
# Insight into oxidative stress mediated by nitric oxide synthase (NOS) isoforms in atherosclerosis

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**By**

**P.Padmapriya**

**Coimbatore, TN, INDIA**

**Würzburg, GERMANY**

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**Mitglieder der Promotionskommission:**

Vorsitzender : Prof. Dr. Martin. J. Müller

Gutachter : PD. Dr. Peter. J. Kuhlencordt

Gutachter : Prof. Dr. Roland Benz

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# Eidesstattliche Erklärungen

Hiermit erkläre ich ehrenwörtlich, dass die vorliegende Dissertation **"Insight into oxidative stress mediated by nitric oxide synthase (NOS) isoforms in atherosclerosis"** selbständig an der Medizinische Klinik I der Universität Würzburg unter der Anleitung von Dr.Peter Kuhlencordt angefertigt wurde und dass ich keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Weiterhin versichere ich, dass die vorliegende Dissertation weder in gleicher oder ähnlicher Form noch nicht in einem anderen Prüfungsverfahren vorgelegen hat und ich bisher noch keine akademischen Grade erworben oder zu erwerben versucht habe.

Hiermit bewerbe ich mich erstmals um den Doktorgrad der Naturwissenschaften der Bayerischen Julius-Maximilians-Universität Würzburg.

**(P.Padmapriya)**

**September, 2008**
**Medizinische Klinik I**
**Universität Würzburg**
**GERMANY**

I dedicate my doctoral thesis....

....to all the tiny little ones, whose lives have been

sacrificed for this work!

"I know I've been hard on you at times, pushed you when you were tired, studied you, probed you, but it was all in the interest of science."

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**“Research is to see what everybody else has seen and to think what nobody else has thought”**

-Albert Szent-Gyorgyi

Hungarian Biochemist,

1937 Nobel Prize for Medicine

# Contents

# Chapter 1

# 1.0 Introduction

Atherosclerosis, the disease of large and medium sized arteries is the most common disease in western countries and is known to be the underlying cause of 50% of all deaths. The prevalence of atherosclerosis is estimated to be 17 per 1000 (*NHIS95*), resulting in 1 death per hour among the general population of the USA. Atherosclerosis is characterised by the chronic accumulation of lipids and fibrous elements in the wall of the blood vessel which may result in progressive narrowing of the lumen and consecutive reduction in blood flow. The reduced blood flow is the underlying cause of chronic *angina pectoris* and *claudication.* Atherosclerosis affecting other arteries causes renal impairment, hypertension, abdominal *aortic aneurysms* and critical limb *ischemia*. On the other hand, rupture of an atherosclerotic plaque may result in an acute thrombotic occlusion of a vessel, which may result in *myocardial infarction,* stroke or acute ischemia of the gut or an extremity.

## 1.1 Pathogenesis of atherosclerosis

Atherosclerosis is a chronic inflammatory disease which progresses with age. The development of atherosclerosis is complex, involving numerous cell types and genes. A normal artery is composed of three different layers, 1) the inner most layer called tunica intima, composed of a thin layer of collagen and proteoglycans covered by a single layer of endothelial cells which line the lumen of the artery 2) the middle layer of smooth muscle cells called the tunica media and 3) the outer most layer called tunica adventitia which consists of connective tissue, fibroblasts and smooth muscle cells (Figure 1).

