Nitric oxide and vascular remodeling

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NITRIC OXIDE AND VASCULAR REMODELING

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Promotores:

Prof. dr. H.J.J. Wellens

Prof. dr. M.J.A.P. Daemen

Co-promotor:

Dr. E.D. de Muinck

Beoordelingscommissie:

Prof. dr. A.P.G. Hoeks (voorzitter)

Prof. dr. C.E. Blanco

Prof. dr. M.M. Kocks (Universiteit Antwerpen, België)

Prof. dr. A.J. Rabelink (Universiteit Leiden)

Prof. dr. J. Waltenberger

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CHAPTER 1

Introduction

It is known that the endothelium is not simply an inert barrier between the blood and the vascular smooth muscle. In addition to its barrier function, the endothelium is actively involved in the regulation of vascular tone and blood flow, in maintaining a balance between blood fluidity and thrombosis, in modulating control by perivascular nerves and in the regulation of cell growth within the vessel wall. One of the most important molecules involved in these processes is nitric oxide (NO), which is constitutively produced by endothelial NO synthase (eNOS).

Hypercholesterolemia and atherosclerosis are associated with endothelial dysfunction which causes a shift in the balance between locally produced vasodilators, such as NO, prostacyclin, bradykinin and endothelium-derived hyperpolarizing factor(s) and constrictors such as endothelin. As a consequence, endothelial dysfunction is characterized by decreased vasodilation and increased vasoconstriction in response to various stimuli. Another hall mark of endothelial dysfunction is the loss of atheroprotective properties of the endothelial lining. Hence, persisting endothelial dysfunction will contribute to atherosclerotic plaque formation. One of the mechanisms that compensate for vessel wall thickening caused by plaque accumulation is outward remodeling of the vessel. When outward remodeling can no longer compensate for vessel wall thickening, luminal narrowing ensues causing impaired perfusion and oxygenation of the tissue down stream from the obstruction. When medical therapy is unsuccessful in alleviating the symptoms of tissue hypoxia, invasive strategies are mandated that either bypass the obstruction with an arterial or venous graft or abolish it by dilating the artery at the site of the obstruction. The surgical approach is termed coronary artery bypass graft (CABG) surgery and abolishment of the stenosis entails a catheter-based approach known as percutaneous intervention. Currently, stents are implanted in > 90% of percutaneous intervention cases. Vessel narrowing recurs (restenosis) after both surgical and percutaneous intervention and the new lesion is the result of a wound healing response triggered by the vascular trauma caused by CABG or percutaneous intervention. It is characterized by a combination of smooth muscle cell proliferation and inward remodeling.

In this thesis the main hypothesis is tested that NO inhibits the remodeling response after vascular injury. In addition the effects of two potentiators of NO

production are tested in the context of vascular injury and atherosclerosis. The specific hypotheses are that:

- endothelium-dependent flow-mediated outward remodeling is impaired in a hyperlipidemic mouse model with endothelial dysfunction.
- estrogen's ability to inhibit vascular remodeling after arterial injury is mediated via eNOS-derived NO.
- local eNOS-dependent delivery of NO inhibits vascular remodeling after arterial damage in hyperlipidemic mice (A) an effect that is fortified by systemic application of folate, a cofactor for eNOS enzyme (B).

NITRIC OXIDE

In 1980, Furchgott (Nobel prize 1998) and Zawadzki observed that the presence of an intact vascular endothelium is a prerequisite for acetylcholine-induced relaxation of smooth muscle cells.¹ Later this relaxation was shown to occur in a Ca^{2+} dependent manner via stimulation of the soluble isoform of guanylate cyclase. The endothelium-dependent relaxing factor (EDRF) was identified as the free radical gas NO, which is highly lipophilic and chemically reactive, allowing it to bind to a large variety of chemical moieties.²⁻⁴ It has become apparent that the simple molecule NO plays an important role in regulating many biological functions such as vascular tone,⁵ platelet activation⁶ and neurotransmission.^{7,8} Also, NO has been shown to be an important immunoregulatory molecule with cytostatic and cytotoxic properties.^{9,10} NO is a gas that is poorly soluble in water. It has a short half-life of approximately 10 to 60 seconds in tissue, because it readily reacts with oxygen, superoxide radicals, and transition metals such as iron and copper.^{11,12} Consequently, NO cannot function at a distance, when it enters the blood stream it combines rapidly with oxyhemoglobin to form methemoglobin and nitrate (NO_3^-).¹³ However, NO is particularly useful as an intracellular messenger because it can easily diffuse through most cells and tissues.¹⁴

NO-chemistry

NO is generated from the terminal guanidino-nitrogen of L-arginine by an N^ω -hydroxyl-L-arginine intermediate yielding citrulline.^{11,15} This reaction is catalyzed by a NO synthase (NOS):

NOS

NOS contains flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN)¹⁶ and NADPH and requires the presence of several oxidative cofactors, including tetrahydrobiopterin (BH_4),¹⁷ reduced glutathione,¹⁸ and a heme complex (Fe) (Figure 1).¹⁹ Three isoforms of NOS have been documented thus far, based on three different genes that have been cloned and sequenced. There are two isoforms of

Figure 1. The eNOS enzyme is a homodimer: each monomer has a reductase domain (FAD, FMN and NADPH). The reductase domain supplies electrons to the oxidase domain of the other monomer, where L-arginine is oxidized at the active site, which incorporates a heme group (Fe). Electron transfer is controlled by the binding of calcium-calmodulin (CaM) to a region between the reductase and oxidase domains. Tetra hydrobiopterin (BH₄) is required for homodimerization and modulates the heme redox state during electron transfer. eNOS is activated by agonists that bind specific G protein-coupled receptors (eg, bradykinin (BK)). These receptors are concentrated in specialized plasma membrane domains, caveolae. Caveolin binds eNOS in an inactive state. Shear stress activates eNOS by phosphorylation at serine 1179. Adapted from Channon et al.²⁷²

constitutive NOS: eNOS, constitutively expressed in vascular endothelial cells and cardiomyocytes, is membrane-bound and activated only when it binds to calmodulin in a reversible, Ca²⁺-dependent manner²⁰ and nNOS (neuronal NOS), present in brain tissue. The third isoform of NOS is the inducible form (iNOS) and is mainly found in macrophages and neutrophils.^{21, 22} It is activated by exposure to bacterial endotoxin or cytokines such as interleukin-1 (IL-1) or γ -interferon. Inducible NOS is regulated at the transcriptional level and not affected by intracellular [Ca²⁺].²³ eNOS is activated by agonists that bind specific G protein-coupled receptors (eg, bradykinin, acetylcholine, serotonin), leading to elevations in intracellular calcium and activation of calmodulin binding.^{24,25} Caveolin, the major structural protein of caveolae, binds eNOS in an inactive state. Caveolin and calmodulin binding are exclusive; activation releases eNOS from the caveolae. Shear stress activates eNOS by phosphorylation at serine 1179 through a phosphatidylinositol 3-kinase-dependent pathway, independent of calcium-calmodulin.²⁶

ANGIOPLASTY AS A TREATMENT FOR CORONARY HEART DISEASE

Atherosclerosis alters the normal structure and function of muscular arteries like the coronary arterial bed as a consequence of the cellular, mineral, fatty, and connective-tissue components that accumulate within the blood vessel wall.^{27,28} Any reduction of blood flow through an atherosclerotic coronary artery below that required by the oxidative metabolism of working heart muscle (myocardium) establishes the disease state of myocardial ischemia.²⁹ If sustained for just a few hours in humans, ischemia may severely compromise cardiac function. This will lead to the formation of an irreversible zone of infarction characterized by dead myocardial tissue.²⁹ Immediate reperfusion of ischemic myocardium by reestablishing the coronary blood flow is the only well-accepted means for preserving postischemic heart function.³⁰ To date, three reperfusion-based interventions have been used for treatment of coronary heart disease: coronary artery bypass grafting, thrombolysis, and percutaneous transluminal coronary angioplasty (PTCA).

CABG is a major surgical procedure that utilizes a vein (or artery graft) to bypass an atherosclerotic coronary obstruction and route blood from the aorta to the ischemic heart muscle at risk.³¹ The failure rate of venous grafts after 1 year approaches 20%.³² The development of neointima or atherosclerosis in the grafted vessel often leads to obliterative stenosis. A quest for less invasive alternatives to CABG supported administration of thrombolytic agents designed to dissolve acute occlusive thrombi, which precede most myocardial infarctions. Unfortunately, tolerance issues, bleeding complications³³ and ineffective reopening or reocclusion^{34,35} of the artery after reperfusion limit the effectiveness of thrombolysis.

In the most conventional form of PTCA, a collapsed, balloon-tipped catheter is passed across the stenosis under visual guidance. The balloon is then inflated with a fluid contrast solution to disrupt the plaque and enhance both luminal dilation and distal coronary perfusion.³⁶ Restenosis still occurs despite advances in treatment i.e. the use of arterial grafts for CABG and stents in percutaneous interventions and is characterized by proliferative and inward remodeling response (see introduction).

RESTENOSIS AND ITS PATHOBIOLOGY

Neointima formation is a characteristic response of vessels to several forms of injury. As such it is one of the processes that contribute to the development of restenosis after a successful balloon angioplasty of a primary stenosis. Restenosis is an occlusive vascular response to trauma characterized by lumen narrowing and is merely a reparative response that has gone on too long or to a greater extent than necessary. Balloon angioplasty stretches the artery wall; in an atherosclerotic vessel this elicits plaque cracking or splitting and exposes the vessel to overexpansion. Immediate consequences of balloon manipulation on the arterial wall include intimal tearing, endothelial cell damage, and exposure of subendothelial connective tissue to blood

Figure 2. Histologic cross-section of a typical proliferative lesion in a human coronary artery several months after PTCA. PL, primary lesion; SL, secondary lesion; OL, old lumen; NL, new lumen. Generously provided by Dr. RJ van Suylen.

components.^{37–41} In response to this trauma, restenosis evolves over several months postangioplasty, usually stabilizing by the sixth month.⁴² Stent implantation has reduced the restenosis rate to approximately 10% depending on vessel type, lesion location, vessel diameter and patient characteristics. However, since all known angioplasty techniques may damage the blood vessel wall, even the most procedurally successful percutaneous intervention invites restenosis.^{37,38}

The pathogenesis of restenosis has been studied most extensively in healthy laboratory animals as the response of normal arteries to mechanical injury.^{37–40,43–47} Complex mechanisms underlying the wound healing response characteristic of restenosis can be identified (Figure 2). These include disruption of the endothelium, platelet activation and thrombus formation, smooth muscle cell migration and proliferation, extracellular matrix changes and remodeling or ‘shrinkage’ of the artery. Disruption of the endothelium increases surface thrombogenicity, facilitates adhesion of inflammatory cells and results in impaired production of NO and other vasodilators that inhibit vasospasm and cellular proliferation.⁴⁸ Platelet activation and thrombus formation are associated with massive growth factor release from the activated platelets, activation of the clotting cascade with thrombin as a central player, and formation of a thrombus/fibrin matrix which serves as a temporary skeleton for smooth muscle migration.^{49,50} Vascular smooth muscle cell (VSMC) migration and proliferation are facilitated by the loss of the antimigratory effect mediated by intact endothelium, growth factor release by platelets and, later on, in an autocrine fashion by smooth muscle cells as well as extracellular matrix degradation and alteration of its composition.^{48,51,52} Accelerated formation of extracellular matrix is a major contributor of restenosis. Before injury, the smooth muscle cell is

Figure 3. Diagram indicating NO's antiatherosclerotic effects in atherosclerosis. Dotted lines indicate an inhibitory effect. L-Cit, L-citrulline; ICAM-1, intercellular adhesion molecule-1. Reprinted from Laufs.

embedded in a dense fibrous extracellular matrix. After injury, this is replaced by fibroproliferative tissue which initially has a loose, myxoid appearance but shows an increase in density over time, associated with an increase in collagen content.⁵³ The smooth muscle cell actively synthesises proteolytic enzymes, glycoproteins and proteoglycans that account for these matrix changes.⁵⁴ In human restenotic lesions, the volume of a typical neointima mainly exists of extracellular matrix, while approximately 10% of the volume consists of cells.^{50,55} Finally, there is evidence that negative remodeling or 'shrinkage' of the artery without an increase in media and intima volume contributes to luminal loss after angioplasty.⁵⁶

NO IN PATHOLOGICAL VASCULAR REMODELING AND CELLULAR PROLIFERATION

Because restenosis involved both proliferation and inward remodeling NO is the ideal candidate for therapeutic intervention. NO produced in the endothelium rapidly diffuses to interact with molecular targets in cells in the vascular wall and lumen.² NO interacts with thiol groups and with metal centers in diverse protein targets, including membrane receptors, G proteins, ion channels, cytosolic enzymes, and transcription factors such as activator protein-1 and nuclear factor- κ B (reviewed in reference⁵⁷). S-Nitrosylation of thiol groups in plasma proteins such as albumin generates a circulating "pool" of NO-donating groups,⁵⁸ whereas S-nitrosylation of

hemoglobin in the lung provides nitrosothiol groups to the peripheral vasculature and regulates oxygen delivery.^{59,60} In the vascular wall, NO activates soluble guanylate cyclase in VSMCs, leading to elevation of cGMP, activation of cGMP-dependent protein kinase (PKG), and vasorelaxation, the primary basis for blood flow and pressure regulation.⁶¹ In addition to regulating vascular tone, a substantial body of evidence suggests that NO has important antiatherogenic effects^{62, 63} (Figure 3). The anti-atherogenic effects of NO are listed below and are all potentially beneficial in the prevention of restenosis as well.

1. antiplatelet effects: NO inhibits platelet adhesion and aggregation⁶ and thrombin-induced expression of platelet-activating factor.
2. antiproliferative effects: NO donors potently inhibit VSMC proliferation,^{64,65} migration,⁶⁶ and extracellular matrix synthesis.⁶⁷
3. anti-inflammatory effects: atherogenic stimuli stimulate endothelial expression of adhesion molecules and chemokines, leading to inflammatory cell recruitment.⁶⁸ NO donors inhibit activation and nuclear translocation of nuclear factor- κ B,^{69,70} block cytokine-stimulated endothelial adhesion molecule expression (VCAM-1),⁷⁰ and reduce adhesion and activation of neutrophils and monocytes.⁷¹ In particular, inhibition of monocyte chemotactic protein-1 (MCP-1) expression appears to be an important aspect of NO's anti-inflammatory effect in atherosclerosis.^{72, 73}
4. antioxidant effects: unmodified LDL undergoes oxidative modifications in the vessel wall that render it highly atherogenic (Figure 3).⁷⁴ Continuous, low-level NO production may directly inhibit lipid oxidation by scavenging free radicals.⁷⁵

From the foregoing, it is clear that NO is an important determinant of vascular homeostasis. As the vascular response to injury involves many of the above mentioned processes in which NO acts as a key modulator, NO may also be a unique and potentially powerful candidate to control the remodeling process.

THE ROLE OF HEMODYNAMIC SHEAR STRESS IN ATHEROSCLEROSIS AND VASCULAR REMODELING

Hemodynamic shear stress, which is the frictional force acting on the endothelial cell surface as a result of blood flow, has been proposed as a factor regulating blood vessel structure^{76, 77} and influencing the development of vascular pathology such as atherosclerosis,^{78–80} aneurysms,⁸¹ poststenotic dilations,⁸² and arteriovenous malformations.⁸³ Despite its associated risk factors, atherosclerosis is a geometrically focal disease that has a propensity to involve the inner edges of blood vessels bifurcations.^{80, 84, 85} In these susceptible areas, blood flow is slow, resulting in a low hemodynamic shear stress. In contrast, vessel regions that are exposed to steady blood flow and higher shear stress are less affected.^{79, 80, 84–87} Animal, molecular, and cellular studies of the endothelium's response to hemodynamic shear stress have

provided new insights into its possible contribution to the pathogenesis of atherosclerosis.^{88–93}

The luminal surface of the blood vessel and its endothelial surface are constantly exposed to hemodynamic shear stress.⁸⁸ In numerous experiments, shear stress has been shown to actively influence vessel wall remodeling.^{76,77,94} Changes in blood flow drive both acute and long term compensatory responses that ultimately result in normalization of wall shear stress. In the short term, vessels either dilate or constrict, usually due to the local release of vasoactive peptides, to quickly accommodate changes in blood flow. In the case of persistent increases or decreases in flow, however, a different process evolves that includes adaptive remodeling of the vessel wall, characterized by the reorganization of cellular and extracellular components. This shear stress stabilizing process is dependent on intact endothelial function and is abolished by prior selective destruction of the endothelial monolayer.⁷⁷

An acute increase in blood flow generates a dynamic vascular response resulting in dilation mediated by the endothelial release of NO,^{1,3} prostacyclin⁹⁵ and endothelial derived hyperpolarizing factor⁹⁶ (EDHF) of which the exact nature remains to be determined. A chronic increase in flow causes structural changes involving proliferative responses and matrix degradation.^{97,98} These alterations of vascular structure could be prevented by L-NAME⁹⁸ indicating a role for NO. This has further been established in endothelial NO synthase knockout mice which lack compensatory arterial remodeling in response to increased flow.⁹⁹ Elevated shear stress has been shown to increase the production of growth factors like platelet-derived growth factor¹⁰⁰ (PDGF) and fibroblast growth factor-2^{101,102} (FGF-2). It has also been shown that NO promotes endogenous FGF-2 production in endothelial cells.¹⁰³ In order to facilitate arterial distensibility and structural remodeling changes in matrix formation must take place. Wong and Langille¹⁰⁴ demonstrated enlarged fenestrae in the internal elastic lamina (IEL) of flow-loaded rabbit carotid arteries. Matrix metalloproteinases (MMPs) are likely the mediators of IEL degradation in the vessel wall. It has been reported that increased shear stress might stimulate MMP production by endothelial cells and that these cells may secrete factors that trigger MMP production by SMCs. These latter cells are capable of producing MMPs in response to cytokines¹⁰⁵ or growth factors, like FGF-2.¹⁰⁶ NO or peroxynitrite directly,^{107,108} or indirectly¹⁰⁹ via induction of the plasminogen/plasmin system to activate MMPs. Chronic inhibition of NO production by L-NAME prevented MMP-2 activation in flow-loaded carotid arteries and also decreased vascular remodeling,⁹⁸ demonstrating that NO (or peroxynitrite) is involved in blood flow-induced vascular remodeling. Increased MMP expression and activity were associated with the development of neointimal arterial lesions and SMC migration after arterial balloon injury in experimental models, whereas MMP inhibition decreases SMC migration in vitro and in situ.^{110–113} In summary, a large body of evidence suggests an important regulatory role for NO in the adaptive struc-

tural changes that occur in the presence of chronic flow increases, both at the level of vascular smooth muscle cell proliferation and extra-cellular matrix degradation.

APPROACHES TO INHIBIT THE VASCULAR RESPONSE TO INJURY USING NO AND NO SURROGATES

NO gas

Two animal studies revealed that inhalation of NO gas inhibited neointimal thickening after vascular damage.^{114,115} However, since hemoglobin inactivates NO with a blood half-life of ~100 ms,¹¹⁶ reduction of neointimal formation following NO inhalation may be a nonspecific response. Furthermore, inhalation of NO for over a few hours may damage mammalian lung tissue.

NOS-related

L-arginine. The NOS substrate, L-arginine, has antiatherogenic properties in laboratory animals and humans. Supplemental L-arginine restores endothelium-dependent vascular function in hyperlipidemic animals¹¹⁷⁻¹²¹ and humans¹²²⁻¹²⁴ and inhibits atherosclerosis in rodents.^{125, 126}

A large body of evidence reports reduction of neointimal formation by exogenous L-arginine application after vascular injury in healthy and hyperlipidemic rodents.¹²⁶⁻¹²⁸

The arginine paradox refers to the discordance between observations made *in vitro* and those made *in vivo* regarding the sensitivity of NO synthesis to L-arginine availability. L-arginine should not be rate-limiting for NO synthesis as the K_m of NOS for L-arginine in a cell-free system is in the micromolar range,¹²⁹ while plasma L-arginine concentration is in the 50 micromolar range and endothelial cell L-arginine concentration is in the millimolar range. However, in hypercholesterolemic plasma levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS, are elevated. The presence of an endogenous inhibitor in plasma might explain the observation that L-arginine administration in hypercholesterolemic animals and patients results in improved endothelium-dependent vasodilation.¹³⁰⁻¹³²

NOS potentiators

Selected chemical agents and physical stimuli have been reported to potentiate NO synthesis by cytokine-stimulated vascular endothelium and/or smooth muscle. These are a hypoglycemic agent (troglitazone),¹³³ an antithrombotic drug (ticlopidine),¹³⁴ angiotensin converting enzyme (ACE) inhibitors (cilazapril and fosinopril),¹³⁵ a cyclic adenosine monophosphate (cAMP) phosphodiesterase III inhibitor (cilostazol),¹³⁶ a nonselective protein kinase C inhibitor (staurosporine),¹³⁷ homocysteine,¹³⁸ adenosine,¹³⁹ and 8-bromo-cGMP.¹⁴⁰

Two NOS potentiators relevant to this thesis, estrogen and folate, will be discussed briefly.

The incidence of atherosclerotic disease is low in premenopausal women, rises in postmenopausal women, and is reduced to premenopausal levels in postmenopausal women who receive estrogen therapy.^{141–143} Despite a wealth of supportive observational data, however, the results of randomized clinical trials are mixed.^{144–146} One of the most recent trials showed no cardiovascular benefit but instead a potential for increased harm with a trend towards more rapid angiographic progression of coronary disease in the hormone replacement therapy (HRT) group and a significant increase in death and myocardial infarction in this group.¹⁴⁶ Another worrisome effect of HRT is the increased risk of breast cancer that is associated with this type of treatment.¹⁴⁷ Mechanistically, the potentiating effect of estrogen on NO production remains intriguing and should be pursued experimentally, while clinical scenarios and pharmacologic regimens are being developed that should make HRT safe and effective.

Estrogen's protection against atherosclerosis was thought to be related to its effects on serum lipid concentrations,¹⁵⁰ but this protective effect accounts for only one third of estrogen-induced protection.^{143,148,149} Besides its effect on serum lipid concentration, estrogen alters coagulation and fibrinolytic systems,^{151–153} antioxidant systems,^{154–159} and the production of other vasoactive molecules,^{160–163} such as NO and prostaglandins, all of which can influence the development of vascular disease.

Reviews of the data suggest that the direct actions of estrogen on blood vessels contributes substantially to the cardiovascular effects of estrogen.^{148,149,164}

Estrogen action on blood vessels can be divided into rapid, short-term and longer-term effects.

