FAK phosphorylation may be important in the regulation of the centrosome-associated microtubule structure to promote nuclear translocation by prevention of the accumulation of FAK at the centrosome [11]. Evidence has been accumulated to show that glycogen synthase kinase 3 (GSK-3) is involved in the regulation of microtubule turnover and stabilization. Results from recent studies suggested that GSK-3beta may function in transporting centrosomal proteins to the MTOC resulting in the regulation of a focused/polarized microtubule organization [12, 13].

The heme oxygenase system catalyzes heme degradation into its metabolites biliverdin (BV) bilirubin (BR), iron and carbon monoxide (CO) [14]. The generated CO is thought to function as a second messenger in various signal transduction pathways. CO activates intracellular signaling pathways that involve activation of soluble guanylate cyclase and generation of cyclic GMP, as well as p38 mitogen-activated protein kinase activation and subsequent downstream phosphorylation of several rho GTPases including CDC42 [15]. Previous studies have shown that HO-1 inhibits VSMC proliferation by causing a cell cycle arrest at the G1/S phase [16]. However the effects of HO-1 on the regulation of directed VSMC migration are still poorly understood.

To define the biological function of HO-1 in VSMC migration, we assessed the effect of HO-1 overexpression on directional VSMC migration, mediated by focal adhesion assembly and MTOC polarization. The current study suggests that HO-1 inhibits directional migration of VSMC by interfering with turnover of focal adhesion complexes through modulation of FAK expression and GSK-3ß phosphorlation. Insight in the mechanism that drives VSMC migration in vascular proliferative disease including atherosclerosis and restenosis could contribute to the development of useful therapeutic remedies.

RESULTS

In order to assess the effects of HO-1 expression on the migration of VSMC HO-1 overexpression was induced by adenoviral transfection in these different in vitro migration assays.

HO-1 inhibited VSMC migration in vitro both in a modified Boyden's Cell chamber assay and in a standard scratch assay under PDGF and FCS chemotactic stimulation (Scratch assay figure 1a, b,c, : 10% FCS: control; 3.05µM +/- 0.20µM, E1A; 2.8 µM +/- 0.33 µM, AdHO-1;0.875 µM +/-0.02 µM vs. 20% FCS: control; 3.8µM +/- 0.08µM, E1A; 3.47 µM +/- 0.11 µM, AdHO-1; 1.6µM +/-0.08 µM vs. PDGF10 ng/ml : control; 3.7µM +/- 0.33µM, E1A; 3.65µM +/- 0.12 µM, AdHO-1; 0.1.40 µM +/-0.08 µM and modified Boyden chamber assay (figure 1g: %change to normal; E1A 100%+/-9.8%,HO-1 56.9%+/-4.8%). These anti-migratory effects were attenuated by a specific inhibitor of sGC (fig1g; HO-1/ODQ 61%+/- 6.8%) but not by NOS inhibition (figure 1g; HO-1/LNAME 43.05% +/- 0.8), which indicates that the effect of HO-1 on VSMC migration is mediated by sGC activation independent of NOS stimulation.

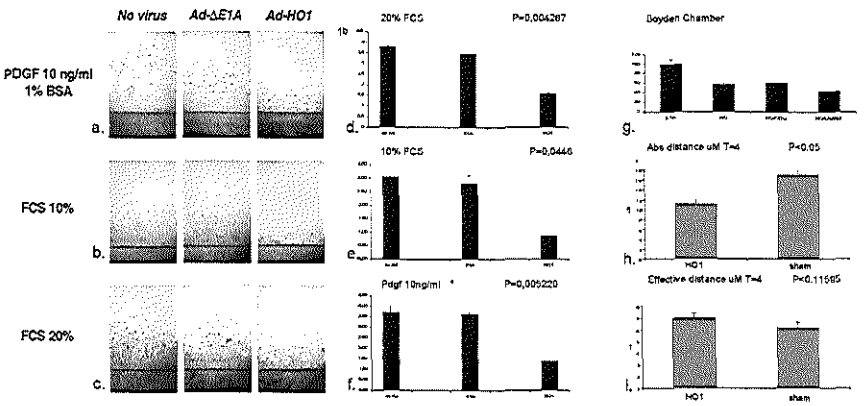

Figure 1 *In vitro primary VSMC migration assays.*

HO-1 inhibited VSMC migration in vitro both in a modified Boyden's Cell chamber assay and in a standard scratch assa under PDGF and FCS chemotactic stimulation (Scratch assay figure 1a, b ,c, : 10% FCS: control; 3.05µM +/- 0.20µM, E1A; 2.8 µM +/- 0.33 µM, AdHO-1;0.875 µM +/-0.02 µM vs. 20% FCS: control; 3.8µM +/- 0.08µM, E1A; 3.47 µM +/- 0.11 µM, AdHO-1; 1.6µM +/-0.08 µM vs. PDGF10 ng/ml : control; 3.7µM +/- 0.33µM, E1A; 3.65µM +/- 0.12 µM, AdHO-1; 0.1.40 µM +/-0.08 µM and Boyden chamber figure 1g: %change to normal; E1A 100%+/-9.8%, HO-1 56.9%+/-4.8%).These anti-migratory effects were attenuated by specific inhibitor of sGC (1g; HO-1/ODQ 61%+/- 6.8%) but not by NOS inhibition (1g; HO-1/LNAME 43.05% +/- 0.8).24 hour time lapse imaging in a barrier migration assay showed significant reduction in absolute migratory distance in VSMC overexpressing HO-1 vs. sham controls (figure 1h,i: Absolute distance; 12.27µm ± 3.22µm vs.13.60 µm ±3.01µm p<0.05; Effective distance; 7.5 ±2.9µm vs 5.6 µm± 2.5µm. p=0.11).

Subsequently, we aimed to evaluate the effect of HO-1 expression on VSMC migration using time-lapse imaging. HO-1 overexpression in VSMC impeded the absolute migration distance (defined as the absolute, non-lineair distance in µm that each individual cell migrated from the starting position over 4 hours as analyzed by computer assisted cell tracking) compared to sham virus transfected cells. No difference was observed in effective migration distance.

(Defined as the lineair distance between the end and starting point after 4 hours migration analyzed with computer-assisted cell tracking) (figure 1h,i: absolute distance; 12.27µm ± 3.22µm vs.13.60 µm ±3.01µm $p<0.05$; effective distance; 7.5 ±2.9µm vs 5.6 µm± 2.5µm. $P=0.11$).

HO-1 adenoviral overexpression enhanced cGMP levels in VSMC in vitro as compared to control VSMC. Co-incubation with a NOS inhibitor L-NAME (1µM lane 3) or cGMP-inhibitor; Rp8Br-GMPs (30µM lane 5) did not reverse cGMP levels, whereas inhibition of HO-1 (ZNPPIX 20µM lane 2) and sGC (ODQ, 3µM; Lane 4) reduced cGMP to control levels.(figure 2: %change to control level: Ad-HO1 228+/-26, +ZnPPIX 113+/-5, +L-NAME 293+/-53, ODQ110+/-6, Rp8Br-GMP 218+/-18).

The decrease in migration in VSMC overexpressing HO-1 was associated with a prominent reduction in focal adhesion kinase pp125 (FAKpp125) protein expression (figure 3a.) Loss of FAK protein expression closely coincided with HO-1 protein expression in a close time dependent manner, suggesting a direct interaction between HO-1 and FAK (figure 3a,b). In addition, HO-1 overexpression was associated with Erk1 MAPK dephosphorylation, while total Erk1 and Erk2 protein levels remained unaffected (figure 4b,e). No differences in SRC phosphorylation were observed.

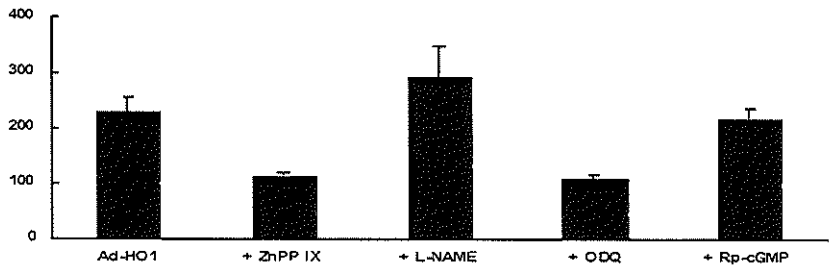

Figure 2 *Steady state cGMP levels adenoviral infected VSMC.*

HO-1 adenoviral expression enhanced steady state cGMP levels in VSMC in vitro compared with control VSMC. Inhibition of NOS by L-NAME (1µM lane 3) or inhibition of cGMP(RP8Br-GMP 30µM lane 5) did not affect cGMP levels, whereas inhibition of HO-1 (ZnPPIX 20µM lane 2) and sGC(ODQ, 3µM; Lane 4) reduced cGMP levels to baseline values (%change to control level: Ad-HO1 228+/-26, +ZnPPIX 113+/-5, +L-NAME 293+/-53, ODQ110+/-6, Rp8Br-GMP 218+/-18)

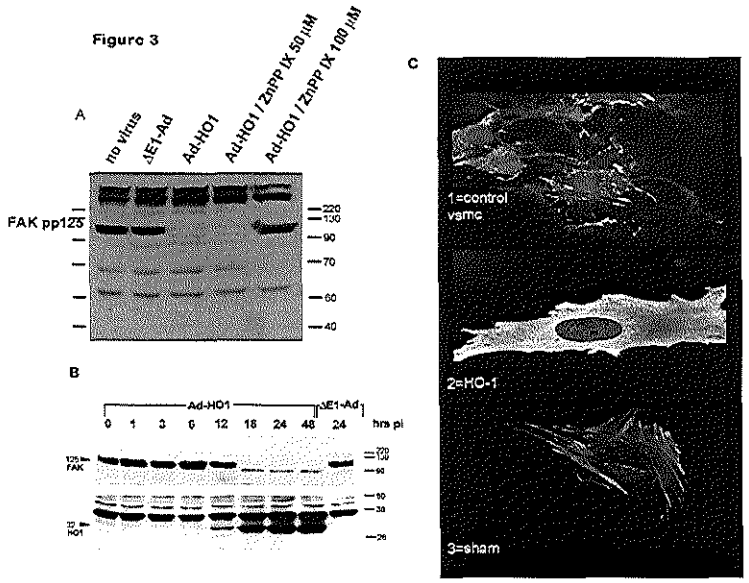

Figure 3 *FAK (pp125) protein expression in HO-1 expressing cells.*

HO-1 was associated with a decrease in FAKpp125 expression (figure 3a) Effects of HO-1 protein expression associated with FAK protein expression in a time dependent manner suggesting a direct relation. (figure 3a, b) Confocal microscopy of vinculin (FITC), phalloidin (TRITC) and the nucleus (DAPI) in (1) control VSMC (2), HO1 transfected VSMC (MOI 1000) and (3) after co-incubation with ZnPPIX.