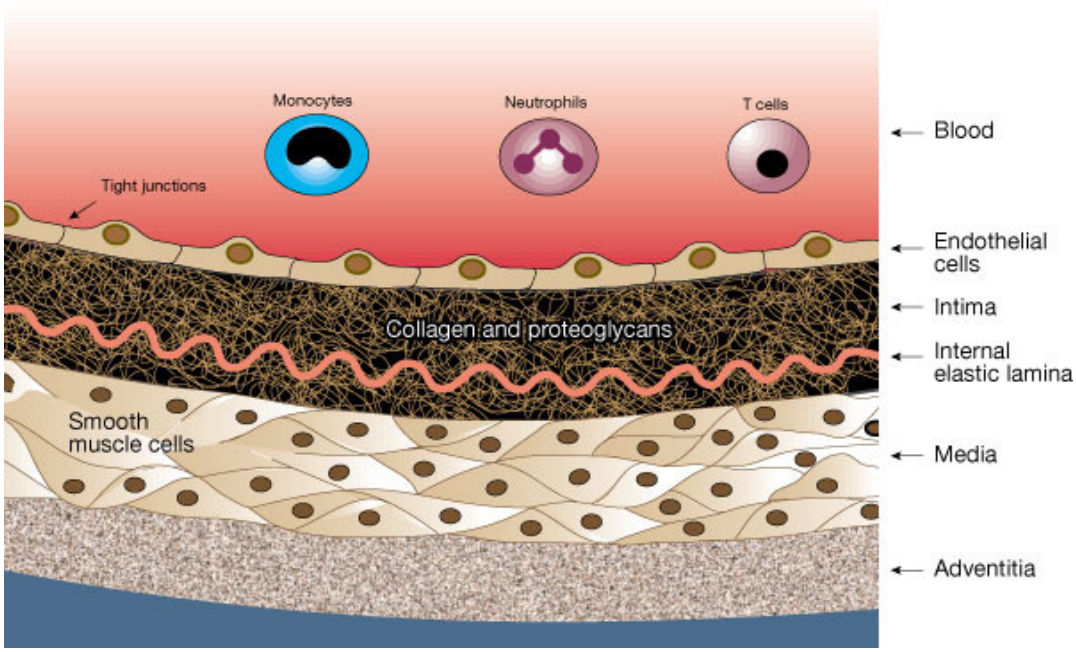


**Figure 1:** Structure of a normal vessel wall. The cross sectional view shows the three distinct layers of the vessel wall: the intima, media and adventitia. (Picture from Lusis AJ, Nature. 2000; 407(6801):233-41)

During the initial stages, lipoproteins and their aggregates accumulate in the intima of the vessel wall, at sites of lesion predilection. These predilection sites are usually the branching points or the inner curvature of the arteries, where normal blood flow dynamics are altered[1]. Subsequently, monocytes and lymphocytes adhere to the endothelium, transmigrate across the endothelial layer into the intima of the vessel where they proliferate, differentiate and take up lipoproteins to form “foam cells”. Though these early plaques or foam cells (also termed as ‘fatty streaks’) are not of clinical significance, they are the precursors of advanced lesions. As the disease progresses the foam cells die and the smooth muscle cells migrate from the medial layer into the intima, where they accumulate and proliferate. The so called advanced lesions are characterised by the accumulation of smooth muscle cells and dead foam cells,

which contribute to the lipid rich “necrotic core”. The smooth muscle cells secrete fibrous elements which form the “fibrous cap”, enclosing the lipid rich necrotic core. Initially the lesions grow towards the adventitia until a critical point is reached after which further growth of the plaques encroaches the lumen. As leukocyte recruitment and smooth muscle cell proliferation continues plaque development progresses. Additional extracellular matrix production, accumulation of extracellular lipid and calcification leads to the development of advanced atherosclerotic lesions (Figure 2).

Depending on the composition, atherosclerotic lesions can be classified into two types, namely stable or vulnerable plaques (Figure 2). A “stable plaque” has a thick fibrous cap, a small lipid pool, few inflammatory cells and a dense extracellular matrix. In contrast, a “vulnerable plaque” is characterised by a thin fibrous cap, an increased number of inflammatory cells, a large lipid pool and fewer smooth muscle cells. Vulnerable plaques are unstable, which may result in plaque rupture and instantaneous occlusion of the vessel. Plaque rupture usually occurs at the shoulder of the lesion, resulting in thrombus formation. Subsequent embolization of the thrombotic material may lead to additional occlusion of distal coronary arteries or cerebral arteries which can further aggravate myocardial or cerebral ischemia.