-Rapid, nongenomic effects

In normal blood vessels, the endothelium releases NO in response to various stimuli, which causes vasodilation.¹⁶⁵ In cell culture studies physiologic concentrations of estrogen activate NOS, which can be blocked by estrogen receptor (ER)- α antagonists.^{166–168} Estrogen rapidly causes coronary vasodilation *ex vivo*^{149,164} and *in vivo* in cholesterol-fed ovariectomized primates¹⁶⁹ and other animals.¹⁷⁰ Estrogen dilates coronary and brachial arteries in postmenopausal women^{171–176} and, in some studies, in men.^{175,177,178} The short-term coronary vasodilatory effects of estrogen in humans are largely mediated by the increased production of NO.¹⁷⁶

-Longer-term effects

Estrogen increases the expression of genes for important vasodilatory enzymes such as prostacyclin synthase and NOS.^{179,180} Estrogen also increased NO bioavailability by increasing the expression of inducible NO synthase gene in endothelium-denuded rat aorta.¹⁸⁰ Long-term administration of estrogen increased acetylcholine-mediated coro-

nary vasodilation in nonhuman primates,^{181,182} postmenopausal women¹⁸³ and postmenopausal women with angina and normal coronary arteries.¹⁸⁴

-Estrogen and vascular remodeling

Estrogen facilitates reendothelialization after vascular injury which may be due to increased local expression of vascular endothelial growth factor.¹⁸⁵ Early restoration of endothelial integrity by estrogen may contribute to the attenuation of the response to injury by increasing the availability of NO, which can directly inhibit the proliferation of smooth muscle cells.¹⁸⁶ Estrogen itself has also been shown to directly inhibit the migration and proliferation of smooth muscle cells in vitro.^{187,188}

Estrogen not only protects against atherosclerotic lesion formation, but it also inhibits the formation of vascular lesions induced by mechanical injury.^{149,164,189-196} The role of vascular estrogen receptors in the protection against mechanical injury was shown in normocholesterolemic mice disrupted of ER- α and ER- β . In these models, protection was lost against some, but not all, vascular injury endpoints. This raised the possibility that either a third ER exists or that the ER- α was only partially disrupted. The latter seemed to be true; in a newly created mouse with complete disruption of ER- α estrogen no longer protected against vascular injury.¹⁹⁷ The role for ER- α in atheroprotection was confirmed in apoE deficient mice in which the ER- α gene also had been silenced.¹⁹⁸

-Folates

The folate tetrahydrobiopterin (BH₄) is required as a cofactor for NOS activity: all NOS isoforms require tetrahydrobiopterin for NOS homodimerization and for electron transfer during arginine oxidation.¹⁹⁹ Production of NO by endothelial cells requires adequate tetrahydrobiopterin levels,²⁰⁰ and NOS does not generate NO in the absence of tetrahydrobiopterin; exogenous tetrahydrobiopterin restores enzyme activity.²⁰¹ More importantly, the enzymatic activity of NOS shifts towards the generation of superoxide rather than NO when tetrahydrobiopterin is lacking.²⁰² Tetrahydrobiopterin levels are decreased in the diabetic rat aorta and underlie the observed reduction in NO production.²⁰³ Exogenous tetrahydrobiopterin partially restores endothelial NO production and reduces NOS-dependent superoxide generation in hypertension,^{204,205} hypercholesterolemia,²⁰⁶ and smokers,²⁰⁷ whereas inhibition of tetrahydrobiopterin synthesis impairs NO-mediated vasorelaxation.²⁰⁸ Thus, tetrahydrobiopterin appears to be an important factor in modulating NOS activity in vascular disease. Furthermore, tetrahydrobiopterin levels in atherosclerotic vessels or in vessels in which the endothelium has been damaged by mechanical injury may augment the beneficial effects of NO in these settings. From this it might be clear that endothelial NOS has a dual role in atherosclerosis; under normal conditions it produces low amounts of NO favoring an antiatherosclerotic milieu. On the other hand, in the diseased state, like hyperlipidemia and atherosclerosis, it may get uncoupled resulting in more superoxide and less BH₄-dependent

NO production.²⁰⁹ 5-methyltetrahydrofolate (5-MTHF), the main form of folate in the circulation, improved endothelial function that was found to be BH₄-dependent²¹⁰ in familial hypercholesterolemia.²¹¹ Several putative underlying working mechanisms for the effects of 5-MTHF on the enzymatic activity of eNOS have been suggested. 5-MTHF might facilitate electron transfer by BH₄ from the reductase domain of eNOS to heme,²¹² enhancing the binding of BH₄ to eNOS, increasing BH₄ availability via chemical stabilization of BH₄²¹³ or stimulate the regeneration BH₄ from the inactive oxidized state.²¹⁴

NO donors

In contrast to NO donors that generate NO directly,^{215–217} compounds^{218, 219} that require either biotransformation or an intermediate to yield NO have been essentially ineffective against arterial injury in normocholesterolemic animals. Recently, beneficial effects of NO-releasing aspirin on postinjury neointimal formation in normo-^{220, 221} and hypercholesterolemic^{222–224} animals have been published.

NOS gene transfer

Gene transfer vectors allow targeted expression of genes in cells in the vessel wall, provide tools to investigate the function of specific genes in disease processes, and may potentially lead to gene therapy strategies for vascular disease.²²⁵ For NOS gene transfer, adenoviral vectors have been most widely used, with a smaller number of studies using high-efficiency plasmid-liposome gene delivery and incorporating coat proteins from hemagglutinating virus of Japan. Whereas adenovirus results in the highest efficiency of gene transfer, liposome-mediated gene transfer is a virus-free system with little host immune response and potentially longer transgene expression. Because NO diffuses from NOS-targeted cells to surrounding cells, very high-efficiency transduction may not be an absolute requirement to exert a biological effect. Experimental studies of NOS gene transfer initially evaluated the expression and activity of recombinant NOS isoforms in cultured cells,²²⁶ then in isolated vascular rings after *ex vivo* infection and culture,^{227–234} and finally in several *in vivo* models of experimental gene therapy.^{235–248} (Table 1)

Table 1. Studies of experimental vascular gene therapy with NOS

Vessel	Species	NOS isoforms		Vector	End points
Carotid artery Adventitia	Rabbit	eNOS	<i>Ex vivo</i>	Ad	Restored/augmented vasorelaxation ^{227, 228}
+Diabetes	Rabbit	eNOS	<i>Ex vivo</i>	Ad	Restored vasorelaxation ²⁵²
Aorta	Rabbit	enos	<i>Ex vivo</i>	Ad	Increased vasorelaxation ²²⁹
+Cholesterol					
+Ang II hypertension	Rabbit	eNOS	<i>Ex vivo</i>	Ad	Restored vasorelaxation ²⁵¹
	Mouse (eNOS-KO)	eNOS	<i>Ex vivo</i>	Ad	Restored vasorelaxation ²⁴⁹
Coronary artery	Pig	eNOS	<i>Ex vivo</i>	Ad	Increased vasomotor relaxation ²³⁰
Basilar artery	Dog	eNOS	<i>Ex vivo</i>	Ad	Increased cGMP, increased vasorelaxation ^{231, 234}
Radial artery	Human	eNOS	<i>Ex vivo</i>	Ad	Reduced vasospasm ²³²
Saphenous vein	Human	eNOS	<i>Ex vivo</i>	Ad	Recombinant NOS ²³³
Carotid artery ± Cholesterol	Rabbit	nNOS eNOS	<i>In vivo</i>	Ad	Increased NO production ²⁴⁰ Reduced endothelial activation and inflammation ²⁴²
Adventitia	Rabbit	eNOS	<i>In vivo</i>	Ad	Restored/augmented vasorelaxation ^{243, 244}
+Balloon injury	Rat	eNOS	<i>In vivo</i>	HVJ	Increased NO production, increased vasorelaxation, reduced intimal hyperplasia ²³⁶
	Rat	eNOS	<i>In vivo</i>	Ad	Increased NO production, reduced intimal hyperplasia ²³⁵
	Rat	eNOS	<i>In vivo</i>	Ad	Reduced intimal hyperplasia, increased paxillin expression ²³⁹
+Hypertension	Rat	eNOS	<i>In vivo</i>	Ad	Restored vasorelaxation ²⁵⁰
Aorta	Rat	iNOS	<i>In vivo</i>	Ad	Reduced intimal hyperplasia ²³⁷
Iliac artery	Pig	iNOS	<i>In vivo</i>	Ad	Reduced intimal hyperplasia ²³⁷
Femoral artery	Rat	eNOS	<i>In vivo</i>	Ad	Restored vasorelaxation ²⁴⁸
+Heart failure					
Coronary artery +balloon injury	Pig	eNOS	<i>In vivo</i>	Ad	Increased cGMP, reduced intimal hyperplasia ²³⁸
Cerebral arteria via CSF	Dog	eNOS	<i>In vivo</i>	Ad	Increased cGMP, increased vasorelaxation ²⁴¹
Aortic allograft	Rat	iNOS	<i>In vivo</i>	Ad	Reduced allograft arteriosclerosis ²⁴⁵
Vein graft	Dog	eNOS	<i>In vivo</i>	HVJ	Reduced intimal hyperplasia ²⁴⁶
	Rabbit	nNOS	<i>In vivo</i>	Ad	Reduced inflammation, reduced intimal hyperplasia ²⁷⁴
Pulmonary artery	Rat	eNOS	<i>In vivo</i>	Ad	Increased NO, increased cGMP, reduced hypoxic constriction ²⁴⁷

Ang II indicates angiotensin II; CSF, cerebrospinal fluid; Ad, adenovirus; HVJ, hemagglutinating virus of Japan liposomes.

The ability to restore endothelial vasomotor function in atherosclerotic or mechanically injured arteries represents a critically important test of NOS gene transfer. *Ex vivo* eNOS gene transfer to isolated vascular rings from normal or hypercholesterolemic rabbit carotid arteries or aorta,²²⁹ canine basilar arteries,²³¹ porcine coronary arteries,²³⁰ or human saphenous veins²³³ improves vascular relaxations to a variety of vasoactive agonists, such as calcium ionophore, acetylcholine, or UTP. *In vivo* gene delivery of eNOS or nNOS confirms that NOS gene transfer can restore or augment regulated NO-mediated vasorelaxation in several settings, including normal,^{240, 242, 243} atherosclerotic,²⁴⁰ balloon-injured,²³⁶ or eNOS-deficient²⁴⁹ arteries. Moreover, eNOS gene transfer reduces the vasoconstrictive response to hypoxia in rat lungs²⁴⁷ and restores NO-mediated endothelial function in models of cardiac failure,²⁴⁸ hypertension^{250,251} or diabetes,²⁵² demonstrating that the functional effects of NOS gene transfer are not limited to pharmacological agonist stimulation *ex vivo*, nor to specific models of endothelial dysfunction such as hypercholesterolemia.

Exogenous NO, regardless of the route and mode of administration increases intracellular cGMP, inhibit VSMC proliferation,⁶⁴ VSMC migration,⁶⁶ and collagen synthesis,^{67,253} all key components of intimal hyperplasia. Exogenous NO-donating compounds such molsidomine⁶⁵ or L-arginine²⁵⁴ also reduce intimal hyperplasia in vascular injury models *in vivo*. These results suggest that endothelial NO may limit VSMC proliferation and neointimal formation *in vivo*. Gene transfer studies provide direct evidence that local augmentation of vascular NO production by NOS isoforms inhibits intimal hyperplasia in several models of vascular injury: balloon-injured rat^{236,237,239} or pig²³⁸ arteries, canine venous bypass grafts²⁴⁶ and rat aortic allografts²⁴⁵ and mechanical injured mouse arteries. The effects of NO on cell proliferation and vascular remodeling both contribute to the attenuation of neointima formation in these models. Inhibition of VSMC proliferation, has been demonstrated by reduced incorporation of bromodeoxyuridine and proliferating cell nuclear antigen staining²³⁵. Changes in VSMC cell phenotype²³⁹ and cell cycle inhibition have also been observed.^{255,256} In addition, NOS gene transfer inhibits VSMC migration and matrix remodeling.²⁵⁷ Additional mechanisms include increased VSMC apoptosis,²⁵⁸ reduced inflammation or platelet activation,²⁵⁹ and promotion of endothelial regrowth.

ANIMAL MODELS FOR INTIMAL THICKENING

In order to unravel the mechanisms of intimal thickening in response to mechanical injury there is a need for appropriate animal models. Intimal thickening occurs in all arteries as a response to a variety of different mechanical traumata, regardless of animal species. Those injuries include such techniques as denudation of endothelial cells by the gentle passage of a balloon embolectomy catheter, the distension of the artery with a balloon angioplasty catheter, stent implantation, a rotating wire,²⁶⁰

photochemical reaction,^{261, 262} electrical stimulation,²⁶³ compression,²⁶⁴ by blowing a stream of air through the artery,^{265, 266} or by a perivascular collar.^{267–270} These strategies may be combined with a high lipid diet to investigate the combined effect of perturbation of vessel homeostasis by hyperlipidemia and mechanical injury.

In regard to this thesis the mouse carotid artery denudation model, developed by Lindner,²⁷¹ will be discussed briefly.

Mouse carotid artery denudation model

In the mouse carotid artery denudation model an intact mouse carotid artery is exposed via a midline incision on the ventral side of the neck. Two ligatures are placed around the external carotid artery, which is tied off with the cranial ligature. The internal carotid artery is temporarily clipped off to prevent back bleeding. A puncture is made between the two ligatures to introduce the denudation guide wire. A curved stainless flexible wire (0.35 mm diameter) is introduced via the incision in the external carotid artery into the common carotid artery and under rotation the wire is passed along the vessel three times. The wire is then removed, and the external carotid artery tied off caudal to the incision hole with the caudal ligature. The internal carotid artery is declipped. For intra-arterial applications of drugs a catheter can be introduced via the incision into the external carotid artery. After closure of the skin incision the animals are allowed to recover for several weeks to evaluate several morphometric endpoints.

In this model the passage of the wire removes all endothelial cells from the injured arteries and the denuded surface is covered with a monolayer of platelets adhering to the exposed subendothelial matrix. Maximal replication of medial and intimal SMC is observed 5 and 8 days after injury and intimal and medial areas are significantly increased two weeks after injury. Complete reendothelialization of the vessel segment (approximately 9 mm in length) occurs within 3 weeks.²⁷¹

Aim of this thesis

The aim of this thesis is to investigate the role of NO in vascular remodeling. The effect of NO on vascular responses to chronically increased flow and mechanical injury are studied in normo- and hyperlipidemic settings. In addition, the effects of the NOS potentiators estrogen and 5-methyltetrahydrofolate (5-MTHF) are evaluated either as adjuvant therapy to adenoviral eNOS gen transfer or as stand alone interventions (estrogen).

In chapter 2 we use the unilateral carotid artery ligation model in hypercholesterolemic atherosclerotic apoE-deficient mice to assess whether acute dynamic responses to agonists and flow alterations are predictive of chronic structural vascular changes in response to increased flow. We characterize agonist induced endothelial responses in a myograph and acute flow-induced responses in an arteriograph. We hypothesize that in a model of endothelial dysfunction, caused by hypercholesterol-

emia, acute flow-induced dilations and outward remodeling to increased shear stress are reduced.

In chapter 3, 4 and 5 we use the mouse carotid artery denudation model as a way to evaluate the structural response to mechanical vascular injury. With the use of this model we examine the protective effects and underlying mechanisms of estrogen, a NOS-potentiator, on remodeling after vascular damage in wild type mice and in mice in which either the iNOS gene or the genes encoding both estrogen receptors have been silenced. We hypothesize that estrogen protection is mediated via a NO-dependent pathway and that ER- α and ER- β are able to complement one another such that each receptor alone is sufficient to mediate estrogen's protection.

In chapter 6, we use carotid artery injury to remove the endothelium as a source for eNOS in hyperlipidemic apoE deficient mice with subsequent rescue by local delivery of adenoviral eNOS cDNA alone or in combination with systemic folic acid administration. We hypothesize that restoration of NO production at the site of injury will result in less intimal thickening. Furthermore, we hypothesize that adjunct systemic folic acid delivery will augment the effect of NO on inhibiting neointimal thickening, since 5-MTHF, the active form of folic acid, has been demonstrated to completely restore endothelial function in patients with hypercholesterolemia.

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CHAPTER 2

Reduced endothelium-dependent relaxations and impaired acute flow-induced vasodilation do not compromise chronic flow-related arterial remodeling in hyperlipidemic mice

Henny M. Schulten, Rob H.P. Hilgers, Paul M.H. Schiffrers, Agnieszka Strzelecka, Gregorio E. Fazzi, Ebo D. de Muinck, and Jo G.R. De Mey.

Abstract

Background – We sought to determine whether mild and severe hyperlipidemia which are associated with endothelial dysfunction are accompanied by an impairment of both acute flow-induced dilation and flow-related arterial remodeling.

Methods and Results – *Ex-vivo* agonist- and flow-induced vasomotor responses were determined in carotid arteries of 6 months old apolipoprotein E deficient mice (apoE^{-/-}) on a Western diet and in wild type (WT) mice on a Western diet in comparison to the responses in WT animals. Structural parameters were measured in carotid arteries of sham-operated animals and in carotid arteries of animals four weeks after unilateral carotid artery ligation. Plasma cholesterol levels were more than 10- and 5-fold increased in respectively apoE^{-/-} and WT both maintained on Western type diet as compared to WT on normal chow. Acetylcholine-induced relaxations were reduced in carotid artery segments of apoE^{-/-} on diet and WT on diet. Indomethacin normalized the acetylcholine-induced relaxations. Flow-induced dilation of isolated carotid arteries was reduced in apoE^{-/-} on diet as compared to both WT and WT on diet at all flow rates ($P < 0.01$). In apoE^{-/-} on diet, indomethacin increased flow-induced dilation at high flow rates. In WT on diet, indomethacin reduced flow-induced dilation at low flow rates ($P < 0.05$), but not at high flow rates. Media cross-sectional area of the carotid artery was significantly smaller in apoE^{-/-} on diet and WT on diet than in WT on normal chow. Unilateral carotid artery ligation stopped blood flow in the ipsilateral vessel and resulted in a significant increase of blood flow in the contralateral vessel. This increase in blood flow was significantly smaller in apoE^{-/-} on diet and WT on diet. In the chronically hyperperfused contralateral arteries outer diameter was significantly increased in all experimental groups. In the occluded artery, outer diameter was markedly and comparably reduced after 4 weeks in all experimental groups. This inward remodeling was accompanied by marked media hypertrophy in apoE^{-/-} on diet and WT on diet while no media hypertrophy was seen in WT on normal chow.

Conclusions – In hyperlipidemic mice chronic flow-related arterial remodeling is preserved despite reduced endothelium-dependent relaxations and blunted acute flow-induced dilation.

INTRODUCTION

Arteries respond to chronic alterations in blood flow by structural luminal diameter adjustments in order to normalize wall shear stress.^{1, 2} In general, chronic blood flow reductions lead to a narrowing of lumen diameter, while chronically elevated blood flow results in a widening of arteries.^{3–6} Flow-related remodeling occurs in skeletal muscle arteries during endurance training⁷ and in uterine arteries during pregnancy.⁸ Also, in pathological situations, flow-related remodeling is involved, for instance, in coronary collateral arteries after ischemia of the heart.⁹ Outward remodeling has been proposed to represent a natural defense mechanism that prevents the impact of a growing neointima or atherosclerotic plaque on blood flow.^{10, 11} Arterial remodeling in response to imposed, developmental or physiological changes in blood flow was demonstrated to be endothelium-dependent.^{5, 12}

On an acute basis, the endothelium responds to increases in blood flow by releasing vasoactive compounds, such as nitric oxide (NO),¹³ prostacyclin,¹⁴ bradykinin¹⁵ and endothelium-derived hyperpolarizing factor (EDHF).¹⁶ It has been proposed that sustained flow-induced dilation ultimately gives rise to structural arterial wall adjustments. In a broad variety of conditions, such as hypercholesterolemia, acute endothelium-dependent vasomotor responses to agonists and blood flow were found to be reduced as a result of decreased bioavailability or functional antagonism of endothelium-derived nitric oxide.^{17–20} Whether an altered endothelium-dependent reactivity predisposes to abnormalities in flow-related remodeling is unclear.

We hypothesized that during hypercholesterolemia flow-related remodeling is decreased because of endothelial dysfunction. To address this issue, endothelial (dys)function was evaluated in young adult (6 months) wild type (WT) mice on a normal diet, wild type mice on high fat “Western-type” diet and apolipoprotein E-deficient (apoE^{-/-}) mice on diet. Arterial remodeling in response to altered blood flow was studied after unilateral carotid artery ligation. Functional and structural analyses were concentrated on the central part of the common carotid arteries which was devoid of neointima and atherosclerotic plaques in all experimental groups. In this study we document (1) reduced endothelium-dependent relaxations in apoE^{-/-} on diet and WT animals on diet (2) impaired acute flow-induced dilation in severely hyperlipidemic apoE^{-/-} mice on diet and altered acute flow-induced dilation in mildly hyperlipidemic WT on diet and (3) comparable structural arterial diameter responses to chronically altered blood flow in the different strain and diet groups.

MATERIALS AND METHODS

ANIMALS

Procedures used in these experiments were approved by the University of Maastricht Animal Care and Ethical Committee. We studied C56BL/6J mice (WT) and homozygous apoE-deficient mice (with a 129xC57BL/6J background and backcrossed 9 generations on a C57BL/6J background; Charles River Laboratories, the Netherlands). Mice had free access to tap water and were maintained from weaning on either regular mouse chow (Hope Farms, Woerden, the Netherlands) or on a high fat “Western-type” of diet (15% cacao butter, 2% cholate, 0.25% cholesterol, 40.5% sucrose, 10% corn starch, 1% corn oil and 5.95% cellulose; Hope Farms, Woerden, the Netherlands).

LIPID PROFILE

Plasma cholesterol and triglyceride levels were determined in duplicate by using a colorimetric assay (CHOD-PAP 1442341 and GPO-PAP 701912, respectively, La Roche) in arterial plasma samples obtained at 6 months of age.

UNILATERAL CAROTID ARTERY LIGATION

Male wild type mice on normal chow (WT, $n=24$), male wild type on a high fat diet (WT on diet, $n=24$), and male apoE^{-/-} on a high fat diet (apoE^{-/-} on diet, $n=24$) were anesthetized with ketamine/xylazine (100 and 10 mg/kg s.c.). At an age of 5 months, both common carotid arteries were exposed through a midline incision in the neck. In half of each experimental group, the left carotid artery was ligated with 5.0 surgical suture just caudal to the carotid bifurcation as described (Figure 1).^{2,3} In the other animals a sham operation was performed in which mice were operated as described above omitting the left carotid artery ligation. Only two animals (1 WT on diet and 1 apoE^{-/-} on diet) died after the ligation procedure.

BLOOD FLOW MEASUREMENTS

For weeks after sham surgery or unilateral carotid artery ligation animals were anesthetized again with ketamine/xylazine and body temperature was maintained at 37.5°C by a thermostatically controlled heating platform. Both the left and right carotid artery blood flow were recorded. We used a transit-time ultrasonic flow-probe (0.5 mm V-series, Transonic Systems, Ithaca, NY, USA) that was mounted on a micro-manipulator positioned halfway between the aortic arch and the carotid artery bifurcation. The pulsatile blood flow signal was recorded and

Figure 1. Scheme of experimental setup. Unilateral left carotid artery (LCA) ligation was performed at an age of 5 months in apoE-deficient mice maintained on a Western type diet (apoE^{-/-} on D), wild type on D (WT on D) and WT on normal chow (WT on NC). For weeks after carotid artery ligation mice *in vivo* blood flow measurements were performed and mice were sacrificed with subsequent *ex-vivo* measurements as indicated in scheme.

mean blood flow was obtained by averaging during a 5-minute period for both the left and right carotid artery of each animal.