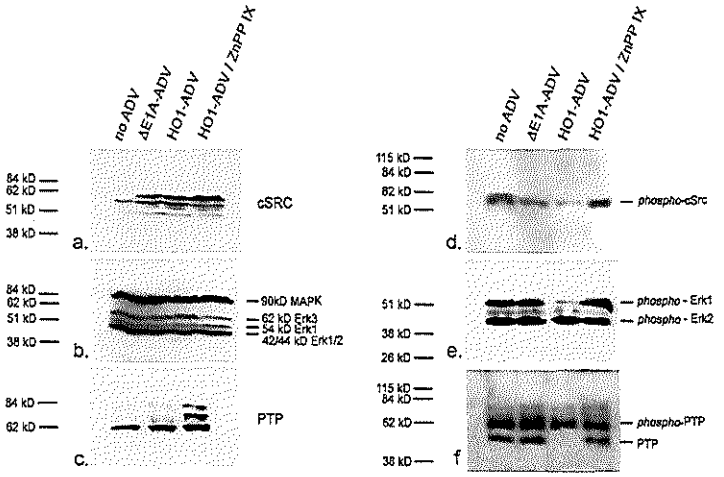

Figure 4. *Western blot showing that HO-1 overexpression was associated with Erk1 MAPK dephosphorylation, but not Erk2. These effects could be reversed upon co- incubation with the HO-1 inhibitor ZNPPIX. No differences in SRC or PtP protein expression were observed.*

HO-1 overexpression in VSMCs increased focal adhesion formation, indicated by confocal microscopical imaging of VSMC stained for vinculin and phalloidin which suggests decreased turnover of focal contact-sites by downregulated FAK (figure 3c). Cellular polarity of VSMC was assessed in the barrier ring assay. Immunofluorescent staining of acetylated and -tubulin showed an increase in stable MT formation (preliminary data not shown) and significant reduction in MTOC polarization directed towards the barrier area in VSMC overexpressing HO-1, two hours after removal of the barrier VSMC (38% vs. 65% p< 0.05).(figure 5a,) This reduction in cell polarity was accompanied by significant increase in GSK3β phosphorylation as determined by Western blot analysis (ratio GSK3βP/total GSK; 0.69 IOD vs. 0.49 IOD; HO-1 vs. sham) as well as with an increase in MRCK mRNA expression in HO-1 overexpressing VSMC compared to controls (Figure 6 supplemental data).

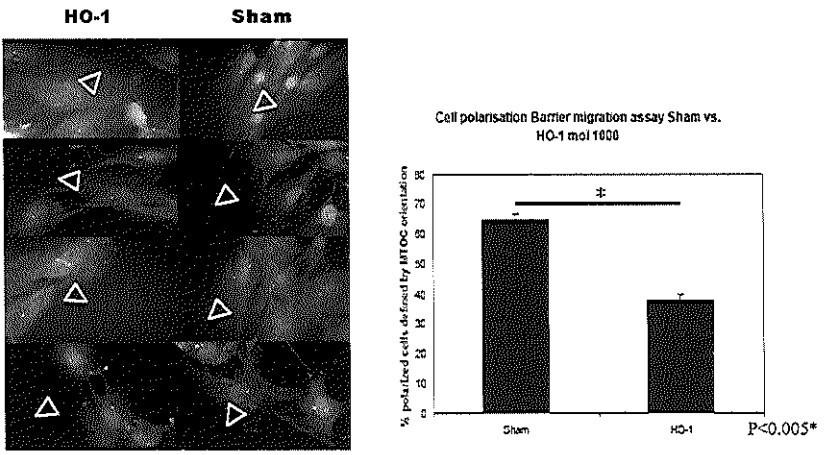

Figure 5 *MTOC orientation in HO-1 overexpressing cells.*

Figure 5a: VSMC transfected with sham (right panel) or HO-1 adenovirus (left panel) 24 hours post transfection and subsequently after migration for 2 hours in barrier ring setup. Cells were fixed and stained for -tubulin to visualize centrosome position. Arrows indicate direction of migration towards the cell free area after the barrier has been removed prior to migration. ***Figure 5b:*** *Analysis of centrosome position from 4 separate experiments of each 12 wells indicating significantly more polarized cells in sham transfected group vs. HO-1 transfected pVSMC two hours after migration.*

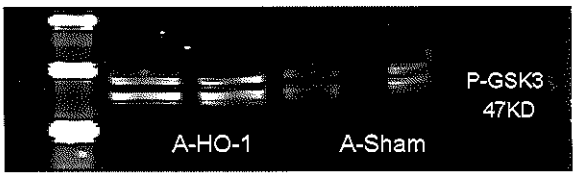

Figure 6 *Effects of HO-1 overexpression on GSK-3 protein phosphorylation.*

HO-1 overexpression leads to increased GSK-3β protein phosphorylation as quantified with Odyssey/Liquor western blot imaging. Ratio GSK3βP/total GSK; 0.69 IOD vs. 0.49 IOD; HO-1 vs. Sham

DISCUSSION

The heme oxygenase system is responsible for the initial step of heme degradation into its metabolites biliverdin, iron and carbon monoxide (CO) [14]. The generated CO, that may function as a gaseous signal transduction messenger, has been demonstrated to share many properties with NO, including signal transduction via activation of guanylyl cyclase [15]. Most of the effects of CO signaling are indeed mediated by the stimulation of the CO-sensitive GC (guanylate cyclase) and the subsequent increase in cyclic guanosine monophophate (cGMP) formation.

Previous studies have shown that CO generated by HO-1 activity triggers cytoprotective signaling pathways rendering tissue specific anti-proliferative, anti-apoptotic, and anti-migratory effects [18, 19].

Moreover, in previous studies it was found that HO-1 inhibited VSMC proliferation by upregulation of $p21^{CIP}$ [16] and induced G1/G0 cell cycle arrest in the context of vascular injury[16] concomitant to an anti migratory effect.

However the effects of HO-1 solely on the directed migration of VSMC remain mainly unclear.

In the current study, HO-1 inhibited VSMC migration in vitro in different migration assays. These effects were diminished by specific inhibition of HO-1 (ZnPPIX) or guanylyl cyclase, but not by NOS inhibition, suggesting that these anti migratory effects occurred independently of NO and were mediated by a cGMP-dependent pathway.

Interestingly, these anti-migratory effects of HO-1 overexpression were associated with a decrease in focal adhesion kinase pp125 (FAKpp125) protein expression, and activation of the Erk1/MAPK pathway and GSK-3ß phosphorylation. HO-1 expression on VSMC was in addition associated with attenuation of cell polarity and MTOC reorientation as compared to sham transfected controls.

This cyclical regulation of focal complex formation and disassembly is critical in the control of cell movement. Several cytoplasmic protein tyrosine kinases, and phosphatases, including Src,PTP, and focal adhesion kinase (FAK), are present in focal complexes [20]. FAK phosphorylates focal adhesion components which leads to focal adhesion disassembly and turnover [21]. The heme oxygenase/ carbon monoxide pathway may alter FAK expression and phosphorylation by cGMP generation.

The relationship between cGMP and FAK already has been suggested in previous work. In rat pancreatic acini, exogenous NO increased tyrosine phosphorylation of pp125FAK which was dependent on cGMP activation [22]. Activation of cyclic Guanylyl Kinase1 (cGK I) by increased levels of cGMP led to inhibition of HUVEC migration through the modulation of focal adhesion assembly. Indeed cGMP-cGK I phosphorylation resulted in loss of the structural components VASP and zyxin from focal adhesions, which led to focal adhesion disassembly [22].

Though cGMP induced by CO may modify FAK pp125 phosphorylation, in our studies heme oxygenase-1 expression led to downregulation of FAK protein expression rather than dephosphorylation. Possibly, supra-physiological HO-1 levels following adenoviral transfection may induce a negative feedback loop between the generated CO and cGMP signaling.

A similar feedback loop was described previously for NO-induced cGMP signaling. For NO-induced cGMP signaling not only depends on cGMP formation, but is also critically determined by the activity of cGMP degradation enzyme phosphodiesterase 5 (PDE5)[23]. Likewise sustained HO-1 overexpression may induce both significant cGMP production and PDE5 activation, which may lead to desensitization within CO/cGMP signaling and alterations in FAK phosphorylation,as observed in our assay.

In addition a tendency in HO1 overexpressing VSMC to loose orientation of MTOC may also account for an attenuated directional migration capacity due to loss of polarization.

MTOC reorientation occurs by a relative movement of the nucleus away from the leading edge of the migrating cell. This complex process of nuclear movement is initiated by myotonic dystrophy kinase-related Cdc42 binding kinase (MRCK) regulated actin-myosin retrograde flow that moves the nucleus in the direction of the leading edge of the cell and par6-PKCζ regulated dynein centration which prevents the MTOC from being swept rearward with the nucleus [24]. In a number of cell types, microtubules (MT) have been shown to selectively stabilize in the migration direction which facilitates polarization of the cell.

The kinase glycogen synthase kinase-3 (GSK-3ß) was first described in a glycogen metabolic pathway [25].Evidence accumulated to suggest the involvement of GSK-3ß in both MTOC orientation, FAK signaling and the regulation of microtubule stabilization during directional migration[26-28].

These findings challenged us to explore whether the observed alterations in FAK phosphorylation and MTOC reorientation after HO-1 overexpression related to GSK-3ß. Previous work indicated a mechanism for regulation of FAK activity during cell spreading and migration, modulated through the competing actions of GSK3ß [29]. Furthermore, reduction of GSK-3ß by RNAi impaired the disassembly of paxillin from focal adhesions [30-31].The phosphorylation of proteins by GSK-3ß at focal adhesions may thus be required to mediate dissembly and assembly.The substrates of GSK-3ß that regulate FAK and MT activity remain to be identified further.

This study demonstrates for the first time that HO-1 in VSMC inhibits directional migration and we propose a signaling cascade in which CO interferes with FAKpp125 expression and phosphorylation by a cGMP dependent signaling pathway. FAK downregulation results in diminished focal adhesion turnover and increased cellular attachment.

Moreover,an increase in GSK-3ß phosphorylation and MRCK expression following HO-1 upregulation may lead to microtubule stabilization, and altered patterns of MTOC orientation. These findings provide insight in the biology of HO-1/CO signaling in vascular disease and may provide new strategies to prevent the formation of neointima in the context of vascular injury.

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7

EFFECT OF HO-1 EXPRESSION ON ANGIOGENESIS AND PLAQUE NEOVASCULARIZATION

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ABSTRACT

Hemoproteins are catabolized by the heme oxygenase family (HO) to yield biliverdin, iron and carbon monoxide (CO). These metabolites have been recently suggested to function as second messengers in multiple signal transduction pathways by modulating redox signaling, heavy-metal-response driven gene transcription or by generating cGMP. Recent reports have shown that HO-1 directly protects endothelial cells from apoptosis, and is involved in blood-vessel relaxation regulating vascular tone,and attenuation of the inflammatory response in the vessel wall.Recent studies have suggested that HO-1 may facilitate neoangiogeneis in HUVEC primary cell culture, however, the exact role of HO-1 in angiogenesis and vasculogenesis remains to be elucidated. Recently we were able to perform a genome-wide microarray analysis to specify the genetic regulation of vasculogenesis by angioblasts during murine embryonic development. Among the candidate genes that may be involved in embryonic vasculogenesis was heme oxygenase-1.To extend our studies to define the biological function of heme oxygenase-1 in adult vessel formation and atherosclerotic plaque neovascularization HO-1 transgene expression was studied in in vitro assay for angiogenic sprouting and *in vivo* in a murine model for vulnerable atherosclerotic plaque formation.