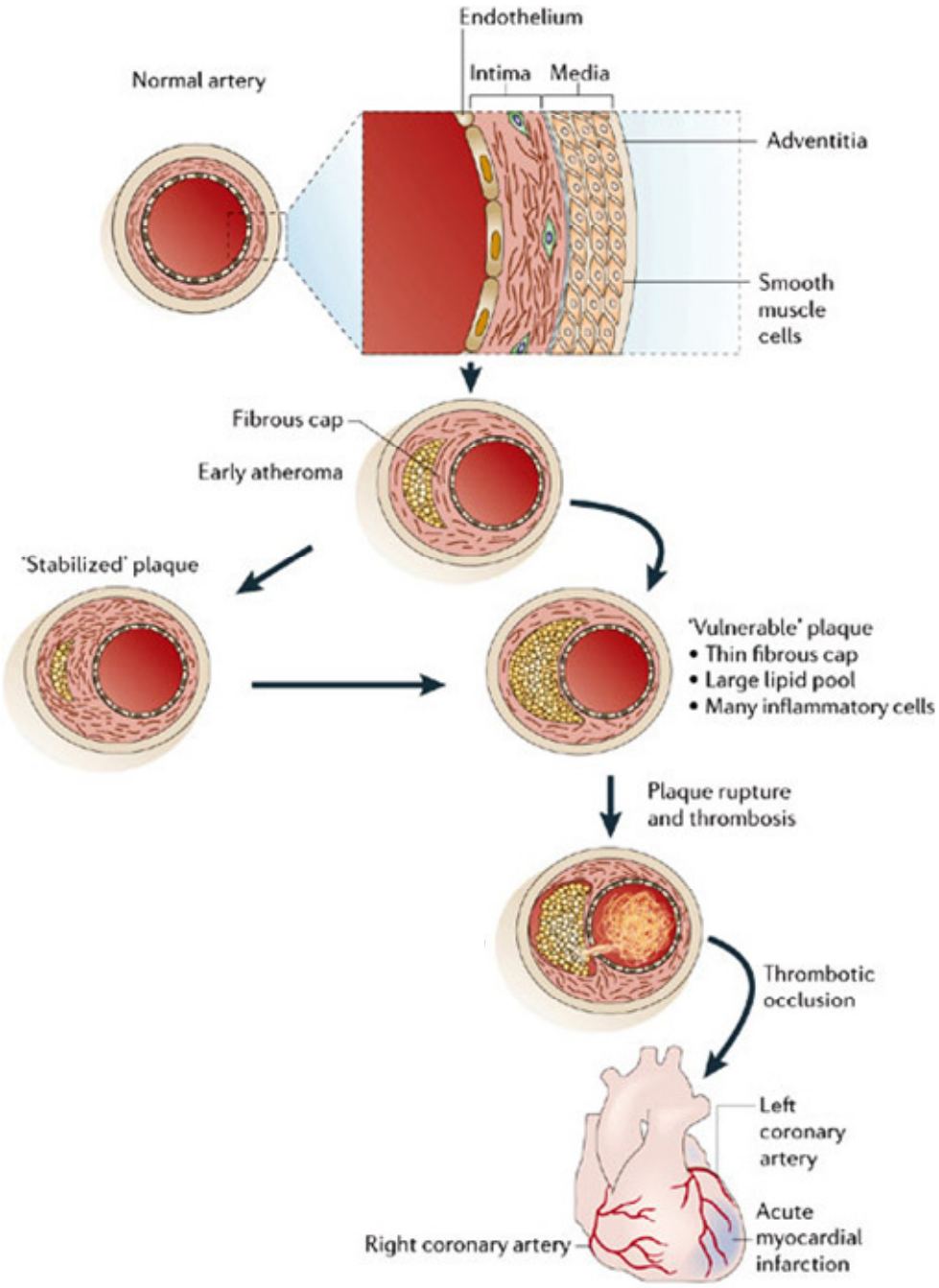


**Figure 2:** Developmental stages of atherosclerosis. (Picture from Hugh Watkins et al., Nature Reviews Genetics. 2006; 7: 163-73)

## 1.2 Risk Factors

Atherosclerosis has a complex aetiology influenced by a number of factors. The cardiovascular risk increases with the number of risk factors of a patient. Factors which influence atherosclerosis development can be grouped into genetic and environmental. In most cases the development of atherosclerosis results from complex interactions between environmental and genetic factors.