ACUTE AGONIST-INDUCED VASOMOTOR RESPONSES

Two-millimeter segments of the freshly isolated carotid arteries were mounted (steel wires, diameter 40 μ m) in a myograph organ bath (Danish Myotechnology Aarhus, Denmark) for isometric tension measurements. Organ baths were filled with a Krebs-Ringer bicarbonate buffer (composition in mmol/L: NaCl, 118.5; KCl, 4.7; MgSO₄·7H₂O, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25.0; CaCl₂, 2.5; glucose, 5.5), maintained at 37°C, and aerated with 95% O₂ and 5% CO₂. The arterial segments were stretched until maximal contractile responses to 63 mmol/L potassium solution were achieved (optimal diameter). After 30 minutes of equilibration, concentration-response curves for phenylephrine (Phe) (10⁻⁸ to 10^{-4.5} mol/L, half log units) were constructed and relaxing effects of ACh (10⁻⁸ to 10⁻⁵ mol/L, half log units) and Na-nitroprusside (SNP) (10⁻¹⁰ to 10^{-4.5} mol/L, half log units) were evaluated. Parallel experiments were performed in the absence and presence of 100 μ mol/L N^G-nitro-arginine-methyl ester (L-NAME) or 10 μ mol/L indomethacin. All drugs were obtained from Sigma Chemical Co (St. Louis, MO, USA).

ACUTE FLOW-INDUCED ARTERIAL DILATION

Carotid arteries of sham operated animals were isolated and placed in a thermostated bath containing Hanks balanced salt solution (HBSS, composition in mmol/L: NaCl, 143; KCl, 4.7; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2; glucose, 5.5; HEPES, 15) aerated with 95% O_2 and 5% CO_2 . They were cannulated at both ends and then perfused continuously in vitro in a system in which flow and pressure could be modified independently.²¹ At the beginning of each experiment, arteries were equilibrated for 45 minutes at 70 mmHg and perfused with an intraluminal flow rate of 10 $\mu\text{L}/\text{min}$. The carotid arteries were maintained under a transmural pressure of 70 mmHg during the experiments. The outer diameter was determined with a binocular lens connected to a video-camera system (Stemmer, VS 450). The pressure at both ends of the segment was monitored using pressure transducers. Intraluminal pressure upstream and downstream of the artery was controlled by means of a pressure servo-control (Living System Instruments, Inc), so that flow could be increased without changing intraluminal pressure. The presence of the endothelium was evaluated by assessing the dilation in response to acetylcholine (ACh; 1 $\mu\text{mol}/\text{L}$) during constriction induced by phenylephrine (Phe; 30 $\mu\text{mol}/\text{L}$).

All experiments evaluating the response to increases in intraluminal flow rate were performed in the presence of Phe (30 $\mu\text{mol}/\text{L}$) with or without indomethacin (10 $\mu\text{mol}/\text{L}$; a cyclooxygenase inhibitor that does not discriminate between COX-1 and COX-2) or indomethacin and L-NAME (100 $\mu\text{mol}/\text{L}$). Each inhibitor combination was incubated for at least 30 minutes in the intraluminal and extraluminal perfusions. When the constriction to Phe was stable for at least 10 minutes, the intraluminal flow rate was increased in a stepwise manner from 10 to 800 $\mu\text{L}/\text{min}$. The flow was augmented to the next level until a stable diameter was reached.

PRESSURE DIAMETER CURVES

The relationship between transmural pressure and diameter was determined in carotid arteries exposed to normal blood flow (sham-operated mice) and after 4 weeks exposure to elevated or drastically reduced blood flow (unilateral carotid artery ligation). Isolated arteries were incubated in vitro for 30 minutes in Ca^{2+} -free HBSS containing SNP (10 $\mu\text{mol}/\text{L}$), which abolished the smooth muscle tone. Passive diameter curves were obtained by increasing intraluminal pressures in 10 mmHg steps from 20 to 150 mmHg. Outer diameter was recorded at 2 minutes after each pressure increment. After reaching the highest pressure, pressure was returned to 100 mmHg and the artery was incubated in phosphate-buffered (pH 7.4) formaldehyde (4%) for overnight fixation. All drugs were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Outer diameter was expressed as a function of distending pressure. To determine the maximal outer diameter, pressure-diameter curves were analyzed by a four-parametric

logistic sigmoidal curve fitting ($Y=Y_{\min}+(Y_{\max}-Y_{\min})/(1+10^{(\text{Log } E_{50}-X) \cdot \text{Hillslope}})$) (Graphpad Prism 2.01, San Diego, CA, USA). The correlation coefficient of each individual curve fit exceeded 0.99 in all cases.

MORPHOMETRY

To concentrate histological analyses on the central part of the carotid arteries, fixed carotid arteries were cut in half and both parts were embedded side by side in paraffin. Cross-sections (4- μm) were stained with Lawson's solution (Boom BV, Meppel, the Netherlands), which highlights the elastic laminae. Media cross-sectional area (CSA) was determined, using video images generated by a Zeiss axioscope (Zeiss, Germany), a standard CCD camera (Sony) and commercial software (JAVA 1.21, Jandell Scientific, Corte Madera, CA, USA).

DATA ANALYSIS

Results are shown as means $\pm$ SEM. Statistical significance of differences between mouse strains and diets was evaluated using analysis of variance (ANOVA) followed by a post-hoc comparison with student Newman-Keuls significant difference test. For arteriograph studies *n* refers to the total number of vessels out of 5–7 mice. Comparisons between two groups were made with the Student's *t*-test. All statistics were performed using SigmaStat software. A *P*-value of <0.05 was considered significant.

RESULTS

GENERAL OBSERVATIONS

Body weight was significantly larger for apoE^{-/-} on diet and WT on diet compared to wild type mice on normal chow (NC) (Table 1). Cholesterol levels were significantly higher in apoE^{-/-} on diet and WT on diet than WT on NC. Plasma triglycerid levels were significantly elevated in the apoE^{-/-} on diet as compared to the other two groups (Table 1). Carotid artery blood flow did not differ in sham-operated animals between the left and right carotid arteries and among the three experimental groups (Table 1). This excludes the possibility that group differences in arterial reactivity to agonists and flow rates were due to different *in vivo* blood flow velocities between groups. Distal ligation of the left carotid artery stopped blood flow in the vessel and resulted in a substantial increase in blood flow through the contralateral artery in all experimental groups. This increase in blood flow was less pronounced in apoE^{-/-} on diet and WT on diet than in WT on NC (Table 1).

Table 1. Characteristics of mice

	ApoE ^{-/-} on D		WT on D		WT	
	Sham	uLig	Sham	uLig	Sham	uLig
Body weight (g)	35±3*	36±2*	31±1*	31±1*	28±1	26±1
Cholesterol (mmol/L)	19.7±2.39*,‡		3.55±0.17*		1.79±0.08	
Triglycerides (mmol/L)	1.80±0.33*,‡		0.71±0.09		0.91±0.11	
BF, mL/min						
LCA (ligated)	0.44±0.03	...	0.39±0.07	...	0.38±0.04	...
RCA (patent)	0.46±0.04	0.77±0.02*,†	0.42±0.05	0.81±0.03*,†	0.42±0.03	1.01±0.05†

Values are means±SEM (n=7-12) and were monitored at 4 weeks after surgery. Points ... indicate BF recording below detection limit. * P<0.05 vs WT; † P<0.05 vs sham ‡ P<0.05 vs WT on Western type diet (D). uLig, unilateral carotid artery ligation; BF, blood flow; LCA, left carotid artery; RCA, right carotid artery.

Despite clear atherosclerosis of the aortic arch in apoE^{-/-} on diet (not shown), central parts of all common carotid arteries of both apoE^{-/-} on diet and WT on diet were free from plaques and neointima.

ACUTE CONTRACTILE REACTIVITY

Sensitivity to the contractile effect of the α_1 -adrenergic agonist phenylephrine was comparable in carotid artery segments of apoE^{-/-} on diet, WT on diet and WT (data not shown).

Acetylcholine reduced phenylephrine-induced contraction in all experimental groups (Figure 2). Micromolar concentrations of acetylcholine resulted in contractile responses in apoE^{-/-} on diet and WT on diet, indicating endothelial dysfunction (Figure 2). The sensitivity and maximal relaxing effect of Na-nitroprusside did, however, not differ between experimental groups, showing unaltered response to nitric oxide in the diet groups (data not shown).

The effect of L-NAME on maximal phenylephrine-induced contraction was less pronounced in WT on diet (from 2.5±0.4 to 2.7±0.2 N/m) than in apoE^{-/-} on diet (from 1.2±0.2 to 2.4±0.2 N/m) and WT (from 1.9±0.2 to 3.1±0.4 N/m), suggesting reduced bioavailability of endogenous NO in WT animals on diet as compared to the other groups (Figure 3). In apoE^{-/-} on diet indomethacin enhanced the contractile response to phenylephrine (from 1.20±0.20 to 1.96±0.2 N/m, P<0.05), but did not change the sensitivity (Figure 3). However, in WT on diet indomethacin significantly lowered the maximal contractile response (maximal contraction 2.49±0.4 to 0.90±0.1 N/m) and sensitivity (-logIC₅₀, 6.7±0.1 to 5.8±0.3, P<0.01) to phenylephrine (data not shown).

In the presence of indomethacin acetylcholine-induced relaxations were improved in the diet groups (Figure 3, maximum values from 37.4±15.8% to 82.6±3.7% (P<0.05) and from 44.0±6.6% to 91.6±2.0% (P<0.05) in apoE^{-/-} on

Figure 2. Endothelial dysfunction. Relaxing effects (expressed as percentage inhibition of vessels contracted with 30 $\mu\text{mol/L}$ Phe) of acetylcholine (ACh) in carotid segments of apoE^{-/-} on D or WT on a high fat Western type diet (WT on D) and of WT mice maintained on normal chow (WT). * $P < 0.05$ vs WT.

Figure 3. Relaxing effects (expressed as percentage inhibition of vessels contracted with 30 $\mu\text{mol/L}$ Phe) of acetylcholine (ACh) in carotid segments of apoE^{-/-} on D (left) or WT on a high fat Western type diet (WT on D, middle) and of WT mice maintained on normal chow (WT, right). Experiments were performed in the absence (open circles) and presence of 100 $\mu\text{mol/L}$ L-NAME (closed circles) or 10 $\mu\text{mol/L}$ indomethacin (triangles). Means $\pm$ SEM ($n = 5-9$). * $P < 0.05$ vs CTL.

diet and WT on diet respectively). Indomethacin had no effect on acetylcholine-induced relaxations in WT on normal diet (Figure 3).

ACUTE FLOW-INDUCED ARTERIAL DILATION

The outer diameter of the carotid arteries maintained at a transmural pressure of 70 mmHg was significantly larger in apoE^{-/-} on diet ($564 \pm 7 \mu\text{m}$, $P < 0.001$) than in WT on diet ($511 \pm 4 \mu\text{m}$) and WT ($518 \pm 6 \mu\text{m}$).

In carotid arteries of apoE^{-/-} on diet, constriction in response to 30 μM phenylephrine was significantly smaller than that observed in the other two groups. In WT

Figure 4. Acute flow-induced changes in diameter (μm) in perfused carotid arteries of WT (open squares), WT on D (closed squares) and apoE^{-/-} on D (triangles). *P<0.01 vs WT and WT on D. Means±SEM (n=6–10 vessels from 5–7 mice). *P<0.05 vs WT and WT on D.

on diet and WT, exposure to phenylephrine caused comparable constriction (data not shown). Dilations in response to Na-nitroprusside (10 μM) were indistinguishable between groups (data not shown).

The increase in diameter in response to increasing intraluminal flows was significantly reduced in apoE^{-/-} on diet and even a flow-dependent constriction was observed at low flow rates (Figure 4; 40–200 μL/min, P<0.05 vs 10 μL/min), while no differences in flow-diameter responses were seen between WT on diet and WT.

In apoE^{-/-} on diet, indomethacin augmented flow-induced dilation at a flow rate of 800 μL/min, suggesting a role for vasoconstrictor cyclooxygenase products (Figure 5). In WT on diet, indomethacin reduced dilation in response to low flow rates (between 10 and 400 μL/min, P<0.01 vs control), suggesting the involvement of flow-induced vasodilator cyclooxygenase products (Figure 5). In WT, this effect was less pronounced (at 40 and 80 μL/min; P<0.05 vs control). The combination of indomethacin and L-NAME did not alter flow-induced dilation as compared to incubations with indomethacin alone in all experimental groups.

CHRONIC FLOW-RELATED CAROTID ARTERY REMODELING

Pressure-diameter curves of left and right carotid arteries of sham-operated mice were identical and did not differ between experimental groups. Therefore, the average values of the left and right arteries were pooled in Figure 6. Irrespective of strain and diet, manipulation of blood flow did not lead to neointima formation in the central portion of the carotid arteries. Media cross-sectional area (CSA) was significantly smaller in sham-operated apoE^{-/-} on diet and WT on diet ($16.7 \pm 0.7 \times 10^3 \mu\text{m}^2$ and $18.1 \pm 0.5 \times 10^3 \mu\text{m}^2$) compared to WT ($24.5 \pm 1.4 \times 10^3 \mu\text{m}^2$, Figure 7). In mice of all experimental groups unilateral carotid artery ligation resulted in (1) a reduc-

Figure 5. Acute flow-induced responses (expressed as percentage of maximal dilation to 10 μM SNP) in carotid arteries of apoE^{-/-} on D (left), WT on D (middle) and WT (right). Experiments were performed in the absence (open circles) and presence of 10 $\mu\text{mol}/\text{L}$ indomethacin (closed circles) or indomethacin and 100 $\mu\text{mol}/\text{L}$ L-NAME (triangles). * $P < 0.05$ vs control. Means $\pm$ SEM ($n = 5-10$ from 5-7 mice).

Figure 6. Effects of unilateral carotid artery ligation on pressure-diameter relationships in ligated arteries (closed circles) and in contralateral (patent) arteries (closed squares) for apoE^{-/-} on D (left), WT on D (middle) and WT (right). * $P < 0.05$ vs control. Findings are shown as means $\pm$ SEM ($n = 6-12$) for ligated (closed symbols) and sham-operated animals (open symbols).

Figure 7. Effects of unilateral carotid artery ligation on media cross-sectional area (CSA) in apoE^{-/-} on D (left), WT on D (middle) and for WT mice maintained on normal chow (right). Findings are shown as means $\pm$ SEM ($n = 6-12$). Sham, sham-operated carotid arteries; Ligated, experimentally ligated left carotid artery; Patent, contralateral right carotid artery. * $P < 0.001$ vs sham, † $P < 0.001$ vs WT.

tion of diameter and an increase in stiffness of the ligated arteries and (2) in diameter increases of the hyperperfused arteries after 4 weeks (Figure 6). In all experimental groups chronic hyperperfusion did not result in a significant change of media CSA (Figure 7). In the ligated arteries of both WT on diet and apoE^{-/-} on diet a significant media hypertrophy was seen while in WT no change in media CSA was observed compared to sham-operated arteries (Figure 7).

DISCUSSION

This study demonstrates that both impaired endothelial function and impaired acute flow-induced vasodilation do not compromise flow-related arterial remodeling in hyperlipidemic mice.

In apoE^{-/-} mice, hypercholesterolemia, atherosclerosis and endothelial dysfunction are markedly increased by a “Western-type” high fat diet.²² Plasma cholesterol levels are markedly elevated because of increased levels of VLDL and LDL, atherosclerosis develops at sites comparable to those in human atherosclerosis, plasma nitrite levels, a marker for NO activity *in vivo*, are reduced, and impaired arterial endothelium-dependent vasodilator responses to agonists have been observed.^{17,18} Here, we addressed whether this entails a reduced capacity to respond to acute and chronic changes in flow.

ENDOTHELIAL DYSFUNCTION

The endothelial dysfunction observed in 6 months adult WT and apoE^{-/-} both on diet was restricted to increased contractile responses to high concentrations of acetylcholine.

The mechanisms underlying endothelial dysfunction in hyperlipidemia and atherosclerosis in general, and in apoE^{-/-} in particular, remain controversial. Expression and protein levels of endothelial nitric oxide synthase and the production of NO in the atherosclerotic rabbit aorta appear to be increased rather than decreased.^{23,24} Inactivation of NO by superoxide anions produced in excess as a result of the incorporation of lipids within the endothelium, a deficiency in co-factors for NO-synthase, such as tetrahydrobiopterin,²⁵ and increased levels of asymmetric dimethylarginine (ADMA)²⁰ could be possible alternatives.

However, increased inactivation of endothelium-derived NO is not supported by the normal relaxing responsiveness to Na-nitroprusside and the identical sensitization to this NO-donor substance by L-NAME (not shown), which we observed in apoE^{-/-} compared to WT. Reported effects of an endothelin receptor antagonist on endothelial dysfunction⁴ rather suggest a role for an endothelium-derived contractile factor, such as endothelin-1.

In this study we show that vasoconstrictor cyclooxygenase products are involved in the increased contractile response to acetylcholine in mice on a Western type diet. Nevertheless, NO may be indirectly involved in this increased contractile response to acetylcholine, because the contractile component in the acetylcholine response was not observed in the presence of L-NAME in carotid artery segments of WT on diet and apoE^{-/-} on diet. Under pathological circumstances such as hypercholesterolemia in which vascular oxidase systems are upregulated, superoxide anions are produced which react with NO to form peroxynitrite (ONOO⁻). Gupte et al²⁶ found that superoxide in the presence of an NO donor elicited thromboxane (TxA₂)-mediated constriction in the rat coronary circulation. A likely mechanism by which ONOO⁻ may interfere with arachidonic acid metabolism is inactivation of PGI₂ synthase,²⁷ thus promoting synthesis of TxA₂, as suggested by Bagi et al.²⁸

ACUTE FLOW-INDUCED DILATION

Flow-induced dilation has been shown to be endothelium-dependent.^{15,29} In line with other studies we found that vasodilator prostaglandins (PGs) and other factors not characterized, possibly EDHF and bradykinin, are involved in flow-induced dilation.^{15,16, 30–33}

In the severely hypercholesterolemic apoE^{-/-} on diet, flow-induced dilation was impaired at all flow rates tested. At low flow rates, flow-induced constricting factors distinct from PGs and NO are involved. At the highest flow rate, however, vasoconstrictor cyclooxygenase products were involved. A candidate constrictor at low flow rates is endothelin-1.³⁴ Lüscher et al¹⁷ reported that endothelin-1 production is increased in apoE-deficient mice. Furthermore, an early study reported evidence for endothelial flow-dependent release of free radicals unrelated to cyclooxygenase or nitric oxide synthase activities in aortae of normocholesterolemic rabbits.³⁵ Under normal and mild hypercholesterolemic conditions these flow-dependent free radicals can be buffered by efficient scavenging systems, but possibly not in excessive free radical production in severe hyperlipidemia. In mildly hypercholesterolemic WT on diet, flow-induced dilation seemed to be maintained by increased production of vasodilator PGs at low flow rates.

CHRONIC FLOW-RELATED REMODELING

We used unilateral carotid artery ligation^{3,4} to measure arterial flow-related remodeling. In this model, a growing neointima develops close to the ligation.³ This involves P-selectin,³⁶ PDGF³⁷ and bFGF^{36–38} and is regionally restricted. In WT C57BL/6J mice, it does not extend into the center of the common carotid artery.³⁹ As judged from our findings, neointima formation in response to the absence of flow is not markedly increased by a high-fat diet or by severe hyperlipidemia resulting from apoE-deficiency.

We focussed on the central part of the vessel because it represents the structural response of the intact wall to altered shear stress without the complicating influences of a neointima or atherosclerotic plaque.⁴⁰

As noted previously,⁴ total carotid artery blood flow was largely maintained in wild type mice after unilateral carotid artery ligation due to a compensatory increase in contralateral blood flow. Pre-existing collaterals, that interconnect the facial, cranial and cerebral areas, perfused by both carotid arteries form the anatomical basis for this shunting.⁴¹ In arteries of immature juvenile animals, chronic increases in blood flow lead to compensatory outward hypertrophic remodeling, i.e., an increase in both lumen diameter and wall mass whereby wall shear stress and circumferential wall stress are normalized.^{12, 42} However, the ability of the vessel wall to respond to altered blood flow diminishes with age.^{12, 42} We recently reported that doubling of blood flow did not alter carotid artery media mass or diameter after 4 weeks in mature 4-month old 129/Sv mice.⁴ Despite mild to severe hyperlipidemia, altered flow-induced dilation and reduced contralateral shunting of blood flow, the flow-loaded carotid arteries of these mice displayed a significant increase in diameter and no media hypertrophy. This structural response tends to normalize wall shear stress, thereby preserving the integrity of the endothelium. These findings in hyperlipidemic mice are in line with our previous observations in vimentin-deficient mice.⁴ In these mice, absence of the intermediate cytoskeleton filament results in impaired endothelium-dependent flow-induced dilation,⁴³ but does not entail a blunting of flow-related arterial remodeling.⁴

IN SUMMARY

In mild and severely hyperlipidemic mice, endothelium-dependent relaxations were reduced and acute flow-induced vasomotor function was altered and markedly impaired, respectively. Neither, however, impeded arterial structural responses to unilateral carotid artery ligation.

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CHAPTER 3

**Effects of estrogen on the vascular injury
response in estrogen receptor α,β (double)
knockout mice**

Richard H. Karas, Henny Schulten, Gary Pare, Mark J. Aronovitz, Claes Ohlson,
Jan-Ake Gustafsson, Micheal E. Mendelsohn

Abstract

The two known estrogen receptors, ER α and ER β , mediate the effects of estrogen in all target tissues, including blood vessels. We have shown previously that estrogen inhibits vascular injury response to the same extent in female wild-type (WT), ER α knockout (ER α KO_{CH}), and ER β knockout (ER β KO_{CH}) mice. We generated mice harboring disruptions of both ER α and ER β genes (ER α,β KO_{CH}) by breeding and studied the effect of 17 β -estradiol (E₂) on vascular injury responses in ovariectomized female ER α,β KO_{CH} mice and WT littermates. E₂ inhibited increases in vascular medial area following injury in the WT mice but not in the ER α,β KO_{CH} mice, demonstrating for the first time that the two known estrogen receptors are necessary and sufficient to mediate estrogen inhibition of a component of the vascular injury response. Surprisingly, as in WT littermates, E₂ still significantly increased uterine weight and inhibited vascular smooth muscle cell (VSMC) proliferation following injury in the ER α,β KO_{CH} mice. These data support that the role of estrogen receptors differs for specific components of the vascular injury response in the ER α,β KO_{CH} mice. The results leave unresolved whether E₂ inhibition of VSMC proliferation in ER α,β KO_{CH} mice is caused by a receptor-independent mechanism, an unidentified receptor responsive to estrogen, or residual activity of the ER α splice variant reported previously in the parental ER α KO_{CH} mice. These possibilities may be resolved by studies of mice in which ER α has been fully disrupted (ER α KO_{S β}), which are in progress.