HO-1 promoted angiogenic sprouting and tube formation of HUVEC in an in vitro matrigel assay whereas systemic induction of HO-1 by cobalt-protoporphyrin (CoPPIX) in a murine atherosclerosis model promoted neovascularization in atherosclerotic plaques, suggestive of plaque destabilization.

We hypothesize that HO-1 facilitates noninflammatory angiogenesis, as well as in embryonic vessel development as well as in vessel formation in atherosclerotic plaques.

INTRODUCTION

Neovascularization is the generation of new blood vessels, either by vasculogenesis (defined as de novo vessel synthesis from progenitor cells) or angiogenesis (defined as new vessel formation from sprouting of pre-existing vasculature). This process is required for embryonic development and plays an important role in the pathogenesis of a variety of adult human diseases[1] including cancer and rheumatoid arthritis, and diabetes mellitus[2]. In atherosclerosis, the role of neovascularization remains controversial and incompletely understood. Atherosclerosis is regarded as an inflammatory disease of the arterial wall in response to intra-vessel lipid deposition. Angiogenesis is a complex multi-step process, where, in response to angiogenic stimuli, new vessels are created from the existing vasculature. These steps include: degradation of the basement membrane, proliferation and migration (sprouting) of endothelial cells (EC) into the extracellular matrix, alignment of EC into cords, branching, lumen formation, anastomosis, and formation of a new basement membrane. Intra-lesional neovascularization is observed in advanced atherosclerotic lesions exceeding a certain thickness in humans and in animal models including the murine ApoE-/- model[3].

Growing evidence suggested an association between intra-lesional angiogenesis with atherosclerosis progression and plaque destabilization [4, 5].

In large mammalian arteries, the main function of the microvasculature in the adventitial layer (vasa vasorum) is to supply nutrients to the vessel wall. Due to the increase in intimal area during atherosclerosis progression, limitations to the diffusion efficiency from both the luminal and adventitial side create hypoxic zones in the atherosclerotic lesion.

A genome-wide microarray analysis for the genetic expression pattern in angioblasts during embryonic vessel development previously identified potential genetic regulators of vasculogenesis. The specific vascular expression and functionality of these genes were validated by gene knockdown studies (morpholino injections) and whole mount in situ hybridization in the zebrafishmodel. Among the candidate genes identified in this screen was heme oxygenase-1 (HO-1) [6]

The heme oxygenase family consists of three HO-1 isoenzymes of which HO-1 and HO-2 are catalysts of heme protein degradation. The generated metabolites from heme degradation; carbon monoxide (CO) iron and biliverdin gained interest over the past years because of their potential role in the cellular stress defense, as important antioxidant, anti apoptotic and anti-inflammatory mediators. HO-1 in the vascular bed was shown to protect endothelial cells from apoptosis, to regulate vasomotor tone [7], and attenuate the local inflammatory response in the vascular wall. Preliminary data suggest that HO-1 may facilitate blood vessel formation by angiogenesis [8]. The latter function links HO-1 expression inevitably to cardiovascular ischemia but also to many other conditions that are dependent on neovascularization, including the vascular repair response, embryological development, metastasis and solid tumor formation.

HO-1 is indeed induced rapidly during hypoxia, ischemia/reperfusion,hyperthermia and during inflammation [9].The importance of this is demonstrated by the severe endothelial damage observed in human HO-1 deficiency [10] and in gene targeted mice deficient of HO-1 [11]. In addition to its role in vascular cytoprotection during ischemia and inflammation, Preliminary data suggest that HO-1 may play a role in angiogenesis as expression of HO-1 increases endothelial VEGF synthesis and endothelial cell proliferation and formation of capillary like structures.

We aimed at understanding the role of HO-1 in the generation of new blood vessels in a well-defined angiogenesis tube formation assay in vitro and *in vivo* to assess neovascularization within atherosclerotic vulnerable lesions.

MATERIALS AND METHODS

Cell culture and tube formation assay

HUVEC's (ATCC) were cultured for 24 hours in fresh EGM-2 (Clonetics, Lonza Walkersville, USA) on 0.1% gelatin coated 10 cm Nunc polyethylene cell culture dishes before transfection.

Ad-HO-1 and control Ad-ΔE1A vectors were constructed by homologous recombination in 293 cells as earlier described (Duckers et.al. Nature, 2001) purified and viral titer was determined by optical densimetry. Cells were infected by adenoviral vectors for 2 hours under low serum conditions, incubated for another 22 hours after serum suppletion and detached from their matrix with Accutase (Ebioscience) and harvested.

siRNA for HO-1 and sham controls were obtained from Dharmacon RNAi technologies (Lafayette, CO, USA) and used according to manufacturers instructions. HO-1 siRNA mix contained:

sense,5'-UGCUGAGUUCAUGAGGAACUU3';

antisense, 5'GUUCCUCAUGAACUCAGCAUU-3'

and sense, 5'CAUUGCCAGUGCCACCAAGUU-3';

antisense, 5'CUUGGUGGCACUGGCAAUGUU-3'

Corresponding negative control sequences were also purchased from Dharmacon RNAi technologies (Lafayette, CO, USA). HUVECs were plated at 4×10^5 cells per well in six-well plates in Endothelial Cell Basal Medium (EBM2) (Cambrex BioScience, Wokingham, UK) without antibiotics and were cultured to obtain 50% confluency. Test and control siRNA (10–50 nM) was transfected into HUVECs using lipid–siRNA complex-based transfection.

ECs were cultured for 48 h in EBM2 and analyzed for target gene expression by quantitative real-time PCR

After transfection cells were counted and transferred to the Biocoat Angiogenesis System tube formation assay according to manufacturer's instructions in a seeding density 4×10^5 cells/ml.

Cells in the tube formation assay were closely monitored at different time points and were photographed after staining with Calceine Am® to increase tube resolution for imaging with fluorescent microscope (Zeiss)

In vivo vulnerable plaque model:

All experiments were performed in compliance with institutional (Erasmus University Medical Center, Rotterdam, The Netherlands) and national guidelines.

ApoE–/– mice on a C57BL/6J background (age 12-15 weeks) were obtained from Jackson Laboratory (Bar Harbor, USA). The protocol for this animal study is described in figure 1. (A) Two weeks before surgery, all animals were placed on a Western type diet containing 15% (w/v) cacoa butter and

0,25% (w/v) cholesterol (diet W, Hope Farms, The Netherlands). (B) Two weeks later, animals were anaesthetized by isoflurane inhalation, and the right common carotid artery was dissected from circumferential connective tissues. The cast was placed around the right common carotid artery, after which the wounds were closed with vicryl sutures and the animals were allowed to recover. (C) Six weeks after cast placement, animals were randomized to either treatment with phosphate buffered saline (Cambrex, UK) (N=10) or cobalt protoporhyrin (CoPPIX, Frontier Scientific Inc., Canada) treatment (N=10) in order to induce endogenous Hmox-1 (ip injection, 5mg/kg q2d, in 0,2M NaOH, ph 7, 4). After 21 days of either PBS or CoPPIX treatment, animals were scarified and carotid arteries were harvested and processed for histological analysis.

Tissue preparation and histological analysis

Carotid arteries were embedded in OCT (Sakura Finetek, The Netherlands) and snap-frozen in liquid nitrogen. Quantification of histological data was performed on 7 µm cryosections, at 140 µm intervals. In order to evaluate the effect of HO-1 induction on neovascularization driven atherosclerotic lesion growth sections were stained with an antibody against PECAM; CD31+ conjugated to Cy-5 (Biomol) to assess vascular ingrowth in the plaque. The percentage of neovascularisation in the atherosclerotic area was calculated as the surface area positive for CD31 expressed as a percentage of the total intimal surface area. Statistical analysis was performed using student's T-test. Data are presented as mean ± SEM. P values less than 0, 05 were considered to be significant.

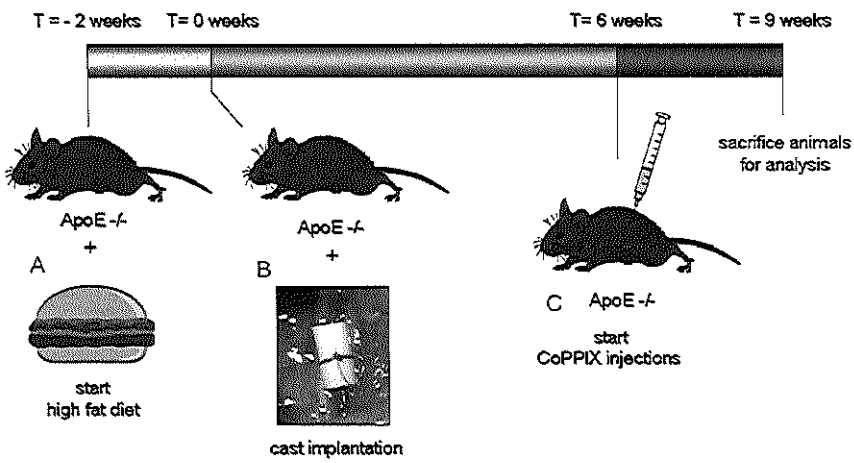
RESULTS

The effect of HO-1 adenoviral overexpression on the formation of angiogenic sprouts, the first stage in angiogenesis after basement membrane degradation was assessed in vitro in a 2- dimensional matrigel.

7 hours after onset of the tube formation assay HUVEC's over expressing HO-1 initially had formed more sprouts compared to HUVEC's transfected with sham vector. However, the shape and thickness of the sprouts formed by the HUVEC expressing HO-1 were slightly deteriorated, sprouts were thinner and there seem to have formed an increased number of side branches in the assay with the HO-1 transfected HUVEC's compared to the sham controls. In contrast sprouts formed by the HUVEC's in the sham transfected group tend to have a more dense shape.

In the left panel of figure 2 (a) four representative examples of each group is depicted at 7 hours after onset of incubation. The right panel (2b) shows the network of angiogenic sprouts at 23 hours after seeding of the HUVEC. The neocapillary network of HO-1 expressing cells contained gaps whereas sprouts are thin and irregularly shaped. The sham transfected HUVEC tend to form a more dense type of sprouts. In figure 2c the preliminary results from the effect of silencing HO-1 gene expression with siRNA are shown. Silencing of HO-1 in HUVEC resulted in a decrease in sprout formation at 24 hours following initiation of the 2D matrigel culture compared to empty-particle siRNA transfected control HUVEC.