### 1.2.1 Factors with a strong genetic component

1. Elevated levels of low density lipoproteins (LDL)

Low density lipoproteins play an important role in transportation of cholesterol and triglycerides from the liver to peripheral tissues and in the regulation of cholesterol synthesis. Elevated levels of LDL usually result from mutations in the LDL receptor gene, causing familial *hypercholesterolemia*[2]. Some of the genetic variants which cause elevated LDL levels are the apolipoprotein E[3] and the apolipoprotein (a) genes[4].

2. Reduced levels of high density lipoproteins (HDL)

High density lipoproteins carry cholesterol from the systemic circulation to the liver, where they are excreted or re-utilized. Polymorphisms in the hepatic lipase encoding gene and the apolipoprotein AI-CIII-AIV gene cluster results in altered levels of HDL[5].

3. Elevated blood pressure

Hypertension is considered one of the major cardiovascular risk factors. Consequently, treatment of hypertension reduces the risk of cardiovascular diseases by 50%, compared to patients whose blood pressure is uncontrolled[6].

4. Elevated levels of homocysteine

Homocysteine, a sulphur containing amino acid is an intermediate product of the metabolism of methionine and cysteine. A single mutation (677C→T) in methylenetetrahydrofolate reductase gene causes increased homocysteine levels[7] associated with premature atherosclerosis.

5. Metabolic syndrome

Metabolic syndrome is a cluster of metabolic disturbances that strongly predisposes to atherosclerosis development[8]. Peripheral insulin resistance seems to be the central phenomenon of the metabolic syndrome, which is characterised by impaired glucose tolerance, dyslipidemia, hypertension and obesity.

6. Male gender and family history

It has been reported that below 60 years of age, men develop coronary heart disease (CHD) at more than twice the rate of women[9]. Individuals with a family history, i.e first degree relatives of patients with early onset of cardiovascular disease are at a higher risk of developing atherosclerosis which may be due to a common genetic predisposition (elevated blood pressure or cholesterol levels) or non genetic effects/environmental factors (smoking or diet)[10, 11].

### 1.2.2 Environmental factors

1. High-fat diet

High fat and high cholesterol diets have been shown to increase atherosclerosis. In experimental models, high fat diets are used to induce plaque formation. In humans, regular consumption of high fat diet results in obesity and subsequent reduction of average life expectancy. Hence, reduction of body weight through diets is one of the main treatment strategies to reduce the individual cardiovascular risk. Mediterranean diets rich in olive oil or nuts have proved to reduce the cardiovascular risk more effectively than a conventional low-fat diet[12]. Additionally, omega-3 fatty acid rich diets reduce the risk of cardiovascular diseases[13].

2. Smoking

It has been calculated that about 30% of cardiovascular deaths are due to smoking[14]. Cigarette smoking increases total cholesterol, triglycerides and LDL-cholesterin and decreases the cardio-protective HDL-cholesterin. Smoking is an established independent risk factor for atherosclerosis development even among young individuals[15].

3. Infectious disease/chronic inflammatory disease

Recent studies have shown that inflammation plays a fundamental role in development of atherosclerosis[16, 17]. The signalling cascades that are triggered in response to inflammation have a proatherogenic role. Epidemiological and basic scientific studies have shown that pathogens like Chlamydia pneumonia[18]

and Porphyromonas gingivalis[19] are associated with atherosclerosis development. Chronic inflammatory disease secondary to infection, like acquired immunodeficiency syndrome (AIDS) due to infection of human immunodeficiency virus (HIV)[20] and auto immune diseases like systemic lupus erythematosus and rheumatoid arthritis also accelerate atherosclerosis development[21].

4. Lack of exercise

Lack of physical exercise and a sedentary life style is an independent risk factor for various cardiovascular diseases. Regular exercise results in reduction of body fat, LDL cholesterol, triglyceride levels and blood pressure and increases atheroprotective HDL cholesterol levels[22]. Physical exercise is of paramount importance as it positively influences many independent cardiovascular disease risk factors.