INTRODUCTION

The cardiovascular effects of estrogen are diverse. Estrogen has both systemic effects on circulating factors (eg, cholesterol, cytokines, coagulation/fibrinolytic factors) and direct effects on the blood vessel wall (eg, regulation of vasomotor tone, vascular cell proliferation; reviewed in Mendelsohn and Karas,¹ Farhat et al,² and Mendelsohn³) Some of the effects of estrogen occur rapidly, whereas others require prolonged estrogen exposure. At physiologically relevant concentrations of estrogen, both the rapid and the longer-term cardiovascular effects of estrogens are mediated by estrogen receptors.^{3–6} To date, two estrogen receptors (ERs) have been described, ER α and ER β (reviewed in Gustafsson [1997],⁷ Katzenellenbogen and Korach,⁸ and Gustafsson [1999]⁹). Although their physiological relevance in the vasculature is incompletely understood, ER α and ER β are expressed in endothelial cells and vascular smooth muscle cells (VSMCs),^{10,11–16} the predominant cells present in vascular tissues. Using wild-type (WT) and estrogen receptor knockout (KO) mice, we have previously studied the role of ER α and ER β in mediating the vascular protective effects of estrogen in a mouse carotid artery injury model.^{10,17,18} Previous studies used mice developed at the University of North Carolina, Chapel Hill, which harbor gene deletions of either ER α (ER α KO_{CH})¹⁹ or ER β (ER β KO_{CH}).²⁰ These studies show that treatment of ovariectomized female mice with nanomolar concentrations of 17 β -estradiol (E₂) inhibits the response to vascular injury to equivalent levels in wild-type,¹⁷ ER α KO_{CH},¹⁰ and ER β KO_{CH} mice.¹⁸ These findings suggest that ER α and ER β are able to complement one another such that each receptor alone is sufficient to mediate the vascular protective effects of estrogen, or that the vascular protective effects of estrogen are mediated by an ER α /ER β -independent pathway. To distinguish between these two hypotheses, we undertook the present study examining the effect of estrogen on the response to vascular injury in ER α , β KO_{CH} (double) estrogen receptor knockout mice.

MATERIAL AND METHODS

ANIMALS

A total of over 820 animals were required to ultimately generate the ER α , β KO_{CH} mice used in this study. These mice, which have been extensively studied, do not express ER proteins in any tissue.^{19,20,21–23} However, ER α KO_{CH} mice have been shown previously to express mRNA for 2 partial ER α transcripts, one of which retains the hormone- and DNA-binding domains of full-length ER α , and can both bind estradiol and mediate hormone-induced gene expression.²³ This partial ER α transcript, detectable only by reverse transcriptase polymerase chain reaction approaches, may account for the low level of residual specific binding of estradiol in

uterine tissue of the $ER\alpha KO_{CH}$ mice.²³ $ER\alpha, \beta KO_{CH}$ mice were generated by extensive crossbreeding in one of our laboratories (C.O.) as follows: male and female double heterozygous ($ER\alpha^{+/-}\beta^{+/-}$) mice were created from the parental $ER\alpha KO_{CH}$ and $ER\beta KO_{CH}$ mice. Female offspring with the wild-type $ER\alpha^{+/+}\beta^{+/+}$ genotype and the double receptor knockout $ER\alpha^{-/-}\beta^{-/-}$ genotype were included in the present study,^{19,20,24} and the latter are referred to as $ER\alpha, \beta KO_{CH}$. More than 830 mice were required to generate a sufficient number of $ER\alpha, \beta KO_{CH}$ female mice for the study. All mice were of mixed C57BL/6J/129 backgrounds. Genotyping of tail DNA was performed at 4 weeks of age and again at the end of the vascular injury experiment on tail snips. The $ER\alpha$ gene was analyzed with the following primer pairs. Primers AACTCGCCGGCTGCCACTTACCAT and CATCAGCGGGCTAGGCGACACG for the WT gene correspond to flanking regions in the targeted exon 2. They produce a fragment of 320 bp. Primers TGTGGCCGGCTGGGTGTG and GGCGCTGGGCTCGTTCTC for the knockout gene correspond to part of the Neo cassette and the flanking exon 2. They produce a 700-bp fragment. Genotyping of the $ER\beta$ gene has been described previously.²⁵ (The primers used for the $ER\beta$ gene were one primer in intron 2 [β NHD4–25; 5'-AGAATGTTGCACTG-CCCC TGCTGC-3'], one in intron 3 [C1wt-27; 5'-GGAGTAGAA-ACAAGCAATCCAGACATC-3'], and one in the Neo cassette [Neo-25; 5'-GCAGCCTCTGTTCCACATACACTTC-3']. A 650-bp product [β NHD4–25 and C1wt-27] was amplified for the homozygous wild-type [WT; +/+] mice, and a 450-bp [β NHD4–25 and Neo-25] product was amplified for the homozygous mutant [-/-] mice; both bands were amplified for the heterozygous [+/-] mice). Animals had free access to fresh water and food pellets.

CAROTID ARTERY INJURY

All procedures and protocols were approved by the New England Medical Center Animal Research Committee. A detailed description of the vascular injury protocol has been published previously.^{10,17,18} Briefly, 7 days following ovariectomy, 26 wild-type and 23 $ER\alpha, \beta KO_{CH}$ female mice, average age 12 to 13 weeks, were randomized to receive either placebo pellets (- E_2) or 17 β -estradiol-containing pellets (+ E_2 ; 0.1 mg/21-day pellets, Innovative Research) at a dose previously shown to produce physiologically relevant (ie, low nanomolar) concentrations of circulating 17 β -estradiol (E_2). A week after pellets were implanted, the mice were anesthetized with inhaled isoflurane and subjected to unilateral vascular injury by intraluminal passage of a wire into the left common carotid artery resulting in endothelial denudation. At this time, osmotic minipumps calibrated to release bromodeoxyuridine (BrdU) over the 14 days of the experiment were implanted subcutaneously, as described.¹⁷ The mice were allowed to recover and return to normal activity.

TISSUE HARVEST

Two weeks following vascular injury, the mice were anesthetized with 2.5% isoflurane and blood was taken for cholesterol and estradiol determinations. Uteri were then extracted and wet weights obtained, followed by harvesting of a small segment of the tail for confirmation of pre-study genotype analysis by reverse transcriptase-polymerase chain reaction. Both carotid arteries were harvested next after perfusion fixation with 10% formalin at 150 mmHg for 4 minutes. A small segment of small intestine was also harvested for use as a positive control for the BrdU stains (see next section).

IMMUNOHISTOCHEMISTRY AND MORPHOMETRY

Following embedding in paraffin, parallel sections from all 98 carotid arteries were stained as previously described for hematoxylin/eosin and for elastin.^{10,17,18,26} Immunodetection of BrdU-labeled cells was also performed as previously described.^{10,17,18} A section of small intestine was included on each slide to serve as a positive control for the BrdU stain. One section of each vessel was not exposed to a primary antibody, serving as a negative control. VSMCs were identified by immunostaining for smooth muscle specific actin, performed on a Vantana automated slide staining machine using clone 1A4 from Sigma as the primary antibody. BrdU-stained VSMCs and unstained VSMC nuclei were counted by two independent observers. A VSMC proliferation index also was calculated by dividing the number of BrdU-labeled VSMCs by the total number of unstained VSMCs for each section. Medial area was determined on serial elastin-stained sections using the ImagePro Plus software. All observations were made by observers blinded to both the genotype and the treatment group of the specimen. Only one vessel developed an occlusive thrombosis, and this mouse from the WT-E₂ group was excluded from analysis.

ADDITIONAL ASSAYS

Circulating concentrations of E₂ were measured by radioimmunoassay using a commercially available kit (Ultra-Sensitive Estradiol RIA, Diagnostic System Laboratories Inc), according to the manufacturer's instructions. Pooled samples of serum (200 μ L) from each of the treatment groups were each assayed in triplicate, and the mean value is reported as representative of the group. Control experiments confirmed the linearity of the assay between the range of 0.018 and 2.78 nmol/L. Circulating concentrations of total and HDL cholesterol, and triglycerides were determined as previously described.¹⁷ At the time of euthanasia, the correct genotype of each mouse was confirmed by a second independent set of PCR studies using tail-snip DNA as described above.

STATISTICAL ANALYSIS

For all analyses, differences between the 4 treatment groups were first examined with a one-way ANOVA after the normality of the data were tested. Post hoc comparisons were made with the Student-Newman-Keuls test. Two group comparisons were made with the Student's *t*-test. All statistics were performed using SigmaStat software. A value of $P < 0.05$ was considered significant.

RESULTS

As in our previous studies, implantation of E_2 -containing pellets in the ovariectomized female mice restored circulating levels of E_2 to physiological levels in both the WT and $ER\alpha, \beta KO_{CH}$ mice (0.43 ± 0.03 nmol/L [115 ± 9 pg/mL] and 0.55 ± 0.03 nmol/L [148 ± 7 pg/mL], respectively; Table 1). There were no significant differences in body weight, blood pressure, or heart rate at baseline or at the time of injury between any of the groups (Table 1). Cholesterol levels also were similar between the WT and $ER\alpha, \beta KO_{CH}$ animals, and not significantly changed by estrogen treatment (Table 1). In the WT animals, E_2 treatment resulted in the expected approximately 10-fold increase in uterine weight compared with the $-E_2$ group (Figure 1; $P < 0.001$ WT+ E_2 versus WT- E_2). The uterine weights were lower in the placebo-treated $ER\alpha, \beta KO_{CH}$ mice than in the placebo-treated WT mice ($P < 0.01$ $ER\alpha, \beta KO_{CH}$ - E_2 versus WT- E_2). Surprisingly, E_2 replacement to physiological levels also significantly increased uterine weight in the $ER\alpha, \beta KO_{CH}$ mice (5.8-fold; Figure 1; $P < 0.01$ $ER\alpha, \beta KO_{CH}$ + E_2 versus $ER\alpha, \beta KO_{CH}$ - E_2), although to a lesser extent than in the WT mice. Uterine weight increased in every animal assigned to receive E_2 -containing pellets, confirming proper placement and function of the pellets. The 2 primary endpoints for the mouse carotid artery injury model are well established^{10,17,18} and include (1) change in medial area and (2) extent of VSMC proliferation, both assessed 14 days after unilateral endothelial denudation injury. The medial-area endpoint reflects the combined effects of alterations in extracellular matrix constituents and cellular volume, whereas the VSMC proliferation endpoint specifically indicates the degree of mitogenesis that occurs in these cells in response to the vascular injury.

Table 1. Effects of E₂ on Wild-Type and ER α , β KO_{CH} Mice

	Wild Type		ER α , β KO _{CH}	
	-E ₂	+E ₂	-E ₂	+E ₂
E ₂ level, nmol/L	<0.02	0.43 $\pm$ 0.03	<0.02	0.55 $\pm$ 0.03
Body weight at injury, g	23.3 $\pm$ 0.5	24.4 $\pm$ 0.4	24.4 $\pm$ 0.5	25.1 $\pm$ 0.7
Body weight at sacrifice, g	24.7 $\pm$ 0.4	24.9 $\pm$ 0.3	25.6 $\pm$ 0.6	26.1 $\pm$ 0.5
Tail-cuff blood pressure, mmHg	105 $\pm$ 2.1	100 $\pm$ 0.5	100 $\pm$ 5.2	98 $\pm$ 3.5
Heart rate, bpm	633 $\pm$ 37	586 $\pm$ 38	693 $\pm$ 11	651 $\pm$ 24
Total cholesterol, mg/dL	78	68	79	76
Triglycerides, mg/dL	167	211	187	199
HDL cholesterol, mg/dL	66	60	69	67

Figure 1. Uterine weights of wild-type and ER α , β KO_{CH} mice treated with placebo or 17 β -estradiol. Wet weights of uteri were determined at the time of euthanasia in wild-type and ER α , β KO_{CH} mice. Mice received either placebo pellets (-E₂) or 17 β -estradiol-containing pellets (+E₂) for a total of 3 weeks before harvest. Bars represent the mean value for each group, $\pm$ SEM. *P<0.001 vs -E₂.

RESPONSE TO VASCULAR INJURY: MEDIAL AREA

Medial areas were determined by computerized morphometric analysis of elastin-stained sections of each carotid artery. A summary of the medial area results is presented in Figure 2. The medial areas of the uninjured carotid arteries were similar in the WT (n=26) and ER α , β KO_{CH} (n=23) mice (15.5 $\pm$ 0.3 $\times 10^{-3}$ mm² versus 15.3 $\pm$ 0.5 $\times 10^{-3}$ mm², respectively; P=NS). In the placebo-treated WT mice, injury induced an increase in the medial area to 21.3 $\pm$ 1.6 $\times 10^{-3}$ mm² (P<0.01 WT-E₂ versus uninjured). Estrogen replacement in the WT mice significantly inhibited the injury-induced increase in medial area, resulting in a medial area of 16.0 $\pm$ 1.4 $\times 10^{-3}$ mm² (P=0.03 WT+E₂ versus WT-E₂). The medial area of the injured carotid in the WT+E₂ animals was indistinguishable from that of the uninjured contralateral vessel (P=0.7 WT+E₂ versus uninjured). In the placebo-treated ER α , β KO_{CH} mice, injury

Figure 2. Medial areas of uninjured and injured carotid arteries from wild-type and ER α , β KO_{CH} mice treated with vehicle or 17 β -estradiol. a through f, Representative elastin-stained sections from uninjured (Uninj) and injured (Inj) carotid arteries from wild-type (WT) and ER α , β KO_{CH} mice treated with placebo pellets (-E₂), or 17 β -estradiol-containing pellets (+E₂) are shown (x400). The medial area of each specific section shown is listed in parentheses. The medial area was determined on the entire section. a, Wild-type, uninjured (15.5x10⁻³ mm²). b, Wild-type, injured, placebo-treated (22.2x10⁻³ mm²). c, Wild-type, injured, E₂-treated (15.4x10⁻³ mm²). d, ER α , β KO_{CH}, uninjured (14.7x10⁻³ mm²). e, ER α , β KO_{CH}, injured, placebo-treated (21.6x10⁻³ mm²). f, ER α , β KO_{CH}, injured, E₂-treated (18.9x10⁻³ mm²). g, Summary of medial area results for all mice. In panel b, the external elastic lamina is identified by the red arrow, and the internal elastic lamina is identified by the black arrow. In panel g, bars represent the mean value for each group, $\pm$ SEM. *P<0.05 vs the inj, -E₂ group within the same genotype.

Figure 3. Vascular smooth muscle cell proliferation of uninjured and injured carotid arteries from wild-type and ER α , β KO_{CH} mice treated with vehicle or 17 β -estradiol. a through f, Representative BrdU-stained sections from uninjured (Uninj) and injured (Inj) carotid arteries from wild-type (WT) and ER α , β KO_{CH} mice treated with placebo pellets (-E₂) or 17 β -estradiol-containing pellets (+E₂) are shown (x400). The VSMC proliferation index (BrdU-labeled VSMCs/unstained VSMCs) of each specific section shown is listed in parentheses. The VSMC proliferation index was determined on the entire section. a, Wild-type, uninjured. b, Wild-type, injured, placebo-treated (0.90). c, Wild-type, injured, E₂-treated (0.54). d, ER α , β KO_{CH}, uninjured. e, ER α , β KO_{CH}, injured, placebo-treated (0.77). f, ER α , β KO_{CH}, injured, E₂-treated (0.46). g, Summary of VSMC BrdU-labeling results for all mice. In panel g, bars represent the mean value for each group, mean $\pm$ SEM. *P<0.05 vs the Inj, -E₂ group within the same genotype.

induced a significant increase in medial area ($20.3 \pm 1.1 \times 10^{-3} \text{ mm}^2$; $P < 0.01$ $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}\text{-E}_2$ versus uninjured), similar to the degree of injury response in the placebo-treated WT mice ($P = 0.6$ $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}\text{-E}_2$ versus WT-E_2). In contrast to the findings in the WT mice, in the $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}$ mice E_2 treatment did not significantly inhibit the injury-induced increase in medial area ($19.0 \pm 0.7 \times 10^{-3} \text{ mm}^2$ in the $\text{ER}\alpha, \beta\text{KO}_{\text{CH}} + \text{E}_2$, $P = 0.5$ versus $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}\text{-E}_2$). Thus, the injury-induced increase in medial area was similar in the placebo-treated WT and $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}$ mice, but E_2 replacement no longer inhibited the increase in medial area following injury in the $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}$ mice. In contrast to any of our previous studies using this model, in over 300 animals (References 10, 17, 18, and unpublished results, 2000–2001), these results in the $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}$ mice represent the first instance that E_2 has failed to protect against an endpoint analyzed to quantify the vascular response to carotid injury.

RESPONSE TO VASCULAR INJURY: VSMC PROLIFERATION

VSMC proliferation was assessed by immunohistochemical detection of BrdU-labeled VSMCs in sections from each carotid artery. A summary of these results is presented in Figure 3. There were no differences in the morphological appearance or total number of nuclei in the uninjured vessels of the WT and $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}$ mice (95 ± 15 versus 93 ± 14 cells/section, respectively; $P = \text{NS}$). This supports that there are no obvious developmental abnormalities in the formation of the vasculature in the $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}$ mice. As expected, BrdU-labeled VSMCs were only rarely detected in the uninjured vessels of either the WT or the $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}$ mice (mean < 1 labeled cell per vessel for WT and $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}$ groups; $P = \text{NS}$ between groups). As in our previous studies using this injury technique, a small amount of neointima was observed in a minority of injured vessels and there were no significant differences in neointimal formation in the WT compared with the $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}$ mice (data not shown). In the placebo-treated WT mice, injury increased the VSMC proliferation index from < 0.1 to 1.1 ± 0.3 ($P < 0.001$ WT-E_2 versus uninjured). This increase in VSMC proliferation was significantly attenuated by E_2 treatment in the WT mice, resulting in a VSMC proliferation index of 0.5 ± 0.1 ($P < 0.05$ $\text{WT} + \text{E}_2$ versus WT-E_2). In the placebo-treated $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}$ mice, injury also induced an increase in the VSMC proliferation index from < 0.1 to 0.8 ± 0.1 ($P < 0.001$ $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}\text{-E}_2$ versus uninjured). The injury-induced increase in VSMC proliferation in the placebo-treated $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}$ mice was similar to that observed in the placebo-treated WT mice ($P = 0.4$ $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}\text{-E}_2$ versus WT-E_2). As in the WT mice, E_2 treatment of the $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}$ mice significantly attenuated the injury-induced increase in the VSMC proliferation index (0.4 ± 0.1 ; $P < 0.05$ $\text{ER}\alpha, \beta\text{KO}_{\text{CH}} + \text{E}_2$ versus $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}\text{-E}_2$; $P = 0.6$ $\text{ER}\alpha, \beta\text{KO}_{\text{CH}} + \text{E}_2$ versus $\text{WT} + \text{E}_2$). Thus, injury induced an increase in VSMC proliferation to similar degrees in the placebo-treated WT and $\text{ER}\alpha, \beta\text{KO}_{\text{CH}}$ mice, and the inhibitory effect of E_2 on the

injury-induced increase in VSMCs observed in the WT animals was retained in the $ER\alpha,\beta KO_{CH}$ mice. These findings demonstrate maintenance of the protective effect of E_2 on VSMC proliferation in the $ER\alpha KO_{CH}$ mice, in surprising contrast to the loss of the protective effect of E_2 on the medial-area endpoint presented above. Recent data demonstrate clearly that estrogen receptors mediate both genomic and nongenomic effects in vascular cells at physiological levels of estrogen.^{3–6} $ER\alpha$ was the first and only known estrogen receptor from 1985, when it was initially cloned,²⁷ until 1996, when $ER\beta$ was discovered.^{28–30} Although $ER\alpha$ and $ER\beta$ are the products of distinct genes, they share relatively high degrees of homology in the DNA binding domain ($\approx 95\%$) and in the ligand binding domain ($\approx 55\%$). Recent studies have begun to define receptor-specific differences in ligand binding affinity,^{28,31–33} receptor interacting proteins,^{34,35} and pharmacologic agonists and antagonists.^{36–38} These findings highlight the potential for differences in the physiological effects of these two receptors. In 1995, we first reported that estrogen inhibits the vascular injury response in normolipemic wild-type mice,¹⁷ as it does in other animal models (reviewed in Mendelsohn and Karas¹). A subsequent study showed equivalent inhibition of the vascular injury response in the $ER\alpha KO_{CH}$ mice created by Lubahn et al¹⁹ compared with their littermate wild-type controls.¹⁰ A more recent study in which $ER\alpha KO_{CH}$ mice were bred with the hyperlipidemic apolipoprotein E knockout mice demonstrated that in this model estrogen no longer decreased plaque size, but continued to significantly alter plaque complexity, decreasing the number of lesions with fibrous caps, calcifications, and cholesterol clefts.³⁹ These studies support the existence of $ER\alpha$ -independent vascular protective effects of E_2 or of residual $ER\alpha$ activity in the $ER\alpha KO_{CH}$ mice.²³ The discovery of $ER\beta$,²⁸ identification of its expression in vascular cells and tissues,¹⁰ and demonstration that vascular injury dramatically enhances vascular cell expression of $ER\beta$ but not $ER\alpha$ ^{13,16} all raised expectations that $ER\beta$ might mediate the vascular protective effects of estrogen. However, a subsequent carotid artery injury study in the $ER\beta KO$ mice demonstrated that E_2 continued to inhibit the response to injury equally well in $ER\beta KO$ and WT mice.¹⁸ These studies, which used the only available ER knockout mice at that time,^{19,20} suggested that neither $ER\alpha$ nor $ER\beta$ by themselves are necessary for the protective effect of estrogen in the vasculature. In the present study, the effect of E_2 on the usual two vascular injury endpoints, medial area and VSMC proliferation, was compared in $ER\alpha,\beta KO_{CH}$ and WT mice. In the wild-type mice, E_2 inhibited the injury-induced increase in medial area as it has in each of our previous studies.^{10,17,18} In contrast, in the $ER\alpha,\beta KO_{CH}$ mice, E_2 did not prevent the injury-induced increase in medial area. Loss of the protective effect of estrogen on the medial-area endpoint in the $ER\alpha,\beta KO_{CH}$ mice supports that $ER\alpha$ and $ER\beta$ mediate this effect of estrogen. Thus, for the first time, $ER\alpha$ and $ER\beta$ are implicated as physiologically relevant mediators of one component of the vascular injury response. Taken together with the results of previous single estrogen receptor knockout experiments,^{10,18} these data support that the $ER\alpha$ and $ER\beta$ can functionally complement