Figure 1



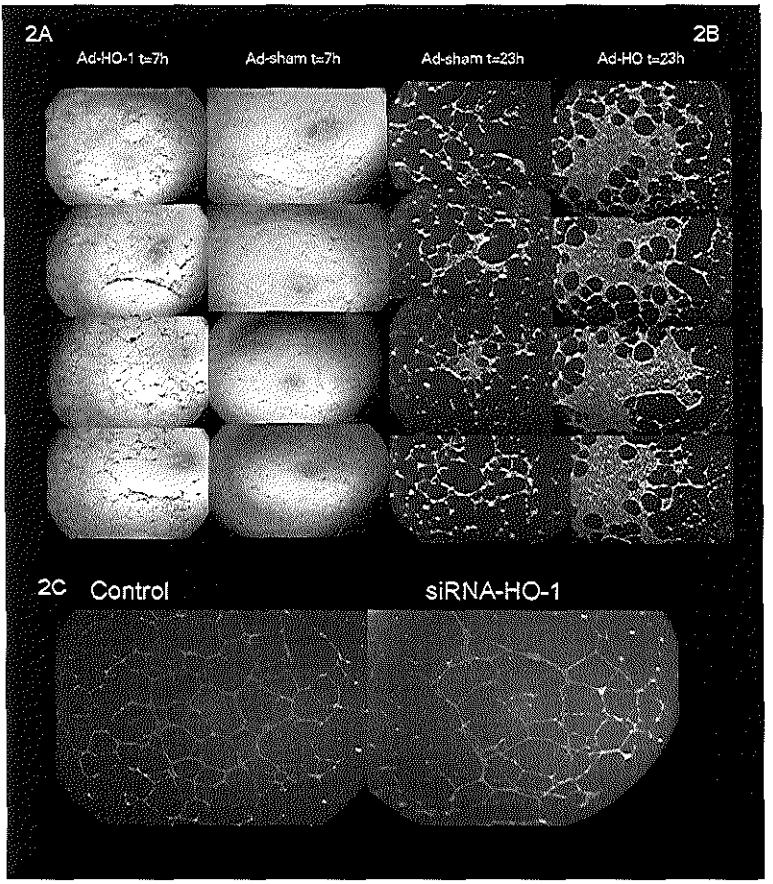


Figure 2 *Four representative examples of each group is depicted at 7 hours after onset of incubation. The right panel (2b) shows the network of angiogenic sprouts at 23 hours after seeding of the HUVEC figure 2c shows preliminary results from the effect of silencing HO-1 gene expression with siRNA are. Silencing of HO-1 in HUVEC resulted in a decrease in sprout formation at 24 hours following initiation of the 2D matrigel culture compared to empty-particle siRNA transfected control HUVEC.*

The brigth field microscopical analysis of HO-1 overexpression or silencing on lumen formation of HUVEC are shown in figure 3.

Eventhough it needs to be addressed that further experiments are necessary to draw valid conclusions these preliminary data suggest that loss of HO-1 gene expression in HUVECs did not affect lumen formation as compared to untransfected HUVEC.

Next, we assessed the effects of HO-1 gene expression on HUVEC to migration in vitro in matrigel. Figure 4 shows that at 4 hours after the incubation of an in vitro transwell assay overexpression of HO-1 significantly inhibits migration of HUVEC compared to sham transfected HUVEC and control. Future experiments will have to be carried out in different animal models for angiogenesis to further specify the effect of HO-1 in angiogenesis formation *in vivo*.

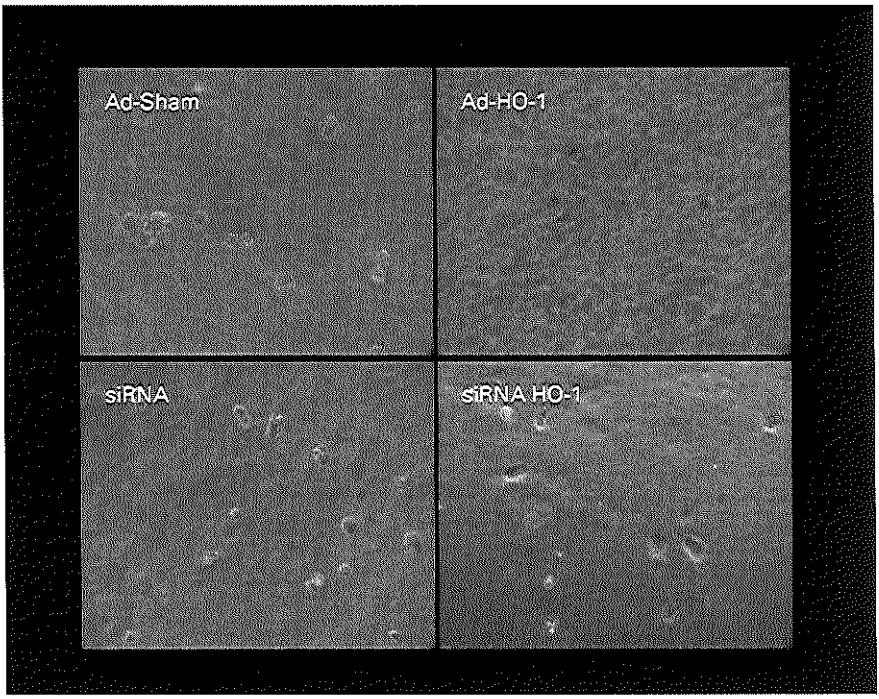


Figure 3 *Analysis of HO-1 overexpression or silencing on lumen formation of HUVEC are shown.loss of HO-1 gene expression in HUVECs did not affect lumen formation as compared to untransfected HUVEC. (preliminary data).*

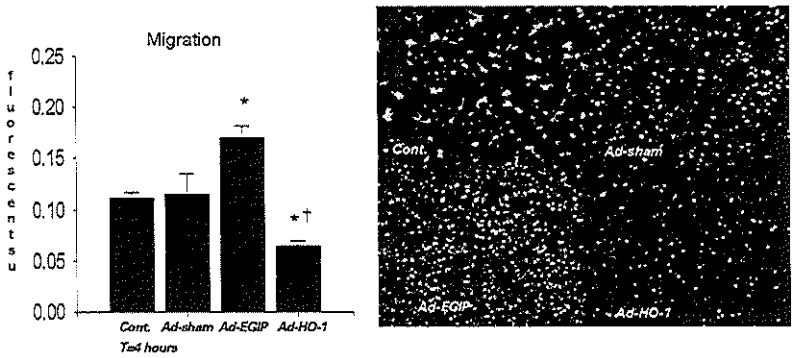


Figure 4 *4 hours after the incubation of an in vitro transwell assay overexpression of HO-1 significantly inhibits migration of HUVEC compared to sham transfected HUVEC and control.*

Finally, the effect of HO-1 gene expression was assessed on atherosclerotic plaque (de)stabilization and intraplaque neovascularisation (Figure 5). In a previous study of human carotid atherectomy material, we showed that high protein levels of HO-1 strongly correlated to a vulnerable atherosclerotic plaque phenotype. High levels of HO-1 were found in lesions with high content of lipids and macrophages, and low abundance of collagen and VSMCs. In a well defined *in vivo* model for vulnerable plaque development, systemic induction of HO-1 by cobalt-protoporphyrin (CoPPIX) significantly promoted neovascularization in the vulnerable plaque although no plaque rupture was observed.

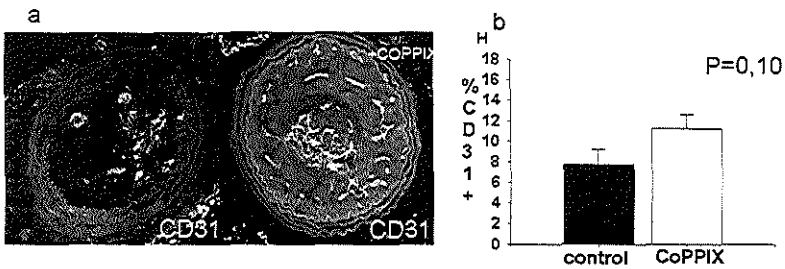


Figure 5 *Systemic induction of HO-1 by cobalt-protoporphyrin (CoPPix) tend to increase neovascularization as indicated by increased CD31 staining (5a) in the vulnerable plaque. (Preliminary data P=0.10*

DISCUSSION

In this study gain-of-function and loss-of-function modifications of HO-1 have been used, including siRNA-targetting Hmox-1 and recombinant viral vector mediated HO-1 overexpression, to develop understanding of the role of HO-1 in angiogenesis in vitro and the effect on intraplaque neovascularization *in vivo*. It should be emphasized that the results presented here are unfortunately preliminarly. Additional studies are needed to clarify the molecular basis of HO-1 more detailed in promoting neovascularization in atherosclerotic disease *in vivo*.

HO-1 was initially identidied as a catabolic enzyme that degrades hemoproteins, but was more recently shown to posses cytoprotective properties against oxidative stress, inflammatory damage and apoptotosis. The role of HO-1 in angiogenesis appears to be a complex one [12]. Studies supporting a role for HO-1 in angiogenesis have shown that several proangiogenic activate HO-1 expression in endothelial cells in vitro[12]. Furthermore, local HO-1 inhibition with competitive inhibitors including ZnPPIX or siRNA blocked tumor angiogenesis *in vivo* [13]. Recent *in vivo* studies reported that vascular endothelial growth factor (VEGF) was able to upregulate HO-1 expression and activity in vascular endothelial cells whereas inhibition of HO-1 abrogated VEGF-induced endothelial activation and neocapillary formation.

Remarkably HO-1 induction was also associated with VEGF-induced monocyte recruitment and inflammatory angiogenesis [14, 15]. Finally HO-1 may also regulate the synthesis and activity of VEGF, resulting in a positive-feedback loop[14]. Indeed, HO-1 activity likewise attenuated (lipopolysaccharide-driven) inflammatory angiogenesis.

Our in vitro experiments corroborate in part the results from earlier reports [16-18] suggesting that HO-1 promotes angiogenic sprouting and tube formation. However, sprouts formed by HO-1 expressing HUVEC in our assays deteriorated over time,suggestive of a possible cytopathic effect following adenoviral transfection. Alternatively, an imbalance between cell growth and capillary network differentiation may underlie these *in vitro* effects. To further specify these results additional experiments are currently being performed.

Neovascularization assures perfusion of nutrients into the growing lesional area, thereby increasing cell proliferation, and accounts for an additional delivery route for lipoproteins and inflammatory cells wich promotes lesion growth [6, 19].