5. Oxidative stress

Increased levels of oxidants or decreased levels of anti-oxidants secondary to dyslipidemia, hypertension, diabetes and smoking are implicated in the pathogenesis of atherosclerosis. Oxidation of LDL is considered to be the critical step involved in 'foam cell' formation[23]. Oxidation of LDL results in many structural modifications and generation of numerous 'oxidation specific epitopes' such as malondialdehyde (MDA)-lysines and 4-hydroxynonenal (4-HNE)–lysine' which are highly immunogenic. Immunization of mice with MDA and native LDL resulted in a significant reduction of atherosclerosis indicating the proatherosclerotic role of oxidized LDL (ox-LDL)[24].

## 1.3 Oxidative Stress

Oxidative stress results from increased production of reactive oxygen species (ROS) in biological systems. ROS include “free radicals” and “non-free radicals” which are produced during electron transfer reactions in oxygen ($O_2$) metabolism. Molecular $O_2$ is essential for the survival of all aerobic organisms and acts as the electron acceptor during various metabolic reactions. The partially reduced, highly reactive metabolites formed during these reactions react more avidly compared to molecular $O_2$. ROS are generally considered to be by-products of metabolism with a potential to cause cellular injury[25]. During evolution organisms have developed several strategies to potentially detoxify ROS. However, under physiological condition ROS are also important signalling molecules[26] and there exists a balance between production and detoxification of ROS. Diseases may cause an imbalance between the production and neutralization of ROS, resulting in altered cell signalling and oxidative stress.

Superoxide anion formation generates a chain of reactions which result in the formation of various highly reactive free radicals and non radicals. In atherosclerosis and other vascular diseases ROS are potent pathological mediators[27], as they cause lipid peroxidation, smooth muscle cell proliferation, protein oxidation, inflammatory cell recruitment and vascular inflammation. For example, oxidative modification of lipoproteins initiates foam cell formation[23] and vascular oxidative stress is a major cause of cardiovascular diseases[28]. ROS are implicated in the process of initiation of foam cell formation until ultimate plaque rupture. Increased oxidative stress results in reduced *endothelial dysfunction*[29].

### 1.3.1 Sources of oxidants in the vasculature

There are numerous enzymatic sources of ROS in the vasculature, including mitochondrial electron transport chain, the arachidonic acid metabolizing enzymes lipoxygenase and cyclooxygenase, the cytochrome P450s, myeloperoxidase[30], xanthine oxidase[31], Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase[32], and the nitric oxide synthases (NOS).

NADPH oxidase and xanthine oxidase are important contributors to oxidative stress in cardiovascular diseases[32, 33]. NADPH oxidase is expressed in endothelial cells, smooth muscle cells, fibroblasts and macrophages, while xanthine oxidase is expressed in endothelial cells and found in plasma. Myeloperoxidase (expressed in neutrophils), the enzyme that converts chloride ($Cl^-$) ion to hypochlorous acid, increases atherosclerosis development[30], as expression of this enzyme was observed in human atherosclerotic lesions. Furthermore, footprints of oxidative modifications of LDL by myeloperoxidase/HOCl were observed in atherosclerotic lesions[34].

NOS enzymes produce nitric oxide (NO) by their catalytic conversion of L-arginine to L-citrulline. This family of enzymes includes the endothelial NOS (eNOS or NOS III), inducible NOS (iNOS or NOS II) and neuronal NOS (nNOS or NOS I). However, NOS not only produces nitric oxide but may directly produce superoxide in special metabolic situations. Under conditions of substrate L-arginine or cofactor, (tetrahydrobiopterin ($BH_4$)) deficiency, NOS "uncouple" directing electron transfer to molecular oxygen rather than to L-arginine, resulting in generation of superoxide[35-37]. In vitro, the generation of superoxide and nitric oxide results in the formation of the strong oxidant

peroxynitrite[38] which by itself causes the formation of a complex array of oxidants leading to lipid peroxidation and protein nitration[39, 40]. Therefore, it has been speculated that the superoxide generated by uncoupled NOS might result in peroxynitrite formation and consecutive oxidative stress.