one another *in vivo*, such that either receptor alone can mediate the inhibitory effect of E_2 on the medial area thickening endpoint (see subsequent caveat). The E_2 -mediated inhibition of VSMC proliferation observed in the WT mice in the present study also is consistent with our previous observations in the WT mice.^{10,17,18} Quite surprisingly, however, estrogen also inhibited VSMC proliferation in the $ER\alpha,\beta KO_{CH}$ mice to a similar extent as in the WT mice. One interpretation of the preserved estrogenic effect on VSMC proliferation in $ER\alpha,\beta KO_{CH}$ mice is the exciting possibility that there are estrogen receptors other than $ER\alpha$ and $ER\beta$ that have not yet been identified. However, to date, extensive searching has failed to identify any likely candidates (authors' unpublished results, 1997–2001). It is important to note that E_2 -mediated inhibition of VSMC proliferation was not the only estrogenic effect observed in the $ER\alpha,\beta KO_{CH}$ mice. Estrogen exposure also resulted in a significant increase in $ER\alpha,\beta KO_{CH}$ uterine weights (Figure 1). This demonstrates that preservation of an effect of estrogen in the $ER\alpha,\beta KO_{CH}$ mice is not confined to, or specific for, the cardiovascular system. The estrogen responsiveness of the uterus in these mice raises an additional consideration. The $ER\alpha,\beta KO_{CH}$ mice used in the present study were produced by breeding $ER\alpha KO_{CH}$ and $ER\beta KO_{CH}$ mice.^{19,20,21,22} Uteri of $ER\alpha KO_{CH}$ mice express mRNA for 2 partial $ER\alpha$ transcripts.²³ Although one of these transcripts is nonfunctional, the other transcript, E1, is a splice variant of the wild-type $ER\alpha$ in which only a portion of exon 2 is omitted.²³ The protein encoded by this mutant sequence appears to bind estrogen with an affinity similar to that of the wild-type receptor, and to be capable of mediating hormone-induced gene expression.²³ In this prior study, immunoblot analyses of $ER\alpha KO_{CH}$ uterus did not detect expression of a protein from the E1 splice variant.²³ However, it remains quite possible that the estrogenic effects observed on VSMC proliferation and uterine weight in the present study are mediated by expression of low levels of this mutant $ER\alpha$. If the E1 transcript mediates the effects of estrogen on uterine weight and/or VSMC proliferation in the $ER\alpha,\beta KO_{CH}$ mice (and the parental $ER\alpha KO_{CH}$ mice¹⁰), this would have widespread implications for the published reports that have used $ER\alpha KO_{CH}$ mice to study various physiological systems (reviewed in Couse and Korach⁴⁰). The mechanism by which estrogen continues to inhibit VSMC proliferation and increase uterine weight in the $ER\alpha,\beta KO_{CH}$ mice thus remains unclear. Receptor-independent effects of estradiol could be involved, although there is little physiological evidence in support of this explanation.¹ The presence of an unidentified, novel estrogen receptor could also account for the persistent ability of E_2 to inhibit VSMC proliferation, although no $ER\alpha$ has been identified despite extensive searching. However, another member of the steroid hormone receptor superfamily⁴¹ (eg, $ERR\alpha$, $ERR\beta$, $ERR\alpha$, or an orphan receptor) with the ability to respond to estrogen could be involved. Alternatively, a low level of expression of the E1 estrogen receptor splice variant identified by Couse et al²³ in the $ER\alpha KO_{CH}$ parental strain could mediate residual estrogen responsiveness in $ER\alpha,\beta KO_{CH}$ mice. The explanation for persistent effects of estrogen in this study may be resolved by our ongoing

studies of the effect of E_2 on the vascular injury response in mice recently created in the Chambon laboratory in Strasbourg, France ($ER\alpha KO_{St}$),⁴² in which $ER\alpha$ has been fully disrupted. Understanding the mechanism by which estrogen responsiveness is retained in the $ER\alpha, \beta KO_{CH}$ mice has important implications for our understanding of estrogen receptor-mediated signaling in all target tissues, including the cardiovascular system.

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CHAPTER 4

Nitric oxide synthase(s) mediate the vascular protective effect of estrogen in the mouse carotid artery injury model

Henny M. Schulten, Richard H. Karas, Mark Aronovitz, Gary Pare, Flore Celestin, Ebo D. de Muinck, and Micheal E. Mendelsohn.

Abstract

Estrogen increases bioavailability of the endogenous vasodilator and vascular protective molecule, nitric oxide. We have previously shown that 17β -estradiol (E_2) inhibits the response to vascular injury in the mouse carotid injury model. We examined the effect of L-NAME, a non-selective nitric oxide synthase (NOS) inhibitor, on E_2 -mediated protection against vascular injury. Forty-one ovariectomized C57BL/6J female mice were randomized to receive vehicle alone, E_2 , L-NAME, or L-NAME+ E_2 . Mice were subjected to unilateral carotid arterial injury and the response to injury was assessed by independent measures of carotid medial area (MA) and vascular smooth muscle cell (VSMC) proliferation two weeks later. As compared to vehicle-treated mice (102.2 ± 0.3 mmHg), L-NAME treatment significantly increased mean systolic blood pressure in both the L-NAME-treated mice (122.9 ± 1.6 mmHg; $P < 0.001$ vs vehicle), and in the L-NAME+ E_2 -treated mice (110.8 ± 2.2 mmHg; $P < 0.001$ vs vehicle). Estrogen decreased blood pressure in both the E_2 -treated mice as compared to control animals (95.9 ± 1.3 mmHg; $P < 0.01$ vs vehicle), and the L-NAME+ E_2 -treated mice, as compared to mice treated with L-NAME alone (110.8 ± 2.2 mmHg; $P < 0.001$ vs L-NAME). Injury induced a significant increase in MA in vehicle-treated mice that was reversed in E_2 -treated mice ($P < 0.05$ vs vehicle-treated). In L-NAME-treated mice, injury caused an increase in MA similar to that seen in vehicle-treated mice ($P = \text{NS}$ vs vehicle-treated). However, E_2 treatment had no protective effect in mice receiving L-NAME. In vehicle-treated animals, carotid injury induced an increase in BrdU-labeled VSMCs ($PI = 0.37 \pm 0.04$; $P < 0.001$) that E_2 inhibited ($PI = 0.21 \pm 0.03$; $P < 0.01$ vs vehicle-treated). L-NAME alone had no significant effect on the degree of injury-induced VSMC proliferation. In L-NAME+ E_2 animals, estrogen no longer suppressed the vascular injury response. Therefore, pharmacological inhibition of nitric oxide synthases abolishes the protective effects of E_2 on medial area and VSMC proliferation in the mouse carotid injury model. These findings support that the vascular protective effects of E_2 *in vivo* are due to estrogen receptor-mediated activation of one or more NOS enzymes.

INTRODUCTION

Recent randomized clinical trials of conjugated equine estrogens and medroxy-progesterone acetate in older women, with or without known atherosclerosis, have shown no benefit and there were some trends toward harm.¹⁻³ In contrast, several large-scale observational studies have shown reduced cardiovascular risk associated with use of hormone replacement therapy.^{4,5} The effects of various hormone replacement regimens on both non-vascular and cardiovascular tissue are complex, underscoring the importance of understanding the molecular mechanisms by which hormones regulate vascular biology.

Animal models provide one useful approach to evaluating mechanisms by which estrogen can exert vascular protective effects.⁶ The mouse model of carotid arterial injury has proven useful in understanding the role of estrogen receptors (ER) α and β in mediating the protective cardiovascular effects of estrogen. Using this model, we have shown previously that administration of physiologic levels of 17 β -estradiol (E_2) completely inhibits the vascular injury response in ovariectomized female WT mice, an effect that is mediated by ER α in this model.⁷⁻¹⁰ Estrogen also accelerates re-endothelization of injured murine carotid arteries through ER α .¹¹ However, the molecular pathways downstream of estrogen receptor α responsible for mediating the protective effects of estradiol/ER α on vascular injury remain unknown.^{6,12}

Nitric oxide (NO), which has multiple important vascular protective effects, is one likely candidate to explain the beneficial effects of estrogen. Estrogen has previously been shown to augment NO bioavailability, acutely and following prolonged exposure, both *in vitro* and *in vivo*.^{6,11,13-16} Nitric oxide is produced by three isoforms of nitric oxide synthase (NOS). All three NOS isoforms are detectable in the vasculature, and data supporting a protective role of each have been reported.¹⁷⁻²² To test the hypothesis that NO produced by one or more of these NOS isoforms mediates estrogenic protection against vascular injury, we examined the effects of a non-selective pharmacologic NOS inhibitor on estrogen protection against the vascular injury response in the mouse carotid injury model.

MATERIALS AND METHODS

ANIMALS

Ten-twelve week old, female C57BL/6J mice (45) were purchased from Jackson Laboratories (Bar Harbor, ME). Four animals were housed per cage in a temperature-controlled facility on a 12 hour light-dark cycle and were fed a normal diet *ad libitum*, as previously described.^{7,8} Institutional guidelines of the Institutional Animal Care and Use committee, and of the Department of Laboratory Animals of

Tufts University School of Medicine and the Tufts New England Medical Center were followed.

MOUSE MODEL OF CAROTID ARTERIAL INJURY

The mouse carotid injury model used in this study has been described in detail previously.^{7-9,23,24} Forty-five adult female mice were ovariectomized and allowed to recover for 7–10 days. On Day–7, mice were randomly assigned to receive vehicle alone (vehicle), E₂-releasing pellets (E₂; 0.1 mg, 21-day release pellets, Innovative Research of America), N^G-nitro-arginine-methyl ester (L-NAME; Sigma Chemical Co., St. Louis, Missouri, USA), a non selective inhibitor of nitric oxide synthase inhibitor (1.5 gram/L in drinking water; refreshed every two days) or both L-NAME and E₂ (L-NAME+E₂). On day 0, the mice underwent unilateral common carotid artery injury by intraluminal passage of a fine wire, denuding the vascular endothelium and inducing a characteristic increase in medial area and VSMC proliferation. On the same day as the carotid injury was performed, the mice were implanted subcutaneously with an osmotic minipump (Alzet Inc., Palo Alto, California, USA) that continuously releases bromo-deoxy-uridine (BrdU; 25 mg/kg/d) throughout the experimental period.

BLOOD PRESSURE MEASUREMENTS

Blood pressure was measured in conscious animals on day–7, 0, 3, 10 and 14. Systolic blood pressure was measured by the tail-cuff method using a BP-2000 Specimen Platform (Visitech System, Cory, NC). Before the start of the experiment all animals were acclimated to the procedures involved with measuring blood pressure. Blood pressures were measured during 4 intervals, each lasting 5 minutes, during which 10 blood pressure measurements were taken. The reported blood pressures represent the means of all of these measurements. An investigator blinded to the animals treatment group assignment performed all measurements.

MORPHOMETRY AND IMMUNOHISTOCHEMISTRY

Two weeks following vascular injury, the mice were anesthetized again with 2.5% isoflurane and both carotid arteries were harvested after perfusion fixation with 10% formalin at 150 mmHg for 4 minutes. Uteri were also removed and wet weights were obtained. The response to vascular injury was assessed using two measures: MA (medial area) and VSMC proliferation. Parallel sections from all carotid arteries were stained as described previously for hematoxyline/eosin and for elastin, and MA measurements were made using a computerized morphometric analysis system on the elastin-stained sections (Image Pro Plus software).^{8,9,23} Immunostaining also was performed on parallel carotid artery sections using anti-BrdU antibodies (Sigma

Chemical Co., St. Louis, Missouri, USA), and VSMCs that were labeled with BrdU were quantified as described.^{8,9,23} Adventitial cells were not included in any of these analyses. Proliferation Index (PI) was calculated as the ratio of BrdU-positive VSMC to total VSMC per section. Two observers blinded to treatment group performed all analyses.

STATISTICAL ANALYSES

All values are expressed as mean $\pm$ SEM. For statistical analyses of blood pressure, time and treatment group were considered independent variables and a 2-factor ANOVA was performed. Post-hoc pairwise comparisons were made with the Student-Newman-Keuls test. All other statistical analyses were tested by 1-factor ANOVA, with post-hoc pairwise comparisons made with the Student-Newman-Keuls test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Four animals died during the surgery and three animals (2 E_2 -treated and 1 L-NAME-treated) had completely thrombosed arteries at the time of sacrifice. These animals were excluded from further analysis. At the time of injury there were no differences in body weight between the experimental groups (placebo, 23.4 ± 0.4 g; E_2 , 23.8 ± 0.3 g; L-NAME, 22.9 ± 0.4 g; L-NAME+ E_2 , 23.0 ± 0.4 g, $P = \text{NS}$). Estrogen treatment resulted in the expected increase in uterine weight^{9,10} from $9.5 \pm 0.8 \times 10^{-3}$ and $9.9 \pm 1.0 \times 10^{-3}$ g (placebo and L-NAME, respectively) to $74.1 \pm 5.6 \times 10^{-3}$ and $75.1 \pm 5.7 \times 10^{-3}$ g (respectively, E_2 and L-NAME+ E_2 groups, $P < 0.001$). These data confirm the appropriate delivery of and a physiologic response to E_2 in the E_2 -treated animals.

The mean systolic blood pressure in the vehicle-treated mice was 102.2 ± 0.3 mmHg. L-NAME treatment significantly increased mean systolic blood pressure in both the L-NAME mice (122.9 ± 1.6 mmHg; Figure 2; $P < 0.001$ vs Vehicle), and in the L-NAME+ E_2 mice (110.8 ± 2.2 mmHg, $P < 0.001$ vs Vehicle). Estrogen significantly decreased mean blood pressure in both the E_2 mice (95.9 ± 1.3 mmHg; Figure 2; $P = 0.008$ vs vehicle), and the L-NAME+ E_2 mice, as compared to mice treated with L-NAME alone (110.8 ± 2.2 mmHg; Figure 2; $P < 0.001$ vs L-NAME).

EFFECTS OF E_2 ON MEDIAL AREA

The MA of the uninjured (uninj) vessels was $13.1 \pm 0.4 \times 10^{-3}$ mm². Injury induced an increase in MA to $18.4 \pm 0.6 \times 10^{-3}$ mm² in the vehicle-treated mice (Figures 3 and 4, $P < 0.001$). As in previous studies,^{7-10,23} E_2 replacement inhibited the injury-induced increase in medial area to levels comparable to the uninjured vessels

Figure 1. Uterine weights of C57BL/6J mice treated with vehicle, E₂, L-NAME or L-NAME+E₂. The increases in uterine weights in the estrogen-treated groups is highly significant compared with the non-estrogen-treated groups. Bars represent the mean±SEM for each group. *P<0.001 vs vehicle or L-NAME.

Figure 2. Mean systolic blood pressure measurements in study groups over time. Bars represent the mean±SEM for each group. *P<0.001 vs vehicle-treated animals; # P<0.001 vs L-NAME.

($15.1 \pm 1.1 \times 10^{-3} \text{ mm}^2$, $P < 0.05$ vs vehicle; $P = \text{NS}$ vs uninj). L-NAME alone did not affect the magnitude of injury-induced increase in MA ($20.8 \pm 1.6 \times 10^{-3} \text{ mm}^2$; $P = \text{NS}$ vs vehicle-treated). However, E₂ was not able to inhibit the injury-induced increase in MA in the L-NAME+E₂ animals (Figures 3), $20.0 \pm 1.5 \times 10^{-3} \text{ mm}^2$; $P = \text{NS}$ vs L-NAME alone; $P < 0.01$ vs vehicle-treated).

EFFECTS OF E₂ ON VSMC PROLIFERATION

As in all previous studies with this model⁷⁻¹⁰ BrdU-labeled cells were detected only rarely in the uninjured arteries (mean < 1 cell per section). Carotid injury induced a pronounced increase in BrdU-labeled VSMCs in the vehicle-treated animals (proliferation index (PI)= 0.37 ± 0.04 ; $P < 0.001$). E₂ significantly inhibited this increase (PI= 0.21 ± 0.03 ; $P < 0.01$ vs vehicle-treated, Figure 5), as expected.^{9,10} L-NAME

Figure 3. Representative sections stained for measurement of medial area. (A-E) Representative segments from elastin-stained carotid artery sections are shown. All area measurements were made on complete sections. (A) uninjured, (B) injured, vehicle-treated, (C) injured, E₂-treated, (D) injured, L-NAME-treated, (E) injured, L-NAME+E₂-treated.

Figure 4. Medial area of uninjured (uninj) and injured (inj) carotid arteries from mice receiving vehicle, E₂, L-NAME or L-NAME+E₂. E₂ replacement significantly inhibited the injury-induced increase in medial area to levels comparable to the uninjured animals in the absence of L-NAME. E₂ replacement had no effect on the injury-induced increase in the presence of L-NAME. Bars represent the mean $\pm$ SEM for each group. * $P < 0.001$ vs uninj.

Figure 5. Vascular smooth muscle cell proliferation of uninjured (uninj) and injured (inj) carotid arteries from mice treated with vehicle, E₂, L-NAME, or L-NAME+E₂. E₂ inhibited VSMC proliferation significantly, in the absence of L-NAME. E₂ had no effect on injury-induced increases in VSMC proliferation in the presence of L-NAME. Bars represent the mean±SEM for each group. *P<0.001 vs uninj.

alone had no significant effect on VSMC proliferation following injury (0.47 ± 0.06 ; $P=NS$ vs vehicle-treated). As with the medial area, estrogen's ability to protect against vascular injury was completely abolished in the L-NAME+E₂ animals ($PI=0.44 \pm 0.04$; $P=NS$ vs L-NAME alone; $P<0.01$ vs vehicle-treated, Figure 5).

DISCUSSION

This study used the mouse carotid injury model to examine the mechanism by which estrogen inhibits medial thickening and VSMC proliferation following vascular endothelial denudation injury in ovariectomized female mice. Our data support that the vascular protective effects of estrogen in this model are mediated by one or more of the three nitric oxide synthase isoforms, eNOS, iNOS and/or nNOS, through which specific isoform(s) are responsible remains unclear.

It is important to consider the potential impact on our findings of differences in blood pressures between the treatment groups. As expected, both groups of mice that received L-NAME had significant elevations in mean systolic blood pressures. Though the presence of hypertension could offset estrogen's protective effect, hypertension-mediated inhibition of the response to injury in the L-NAME+E₂ mice seems unlikely for at least two reasons. First, despite the significant increase in blood pressure in the L-NAME mice, there was no significant difference in the magnitude of the response to vascular injury between the L-NAME and the vehicle-treated mice. Second, the addition of E₂ treatment in the L-NAME+E₂ group significantly decreased blood pressure compared to the L-NAME group, but this had no effect on the response to vascular injury. Thus, it seems unlikely that L-NAME's effects on blood pressure contributed in any important way to the current findings. It is of interest that E₂ treatment was able to lower blood pressure even in the L-NAME-treated animals. Though it is possible that this was due to an effect of E₂

on NOS activity despite the presence of high-dose L-NAME, it seems more likely that this is due to NOS-independent effects of E_2 on blood pressure regulation. One potential pathway by which this could occur is by E_2 -mediated inhibition of central sympathetic nerve activity, which can be induced by L-NAME.^{25,26} E_2 has previously been shown to inhibit sympathetic outflow in ovariectomized rats,²⁷ and E_2 -receptors have been identified in brain centers involved in cardiovascular regulation.^{28,29}

Estrogen causes an estrogen receptor-dependent but non-genomic increase in eNOS activity.^{13,15,30-34} Estrogen also induces the expression of eNOS,³⁵ iNOS,^{36,37} and nNOS^{35,38} genes in vascular cells. Furthermore, estradiol increases iNOS expression in macrophages³⁹ and neuronal nitric oxide protein expression in neutrophils.⁴⁰ There are therefore multiple explanations for the effects of L-NAME noted in the present study. The mouse carotid injury model is based on quantification of the vascular response to an endothelial denudation injury in normolipidemic animals. Very few, if any, white blood cells are noted in the injured vessels in this model (RKH and MEM, unpublished observations). In addition, in mice there is no published evidence at present for the expression of nNOS in the vessel wall and repeated attempts made to detect nNOS in the vasculature in one of our prior studies were unsuccessful.²² Therefore, the most plausible mechanism for estrogen-mediated protection against vascular injury response in this model is that estrogen-estrogen receptor alpha¹⁰ induced eNOS and/or iNOS in the vasculature of wild-type mice. Because the non-specific NOS inhibitor L-NAME was used in this study, it will be necessary to address whether eNOS and/or iNOS are responsible for mediating protection in subsequent studies with NOS-specific knockout mice.

In conclusion, the current findings demonstrate that estrogen-mediated protection against vascular injury is blocked by co-administration of the non-specific NOS inhibitor L-NAME. This observation supports that E_2 -mediated protection from vascular injury is mediated by one or more NOS isoforms. These findings have important implications of the *in vivo* molecular pathways that mediate the vascular protective effects of estrogen.

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CHAPTER 5

**Disruption of the iNOS gene abolishes
estrogen-mediated protection against
vascular injury in mice**

Daniela Neu, Henny Schulten, Mark Aronovitz, Flore Celestin, Richard H. Karas,
and Michael E. Mendelsohn

Abstract

Introduction – We have previously shown that estrogen inhibits the response to vascular injury in the mouse carotid artery injury model by a nitric oxide synthase-dependent pathway. In the current study, we tested the hypothesis that iNOS mediates estrogen's vascular protective effects.

Methods – Twenty-four ovariectomized, female wild-type (WT) and iNOS knockout (iNOS^{-/-}) mice, randomized to receive vehicle alone or 17 β -estradiol (E₂), were subjected to unilateral carotid artery injury. Two weeks later, the response to vascular injury was assessed by quantification of vascular smooth muscle cell (VSMC) proliferation, measured by immunohistochemical detection of BrdU-labeled cells (Proliferation Index, PI: BrdU-labeled cells/total cells), and medial area (MA), measured by computerized morphometry.

Results – Ovariectomy, estrogen replacement or iNOS deficiency did not alter mean systolic blood pressure. BrdU-labeled cells were only rarely detected in uninjured vessels. In the WT mice, carotid artery injury increased the PI in the vehicle-treated mice (PI=0.19 $\pm$ 0.05; P<0.01 vs uninjured), and this was significantly attenuated by E₂ treatment (PI=0.10 $\pm$ 0.03; P<0.05 vs WT vehicle-treated). In the iNOS^{-/-} mice, carotid artery injury also increased VSMC proliferation in the vehicle-treated mice (PI=0.12 $\pm$ 0.04; P<0.01), but this was not significantly diminished in the E₂-treated mice (0.12 $\pm$ 0.02; P=NS vs iNOS^{-/-} vehicle-treated). The MA of the uninjured vessels averaged 18.8 $\pm$ 0.4 $\times 10^{-3}$ mm². In the WT mice, injury induced an increase in MA in the vehicle-treated animals (MA=26.3 $\pm$ 0.4 $\times 10^{-3}$ mm²; P<0.001 vs uninjured), and this was completely blocked in the E₂-treated mice (MA=20 $\pm$ 0.9 $\times 10^{-3}$ mm²; P<0.01 vs vehicle-treated). In contrast, in iNOS^{-/-} mice, injury did not induce a significant increase in MA in the vehicle-treated mice (MA=20.7 $\pm$ 1.1 $\times 10^{-3}$ mm²; p=NS vs uninjured), and was subsequently unaffected by E₂ (MA=20.0 $\pm$ 0.7 $\times 10^{-3}$ mm²; P=NS vs iNOS^{-/-} vehicle-treated).