Recent studies in animal models have indicated that pro-angiogenic factors (including VEGF and PDGF) were able to promote angiogenesis in ischemic cardiac tissues and partially recover heart function, but concomitantly promoted atherosclerosis development. In contrast, treatment of atherosclerosis with inhibitors of angiogenesis (angiostatin, VEGF receptor inhibitors) reduced plaque growth [20-22]. Plaque instability due to intra-lesional neovascularization is more prominent in advanced atherosclerotic plaques [23]. These so-called vulnerable plaques consist of a large, lipid-enriched necrotic core, surrounded by activated macrophages, and a thin fibrous cap of vascular smooth muscle cells (VSMCs). Due to their morphology, they are more likely to rupture and cause

acute thrombotic occlusion. In humans, intra-lesional neovascularization is strongly correlated with both the incidence and the location of vulnerable plaque rupture[24]. However, the mechanism by which intra-plaque angiogenesis could affect plaque stability remains unclear. Based on correlation studies, it was postulated that haemorrhages from the frail neovasculature could trap inflammatory cells in the lesion, secreting pro-inflammatory factors that amplify the general immune response, and contribute to the morphological change of the lesion into a vulnerable plaque [25, 26]. In addition, progressive migration of endothelial cells (ECs) and inflammatory cells increase breakdown of the extra cellular matrix, thereby weakening the collagen based cap, which renders the lesion more likely to rupture [27, 28]. Our results in the murine cast model show that systemic induction of HO-1 by cobalt-protoporphyrin (CoPPIX) increased neovascularization in the vulnerable plaque. However, this occured simultaneously with a stabilizing effect on lesion morphology (Chapter 3), with increased VSMC and collagen content of atherosclerotic plaque. We hypothesize that HO-1 induction during atherosclerotic lesion formation has multiple roles, primarily an anti-inflammatory response inhibiting leukocyte infiltration into the atherosclerotic lesion, as well as a pro-angiogenic response, which protects against hypoxia-induced cell death and benefits tissue repair.

Considering the evidence supporting a role of plaque neovascularization to stimulate atherogenesis and plaque vulnerability, it is crucial to achieve a better understanding of the molecular mechanisms that drive HO-1 induced proangiogenic effects, before attempting to apply pro-angiogenic factors in cardiovascular therapy in general.

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8

DISCUSSION AND FUTURE PERSPECTIVES

DISCUSSION AND FUTURE PERSPECTIVES

Heme oxygenases are enzymes involved in the breakdown of heme containing proteins. Physiological heme degradation is catalyzed by the two functional isoenzymes, HO-1 and HO-2, yielding carbon monoxide, (CO) iron and biliverdin in this process [1]. For many years, these degradation products of heme catabolism were considered to be cellular waste products with even toxic implications.

The free radical scavenging and anti-oxidative properties of bilirubin and the ability of CO as an important gaseous second messenger and activator of soluble guanylate cyclase (sGC) [2, 3] evoked attention to the role of these metabolites as second messengers to mediate cytoprotective properties. Today, the anti-inflammatory, antiapoptotic, and anti-oxidant properties of HO and its metabolites to maintain vascular homeostasis and cellular protection have been described in multiple reports [4-11]. However, the exact molecular background of HO-1 signaling remains incompletely understood.

In this thesis, we sought to understand the role of HO-1 gene expression in cardiovascular related disease and attempt to elucidate the molecular signaling pathways by which these effects are exerted.

Cardiovascular related diseases are the leading cause of mortality in western Society. Atherosclerotic lesion progression into plaque rupture with subsequent acute thrombus formation, and coronary artery occlusion is the main mechanism in the pathogenesis of myocardial infarction and sudden coronary death [12-14].

In **chapter 2 and 3** the role of HO-1 gene expression in the development and destabilization of atherosclerotic lesions was assessed. In progressive atherosclerotic lesions, endogenous HO-1 expression is localized in endothelial cells, macrophages and foam cells [15, 16]. In animal models, induction of HO-1 has been shown to abrogate the development of atherosclerotic lesions, whereas systemic inhibition of HO-1 gene expression by Zinc protoporphyrin IX stimulated atherogenesis [16, 17]. Likewise, ApoE/HO-1 double knockout mice subjected to a high cholesterol diet (HCD) developed accelerated atherosclerosis as compared to ApoE knockout mice on HCD [18]. One explanation for the antiatherogenic property of HO-1 may be that the induction of HO-1 promotes heme degradation and subsequent inactivation of heme proteins, including NADPH, a major source of reactive oxygen species (ROS) in the vascular cells [19]. Biliverdin and ferritin have been shown to posses potent antioxidant activity capable of inhibiting LDL-oxidation and reducing ROS underlying atherogenesis [20, 21].

In addition, HO-1 may inhibit atherosclerosis by activation of specific immune-modulatory signaling pathways. Carbon monoxide, the gaseous metabolite from HO-1 activity has been described earlier to induce a cGMP/p38MAPK dependent signalling cascade leading to a general down regulation of pro-inflammatory cytokines and release of anti inflammatory cytokines as IL10 [22].

The potential physiologic effects of HO-1/CO signaling that could contribute to inhibition of atherosclerotic lesion progression include further tissue specific inhibition of apoptosis [23-26], promotion of cell cycle arrest and inhibition of VSMC proliferation by induction of $p21^{Cip1}$ [27, 28].

Taken together, we hypothesized that specific HO-1/CO signaling, while affecting regulation of atherosclerotic lesion progression, may play an important role in the deterioration from stable to vulnerable lesion types as well.

In collaboration with a research group from the Gulbenkian Institute in Portugal

(prof. M. Soares) we assessed the effects of HO-1/CO inhalation on progression of atherosclerotic lesions in ApoE-/- mice. In this study described in chapter 2, we observed that CO inhalation starting either before or simultaneously with high cholesterol diet (HCD) suppressed the development of atherosclerotic lesions in the aorta and in the supra valvular area significantly with 32 -54% as compared to control mice. An equipotent inhibition of lesion formation was observed when CO inhalation was initiated 4 weeks after onset of HCD. This would suggest that the HO-1 /CO mediated anti-atherogenic effects could is also effective late during lesion development. These data indicate that there may be therapeutical options for the use of CO inhalation to arrest the progression of atherosclerosis development.

Inhalation of CO has already been demonstrated to be effective in models of inflammation, hypertension, and organ transplantation [29-31]. Because of the potential toxic effects of CO inhalation, finding a window of opportunity for safe CO inhalation would be a prerequisite. In a recent report Wistar rats were exposed to either CO inhalation at a dose comparable to the dose we used in our setups, for 20 h/day or air for 72 weeks [32].

It was shown that carboxy-haemoglobin concentration was 14.7+/-0.3% in CO exposed animals and 0.3+/-0.1% in control animals. In the lungs, no signs of pathology were observed between the groups after these 72 days of exposure. However chronic CO inhalation was also associated with a 20% weight increase of the right ventricle and a 14% weight increase of the left ventricle indicating that the effects of long term CO inhalation on the cardiovascular system seem to involve myocardial hypertrophy

Human trials have to place a verdict of whether HO-1/CO signaling can be used as therapeutic strategy for atherosclerosis in the future [33]. The pharmacological administration of CO to tissues using for instance carbon monoxide releasing molecules (CO-RM) may present an acceptable alternative to the inhalation of gas. However safety, tissue site specificity and optimal dosage of these compounds for human delivery remain to be further explored [34]. In the future, the development of improved versions of CO-RM, as well as the ongoing development of other effective methods utilizing the cytoprotective properties of the HO/CO signaling system will inevitably lead to discovery of novel therapeutic strategies.

In **chapter 3** of this thesis the role of HO-1 on atherosclerotic plaque destabilization was assessed in human carotid endatherectomy specimen as well as in a murine model for vulnerable atherosclerotic plaque formation.

In human carotid endatherecthomy material HO-1 protein expression was strongly correlated with a vulnerable plaque morphology: HO-1 protein expression was shown to be specifically upregulated in vulnerable type atherosclerotic lesions with high lipid depositions and macrophage accumulation and conversely low collagen and VSMCs content. Furthermore, these lesions expressed high levels of pro-atherogenic cytokines including IL-6 and IL-8 as well as proteolytic factors like MMP9 and MMP2. The oxidative stress associated with progression of atherosclerosis may involve upregulation of HO-1 expression. On the other hand, we hypothesize that endogenous upregulation of HO-1 expression may function as a compensatory and cytoprotective response resulting in modulation of plaque morphology to a stable phenotype.

To address these questions we used a well validated murine vulnerable plaque model to elucidate whether the expression of HO-1 in this human vulnerable lesion type was secondarily to adverse and progressive plaque development or in contrast, was primarily endogenous mechanism to enhance cytoprotection and prevent plaque morphology from deteriorating further. Systemic HO-1 expression induced by CoPPIX injection effectively increased HO-1 protein expression levels 5-fold. Histomorphological analysis showed that atherosclerotic lesion progression into vulnerable plaques was attenuated, as suggested by a significant reduction of necrotic core formation and intraplaque lipid deposition, whereas cap thickness and the amount of intra lesional VSMCs were increased. These findings are indicative of stabilization of these rupture-prone lesions by the induction of systemic HO-1 expression. Based on these results, we postulate that HO-1 expression not only is involved in the inhibition of atherosclerotic plaque formation (chapter 2) but may also stabilize the vulnerable, rupture prone, lesion type.

The molecular pathways underlying these effects still remain subject of investigation. Recently an interesting report by Watari and colleagues [35] postulated that silencing of the transcriptional factor Bach1, which normally inhibits HO-1 expression, upregulated HO1 in the vasculature coinciding with the inhibition of atherogenesis in ApoE knockout mice. These investigators propose that the inhibition of Bach1 may provide a promising therapeutic option for local vascular enhancement of HO-1 activity and site specific inhibition of atherosclerosis. Conditional modulation of Bach1/HO-1 enables the exploration of local and more focussed HO-1 upregulation without administering toxic pro-oxidants (such as hemin) or the possible adverse side effects of pharmacological HO-1 inducers (like CoPPIX).

One of the interesting perspectives would be to study the effects of Bach1 deletion in our vulnerable plaque model to see whether the effects of HO-1 expression on plaque destabilization are mediated by this transcription factor.

After allogeneic organ transplantation, three main types of rejection may occur; including a hyperacute, an acute and a chronic rejection response. Hyperacute rejection is due to preformed IgG antibodies in the recipient that react to HLA antigens in the transplanted organ. Acute rejection occurs most frequently in the first 6 months after transplantation and is mainly mediated by

T-cells infiltrating into the allograft [34]. Most of the clinical used immunosuppressive drugs are active against this acute rejection phase; unfortunately these pharmacotherapy's have significant deleterious side effects. Chronic rejection is the term used when allograft function slowly deteriorates over time, and it is characterized by vascular neointimal proliferation and fibrosis. Chronic rejection characterized by development of transplant arteriosclerosis and interstitial fibrosis, is a major cause of long term organ transplant failure, particularly in heart transplant recipients since conventional immune suppressive agents fail to arrest neointima formation.

Accumulating evidence suggests that in the context of allogeneic organ transplantation HO-1 triggers cytoprotective responses that may sustain graft survival by reducing (peri-operative) oxidative tissue injury. In addition HO-1 was shown to mediate immunogenicity of the graft and to promote suppressive immune responses determining graft survival, suggesting that modulation HO-1/CO signaling might be a valuable therapeutic option adjacent to allograft transplantation [26, 34, 37-43].

Insight in the exact molecular mechanism and cell and tissue specific effects of HO-1 signaling resulting in the allograft protection is eminent to develop novel and site/tissue specific therapies against chronic allograft rejection.

In vivo models, demonstrated that prolonged graft survival in itself is not indicative of tolerance induction, as many procedures producing indefinite graft survival do not prevent chronic rejection [44]. In addition it was shown that blocking T-cell co-stimulation with CTLA4-Ig or CD40L monoclonal antibodies actually induced indefinite allograft survival. Furthermore, induction of HO-1 in allograft recipients treated with CD4 monoclonal antibody prevented chronic rejection [45]. It was also reported that xenografts were able to survive in the presence of anti-graft antibodies and complement, a situation which is referred to as 'accommodation', and that grafts surviving long-term in accommodated recipients show vascular expression of HO-1,in the context of a Th2 dominant cytokine profile [46].