### 1.3.2 Functional role of superoxide in atherosclerosis

Superoxide functions as a signaling molecule in cell division, differentiation and cell survival[41, 42]. Additionally, increased production of superoxide is observed in hypertension, myocardial infarction, diabetes and atherosclerosis. Moreover, the severity of atherosclerosis correlates with the activation of NADPH oxidase in human carotid arteries[43] and NADPH oxidase deficient apolipoprotein E knockout (apoE ko) animals showed reduced atherosclerosis suggesting an important role of superoxide in atherosclerosis[44]. Increased expression of xanthine oxidase has also been observed in atherosclerotic plaques[45].

Superoxide production reduces nitric oxide bioavailability not only by direct inactivation of nitric oxide but also by oxidizing the NOS co-factor $BH_4$, resulting in NOS “uncoupling”. Reactive oxygen and nitrogen species play a central role in the maintenance of vascular homeostasis. Nitric oxide dependent cell signaling, including endothelial dependent relaxation, is modulated by both superoxide and superoxide dismutase (SOD)[46, 47]. Alterations in both the rate of formation and the extent of superoxide scavenging have been implicated in vascular dysfunction, hypertension, diabetes, as well as in chronic nitrate tolerance[39, 48, 49]. The evidence for the involvement of superoxide in impaired endothelium dependent relaxations is shown by the restoration of endothelium

dependent relaxations using SOD and antioxidants[50, 51]. Further, deficiency of vascular SOD results in impaired endothelial functions[47]. In addition to reducing the bioavailability of nitric oxide, superoxide generation may also promote endothelial cell apoptosis[52].

Superoxide generation causes platelet adhesion and aggregation[53]. NADPH oxidase mediated superoxide production causes increased leukocyte/endothelial cell interaction in hypercholesterolemic mice[54]. Further, SOD inhibits the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) in endothelial cells[55], suggesting that superoxide modulates the expression of adhesion molecules. Additionally, superoxide promotes vascular smooth muscle cell migration and proliferation[52]. Both superoxide and hydroxyl radical contribute to LDL-oxidation which induces cholesterol accumulation in macrophages and leads to foam cell formation[56, 57]. Ox-LDL acts as a chemotactic factor for monocytes and T-cells, the predominant population of blood cells found in the atherosclerotic lesions.

### 1.3.3 Functional role of nitric oxide in atherosclerosis

Nitric oxide, named the 'molecule of the year in 1992' is an important cell signaling, effector and vasodilator molecule with potentially strong antiatherogenic actions. Of all its functions, it's role as endothelium dependent relaxing factor (EDFR) is the most recognized one, thought to reflect vascular homeostasis. Nitric oxide mediates vascular smooth muscle cell relaxation by a calcium-ion channel mediated activation of the cyclic guanosine monophosphate (cGMP) pathway. Further, nitric oxide inhibits smooth cell proliferation, leukocyte/endothelial cell interactions and platelet aggregation.

Pharmacological inhibition of nitric oxide production by NOS results in increased leukocyte adhesion to microvascular endothelium[58] and expression of endothelial surface adhesion molecules, including P-selectin and VCAM-1[59, 60]. Nitric oxide regulates platelet activation, platelet aggregation and platelet/endothelial cell-interactions[61-63]. It was shown *in vitro* that nitric oxide generated in the coronary and pulmonary vasculature inhibits platelet adhesion under constant flow conditions[64]. By it's regulation of leukocyte and platelet adhesion to the endothelium, nitric oxide contributes to the maintenance of microvascular barrier integrity and may decrease local inflammation and vascular permeability.