Conclusions – The response to vascular injury is absent in mice that lack the iNOS protein. These findings identify iNOS as an important target for further investigation of the mechanisms that mediate the vascular protective effects of estrogen.

INTRODUCTION

There is currently considerable controversy, and hence considerable interest, in the cardiovascular effects of steroid sex hormones like estrogen. Several observational studies suggest that estrogen supplementation is associated with a marked reduction in cardiovascular risk in postmenopausal women. In contrast, recent randomized clinical trials have demonstrated no benefit of postmenopausal hormone replacement therapy, and, have suggested further that hormone replacement therapy may increase cardiovascular risk in some women. These discordant findings have highlighted the need for further understanding the molecular mechanisms by which these hormones affect the cardiovascular system. The cardiovascular effects of estrogen are mediated by effects on systemic, circulating factors and by direct effects on the blood vessel.^{1,2-4} We have shown previously that estrogen inhibits the response to vascular injury in normocholesterolemic mice.⁵⁻⁷ However, the molecular mechanisms underlying this process are not well understood.⁸⁻¹⁰ We recently observed that treatment of mice with the non-selective inhibitor of nitric oxide synthases, L-NAME, abolished the inhibitory effects of estrogen on the response to vascular injury. There are 3 known isoforms of nitric oxide synthase (NOS), and both inducible NOS (iNOS), and endothelial NOS (eNOS) have been shown to play important roles in regulating vascular biology. iNOS is generally considered to be a pro-inflammatory factor, induced in cells such as macrophages and vascular smooth muscle cells (VSMC) by cytokines and other inflammatory stimuli. The expression of iNOS is increased in injured arteries,¹¹⁻¹⁵ and the response to vascular injury is diminished in mice lacking iNOS.^{16,17} Taken together with previous reports demonstrating that estrogen regulates iNOS expression in mouse and rat aorta,^{18,19} as well as in cultured cells,^{20,21} these observations led us hypothesize that estrogen's vascular protective effects are in part mediated by its effects on iNOS gene expression. Therefore, as a first step to explore further the role of specific NOS isoforms in the vascular protective effects of estrogen, we examined the effects of estrogen on the vascular injury response in iNOS knockout (iNOS^{-/-}) mice.

MATERIAL AND METHODS

ANIMALS

All procedures involving experimental animals were performed in accordance with the Public Health Service animal welfare policy and the American Association for the Accreditation of Laboratory Animal Care. Animal protocols were approved by the Institutional Animal Care Use Committee at Tufts-New England Medical Center. Animals were housed at four per cage in a temperature-controlled facility on a 12 hour light-dark cycle, and fed regular rodent chow ad libitum. Female iNOS^{-/-}

mice (n=24) and their respective littermate wild-type (WT) controls (n=24) were obtained from Jackson Laboratories, Bar Harbor, ME.²² The genotype of all animals was confirmed by PCR analysis of genomic DNA isolated from tail snips taken at the time of harvest following the protocol provided by the vendor.

BLOOD PRESSURE MEASUREMENTS

Blood pressure was monitored in conscious animals well-acclimated to the procedure. Systolic blood pressure was measured by the tail-cuff method using a BP-2000 Specimen Platform (Visitech Systems, Cory, NC). Groups of four animals were measured over 45 min in intervals of 5 min, each interval consisting of 10 measurements. Results obtained during the first 15 minutes were excluded from the final analysis and the remaining results were averaged. All measurements were done at the same time on two consecutive days by an investigator blinded to the genotype of the mice.

ESTRADIOL RADIO-IMMUNOASSAY

Circulating E₂ levels at the time of harvest were measured in triplicate from 200 μ L pooled serum using a commercially available radio-immunoassay kit (Estradiol RIA, Diagnostic Systems Laboratories, Inc., Webster, TX) according to the manufacturer's instructions. Control experiments confirmed the linearity of the assay between the ranges of 0.018 nM and 2.78 nM.

CAROTID ARTERY INJURY

The carotid artery injury was performed as described previously.^{5-7,23-25} Briefly, thirteen week-old mice were ovariectomized and allowed two weeks to recover. They were then randomized to receive subcutaneously either vehicle alone (Veh), or 21-day sustained release pellets containing 100 mg of 17 β -estradiol (E₂; Innovative Research of America, Sarasota, FL). One week after pellet implantation, gas-anesthetized (2.5% Isoflurane, Abbot Laboratories, North Chicago, IL) mice underwent common carotid artery injury by intraluminal passage of a fine wire, denuding the vascular endothelium and inducing a characteristic increase in medial area and VSMC proliferation. On the same day as the carotid injury, an osmotic minipump was implanted subcutaneously that releases bromodeoxy-uridine (BrdU: 25 mg/kg/d) throughout the experiment.

TISSUE HARVEST

Fourteen days after vascular injury experimental tissues were harvested. The vasculature was perfusion fixed with 10% buffered formalin under constant pressure

of 125 mmHg for 4 min. Both common carotid arteries were then harvested, embedded in paraffin and processed as previously described.^{5-7,23-25}

MORPHOMETRY AND BrdU LABELING

All morphometric analyses were made by two independent observers blinded to genotype and treatment. The response to vascular injury was assessed by 2 methods. VSMC proliferation was assessed by identifying BrdU-labeled cells by immunostaining as previously described.^{5-7,23-25} A Proliferation Index (PI) was calculated as the ratio of BrdU-positive VSMC to total VSMC in each section. To distinguish between VSMC and endothelial cells, immunostaining for vascular smooth muscle cell specific actin and the endothelium specific Von Willebrand Factor was performed as described.^{5-7,23-25} The response to vascular injury was also assessed by measuring vascular medial area (MA) from elastin-stained sections from each artery as described.^{5-7,23-25} Area measurements were made using a computerized morphometric analysis system (ImagePro Plus software). Medial area was defined as the area within the external elastic lamina minus the area contained within the internal elastic lamina.

DATA ANALYSIS

One mouse (iNOS^{-/-}) that developed signs of an acute ischemic stroke at the time of vascular injury was sacrificed. At time of harvest, four arteries were thrombosed (three WT vehicle-treated, one iNOS^{-/-}; E₂-treated). These animals were excluded from further evaluation. Furthermore, three of the injured arteries (one WT; vehicle-treated, one WT E₂-treated, one iNOS^{-/-}; vehicle-treated) were excluded due to incomplete injury, as evidenced by the lack of BrdU-labeled endothelial cells.

All values are expressed as mean ± SEM. When only two groups were compared, differences were assessed by the unpaired Student's *t*-test. When multiple group comparisons were required, differences between the means were tested by one- or two-way-ANOVA. Where appropriate post-hoc analysis was performed using Student-Newman-Keuls multiple comparison test. All statistics were performed using GraphPad software. *P* < 0.05 was considered to be statistically significant.

RESULTS

E₂ LEVELS AND UTERINE WEIGHTS

E₂ treatment provided similar serum levels of E₂ in the WT (0.68 ± 0.01 nmol/L) and iNOS^{-/-} mice (0.57 ± 0.01 nmol/L) (*n* = 8). In the placebo-treated animals the E₂ levels were below detection. E₂-treated animals had uterine weights of 98 ± 5 mg

($n=24$), while in placebo-treated animals the uterine weights were reduced to 8 ± 0.3 mg, ($n=23$) ($P<0.001$). A subgroup analysis revealed no differences in uterine weights between the WT and iNOS^{-/-} animals in their respective groups (Figure 1).

BLOOD PRESSURE MEASUREMENTS

To determine whether there were differences in blood pressure between the iNOS^{-/-} and wild type mice that might alter the response to vascular injury, arterial blood pressures were evaluated. At baseline prior to ovariectomy, mean arterial blood pressure did not differ between the WT and iNOS^{-/-} mice (WT, 114 ± 3 mmHg, $n=24$; iNOS^{-/-}, 112 ± 3 mmHg, $n=23$, $P=NS$). Similarly, at the time of harvest, mean systolic pressure did not differ between the wild type and iNOS^{-/-} mice in either the placebo-treated or E₂-treated groups. There was a trend towards an effect of treatment on blood pressure in that the ovariectomized placebo-treated animals of both genotypes had somewhat higher blood pressures than prior to ovariectomy or after ovariectomy with E₂ treatment, but this did not reach statistical significance.

RESPONSE TO VASCULAR INJURY

BrdU-labeled cells were rarely detected in the uninjured arteries (mean < 1 cell per section). As shown in Figure 3, in the WT mice, carotid injury induced a pronounced increase in BrdU-labeled VSMCs in the vehicle-treated animals $PI=0.19\pm0.05$ ($P<0.01$ vs uninjured). E₂ attenuated this increase, although not completely to the level of uninjured vessels ($PI=0.10\pm0.03$; $P<0.05$ vs uninjured; $P<0.05$ vs WT; vehicle-treated). Vascular injury induced a similar increase in VSMC proliferation in the iNOS^{-/-} animals ($PI=0.12\pm0.04$, $P<0.01$ vs uninjured, $P=NS$ vs injured WT; vehicle-treated). In contrast to what was found in the WT mice, E₂ treatment had no effect on the injury-induced increase in VSMC proliferation in the iNOS^{-/-} mice ($PI=0.12\pm0.02$; $P=NS$ vs iNOS^{-/-}; vehicle-treated).

In the WT animals, injury significantly increased medial area (uninjured $18.8\pm4.1 \times 10^{-3}$ mm², $n=47$ vs WT injured vehicle-treated $26.3\pm3.6 \times 10^{-3}$ mm², (Figure 4) $n=7$, $P<0.001$) in wild-type animals and E₂ treatment inhibited this increase in medial area (WT injured; E₂-treated = $20.82\pm8.91 \times 10^{-3}$ mm², $n=11$, $P=NS$ vs uninjured; vehicle-treated). In the iNOS^{-/-} mice, injury did not induce an increase MA in the vehicle-treated mice (uninjured $18.8\pm4.1 \times 10^{-3}$ mm², $n=47$ vs $20.7\pm1.1 \times 10^{-3}$ mm² iNOS^{-/-}; vehicle treated, $n=10$, $P=NS$) and was for this reason unaffected by E₂ treatment (iNOS^{-/-}; E₂-treated $20.0\pm0.7 \times 10^{-3}$ mm², $n=11$, $P=NS$).

Figure 1. Estrogen increased uterine weight to a similar degree in both the WT and iNOS^{-/-} mice. * $P < 0.01$ vs vehicle-treated animals of the same genotype. Bars represent means $\pm$ SEM.

Figure 2. Summary of mean systolic blood pressure for all wild-type and iNOS^{-/-} mice ($n = 12 + 12$ and $n = 11 + 12$, respectively) related to vehicle-treated or E₂-treated animals. There were no significant differences in mean systolic blood pressure between the groups ($n = 11-12$ per group). All data are shown as mean $\pm$ SEM.

Figure 3. Vascular smooth muscle cell proliferation of uninjured and injured carotid arteries from wild-type and iNOS^{-/-} mice treated with vehicle or 17 β -estradiol. Estrogen reduced VSMC proliferation ($P < 0.05$ vs inj+V). There were no significant differences in VSMC proliferation between vehicle-treated and E₂-treated mice. * $P < 0.01$ vs vehicle-treated animals of the same genotype. All data are shown as mean $\pm$ SEM. Uninj, uninjured; inj, injured; V, vehicle; E₂, 17 β -estradiol.

Figure 4. Medial areas of uninjured and injured carotid arteries from wild-type and *iNOS*^{-/-} mice treated with vehicle or 17 β -estradiol. Estrogen inhibited injury-induced increases in medial area in wild-type animals (P =NS, vs uninj). There were no significant differences in medial areas between the groups of *iNOS*^{-/-} mice. * P <0.01 vs vehicle-treated animals of the same genotype. All data are shown as mean $\pm$ SEM. Uninj, uninjured; inj, injured; V, vehicle; E₂, 17 β -estradiol.

DISCUSSION

This study shows that E₂ inhibits vascular-injury-induced medial thickening and VSMC proliferation in ovariectomized female mice as it has been reported previously.^{5–7,23–25} We observed that the E₂-mediated inhibition of VSMC proliferation in response to vascular injury is no longer present in *iNOS*^{-/-} mice. These results support the hypothesis that the regulation of iNOS expression plays an important role in the E₂-mediated protection against vascular injury.

To compare the results presented here with other studies it is important to identify the different models of vascular injury currently used. Each model emphasizes different aspects of the development of atherosclerosis, like the formation of neointima or vascular remodeling.^{12,26–28} We used a technique of carotid injury by endothelial denudation with a wire. This model is known to lead mainly to medial remodeling and only rarely to minimal neointima formation.²⁶ The two endpoints of response to vascular injury that can be assessed with this model are increase in medial area and medial VSMC proliferation.

In our study the lack of iNOS expression completely blunted injury-induced medial area increase. In contrast, the deficiency of iNOS did not lead to a significant inhibition of VSMC proliferation (WT injured vs *iNOS* injured, P =0.25), suggesting that iNOS-derived NO affects more the other parameters involved in medial thickening, like for example matrix formation or SMC migration from adventitia to media. However, we did not find differences in total medial cell numbers between WT and *iNOS* KO mice (101 ± 10 vs 96 ± 3 respectively, P =NS). The fact that the majority of the endothelial cells stained positive for BrdU proves that the vessels have been deendothelialized by the wire injury to same extend as the wild-type animals. These results are in contrast to earlier studies showing that therapeutic overexpression of iNOS^{29–32}, has a protective effect on the response to

vascular injury. In these studies the acute introduction of iNOS, leading to a transient boost of NO, may have prevented the initiation of the remodeling after injury and as well as inhibition of the subsequent induction of the host's iNOS. This may be important because upregulation of the host's iNOS expression may be detrimental because this results in a prolonged production of huge quantities of NO leading to an inflammatory response with the subsequent induction of growth factors.

Besides this, all the currently published data in iNOS^{-/-} mice using different vascular injury models are in concordance with our findings that iNOS promotes the response to vascular injury.^{16,17} In regard to the study from Chyu et al¹⁶ it is important to notice that iNOS was considered to be vasoprotective regarding the intima/media ratio. However the raw data about medial and intimal thickening confirm our data suggesting a protective role for iNOS-deficiency. This hypothesis is also supported by the fact that iNOS expression is induced after vascular injury¹¹ and that iNOS is present in atherosclerotic plaques colocalizing with oxidized LDL, which is known to have a proatherogenic effect.¹³ We conclude that iNOS which is known to be expressed in the arterial wall after vascular injury promotes vascular remodeling by increase in extracellular matrix and proliferation of VSMC.

Several studies have demonstrated that E₂ treatment can inhibit the cytokine-induced induction of iNOS in cultured VSMC and macrophage-like cells of the CNS.^{18,20,21,33,34} Furthermore, it has been shown in a model of transplant atherosclerosis that physiologic doses of E₂ can inhibit the upregulation of iNOS.³⁵ Interestingly the response to injury in the vehicle-treated iNOS deficient mice closely resembled the values in the E₂-treated WT mice, and E₂ treatment of the iNOS^{-/-} mice did not show any additional effect, suggesting that E₂ mediated downregulation of iNOS is involved in its vasculoprotective action. This pathway could also be responsible for the finding that pregnancy, which is known to be associated with elevated E₂ levels, protects WT as well as NOS3-KO mice from vascular injury, suggesting that E₂ has NOS3-independent effects on vascular remodeling.³⁶

Taken together these findings suggest that E₂'s vasculoprotective effects are mediated by the downregulation of injury-induced iNOS expression. Further studies, using different vascular techniques and mouse models of atherosclerosis will be necessary to confirm the general importance of these findings. In addition studies using specific iNOS inhibitors would be helpful to investigate, if these compounds mimic E₂'s vasculoprotective effects. This is of crucial importance, since the risks associated with hormone replacement therapy have been highly publicized recently and heightened the interest in alternative therapies.³⁷

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CHAPTER 6

**Endothelial nitric oxide synthase inhibits
neointimal hyperplasia after vascular injury
in hyperlipidemic mice**

Henny M. Schulten, Bianca H. Schrans-Stassen, Denny G.P. Sonnemans,
Paul M.H. Schiffers, Mark J. Post, and Ebo D. de Muinck.

Abstract

Objective – The purpose of the current study was to examine the effects of local endothelial nitric oxide synthase (*eNOS*) gene transfer on neointimal hyperplasia in a hyperlipidemic, atherosclerotic model. We hypothesized that in this milieu which is conducive to *eNOS* uncoupling and rich in superoxide, *eNOS* transfer alone may not inhibit neointimal hyperplasia and that its effect could be restored by adjunctive administration of folates.

Methods and Results – ApoE^{-/-} mice on a Western diet were subjected to unilateral carotid artery endothelial denudation injury and randomized into 4 groups receiving vehicle alone, adenovirus containing β -galactosidase cDNA, adenovirus with *eNOS* cDNA, or the combination of adenovirus containing *eNOS* cDNA with folate treatment. Two weeks later, carotid arteries were harvested and the response to injury was assessed by computerized morphometry of carotid arteries total area, medial area and intimal area. Carotid artery injury resulted in reduced intimal area and intima/media ratio in mice receiving AdeNOS with or without folates, while total area and medial area were not affected.

Conclusions – *eNOS* reduced neointima formation after arterial injury, even in the setting of atherosclerosis and hyperlipidemia, hence folate was not required to restore its activity.

INTRODUCTION

Clinical data indicate that stents reduce postangioplasty restenosis by about 40%¹ in spite of augmented neointima formation. Clinically significant in-stent restenosis occurs in 20–40% of percutaneous transluminal coronary angioplasty (PTCA) cases.^{2,3} In-stent restenosis is a disease characterized exclusively by neointimal hyperplasia, since the rigid device virtually precludes the recoil and constrictive remodeling that contribute to balloon angioplasty-related restenosis.⁴ Coated stents can be used as tools for local delivery of drugs or genes that inhibit neointimal hyperplasia, and this technique has opened new avenues to pursue pharmacologic or gene therapy targets that are considered unsafe when given systemically.

Although the pathophysiological mechanisms underlying neointimal hyperplasia and vascular remodeling have been studied extensively, pharmacological and mechanical approaches remain notoriously unsuccessful in mitigating the process. Several mechanisms can be identified: disruption of the endothelium,⁵ platelet activation and thrombus formation,^{6,7} smooth muscle cell (SMC) migration and proliferation,^{5,8,9} extracellular matrix changes and remodeling^{7,10–12} or 'shrinkage' of the artery.¹³ Proliferative and migratory responses of SMCs and myofibroblasts, together with extracellular matrix deposition are central to the process. These responses are driven by a variety of cytokines and chemokines, including platelet-derived growth factor, fibroblast growth factor, endothelial cell growth factor, insulin-like growth factor, angiotensin II, and endothelin.¹⁴

The normal protective barrier function of endothelial cells is disrupted after PTCA resulting in the loss of constitutively expressed endothelium-derived vasoactive factors, including nitric oxide (NO), prostacyclin, and bradykinin.¹⁵ NO stimulates soluble guanylate cyclase in the SMC which leads to vasorelaxation, inhibition of cell proliferation and migration, and extracellular matrix production.^{16,17} NO also affects circulating platelets and inhibits platelet adhesion to the vessel wall. In animal models of arterial injury, administration of NO-donor compounds or stimulation of endogenous NO production by administration of its precursor L-arginine reduces the vascular response.^{18,19}

eNOS consists of a flavin-containing reductase domain and a heme-containing oxidase domain.²⁰ In the presence of Ca^{2+} / calmodulin, an electron is transferred from NADPH to the heme group, leading to reduction of oxygen, which is then used to oxidize L-arginine to NO and citrulline. BH_4 is an eNOS cofactor that directs the electron flow to L-arginine. Under certain pathophysiological conditions, such as oxidative stress in hyperlipidemia, depletion of BH_4 results in uncoupling of oxygen reduction and L-arginine oxidation,²¹ thereby generating superoxide.²² L-5-methyltetrahydrofolate (L-5-MTHF, the main form of folate in the circulation²³) reverses this uncoupling of eNOS supposedly by regeneration of BH_4 .²⁴

Intravascular *eNOS* gene transfer at the site of balloon dilation or stent implantation has been shown to reduce neointimal hyperplasia in normal arteries of

non-hyperlipidemic animals.^{25–28} However, eNOS gene transfer in atherosclerotic arteries of hyperlipidemic animals could lead to uncoupling of eNOS enzymatic activity. Hence, we studied the efficacy of *eNOS* gene transfer under conditions that could be conducive to eNOS uncoupling and the potential rescue by exogenous folate supplementation. We performed these studies in the carotid artery injury model in apolipoprotein (apo) E knockout mice.

MATERIAL AND METHODS

ANIMALS

Procedures used in these experiments were approved by the University of Maastricht Animal Care and Ethical Committee. We studied 12 months old male homozygous apoE-deficient mice (with a 129xC57BL/6J background and backcrossed through 9 generations on a C57BL/6J background; Charles River Laboratories, the Netherlands). Mice had free access to tap water and after weaning were maintained on a high fat Western diet deficient of folic acid (15% cacao butter, 2% cholate, 0.25% cholesterol, 40.5% sucrose, 10% corn starch, 1% corn oil and 5.95% cellulose; Hope Farms, Woerden, the Netherlands). One week prior to injury, animals which were randomized to receive the combination AdeNOS and folate were changed to high fat diet containing L-5-methyltetrahydrofolate (L-5-MTHF) and received supplementary L-5-MTHF in drinking water (1%, w/v).

LIPID PROFILE AND L-5-MTHF DETERMINATIONS

Plasma cholesterol and triglyceride levels were determined in duplicate by using a colorimetric assay (CHOD-PAP 1442341 and GPO-PAP 701912, respectively, La Roche) on arterial plasma samples obtained at 12 months of age.

Plasma L-5-MTHF levels were measured by fluorescence after deproteinization with perchloric acid and separation by high-performance liquid chromatography (HPLC).²⁹

Plasma prostaglandin F_{2α} (PGF_{2α}) levels were determined by using a competitive enzyme immunoassay according to the manufacturer's instructions (Cayman Chemicals).

CAROTID ARTERY INJURY AND APPLICATION OF VIRAL CONSTRUCTS

Sixty-five apoE^{-/-} mice were anesthetized with 2.5% isoflurane and subsequently subjected to unilateral carotid artery injury as described by Lindner.³⁰ Briefly, the left carotid artery was exposed via a midline neck incision. The bifurcation of the carotid artery was located and two ligatures were placed around the external carotid

artery, which was subsequently tied off with the distal ligature and the internal carotid artery was temporarily clipped off to prevent back bleeding. A puncture was made between the ligatures to introduce a curved flexible wire (0.35 mm diameter, Cooke, Bloomington, IN, USA) into the common carotid artery and was passed along the vessel three times while rotating. After the wire had been removed, the common carotid artery was flushed several times with saline to get rid of debris (loose endothelial cells) and remaining blood cells. Then, a catheter was introduced into the common carotid artery and 10 μ L of vehicle (saline), 10 μ L 5.0×10^6 pfu/ μ L of AdLacZ or AdeNOS was instilled for 20 minutes and rinsed out. Flow was restored, the external carotid artery ligated and the skin wound closed with silk sutures.