We gained interest in the effects of HO-1 expression specifically in DC regulated T-cell priming in the allo-immune response directed against vascular allografts. **(chapter 4)**

In this study HO-1 deletion in DCs was shown to increase STAT1 phosphorylation and subsequent CIITA expression, leading to a rise in MHC class II surface expression in DCs without modulation of MHC class I expression. These effects are associated with a preferential CD4+ T-cell alloimmune response in DC/T-cell co-cultures. *In vivo* ablation of HO-1 in DCs was shown to promote transplantation atherosclerosis and increased perivascular infiltration of CD4+ T-cell and IgG deposition in the vascular graft, suggestive of a chronic phase of vasculopathy. These data suggest for the first time that HO-1 is an important regulator of dendritic cell-induced T-cell priming in the alloimmune response.

A key challenge in the future would be to translate these discoveries into new therapeutic modalities. Several studies attributed beneficial effects of HO-1 in transplantation and atherosclerosis formation to the immune modulatory effects of the HO-1/CO signaling cascade. Even though these effects are well described we propose that in addition HO-1 signaling has an important role as a genetic

regulator of myeloid DC function and subsequent T-cell priming in the allo-immune response, influencing the long-term survival of transplanted grafts.

Conditional/site-specific HO-1 (over) expression in DC would be an interesting target to further explore the effects of HO-1 gene expression in the T-cell mediated alloresponse

Future research will have to find the answer whether these findings are applicable and beneficial in the context of human allogeneic organ transplantation. Previous studies have explored the effect of endogenous HO-1 expression in transplant organ recipients on graft survival

In humans, the promoter region of HO-1 displays length polymorphism due to a variable number of GT repeats. Individuals exhibiting 29 or fewer GT repeats express higher levels of HO-I on cellular stress compared with individuals with 30 or more GT repeats. Previous work demonstrated a relation between HO-1 promoter length and graft survival [40, 47-49].

However, in a previous analysis of functional GT repeat polymorphism in the promoter of the HO-1 gene, no associated with graft survival or the development of transplant atherosclerosis after heart transplantation was observed [Balk,Holweg 2004]. It was postulated that even though these results may indicate that the protective effects of HO-1 expression in cardiovascular allografting obtained in animal models are possibly not applicable in humans, investigators postulated that these results could in fact be influenced by inequalities in some baseline characteristics including statin use, as well as differences in HLA- mismatching between groups. We hypothesize in addition that the mechanism by which effects of HO-1 on the alloimmune response are exerted are complex and not only determined by promoter length.

It was previously reported that signal transducer and activator of transcription (STAT) DNA binding sites located in the promoter region of HO-1 and activator protein (AP)-1 DNA binding sites in the distal enhancer element SX2 were necessary for optimal HO-1 gene activation after oxidative stress. Interestingly, a second 5' distal enhancer element, AB1, located 10 kb upstream from the HO-1 promoter, was activated after hyperoxia but could not confer maximal hyperoxia-induced HO-1 gene transcription. These results suggest that the 5' distal enhancer elements of the HO-1 gene act in concert with the promoter to regulate HO-1 gene induction. This is an example that highlights the complexity of HO-1 gene transcription in response to cellular stress. We hypothesize that the role of HO-1 expression in DC regulated T-cell differentiation resembles this complexity and future research exploring HO-1 gene induction and function in the context of the alloimmune response will be necessary.

In **chapter 5** the effects of HO-1 in the regulation of the dendritic cell induced T-cell differentiation response was explored in depth. A well described *in vivo* model of DC sensitization based on intratracheal transfer of ovalbumin (OVA)-pulsed DCs [50] was utilized, to investigate the possibility that HO-1 expression in dendritic cells may be involved in the regulation and differentiation of Th2 helper cells. Previously Heme oxygenase-1 expression has been shown to exert anti-inflammatory effects in animal models of asthma [51-54]. These observed effects are suggested to be due to cell specific HO-1/CO triggered signaling cascades [55].

We were able to show that antigen presentation by HO-1 deficient OVA-pulsed DCs to naïve OTII specific T-cells abolished the Th2-response as compared to control mice sensitized with OVA-pulsed wild type DCs. These findings were characterized by a decrease in the production of the Th2 specific cytokines IL4, IL5, IL10, IL13, without affecting production of the typical Th1 cytokine INF- in vitro.

The obtained results seemed mainly independent of DC maturation, as flow cytometric analysis revealed that expression levels of the maturation markers CD40, CD80, and CD86 remained unaffected after HO-1 deletion.

In vivo, in a murine Th2-dependent asthma model, HO-1 knockout DCs failed to induce the typical Th2 mediated bronchoinflammatory response. HO-1 null Dc treated mice displayed a lack of peribronchial and perivascular infiltration of eosinophilic inflammatory cells, absence of goblet cell hyperplasia, and a substantial decrease of total cell number and eosinophils in the broncho-alveolar lavage fluid (BAL) fluid as compared to controls. These new findings for the first time attribute the immuno- modulatory effects of HO-1 on inflammatory airway disease to its expression in the predominant antigen presenting cell, the DC.

In order to extend the molecular basis for these effects of HO-1 knockdown in DC on inflammation we focused on the transcription factor forkhead box-P3 expression (FOXP3). This transcription factor has been reported earlier to be crucial for $CD4^+CD25^+$ Treg cell development and function [56]. Induction of $CD4^+CD25^+$ Treg cells inhibited the infiltration of eosinophils in the airway[57]. However, in our study the lungs of mice treated with HO-1 knockout DC displayed decreased levels of FOXP3 mRNA expression as determined with qPCR analysis. Therefore, it seems that HO-1 deletion in DC does not counteract the current findings in this murine Th2 model via induction of T-regulatory T cells but interferes directly with Th2 –T cell differentiation. In the future the effect of HO-1 expression on molecular pathways involved in Th1/Th2 differentiation in DC will be further explored to depth. Based on current reports, the Notch signaling pathway and particularly delta-like 4 and jagged-1 expression are interesting targets for future research in this respect [58].

In **chapter 6** we explored the effects of HO-1 expression on directional migration and polarization of vascular smooth muscle cells (VSMC).

Directed migration of vascular smooth muscle cells from the media is thought to play a key role in the pathogenesis of many vascular diseases including neointima formation after vascular injury [59]. This process is regulated by growth factors and chemo attractants and is dependent on multiple interactions with the extra cellular matrix.

One laboratory was already able to show that HO-1 directly inhibits VSMC proliferation (and subsequent neointima formation) in the course of vascular injury in a pig balloon injury model. These effects of HO-1 on vascular smooth muscle cell growth were shown to be mediated by cell-cycle arrest involving $p21^{Cip1}$ [27].

However, effects of HO-1 on the regulation of VSMC migration remain unclear but could contribute to a more complete understanding of the inhibitory effects of HO-1 on neointma formation [60, 61].

GENERAL FUTURE PERSPECTIVES

Increasing evidence demonstrates that HO-1 expression and the degradation products of heme catabolism are crucial in the cytoprotective response against various types of cellular stress and exert inhibitory effects against inflammation, apoptosis and cellular proliferation. Consequently, the role of the HO system in diabetes, heart disease, hypertension, neurological disorders, transplantation, endotoxemia among other pathologies constitute a growing area of scientific research [68]. In this regard it is interesting that humans differ in their ability to up regulate HO-1 in response to stressful stimuli, attributable to variability in two distinct HO-1 promoter polymorphisms[69].

In our study several new beneficial effects of HO-1 expression in cardiovascular related disease models have been observed and described.

These results contribute to our increasing knowledge of the molecular background of the heme oxygenase / carbon monoxide signalling system.

In the near future accumulating insights in these molecular backgrounds of HO-1 signalling, associated with results of administration of exogenous CO, biliverdin or bilirubin in *in vivo* research models will be the driving force for the ongoing development of approaches that might hold great therapeutic potential in human.

However, before the effects of HO-1 or its metabolites actually are assessed within clinical trials there are also some major concerns to overcome.

One of the major challenges of the feasibility of the clinical application of HO-1 will be to identify the particular disease states that in particular will benefit from altered HO-1 activity or administration of one of its metabolites. Scott and Otterbein recently proposed a general valuable approach for the determination of therapeutic applicability of HO-1 in human, depending on the disease and abilities to induce tissue specific HO-1 expression [70].

It is important to note that all HO-1 metabolites have both a toxic and a therapeutic range. In addition most of the pharmacological inducers of HO-1 including hemin and heavy metals, may cause concomitantly adverse cellular effects. The key will be to find the right window of opportunity of these compounds.

Another challenging part is formed by the observation that the beneficial effect of HO-1 may vary depending on the level and tissue specific site of expression. The use of e.g carbon monoxide releasing molecules (CORMs) in this respect is promising since it enables additional routes of CO administration compared with gas delivery by inhalation [70].

To bypass the need for pharmacologic inducing compounds and their possible adverse side effects, a "gene therapy" approach depends on the introduction of the HO-1 gene by viral vector mediated overexpression. However, it should be emphasized that expression of HO-1 at supra-physiological levels may have counter protective effects, so this approach should be explored extensively

and would require additional strategies that enable control of the site and level of expression. The generation of inducible or tissue-specific vectors in this respect, will provide challenging opportunities for therapeutic applicability of HO-1 in the future.

Taken together, the benefit from therapeutically use of HO-1 will be tremendous, and may hold great promise for the future treatment of cardiovascular diseases.

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9

SAMENVATTING HOOFDSTUKKEN

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Heme-Oxygenase-1 (HO-1) katalyseert de reactie waarbij eiwitstructuren die een heem groep bevatten worden afgebroken, zoals hemoglobine. Tijdens deze reactie worden degradatie producten gevormd, te weten; carbon monoxide (CO), ijzer (Fe^{2+}) en biliverdine (dat uiteindelijk wordt geconverteerd in bilirubine).

Jarenlang was er weinig wetenschappelijke interesse in deze degradatieproducten en werden ze beschouwd als potentieel cytotoxisch, totdat onderzoek aantoonde dat ze dankzij hun sterkte anti-oxidatieve eigenschappen een belangrijke functie hadden in het waarborgen van de cellulaire homeostase en verdediging tegen vrije zuurstof radicalen. Van grote waarde was de bevinding dat cabon monoxide niet alleen toxische eigenschappen heeft, door een competitieve binding aan te gaan met zuurstof aan hemoglobine, maar in lagere concentratie functioneert als een belangrijke second messenger in verschillende intra-cellulaire signaal transductiesystemen.