TISSUE HARVEST

Two weeks following vascular injury, the mice were anesthetized again with 2.5% isoflurane and blood was drawn for cholesterol, triglyceride and L-5-MTHF determinations. Both carotid arteries were harvested after perfusion fixation with 10% formalin at 150 mmHg for 4 minutes.

HISTOLOGY AND MORPHOMETRY

Following embedding in paraffin, parallel cross-sections (4- μ m) from the carotid arteries were stained with Lawson's solution (Boom BV, Meppel, the Netherlands), which highlights the elastic membranes. Intimal, medial and total areas were determined on serial sections using Image Pro Plus software. The intimal surface area was calculated by subtracting the free lumen area from the area circumscribed by the internal elastic lamina, whereas the medial area was defined as the area between the internal lamina and the external lamina. The intima/media ratio was determined by dividing the intimal area by the medial area. Total area was defined as the area circumscribed by the external lamina. All observations were made by an observer blinded to treatment group.

For histochemical detection of β -galactosidase activity an AdLacZ transfected carotid artery was harvested two days after vascular injury and placed in a solution containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$ and 1 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) for overnight incubation at 37° C. After fixation in 10% formalin and embedding in paraffin, 4- μ m cross-sections were examined under a microscope for blue-stained cells.

DATA ANALYSIS

Results are shown as means $\pm$ SEM. Statistical significance of differences between experimental groups were evaluated using analysis of variance (ANOVA) followed by a

post-hoc comparison with LSD Fischer significant difference test. All statistics were performed using SigmaStat software. A P-value of <0.05 was considered significant.

RESULTS

GENERAL OBSERVATIONS

Body weight, plasma cholesterol and triglyceride levels did not differ between experimental groups (Table 1). Mice on folates, i.e. 1% L-5-MTHF into the drinking water and folic acid supplemented chow, had elevated L-5-MTHF-plasma levels as compared to animals on tap water and folic acid free chow. Folate treatment did not influence plasma cholesterol and triglyceride levels in the animals (Table 1). Furthermore, folate administration did not affect plasma levels of prostaglandin $F_{2\alpha}$, a biomarker for oxidative stress. Peri-operative and follow-up mortality rates were high in all groups (Table 1).

To confirm effective transduction of the viral vector containing β -galactosidase, we first examined X-gal-stained sections of the transduced injured carotid artery harvested two days after mechanical denudation (Figure 1). Expression of β -galactosidase activity, as reflected by blue staining, was observed in the intimal and medial surface and some scattered blue spots were seen in the adventitia.

RESPONSE TO VASCULAR INJURY

Uninjured carotid arteries showed minimal intimal thickening ($3.3 \pm 1.9 \times 10^{-3} \text{ mm}^2$). Unilateral carotid artery injury resulted in an expected increase in total vessel area as compared to uninjured vessels, however, eNOS treatment alone or in combination with folates did not affect this increase (Table 2).

Table 1. Characteristics of mice

	Vehicle	β -gal	eNOS	eNOS+folate
Weights (g)	38 $\pm$ 3	33 $\pm$ 1	35 $\pm$ 2	34 $\pm$ 2
Cholesterol (mmol/L)	24 $\pm$ 9	20 $\pm$ 3	21 $\pm$ 3	18 $\pm$ 4
Triglycerides (mmol/L)	.56 $\pm$.20	.41 $\pm$.05	.57 $\pm$ 0.07	.54 $\pm$.01
L-5-MTHF (nmol/L)	76 $\pm$ 18	118 $\pm$ 48	N.D.	977 $\pm$ 139*
PGF _{2α} (ng/ml)	3.1 $\pm$.3	3.0 $\pm$.6	3.0 $\pm$.3	3.2 $\pm$.3
Mortality (%)	53	62	36	62

Values are means $\pm$ SEM (n=5–9). N.D. not detectable. * p<0.001 vs vehicle, β -gal and eNOS.

Table 2. Effect of treatment on vessel wall morphometry

Treatment	NI area ($\times 10^{-3}$ mm ²)	Medial area ($\times 10^{-3}$ mm ²)	I/M ratio	Total area ($\times 10^{-3}$ mm ²)
Uninjured	3.3 $\pm$ 1.9	23.3 $\pm$ 1.0	.05 $\pm$.03	78.1 $\pm$ 4.5
Injured, vehicle	39.7 $\pm$ 13.9 *	46.6 $\pm$ 3.9 *	.64 $\pm$.21 *	133.5 $\pm$ 7.5 *
Injured, β -gal	31.2 $\pm$ 11.3 *	44.9 $\pm$ 4.4 *	.49 $\pm$.23 *	153.8 $\pm$ 8.0 *
Injured, <i>eNOS</i>	12.6 $\pm$ 3.2 †	50.6 $\pm$ 3.5 *	.22 $\pm$.05 †	133.8 $\pm$ 6.1 *
Injured, <i>eNOS</i> +folate	17.3 $\pm$ 7.0 †	52.3 $\pm$ 5.8 *	.23 $\pm$.05 †	146.1 $\pm$ 8.5 *

Values are means $\pm$ SEM (n=5-9). NI neointima, * P<0.05 vs uninjured, † P<0.05 vs vehicle.

Figure 1. β -gal expression in an injured carotid artery of an apoE^{-/-} mouse two days after gene transfer and carotid artery denudation. Transgene expression is present in neointima, medial area and rarely spotted in the adventitia. Sections were counterstained with nuclear fast red.

Neointima formation was significantly inhibited by 68% in animals treated with *eNOS* gene transfer, and by 56% in animals treated with AdeNOS and L-5-MTHF (Figures 2, 3 and Table 2). Vascular injury resulted in significant increases in carotid medial area in animals receiving vehicle alone (46.6 $\pm$ 3.9 $\times 10^{-3}$ mm²), β -galactosidase (44.9 $\pm$ 4.4 $\times 10^{-3}$ mm²), *eNOS* (50.6 $\pm$ 3.5 $\times 10^{-3}$ mm²) or the combination of *eNOS* and L-5-MTHF/folic acid (52.3 $\pm$ 5.8 $\times 10^{-3}$ mm²) as compared to the uninjured vessels (23.3 $\pm$ 1.0 $\times 10^{-3}$ mm²) (Figure 2, 4 and Table 2). Unexpectedly, and in spite of the robust medial thickening by injury, medial area was not affected by treatment. To correct for initial changes in arterial size, intima/media (I/M) ratios were calculated (Figure 5 and Table 2). AdeNOS alone or with supplemental L-5-MTHF/folic acid significantly reduced intima/media ratios of injured vessels.

Figure 2. Representative sections stained for measurement of neointimal, medial and total area. Neointimal areas of uninjured and injured mice treated with vehicle, β -gal cDNA gene transfer, *eNOS* cDNA gene transfer or in combination with systemic folate administration. (A-E) Representative segments from elastin-stained carotid artery sections are shown. All area measurements were made on complete sections. (A) uninjured, (B) injured, vehicle-treated, (C) injured, adeno β -gal-treated, (D) injured, adeno *eNOS*-treated, (E) injured, adeno *eNOS*-treated + systemic folate administration (one week prior to injury).

Figure 3. Neointimal area in uninjured (uninj) and injured (inj) carotid arteries from mice receiving vehicle (inj, vehicle), adeno β -gal cDNA (inj, β -gal), adeno *eNOS* cDNA (inj, *eNOS*) or in combination with systemic folate (F) administration (inj, *eNOS*+F). *eNOS* gene transfer, and in combination with folate administration (one week prior to injury) significantly inhibited the injury-induced increase in neointimal area to levels comparable to the uninjured animals. The neointimal area was determined by computerized morphometric analysis of full circumference sections from each animal. Bars represent the mean $\pm$ SEM for each group. * $P < 0.05$ vs uninjured, † $P < 0.05$ vs vehicle.

Figure 4. Medial area in uninjured (uninj) and injured (inj) carotid arteries from mice receiving vehicle (inj, vehicle), adeno β -gal cDNA (inj, β -gal), adeno *eNOS* cDNA (inj, *eNOS*) or in combination with systemic folate (F) administration (inj, *eNOS*+F). Treatment did not inhibit the injury-induced increase in medial area. The medial area was determined by computerized morphometric analysis of full circumference sections from each animal. Bars represent the mean $\pm$ SEM for each group. * $P < 0.05$ vs uninjured.

Figure 5. Intima/media ratio in uninjured (uninj) and injured (inj) carotid arteries from mice receiving vehicle (inj, vehicle), adeno β -gal cDNA (inj, β -gal), adeno *eNOS* cDNA (inj, *eNOS*) or in combination with systemic folate (F) administration (inj, *eNOS*+F). I/M ratio was significantly reduced in animals treated with *eNOS* or in combination with folate administration (one week prior to injury). Bars represent the mean $\pm$ SEM for each group. * $P < 0.05$ vs uninjured, † $P < 0.05$ vs vehicle.

DISCUSSION

In this study in hyperlipidemic atherosclerotic mice we showed that local adenoviral gene transfer of *eNOS* reduces neointima formation after arterial injury robustly so that additional benefit of adjuvant folic acid treatment could not be ascertained. This result is in accordance with NO suppletion studies in non-atherosclerotic models of arterial injury^{18,19,25–28} and has important clinical implications in that it shows the efficacy of *eNOS* gene transfer in an environment conducive to uncoupling of eNOS function and generation of excess superoxide, i.e. the environment in which percutaneous interventions typically are performed.

VSMC proliferation and macrophage activation at the site of injury are central to the remodeling after angioplasty^{6,7} and both are strongly affected by NO. NO inhibits VSMC growth in vitro and experimental neointimal hyperplasia *in vivo*, indicating a role for NO as a regulator of VSMC proliferation.^{16,17} NO is also involved in the control of several other pathophysiological responses, including platelet function and inflammatory cell adhesion, vascular reactivity, and endothelial permeability.^{16,17} Local delivery of the NO donor molsidomine improved hemodynamics, lowered cell proliferation and prevented late lumen loss in femoral arteries of atherosclerotic pigs after balloon angioplasty.³¹ Likewise, systemic delivery of NO by a NO-releasing aspirin showed decreased neointimal hyperplasia after vascular injury in hyperlipidemic^{32,33} and non-hyperlipidemic¹⁹ rodents. Thus, a large body of data supports the rationale for augmenting NO availability as a strategy to prevent neointimal hyperplasia. In the presence of atherosclerosis, NO suppletion remains effective, although in a large clinical study, nitric oxide suppletion by linsidomine followed by oral molsidomine did not affect restenosis rates after balloon angioplasty (measured as 'loss index') in patients with single vessel coronary disease.³⁴

Exogenous administration of endothelial NO synthase is an alternative and possibly more controlled way to deliver NO to the vascular wall. The proof of concept was provided by studies on intra-arterial adenoviral *eNOS* transfer that showed a 70% inhibition of neointima formation in the rat carotid artery balloon injury model.^{26,28} We know of no other studies however, that tested the efficacy of eNOS suppletion in the setting of hypercholesterolemia and there are reasons to believe that the enzymatic activity of eNOS is compromised under condition of augmented oxidative stress.

In hypercholesterolemia with elevated vascular superoxide (O_2^-) production by increased expression of xanthine oxidases, NO combines with O_2^- to form peroxynitrite, thereby reducing the bioavailability of NO. Furthermore, the formed peroxynitrite oxidizes tetrahydrobiopterin (BH_4), a folic acid compound, causing the uncoupling of NO synthase (uncoupling of NADPH oxidation and NO synthase). Isoprostanes, like prostaglandin $F_{2\alpha}$, are chemically stable prostaglandin isomers that result from oxidative modification of arachnidonic acid through a

mechanism catalyzed by free radicals.³⁵ These isoprostanes accumulate in human atherosclerotic plaque,³⁶ a tissue in which oxidative stress with consequent eNOS uncoupling is thought to occur. In our study, we used the apoE deficient mouse as a model of hypercholesterolemia and atherosclerosis. Plasma levels of prostaglandin $F_{2\alpha}$ were higher in this model compared to age-matched C57BL/6J mice, even on a normal chow diet.³⁷ In the older apoE deficient mice on a high cholesterol diet that were used in the present study, $PGF_{2\alpha}$ levels were even higher than in these historic controls, thus providing evidence for augmented oxidative stress.

In spite of elevated oxidative stress, *eNOS* transfection still effectively blocked neointima formation, suggesting that uncoupling of the enzyme either does not occur in this model or is not sufficient to block its effect. The observation that folate addition did not increase the effect of *eNOS* supplementation, further supports absence of uncoupling in this system through BH_4 oxidation. Vascular superoxide production appears to be at least partially mediated by BH_4 -dependent eNOS uncoupling in apoE deficient mice³⁸ so it was to be expected that folate administration would lower vascular superoxide and systemic $PGF_{2\alpha}$ levels. However, in our study, folate supplementation did not lower plasma $PGF_{2\alpha}$ levels, indicating that the oxidative stress status of the animal remained unchanged suggesting that uncoupling of eNOS if present, did not contribute to the vascular pool of superoxide. Other oxidase systems like, NAD(P)H and xanthine oxidases are also active in the formation of ROS (including superoxide) so uncoupling of eNOS is definitely not required to increase oxidative stress in the vascular wall. It is conceivable, that exogenous *eNOS* for instance transfected into vascular smooth muscle cells may not be subject to uncoupling, or to increased oxidative stress. The smooth muscle cell milieu, as compared to that of the endothelial cell, may differ in oxidative stress level (less) or differ in other factors that may alter eNOS enzymatic activity. For example, hypercholesterolemia-induced caveolin-mediated inhibition of eNOS activity in endothelial cells³⁹ may not occur in smooth muscle cells as these cells lack caveolin. The general, not endothelial specific, antioxidant activity of L-5-MTHF,^{40,41} is apparently also not strong enough to reduce neointima formation. Recently, two studies revealed that long term vitamin E and C treatment could reduce atherosclerosis by reducing oxidation in apoE deficient mice.^{37,42} The antioxidant potency of L-5-MTHF was reported to be about 20-fold lower than the scavenging effects of vitamin C, therefore the relevance of a direct antioxidant effect of folate is probably limited. This was supported by a failure of folates to lower plasma isoprostane level reduction in this study.

Recent evidence suggests that uncoupling of eNOS may not be folate-dependent but related to peroxynitrite's ability to disrupt the zinc-thiolate cluster of eNOS enzyme. Zou et al⁴³ reported peroxynitrite-induced uncoupling of purified eNOS by disrupting the zinc-thiolate cluster of the enzyme while the essential cofactor BH_4 was not oxidized. Addition of high concentrations (0.1 mmol/l) of BH_4 did not

result in preventing eNOS from oxidation by low concentrations (50 $\mu\text{mol/l}$) of peroxynitrite.

GENERAL CONCLUSIONS

The current study is the first to demonstrate effective inhibition of neointimal hyperplasia after local vascular gene transfer of *eNOS* in a hyperlipidemic, atherosclerotic model. The maintained efficacy of the enzyme despite its transfer into an environment conducive to uncoupling of eNOS function and rich in superoxide remains unexplained. However, the clinical implications of these findings are that *eNOS* transfer after stent implantation using the stent as a delivery platform, may prove an effective strategy to prevent in-stent restenosis.

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CHAPTER 7

General discussion

INTRODUCTION

In this thesis, the main hypothesis tested is that NO inhibits the remodeling response after vascular injury. To achieve this, we characterized endothelial function and vascular remodeling in response to acute and chronic increased flow in a hyperlipidemic mouse model. Furthermore, we studied the effect of eNOS gene therapy and factors known to enhance eNOS enzyme activity, i.e. folates and estrogen, on vascular remodeling after arterial damage in several mouse strains.

The main findings of these studies are that:

- hyperlipidemia reduces endothelium-dependent NO-mediated relaxations. We demonstrate mechanistic evidence that vasoconstricting cyclooxygenase products contribute to this impairment in relaxation.
- outward remodeling in response to chronic elevated blood flow is not compromised in mild and severe hyperlipidemia despite indications that acute flow-induced endothelium-dependent dilation is impaired in severe hyperlipidemia.
- estrogen's protective effect against medial hyperplasia and vascular smooth muscle cell proliferation in response to mechanical injury are mediated through a NO-dependent pathway. More specifically, we find that regulation of NOS-2 expression plays an important role in the estrogen-mediated effect in mitigating the wound healing response after vascular injury.
- in our vascular gene transfer studies in hyperlipidemic, atherosclerotic mice, we show for the first time that the protective effects of eNOS against neointimal hyperplasia are maintained in a milieu that is conducive to eNOS uncoupling and that is rich in superoxide. The NOS potentiator folic acid, did not significantly enhance this effect.

Table 1. Overview experiments flow-related vascular remodeling

		Chapter 2; effects hypercholesterolemia on endothelial function, acute flow-induced dilation and flow-related remodeling		
Strain		C57BL/6J (WT)/apoE KO (KO)		
Gender		Male		
Age		6 months		
Diet		NC/Western (D)		
Injury		CA ligation		
Groups		WT+NC	WT+D	KO+D
Medial Area (CA)	LF	↑	↑	↑
	NF	ref	=	↓
	HF	↑	=	=
Endothelial dysfunction (CA)		No	Yes	Yes
Pressure-distension (CA)	LF	↓	↓	↓
	HF	=	↑	↑

Scheme overview experiments of chapter 2. ApoE KO, Apolipoprotein E knockout; CA, carotid artery; WT, wild type; NC, normal chow; D, Western type diet; LF, low flow; NF, normal flow; HF, high flow, ref, used as reference. Media area measurements is for LF and HF versus sham-operated animals of same group and for NF versus WT on NC. LF and HF vessels in the pressure-distension measurements are compared to vessels of sham-operated animals.

ENDOTHELIAL DYSFUNCTION, FLOW-INDUCED DILATION AND FLOW-INDUCED VASCULAR REMODELING

The data of this thesis show that mild and severe hyperlipidemia impair endothelium (ACh)-dependent relaxation (Table 1). This endothelial dysfunction, which is also observed in hyperlipidemic and atherosclerotic human subjects, is associated with a decreased NO bioavailability. Furthermore, the classic endothelial dysfunction observed in the static wire-myograph setup, involved vasoconstricting cyclooxygenase products. The impaired endothelial vasorelaxations may be due to decreased expression of eNOS,¹ a lack of substrate or cofactors for eNOS,² alterations of cellular signaling such that eNOS is not appropriately activated,³ accelerated NO degradation by reactive oxygen species (ROS),⁴ and increased levels of asymmetric dimethylarginine (ADMA).⁵

The classic endothelial dysfunction demonstrated in mild and severe hyperlipidemic mice was not completely paralleled with the results obtained in a dynamic set up in which *ex-vivo* acute flow-induced dilations (FID) were measured. Acute flow-induced dilation was only impaired in the severe hyperlipidemic apoE-deficient mice on a Western diet. The factor(s) causing this impairment could not be elucidated in FID at low flow rates, but at higher flow rates FID was normalized by indomethacin suggesting the involvement of constricting COX-related products.

Table 2. Effects of COX inhibition on dynamic vascular response under low and high flow conditions in mild and severe hyperlipidemia.

		FLOW	
		low	high
HYPERLIPIDEMIA	mild	constriction	no effect
	severe	no effect	dilation

In hyperlipidemic mice, indomethacin elicited a response that could be viewed as biphasic (Table 2). Under low flow conditions in mildly hyperlipidemic mice COX-inhibition caused vaso-constriction, indicating a role for vasodilatory COX adducts in dilation under these conditions. Under high flow and severe hyperlipidemia vasoconstrictive COX adducts seem to dictate vasomotion because inhibition of COX enhanced vasodilation. There is controversy over whether chronic medication with COX inhibitors belonging to the group of non-steroid anti-inflammatory drugs (NSAID) increases the risk for MI and cardiac death. Several studies have shown an increase, however in a recent meta-analysis this could not be substantiated.⁶ The increased risk has generally been attributed to an increased incidence in coronary thrombosis^{7,8} but we show a vasodilatory effect of COX inhibitors under hyperlipidemia and high flow, a situation found in exercising coronary disease patients, that could counteract this thrombosis risk. The dilating vessel could prevent the thrombus from becoming occlusive. Potentially, the net effect of COX inhibitors in coronary patients may depend upon the balance between pro-thrombotic and vasodilatory effects of the NSAID in a particular patient.

When NOS was blocked in addition to COX, vasodilation in response to high flow still occurred in both hyperlipidemic groups. This suggests that other vasodilators contribute to FID as well. A likely candidate could be endothelial derived hyperpolarizing factor(s) (EDHF) of which the exact nature is not known yet. Also, in the severely hyperlipidemic mice FID at high flow was less pronounced when both enzymes were blocked compared to COX blockade alone. This may point to some degree of preserved NO generation in the presence of severe dyslipidemia. The observation that even in severely hyperlipidemic animals, an artery dilates in response to high flow *ex vivo*, correlates well with the *in vivo* finding of preserved outward remodeling in response to chronically increased flow in hyperlipidemic animals. The classic endothelial dysfunction as demonstrated in the myograph studies did not predict the remodeling response *in vivo*. The fact that this complex response does not correlate with agonist induced responses in a myograph should not surprise us. Flow-induced remodeling occurs rapidly, i.e. within several weeks in animal models, and is age-dependent.⁹ Particularly, endothelial NO seems to be involved, based on studies with inhibitors of endothelial NO synthase (eNOS)¹⁰ and eNOS knockout mice.¹¹ In contrast, this thesis suggests that eNOS-derived NO is not a dominant mediator of arterial remodeling in response to chronic altered blood flow (Chapter 2). Furthermore, several other studies also suggest that the endothe-

Figure 1. Diagram showing blood flow mediated mechanisms leading to arterial remodeling. Adapted from Lehoux et al.⁵⁸ Dotted arrows indicate the condensation of NO and superoxide to peroxynitrite.

lium¹² or NO¹³ are not involved in structural remodeling to increased chronic shear stress. Other proteins that may potentially influence remodeling include matrix metalloproteinases and their inhibitors,¹⁴ cytokines such as transforming growth factor β (TGF β),^{15,16} growth factors such as platelet derived growth factors (PDGF),¹⁷ cytoskeletal proteins such as vimentin,¹² and proteins associated with apoptosis or cell proliferation.^{18,19} This thesis does not answer if other NOS isoforms, in particular iNOS, or NO-derived products like peroxynitrite are involved in flow-induced remodeling. In hyperlipidemic mice also used in this thesis, iNOS expression as well as superoxide production^{20,21} are upregulated, leading to the formation of peroxynitrite. iNOS-derived NO, peroxynitrite and other reactive species such as hydrogen peroxide, which can be generated from superoxide through the action of dismutases, can induce metalloproteinase activation¹⁴ and possibly implicate the induction of growth factor mitogenic activity (Figure 1). Additionally, one report showed endothelium-dependent flow-induced increases in superoxide production.²² Furthermore, MMP activation is required for adaptive arterial remodeling (fragmentation of internal elastic lamina and arterial enlargement) to occur. These observations are significant since adaptive vascular enlargement and remodeling are known to accompany early human coronary atherosclerosis²³ and stenosis in several hyperlipidemic mice,²⁴ and exaggerated expression of MMPs, in particular MMP-2 and MMP-9, is known to be a marker for aortic aneurysms,^{25,26} and could reflect abnormal flow-induced vessel remodeling. The in this thesis observed intact outward vascular remodeling in response to increases in shear stress in mild and severe hyperlipidemia (Chapter 2), with subsequent elevated (flow-dependent) oxidases systems in combination with upregulated expression of inducible NOS, could be the result of MMP activation by NO/peroxynitrite or other reactive oxygen species formed in the vessel wall.