Thans zijn er vele publicaties die het belang van HO-1 en de anti-inflammatoire, anti-apoptotische en anti-oxidatieve eigenschappen van de door HO-1 gegenereerde metabolieten onderschrijven. HO-1 kan in dit licht worden gezien als een cellulaire chaperonne tegen schadelijke invloeden vanuit het millieu exterieur. De moleculaire basis van de door HO-1 gegenereerde cytoprotectieve effecten is echter nog niet volledig bekend. Het verkrijgen van inzicht in de functie van HO-1/CO als second messenger in celbeschermende signaaltransductie systemen kan uiteindelijk leiden tot ontwikkeling van nieuwe therapeutische strategieën.

In dit proefschrift is getracht het belang van HO-1 gen-expressie in verschillende cardiovasculair gerelateerde ziekte modelien aan te tonen. Voorts is het moleculaire mechanisme van de door HO-1 expressie of deletie gegenereerde effecten geëxploreerd.

Hart- en vaatziekten zijn nog steeds de belangrijkste doodsoorzaak in de westerse wereld.

Progressie van atherosclerotische laesies in de vaatwand kan in sommige gevallen leiden tot ontwikkeling van een instabiele, vulnerabele plaque die makkelijk kan scheuren. Ruptuur van deze plaque leidt tot acute thrombus formatie, coronair occlusie en plotse hartdood.

In **hoofdstuk 2 en 3** van dit proefschrift wordt het effect van HO-1 genexpressie en diens metabolieten op de ontwikkeling van vulnerabele atherosclerotische plaque beschreven.

Het is aangetoond met behulp van dierexperimenteel onderzoek dat inductie van HO-1 genexpressie een inhiberend effect heeft op de vorming van atherosclerotische plaque en dat remming vanHO-1 genexpressie, bijvoorbeeld met de farmacologische HO-1inhibitor

Zinc Protoporphyrine IX, laesie vorming juist stimuleert. Een mogelijke verklaring voor het effect van HO-1 genexpressie op de vorming van atherosclerose is de reductie van de oxidatieve stress op de vaatwand, onder meer door een verhoogde afbraak van nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), een belangrijke bron reactieve zuurstofmoleculen in de vaatwand. Biliverdine en ferritine treden voorts op als sterke anti-oxidanten en hebben een limiterende invloed op de oxidatie van LDL. Naast vermindering van de oxidatieve stress op de vaatwand inhibeert HO-1expressie de vorming van atherosclerose door activatie van immuun-modulerende signaaltransductie systemen.

Uit een aantal studies komt naar voren dat CO via cGMP/p38MAPK afhankelijke, immuunsysteem regulerende signaalcascades, de productie van pro-inflammatoire cytokines remt en het vrijkomen van anti-inflammatoire cytokines als IL-10 juist stimuleert. Daarnaast heeft CO een anti-apoptotische werking en is beschreven dat CO gladde spiercel proliferatie remt door inductie van $p21^{cip1}$. Op basis van deze bevindingen die eerder werden beschreven stelden we de hypothese dat HO-1, en in het bijzonder de HO-1 metaboliet CO, niet alleen een remmend effect heeft op de vorming van de atherosclerotische plaque, maar eveneens invloed heeft op de ontwikkeling van een stabiele in een vulnerabele plaque. In samenwerking met prof. M.Soares van het Gulbenkian Institute in Portugal werd het effect van CO inhalatie op ontwikkeling van atherosclerotische laesies in ApoE -/- muizen die een zgn Western diet kregen gedurende 8 weken onderzocht. Deze studie die wordt beschreven in **hoofdstuk 2** toont dat continue CO inhalatie gestart op verschillende tijdstippen tijdens het experiment de vorming van atherosclerotische laesies met 32-54% verminderde wanneer werd vergeleken met een controle groep dieren. Opvallend was dat dit effect aanwezig was zelfs wanneer CO inhalatie werd gestart 4 weken na aanvang van het Western diet . Dit zou betekenen dat CO inhalatie niet alleen het ontstaan van atherosclerose inhibeert maar dat het ook effectief is wanneer zich al laesies hebben gevormd. Deze bevinding is interessant voor eventuele toekomstige therapeutische mogelijkheden in de strijd tegen atherosclerose. Klinische trials zullen in de toekomst een veilige concentratie met effectieve werking moeten aantonen. De voortgaande ontwikkeling van de CO-releasing molecules kan een van die veilige en effectieve toedieningsvormen vertegenwoordigen.

In **hoofdstuk 3** wordt het effect van HO-1 op de ontwikkling van vulnerabele plaques beschreven. Er werd gebruik gemaakt van de AtheroExpress database in samenwerking met de afdeling Experimentele cardiologie van het UMC Utrecht. HO-1 eiwit expressie werd bepaald in menselijk atherectomie materiaal. HO-1 eiwitexpressie bleek sterk te correleren met het vulnerabele type laesie, dat werd gedefinieerd door een hoog lipide en macrofaag gehalte en lage hoeveelheid aan collageen en gladde spiercel. Dit type laesie toonde tevens een hoge expressie van interleukine-6 (IL-6) en interleukine-8 (IL-8) en matrix metalloproteinase (MMP) 9 en 2, in vergelijking met de stabiele plaque regio's.

We stellen de hypothese dat de oxidatieve stress die gepaard gaat met de progressie van aherosclerose een van de oorzaken is voor de verhoogde HO-1 eiwitexpressie in de vulnerabele regionen van de atherosclerotische plaque. Het is hierbij mogelijk dat er tevens compensatoire endogene inductie van HO-1 expressie optreed ten einde cytoprotectieve effecten en daardoor verdere plaque destabilisatie te voorkomen.

Om deze stelling te onderzoeken maakten we gebruik van een goed gevalideerd muismodel voor de ontwikkeling van vulnerabele atherosclerotische plaque, het cast model. Systemische HO-1 inductie werd bereikt door injectie met kobalt protoporphyrine (CoPPIX).

Histomorfologische analyse toonde aan dat er door systemische HO-1 inductie significant minder vulnerabele plaques werden gevormd. Gebaseerd op deze bevindingen stellen we dat HO-1 expressie niet alleen leidt tot een vermindering van het aantal atherosclerotische plaques maar ook een belangrijke en beschermende rol vervult in het voorkomen van de progressie van een stabiele laesie in een vulnerabele plaque.

Er is toenemend bewijs dat HO-1 expressie erg belangrijk is in het kader van orgaan transplantatie. HO-1 inductie leidt tot activatie van cytoprotectieve responsen die de overlevingsduur van een allograft kunnen verlengen, enerzijds door reductie van (peri-operatieve) oxidatieve stress en anderzijds door het onderdrukken van de afstotingsrespons . Het is echter nog onbekend welke rol HO-1 expressie speelt in antigen presentatie van de dendritische cel binnen het kader van de allograft immuun respons.

In **hoofdstuk 4** worden de effecten van HO-1 genexpressie in een model voor allogene vaat-transplantatie beschreven. HO-1 deletie in dendritische cellen resulteerde in een toename van STAT1 phosphorylatie en verhoogde expressie van CIITA, met een verhoging van de MHC klasse II oppervlakte expressie tot gevolg. Dit effect bleek samen te gaan met een dominante CD4+ T-cel alloimmuun respons in DC/T-cel co-culturen. In een in vivo model voor vasculaire allograft transplantatie bleek dat HO-1 deletie in DC het ontstaan van transplantatie atherosclerose bespoedigde, dit bleek uit ondermeer toegenomen neo-intimavorming, perivasculaire infiltratie van CD4+T cellen en verhoogde IgG depositie in de allografts. Deze data tonen aan dat HO-1 belangrijk is voor de T cel priming in een allo-immuun respons.

Uitdaging voor de toekomst is het vertalen van deze bevindingen naar klinisch toepasbare strategieën.

Uit recente publicaties blijkt dat HO-1 expressie een inhiberend effect heeft op het optreden van bronchiale hyperreactiviteit. Hierbij is een voornamelijk Th2-gemedieerde immuunrepons belangrijk bij het ontstaan. Het remmende effect van HO-1 op het ontstaan van dergelijke hyperreactiviteit lijkt gerelateerd aan door HO-1 geactiveerde celspecifieke signaal cascades . Het basaal moleculaire mechanisme hiervan is echter nog onbekend.

In **hoofdstuk 5** beschrijven we de bevindingen van het onderzoek naar het effect van HO-1 expressie in dendritische cellen in het kader van Th1/Th2-cel differentiatie bij bronchiale hyperreactiviteit.

Antigen presentatie door HO-1 knockout DC faalde in het ontlokken van een Th2-T cel respons in een gevalideerd muismodel voor het ontstaan van bronchiale hyperreactiviteit. Dit kwam onder meer tot uiting in de histologie van de long waarin bij de met HO-1 knockout DC behandelde muizen geen peribronchiale en perivasculaire infiltratie van eosinofielen kon worden aangetoond en goblet cel hyperplasie ontbrak. In de broncho alveolaire lavage (BAL) vloeistof was het aantal eosinofielen substantieel lager dan bij de groep met wild type DC behandelde muizen. Een en ander werd verder onderschreven door een verlaagde productie van Th2 specifieke cytokines, terwijl de productie van Th1-gerelateerde cytokines gelijk bleef. Deze resultaten bleken voorts onafhankelijk van de mate van maturatie van de DC gezien het feit dat HO-1 deletie geen effect had op de oppervlakte expressie van maturatie markers CD40, CD80 en CD86.

Om een mechanisme te vinden voor deze effecten werd onder meer gekeken naar de FOXP3 expressie, een marker voor T-regulatoire T cellen (Treg), in de longen van de onderzoeksgroepen. Op basis van deze bevindingen is het onwaarschijnlijk dat HO-1 deletie in DC leidt tot inductie van Treg. In de toekomst zal worden onderzocht of er na HO-1 deletie in de DC veranderingen optreden in de Notch pathway signaal eiwitten delta-like 4 en jagged-1, welke wellicht verklarend kunnen zijn voor het ontbreken van de Th2 immuun respons na HO-1 deletie in de DC.

In **hoofdstuk 6** wordt het effect van HO-1 expressie op migratie en polarisatie van gladde spiercellen onderzocht. We hebben op dit moment aanwijzingen gevonden voor een inhiberend effect van HO-1 overexpressie op de migratie van gladde spiercellen. Er wordt met behulp van fluorescentie microscopie een effect gezien op de polarisatie en MTOC orientatie van de migrerende spiercel, en uit western blot analyse blijkt dat dit effect mogelijk samengaat met veranderingen in GSK-3 activiteit. Verder hebben we gevonden dat HO-1 overexpressie leidt tot veranderingen van de focal adhesion kinase activiteit. In de toekomst zal met behulp van time-lapse microscopie worden onderzocht hoe de turnover van focal adhesions door de verandering van focal adhesion kinase activiteit wordt beïnvloed.

In **hoofdstuk 7** staat de rol van HO-1 in de ontwikkeling van plaque neovascularisatie in atherosclerose centraal. Uit analyse van in vitro matrigel-assay's blijkt dat overexpressie van HO-1 in HUVEC de nieuwvorming van bloedvaatjes stimuleert in vergelijking met overexpressie met een sham virus. Inhibitie van HO-1 genexpressie met siRNA had in overeenkomst hiermee een remmend effect op de bloedvat nieuwvorming. Lichtmicroscopie toonde echter aan dat de nieuwgevormde bloedvaatjes na HO-1 overexpressie een wat afwijkende vorm hadden. Mogelijk wordt dit veroorzaakt door een

cytopatisch effect tengevolge van een hoge dosering virus partikels. Toekomstig onderzoek zal moeten aantonen wat dit betekent voor de functionaliteit van deze vaatjes.

In een in vivo muismodel voor de ontwikkeling van vulnerabele plaque werd HO-1 systemisch tot overexpressie gebracht door injectie met CoPPIX.

In de groep dieren waarin HO-1 op deze wijze werd geïnduceerd, bleek de mate van vaatnieuwvorming in de plaques toegenomen, hoewel dit niet leidde tot toename van het aantal bloedingen in de plaque. Een toename van plaque neovascularisatie is een kenmerk van ontwikkeling van vulnerabele laesies. Opmerkelijk hierbij is dat in hoofdstuk 3 al werd vermeld dat HO-1 inductie in dit model eveneens tot gevolg had dat gevormde vulnerabele laesies stabiliseerden na HO-1 inductie, wat bleek uit toename van het aantal VSMC's en toename van de relatieve fibrous cap-dikte.

Uit onderzoek van anderen kwam al eerder naar voren dat tijdens een toestand van chronische ontsteking HO-1 expressie de infiltratie van leukocyten in het weefsel remt en weefsel herstel bevordert door VEGF gedreven non-inflammatoire angiogenese na HO-1 deletie in de DC te stimuleren.

Onze hypothese is dat HO-1 overexpressie de intra plaque neovascularisatie bevordert als onderdeel van een algemeen cytoprotectieve respons. Toekomstig onderzoek zal moeten uitwijzen of dit inderdaad het geval is.

10

DANKWOORD

DANKWOORD

Dit proefschrift had natuurlijk nooit geschreven kunnen worden zonder de zeer waardevolle hulp van velen waarvoor ik een ieder dan ook zeer erkentelijk ben.

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Caroline Cheng, zonder jouw hulp was het simpelweg niet gelukt. Ik ben je daar heel erg dankbaar voor. Je hebt veel talent, en niet alleen op het wetenschappelijke gebied. Ik vind je een bijzonder persoon, en ik zou graag dat eerste boek van je willen lezen. Heel veel geluk samen met David in jullie nieuwe huis. Er ligt een prachtige carrière voor je en ik denk dat we nog veel van je zullen horen.

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Professor Dirk-Jan Duncker, ik wil u bedanken voor de support en de leerzame discussies over in het bijzonder het allograft transplantatie werk. Bedankt dat we gebruik mochten maken van alle faciliteiten van het muizenlab, zonder uw steun was het niet mogelijk geweest.

Professor Miguel P. Soares, obrigado para tudo. Era impossível fazê-lo sem você.

Sua ajuda era muito importante.

Sofia, Isabel e Sylvia, obrigado muito para seu trabalho duro.

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Professor Gerard Pasterkamp, bedankt voor de prettige samenwerking.

Professor Bart Lambrecht ik wil u bedanken voor uw medewerking en wetenschappelijke visie op het geheel. Uw ideeën brachten ons steeds weer verder.

Hamida, thank you very much for your help with the experiments. All the best for the future and good luck in Gent.

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Elza, toen ik aan kwam zetten met een filmpje van 5 minuten van een vasculaire allograft transplantatie en vroeg of het mogelijk was om dit ook in Rotterdam te doen zei je meteen dat als iemand ergens op de wereld het voor elkaar gekregen had, het jou ook zou lukken. Er zijn een aantal "keesjes" overheen gegaan maar je had gelijk en het lukte. Ontzettend bedankt voor je inzet, tijd, expertise en geduld. Zonder jou was het een "mission impossible" geweest.

Ineke Hekking, uiteindelijk kwam u op het briljante idee om de tubing onder warm water op te rekken zodat het kaliberverschil tussen carotis en aorta overkomen kon worden. Dit bleek een gouden zet en tevens de doorbraak in het opzetten van het transplantatiemodel. Bedankt voor al uw steun en vakkennis.

Esther, je bent een gouden persoon, de spil van het lab, en je hebt me met veel geduld de basics geleerd. Bij jou kon ik altijd wel even terecht voor wat opbeurende woorden of gewoon om even mn hart te luchten, als de antilichamen niet werkten, de pcr mislukt was, of de virussen niet precies deden wat ik eigenlijk van plan was. Het was altijd erg gezellig met je en ik wil je heel hartelijk bedanken voor je support.

Desiré, jij ook heel hartelijk bedankt voor alle hulp, je geduld en de gezellige etentjes.

Dennie, een speciaal bedankje voor jou voor al je hulp bij de confocal en voor het invallen op de laatste operatiedagen tijdens mijn zwangerschapsverlof.

Ik hoop dat je vlug je eigen dankwoord mag gaan schrijven.

Heel erg veel succes.

Alle Aio's van het moleculaire lab, en Aio's van de afdeling Experimentele Cardiologie, heel veel sterkte met jullie promoties, ik hoop dat jullie nog veel mooie resultaten behalen in de toekomst.

Monique, bedankt dat er altijd iets te regelen viel als een bestelling te laat kwam of niet was geleverd.

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Lieve Inge, we zijn al 30 jaar vriendinnen, en ik hoop dat we er zeker nog 30 jaar bij mogen doen. Ik vind het helemaal geweldig dat je in januari je dochtertje Donna eindelijk in je armen kon sluiten. Wat een prachtige wending van het leven. Ik hou van je en wens jullie alle geluk van de wereld met zn drietjes.

Lieve Maryse, ook jij bent al bijna 30 jaar een vriendin.

Het begon toen we zes waren en ik herinner me schoenendozen vol brieven, dagenlang rollerskaten, samen op ponykamp. Toen wilde ik al dokter worden en dacht ik dat ik met artisjokken (want dat klonk wel heel erg als een medicijn) je hooikoorts klachten kon genezen. We waren vol verwachting over wat de toekomst zou brengen.

Toen die toekomst eenmaal daar was deelden we lief en leed over de perikelen in De Liefde en Het Leven. Er is niemand met wie ik daar over kan praten zoals met jou, je humor is messcherp en laat me altijd weer lachen. Je hebt een moeilijke tijd achter de rug en hebt je ongelooflijk sterk gehouden, iets waar ik veel bewondering voor heb. Ik en er erg trots op dat je me vandaag terzijde staat en mijn paranimf wilt zijn.

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Marieke en Sjeng, hier is dan voor mij de gelegenheid om jullie te bedanken voor alles wat jullie voor mij hebben gedaan. Jullie warmte was voor mij in die tijd erg belangrijk en ik zal dat nooit vergeten.

Elleke, veel succes met je opleiding tot radioloog, ik hoop dat ik je nog vaak zal zien. Veel geluk samen met Bas.

Gerard, je bent een heel lieve opa voor Thijmen, bedankt ook voor de belangrijke steun afgelopen jaar toen hij in het ziekenhuis lag. Dat er nog maar veel succesvolle vierdaagsen mogen volgen!

Lieve Elise, Lieve Arjan, mijn zus en mijn broer, ik hou heel veel van jullie en ben erg blij dat jullie er vandaag bij zijn. Ik ben erg trots op jullie.

Mama, het is niet altijd allemaal even gemakkelijk geweest maar we zijn er toch gekomen. Bedankt voor alles wat je me gegeven hebt en altijd voor ons hebt gedaan.

Papa, je bent al enige tijd erg ver weg maar je blijft m'n vader. Ik hoop dat er een tijd komt dat er ondanks het verdriet uit het verleden ruimte ontstaat voor een toekomst.

Lieve Geert-Jan, deze laatste regels zijn natuurlijk voor jou.

Wat ben ik blij dat ik die ene oktobernacht toch nog even ben meegegaan naar het "Feest van Sinterklaas".

"Gewoon, gezellig"... en voor ik het wist zat ik bij je achter op de motor en reden we in volle vaart langs de (Ierse) kust de toekomst tegemoet. Ik ben heel erg blij met jou. We hebben al veel mooie momenten beleefd samen, met als hoogtepunt de geboorte van onze prachtige zoon Thijmen.

Jouw warmte, kracht en optimisme maken mij heel erg gelukkig. Bedankt voor al jouw steun.

Ik heb je lief

Annemarie

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CURRICULUM VITAE

Annemarie Noordeloos was born on the 3rd of April 1974 in Alkmaar, the Netherlands.

After finishing high school in 1992 at the Han Fortmann College in Heerhugowaard she studied Health Sciences at the University of Maastricht for 3 years. During her fourth year, she got admitted to study medicine and graduated in 2001.

After 2 years working as a cardiac resident in the Maxima Medical Center in Veldhoven (the Netherlands) she started working as a cardiac resident at the CCU in the Eramsus Medical Center in Rotterdam (the Netherlands).

In January 2004 she was employed as a research fellow at the laboratory of Molecular Cardiology (department of Experimental Cardiology). Under leadership of Dr. H.J. Duckers, she studied the cardiovascular effects of HO-1 gene expression in cardiovascular disease. This study resulted in this PhD thesis.

Currently she is involved in her cardiology training, starting a 2 year residency at the department of Internal Medicine at the Sint Fransciscus Gasthuis in Rotterdam, which will be followed by one year of cardiology training in a community hospital and three years of residency at the Thoraxcenter in Rotterdam.

Annemarie currently lives in The Hague, together with Geert Jan and their son Thijmen Geert.

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PhD PORTFOLIO

SUMMARY OF PHD TRAINING AND ACTIVITIES

Name PhD student:	Annemarie Noordeloos
Erasmus MC departement:	Cardiology
Research school:	COEUR
PhD period:	2004-2009
Promotor(s):	Prof. Simoons / Dr. Duckers
Supervisor:	Dr. Deckers

1. PhD Training

	Year	ECTS
General academic skills		
Biomedical English Writing and Communication	2004	3.0
Research skills		
Statistics	2004	6.0

In-depth courses (e.g. Research school, Medical Training)

COEUR		
(Cardiovascular Research school ErasmusmMC Rotterdam)	**2004-2006**	
Cardiovascular imaging and diagnostics		1.5
Pathophysiology of ischemic heartdisease		1.5
Molecular biology in atherosclerosis and cardiovascular research		1.5
Cardiovascular medicine		1.5
Animal experiments		4.5
Radiation hygiene		4.5

Presentations:

Heme oxygenase -1 konockoutdendritic cells skew towards A Th1 response.		
Nederlandse Vereniging van Cardiologie (NVVC) voorjaarscongres **Awarded with first prize*	2005	0.6
Heme Oxygenase-1 inhibits directional migration of vascular smooth muscle cells		
European Society of Cardiology	2006	0.6
Heme Oxygenase 1 in dendritic cells regulates the development of transplantation atherosclerosis		
American Heart Association	2007	0.6
Heme Oxygenase 1 Determines the Outcome of Vulnerable Plaque		
America Heart Association	2007	0.6
Heme Oxygenase 1 Expression In Dendritic Cells drives Cd4+ T Cell Activation In Transplantation Atherosclerosis		
America Heart Association	2008	0.6