INJURY-INDUCED REMODELING AND ITS INHIBITION BY ESTROGEN

The response to injury after vascular injury is followed by neointimal migration of smooth muscle cells (SMC), SMC proliferation and matrix formation by synthetic state SMC. In carotid, aortic, and iliac arteries of normolipidemic, ovariectomized rabbits, rats, and monkeys, estrogen (E_2) reduces neointimal and medial growth by 60% or more after injury to the endothelium by balloon catheter or passage of a fine wire (for review,²⁷). Estrogen receptor α (ER α) KO and ER β KO mice have also been used to clarify the roles of ERs in the protective mechanism of E_2 after vascular injury. The first two such studies,^{28, 29} using a fine wire to denude carotid endothelial cells, demonstrated that E_2 protects against vascular injury in normolipidemic chimeric ER α KO and ER β KO mice. These findings suggest that ER α and ER β are able to complement one another such that each receptor alone is sufficient to mediate the vascular protective effects of estrogen, or that the vascular protective effects of estrogen are mediated by an ER α /ER β -independent pathway. In this thesis, E_2 lost its protection on medial area but not on vascular smooth muscle cell proliferation (VSMC) in ER α,β (double) KO mice (Table 3). This raises the questions whether there are ERs other than ER α and ER β mediating the observed protective effect of E_2 on VSMC proliferation, whether there is a residual function of protein from an ER α splice variant known to be expressed in the parental chimeric ER α KO mice³⁰ or that this effect is receptor independent. A subsequent study using newly created ER α KO mice in which ER α has been fully disrupted solved this question.³¹ In this study estrogen no longer protected against vascular injury on both medial area and VSMC proliferation, indicating that ER α mediates the protective effects of estrogen.

The atheroprotective effects of E_2 in the vasculature have been widely attributed to its ability to increase the bioavailability of NO. Physiologic levels of E_2 stimulate eNOS mRNA and protein expression through long-term, receptor-dependent mechanisms and upregulate eNOS activity through short-term, nongenomic mechanisms.³² Absence of eNOS in apoE KO mice results in hypertension and increased atherosclerotic lesion size.³³ In contrast, exogenous delivered E_2 to gonadectomized female and male mice deficient in eNOS and apoE resulted in reduced lesion size of more than 75% as compared with control-treated mice deficient of both eNOS and apoE or apoE alone, demonstrating that the atheroprotective effects of exogenous E_2 do not depend on the presence of eNOS.³⁴

In contrast to these observations, we showed that the response to carotid endothelial denudation in normocholesterolemic mice was inhibited by exogenous E_2 and that this protective effect of E_2 was completely blocked by systemically administered L-NAME, demonstrating that E_2 protection in this model is mediated via a NO-dependent pathway (Table 3 and Chapter 4). A recent study using five inbred strains of mice demonstrated that the genes dictating susceptibility to injury-induced neointimal hyperplasia are distinct from those that determine susceptibility to

Table 3. Overview of experiments mouse carotid wire injury

	Chapter 3; ER dependency in E ₂ protection	Chapter 4; NO involvement in E ₂ protection	Chapter 5; role of iNOS in response to injury	Chapter 6; eNOS inhibits neointimal formation
Strain	ER α , β KO	C57BL/6J	NOS-2 KO	ApoE KO
Gender	Female, ovariectomized	Female, ovariectomized,	Female, ovariectomized	Male
Age	3 months	3 months	3 months	12 months
Diet	Normal	Normal	Normal	Western
Injury	CA wire-injury	CA wire-injury	CA wire-injury	CA wire-injury
Groups	WT+V WT+E ₂ KO+V KO+E ₂	V E ₂ L-NAME L-NAME+E ₂	WT+V WT+E ₂ KO+V KO+E ₂	V β -gal eNOS eNOS+FA
BP	ref = ref =	ref $\downarrow$ $\uparrow\uparrow$ $\uparrow$	ref = ref =	ND ND ND ND
Uterine weight	ref $\uparrow\uparrow$ $\uparrow$	ref $\uparrow\uparrow$ = $\uparrow\uparrow$	ref $\uparrow\uparrow$ ref $\uparrow\uparrow$	$\uparrow$ $\uparrow$ $\uparrow$ $\uparrow$
Medial area	$\uparrow$ = $\uparrow$	$\uparrow$ = $\uparrow$	$\uparrow$ = =	$\uparrow$ $\uparrow$ $\uparrow$ $\uparrow$
Neointimal area	$\uparrow\uparrow$ $\uparrow$ $\uparrow\uparrow$ $\uparrow$	$\uparrow\uparrow$ $\uparrow$ $\uparrow\uparrow$ $\uparrow\uparrow$	$\uparrow$ $\uparrow$ $\uparrow\uparrow$ $\uparrow\uparrow$	ND ND ND ND
SMC proliferation	$\uparrow\uparrow$ $\uparrow$ $\uparrow\uparrow$ $\uparrow$	$\uparrow\uparrow$ $\uparrow$ $\uparrow\uparrow$ $\uparrow\uparrow$	$\uparrow$ $\uparrow$ $\uparrow\uparrow$ $\uparrow\uparrow$	ND ND ND ND

Schematic overview of experiments. ApoE KO, Apolipoprotein E knockout; CA, carotid artery; ER, estrogen receptor; iNOS, inducible nitric oxide synthase; WT, wild type; E₂, 17 β -estradiol, V, vehicle; β -gal, β -galactosidase; eNOS, endothelial nitric oxide synthase; FA, folic acid; BP, blood pressure; SMC, smooth muscle cell; ND, not determined; ref, used as reference. BP and uterine weight determinations is versus vehicle-treated animals same genotype. Medial area and SMC proliferation measurements are versus uninjured animals same genotype.

diet-induced atherosclerosis,³⁵ which may explain the apparent discrepancy of the E₂ protection in normo- and hypercholesterolemic mice.

ER α -related genes that might be involved in estrogen's inhibitory effects on the responses to vascular injury include eNOS, iNOS, cyclooxygenase-1, matrix metalloproteinase-2, and estrogen-inhibited genes such as endothelin-1, vascular adhesion molecules, and the angiotensin II receptor AT-1 (review see ref³⁶). The data of this thesis provide evidence that estrogen's protective effects are mediated by downregulation of injury-induced iNOS expression as the response to injury in vehicle-treated mice deficient of iNOS closely resembled the values of E₂-treated WT mice (Chapter 5) and E₂ treatment did not show any additional effects in iNOS deficient mice (Chapter 5). Although medial area was not increased in response to arterial injury in iNOS deficient mice, medial VSMC proliferation was not completely inhibited by iNOS deficiency (Table 3). This suggests, that other phenomena like extracellular matrix formation³¹ and adventitial myofibroblast migration³⁷ to the media are prominent in medial thickening.

THE ROLE OF eNOS IN INJURY-INDUCED VASCULAR REMODELING IN HYPERLIPIDEMIA

In hyperlipidemia with elevated vascular superoxide production by increased expression of ROS, NO combines with superoxide to yield peroxynitrite, resulting in decreased bioavailable NO. The formed peroxynitrite oxidizes tetrahydrobiopterin (BH₄), an essential cofactor for eNOS. Depletion of this cofactor results in uncoupling of eNOS activity and a switch from production of NO, from L-arginine, to generation of superoxide.³⁸ L-5-MTHF, the main active form of folic acid in the circulation, reverses the oxidation of BH₄ and restores endothelial function in familial hypercholesterolemia.³⁹ In vitro, L-5-MTHF demonstrated to be capable of directly scavenging superoxide, increasing NO production by eNOS and also reducing superoxide generation by eNOS.⁴⁰ In animal models of arterial injury, exogenous delivery of NO compounds or enhancement of endogenous NO production by administration of L-arginine reduces the vascular response.^{41, 42} In this thesis we provide data indicating that local gene delivery of eNOS completely inhibits neointimal remodeling after carotid endothelial denudation in hyperlipidemic mice (Table 3 and Chapter 6). Subsequently, additional effects of L-5-MTHF on neointimal inhibition by eNOS could not be observed, suggesting that eNOS enzymatic activity does not get uncoupled in hyperlipidemic mice. Surprisingly, eNOS treatment with or without L-5-MTHF administration was not able to inhibit the medial response to injury. This may be in apparent contrast to the other studies described in this thesis in which eNOS activity potentiating agents like E₂ inhibited the medial response to injury (Chapters 3–5). However, E₂-induced protection on medial area after vascular injury in iNOS KO mice seemed to be independent of eNOS (Chapter 5). Furthermore, it is possible that eNOS-derived NO in medial

area of transduced carotid arteries is overwhelmed by superoxide produced by elevated oxidase systems. Hence no effect of NO can be expected. On the luminal border eNOS-derived NO might not or not completely be scavenged by superoxide and may therefore exert its protective effect by inhibiting the release of growth factors involved in neointimal formation, like PDGF, or endothelial adhesion molecules like, von Willebrand factor (vWF). Recently, a study reported that an antibody raised against vWF prevented neointimal formation in balloon-injured carotid arteries of normocholesterolemic guinea pigs but could not inhibit cultured VSMC proliferation.⁴³

CONCLUDING REMARKS

In the second chapter of this thesis we proved endothelial dysfunction in hyperlipidemia characterized by a diminished ability of the arteries to relax in response to increasing concentrations of acetylcholine, suggesting reduced eNOS enzymatic activity. Furthermore, we observed, making use of a more dynamic experimental setting, that acute flow-induced dilations was impaired in severe hyperlipidemic mice. However, in these experiments we could not attribute a prominent role for endothelial-derived NO in mediating flow-induced dilations. In general, acute flow-induced dilations seemed to involve non- NO and cyclooxygenase-related products that may be endothelium-derived hyperpolarizing factor(s). Surprisingly, the observed diminished activity of the endothelium to respond to vasodilating substances and mechanical stimuli (shear stress) did not result in impaired outward arterial remodeling in response to chronic increased shear stress. This suggests that the endothelium, in particular eNOS, may not be involved in mediating arterial remodeling in a hyperlipidemic mouse model of chronic elevated shear stress. This does not imply that NO does not play part in vascular remodeling in this model. One of the features of hyperlipidemia is increased expression of superoxide production and elevated production of iNOS-derived NO. Both iNOS-NO or superoxide or the condensation product of these molecules, peroxynitrite, may facilitate induction of MMP expression. Adaptive vascular remodeling is accompanied with enhanced expression of MMPs, in particular MMP-2. However, exaggerated induction of MMPs may lead to abnormal vascular remodeling giving rise to incidental aneurysms.

In chapter 3 we show that estrogen protection against vascular damage on medial area is mediated via one of the known estrogen receptors and that this involves NO synthase(s) (Chapter 4). The 'bad' factor involved in injury-induced medial thickening is iNOS (Chapter 5). It has repeatedly been reported that estrogen induces release of NO via eNOS activation and that this may be a potential pathway in estrogen protection against atherosclerosis and in 'the response to injury' models in mice. The fact that downregulation of iNOS by estrogen is involved in estrogen-mediated protection on medial thickening after vascular injury in

normocholesterolemic mice (Chapter 5) is in contrast with the findings that blockade of all NOS isoforms, including iNOS, by L-NAME resulted in an increase of medial area in animals treated with estrogen (Chapter 4). The question by which mechanism(s) estrogen regulates iNOS expression remains unanswered. There is little evidence for a direct interaction of estrogen with the iNOS promotor, which lacks estrogen responsive elements.⁴⁴ The question remains whether eNOS and/or nNOS is involved primary to iNOS activation. As proposed for the effect of estrogens on other molecules of the inflammatory cascade, it is conceivable that iNOS mRNA synthesis is controlled by estrogen by interaction with transcription factors such as nuclear factor- κ B.⁴⁵ Furthermore, NO has been shown inducing and stabilizing I κ B α , which in turn, blocks activation of the proinflammatory transcription factor NF- κ B.^{46,47} Furthermore, eNOS-derived NO reduced ischemia-reperfusion injury by inhibiting NF- κ B activation in a rabbit heart transplant model.⁴⁸ This still does not explain why L-NAME blocked estrogen protection to vascular injury where iNOS-related products are responsible for medial thickening. One explanation may be that L-NAME did not fully inhibit iNOS activity. Injury-induced iNOS expression may be elevated to such an extent that L-NAME is not able anymore to completely inhibit iNOS activity or the affinity of L-NAME for iNOS may be lower than for the other NOS isoforms. In contrast, however, L-NAME might have inhibited the activity of eNOS/nNOS protein completely resulting in the activation of NF- κ B and subsequently iNOS activation. Blocking the transcription factor NF- κ B for iNOS by eNOS/nNOS might be more efficient in inhibiting the response to injury than blocking iNOS protein activity by L-NAME.

In chapter 6 we showed that eNOS is involved in inhibiting neointimal formation but was not able to inhibit medial thickening in response to arterial endothelial denudation in male hypercholesterolemic mice. It is not clear if eNOS mediated protection acts through (down)regulation of iNOS expression in this model. If that would be the case then one would expect inhibition of medial thickening as well. Nevertheless, eNOS might have down-regulated NF- κ B and other factors like, adhesion molecules (VCAM-1, ICAM-1, E-selectin),⁴⁹⁻⁵¹ chemotactic agents (MCP-1)⁵² and thrombogenesis (tissue factor)⁵³ involved in neointimal formation on the luminal border, specifically platelets⁵⁴ and monocytes⁵⁵ but might not have been able to modulate NF- κ B induced expression of iNOS in the inner layers of the media where the oxidative stress is higher.

FUTURE ASPECTS

Vascular remodeling after PTCA is characterized by neointimal formation leading to lumen loss. In this thesis we showed that local eNOS gene transfer inhibits arterial injury-induced neointimal growth, but not medial thickening, in a hyperlipidemic mouse.

Recently, the implantation of stents to prevent constrictive remodeling of the vessel wall has become the most common coronary percutaneous revascularization procedure. e-NOS gene transfer may prove very effective in the prevention of in-stent restenosis which is characterized primarily by a proliferative response as inward remodeling is being prevented by the rigidity of the stent. Moreover, stents can be implemented as delivery platforms for genes or drugs. The first multicenter trial with stents coated with an immunosuppressant completely prevented angiographic restenosis at 6 months follow up.⁵⁶ Other drug-eluting stent systems like vascular endothelial growth factor and 17- β estradiol are currently under evaluation.⁵⁷

Future experiments are warranted to examine whether eNOS gene delivery at site may prove useful against restrictive remodeling in other animal species and humans.

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Summary

Vascular endothelial cells are crucial in preserving the patency of blood vessels. Nitric oxide (NO), is constitutively produced by endothelial cells and is an important secreted factor and has a prominent role in the maintenance of function and anatomy of the vessel and is considered as an atheroprotective molecule.

A hall mark of endothelial dysfunction is the loss of atheroprotective properties of the endothelial lining. Chronic endothelial dysfunction will result in atherosclerotic plaque formation that leads to a lumen-narrowing stenosis when outward remodeling of the vessel fails to compensate for vessel wall thickening. Restenosis, the pathological remodeling of a vessel, after coronary angioplasty in order to reopen an artery after initial lumen-narrowing stenosis, is caused by endothelial damage and leads to a recurrent narrowing of the vessel, characterized by neointimal growth, in 30–40% of all patients undergoing angioplasty. Less production of NO by the damaged and impaired functioning endothelial cells may be the cause of restenosis which makes NO an interesting potential target for the prevention of restenosis. This thesis studies the role of NO in vascular remodeling in mice with damaged endothelial cells and/or impaired endothelial function.

Diet-induced hypercholesterolemia results in impaired function of endothelial cells that is characterized by less bioavailable NO. Changes in blood flow drive both acute and long term compensatory responses that eventually result in normalization of wall shear stress. In order to accommodate these responses endothelial cells produce substances like NO. Despite observed endothelial dysfunction in hypercholesterolemic mice outward vascular remodeling was not impaired to chronic increases of blood flow, questioning the role of the endothelium in this phenomenon.

Estrogen protects against heart and coronary diseases and inhibits the vascular response to injury. In this thesis data are provided showing that estrogen's protection is NO dependent. The healthy endothelium produces small quantities of NO, but immense production of NO is produced by other cells, only after the expression of an inducible nitric oxide synthase (iNOS). This thesis proves that estrogen modulates iNOS expression and that this causes estrogen protection against vascular damage in normocholesterolemic mice.

One of the features of hypercholesterolemia is a reduced activity of the enzyme, endothelial NO synthase (eNOS), in which both less NO and simultaneously superoxide is produced that combines with NO to the powerful oxidizing molecule, peroxynitrite. In this thesis we show that neointimal growth can be inhibited by local gene transfer of eNOS after vascular damage in hypercholesterolemic mice.

The data of this thesis provide several possible therapies against the pathological responses to vascular injury. Special balloon catheters introducing eNOS gene immediately after angioplasty or coated stents eluting NO or estrogen may serve as potential therapies against restenosis.

Samenvatting

De endotheelcellen van bloedvaten zijn van essentieel belang in de homeostase van bloedvaten. Stikstofoxide (NO), dat door de endotheelcellen continu wordt aangemaakt, speelt een belangrijke rol in het behoud van een normale functie en vaatwandstructuur van bloedvaten.

Restenose, de vasculaire remodulering van een bloedvat, treedt op in 30–40% van de gevallen na een zogenaamde ‘dotterprocedure’ (ballondilatatie) of stentimplantatie waarbij de lumenvernauwende stenose wordt opgeheven maar ook tevens het endotheel wordt beschadigd en ten gevolge hiervan het vat door neointimale ingroei opnieuw dichtslibt. Verminderde NO productie door beschadigde en/of slecht functionerende endotheelcellen ligt mogelijk hieraan ten grondslag en herstel van NO productie kan dus een mogelijke therapie zijn om deze vorm van vasculaire remodulering tegen te gaan. In dit proefschrift wordt bestudeerd wat de rol is van NO in vasculaire remodulering in muizen met beschadigd endotheel en/of verslechterde endotheelfunctie.

Verhoogde cholesterolspiegels in het bloed door een vetrijk dieet leidt tot een verslechterde endotheelfunctie gekenmerkt door verminderde biologische beschikbaarheid van NO. Verhoogde bloedsnelheid door een bloedvat leidt acuut tot het uitzetten van het bloedvat en een chronische blootstelling aan verhoogde bloedsnelheid leidt tot structurele veranderingen in de vaatwand om zo de door het bloed gegenereerde krachten op de endotheelcellen en vaatwand te normaliseren. Hierbij wordt door het endotheel stoffen geproduceerd zoals NO om deze aanpassing te faciliteren. Ondanks de verslechterde aangetoonde endotheelfunctie in hypercholesterolemische muizen zijn deze toch in staat om hun vaatwandstructuur aan te passen aan veranderde bloedperfusie omstandigheden, waarbij de rol van het endotheel in deze ter discussie gesteld kan worden.

Oestrogenen hebben een beschermende functie tegen hart en vaatziekten en inhiberen de pathologische vaatwandremodulering t.g.v. endotheelbeschadiging. De resultaten van dit proefschrift laten zien dat NO een rol speelt in deze bescherming. NO wordt door endotheelcellen in kleine hoeveelheden geproduceerd, maar kan ook in grote hoeveelheden door andere cellen worden aangemaakt, alleen nadat een bepaald enzym, “induceerbaar NO synthase” (iNOS), tot expressie is gekomen

onder invloed van bepaalde prikkels waaronder ontstekingsreacties. In dit proefschrift wordt aangetoond dat oestrogenen de expressie van iNOS negatief beïnvloeden en dat dit zorgt voor oestrogeenprotectie ten gevolge van vaatwandbeschadiging in normocholesterolemische muizen.

Hypercholesterolemie wordt gekenmerkt door een verminderde activiteit van het enzym, endotheliaal NO synthase (eNOS), waardoor naast (verminderde) NO productie ook superoxide wordt geproduceerd dat vervolgens met NO reageert tot het schadelijke peroxynitriet. In dit proefschrift laten we zien dat de neointimale groei van de vaatwand tegengegaan kan worden met behulp van gentherapie waarbij het eNOS gen lokaal wordt toegediend na vaatwandbeschadiging in hypercholesterolemische muizen.

Dit proefschrift biedt verscheidene mogelijke therapieën aan om de pathologische remodelering, restenose, die optreedt na vaatwandbeschadiging t.g.v. een procedurele ingreep om het vat te heropenen te behandelen. Te denken valt aan applicatiemethoden waarbij het eNOS gen bij ballondilatatie met een speciale catheter lokaal toegediend kan worden en of gecoate stents die NO of oestrogenen vrijgeven.

Dankwoord

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Serban, my fellow European and dear friend. They say that good scientists know how to cook. Then you must be an excellent scientist! With you I explored the Asian food court in Chinatown; I even survived Ding Ho's! I hope that you and your family will do great in the land of Uncle Sam.

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Curriculum Vitae

Henny Schulten was born May 14th 1969 in Tilburg, the Netherlands. After passing the VWO (Atheneum-B) exam at the Theresia Lyceum in Tilburg, he studied Medical Biology (Faculty of Medicine) at the University of Utrecht. After graduation, he started a PhD project in the department of Cardiology at the University of Maastricht to study the role of nitric oxide in arterial remodeling after vascular damage. During this PhD project he worked for one year in the laboratory of Dr. Mendelsohn in Boston, United States (Molecular Cardiology Research Institute, Tufts University). Since 2003 he has been working as a post-doc for the group of Mark J. Post in the department of Physiology at the University of Maastricht.

Henny Schulten werd geboren op 14 mei 1969 te Tilburg, Nederland. Na het behalen van zijn VWO (Atheneum-B) diploma aan het Theresia Lyceum te Tilburg werd de studie Medische Biologie aan de Faculteit Geneeskunde van de Universiteit Utrecht aangevangen. In 1998 werd begonnen met het promotieonderzoek naar de rol stikstofoxide in vasculaire remodelering na vaatwandschade bij de capaciteitsgroep Cardiologie aan de Universiteit van Maastricht. Tijdens zijn promotieonderzoek heeft hij voor een jaar gewerkt in het laboratorium van Dr. Mendelsohn te Boston, de Verenigde Staten (Molecular Cardiology Research Institute, Tufts University). Sinds 2003 is hij werkzaam als post-doc in de groep van Mark Post bij de capaciteitsgroep Fysiologie aan de Universiteit van Maastricht.

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