The eNOS (endothelium) mediated nitric oxide production results in vasodilation, increased blood flow and reduced blood pressure. Impairment in the endothelial dependent relaxation, termed "endothelial dysfunction" is considered to be one of the critical steps in atherosclerosis development. Endothelial dysfunction occurs as a result of decreased nitric oxide production, decreased sensitivity to nitric oxide or decreased nitric oxide bioavailability[65]. Decreased nitric oxide production may occur secondary to reduced transcription or increased instability of eNOS mRNA[66]. Additionally, altered eNOS activity observed in hypercholesterolemia decreases nitric oxide production[67, 68]. NOS inhibitors like asymmetric dimethylarginine (ADMA) and N-monometylarginine (NMA) are involved in endothelial dysfunction[69].

The increased production of superoxide observed during condition of atherosclerosis decreases the bioavailability of nitric oxide since superoxide reacts with nitric oxide at a diffusion limited rate, to form peroxynitrite. The reaction rate of superoxide with nitric oxide ($6\text{-}10 \times 10^9$ $M^{-1}sec^{-1}$) is faster than the

rate at which superoxide is degraded by SOD ($2x10^9$ $M^{-1}sec^{-1}$). Further dismutation of superoxide by SOD can occur only if the latter enzyme is present in the same compartment in which superoxide is produced. In addition to being a significant source of eNOS mediated nitric oxide production, the endothelium is also a significant source of superoxide production in atherosclerotic vessels[70]. Since under these conditioms nitric oxide and superoxide are produced in the same cellular compartment, i.e., the endothelial cell, they can immediately react to form peroxynitrite. Peroxynitrite is a strong oxidant which alters the function of biomolecules by protein nitration and lipid peroxidation[71] with secondary tissue injury[39, 40]. Subintimal lipoprotein oxidation by peroxynitrite may initiate the formation of fatty streaks and subsequent plaque development[39]. Peroxynitrite may also contribute to endothelial cell dependant vascular oxidation.

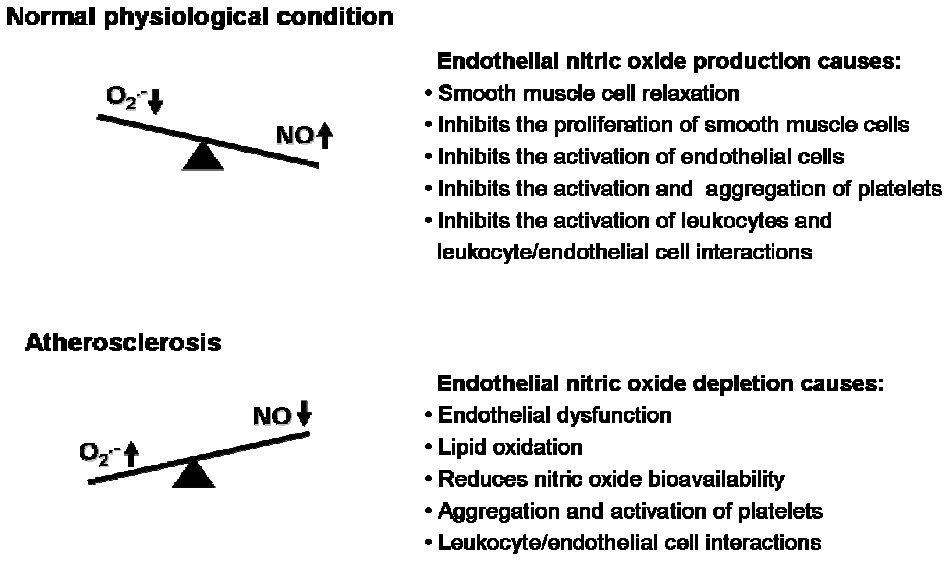


**Figure 3:** Proposed functional role of nitric oxide and superoxide in normal and atherogenic conditions, respectively.

## 1.4 The nitric oxide pathway in atherosclerosis

L-arginine, a non-essential amino acid is utilized by the NOS enzyme to produce L-citrulline and nitric oxide. The nitric oxide synthesized by all NOS may enters one of the following routes: a) activation of *soluble guanylate cyclase* (sGC), which is responsible for most of the physiological effects of nitric oxide b) regulation of expression of adhesion molecules by inducing transcription and stabilization of IκBα[72], an inhibitor of NF-κB through a cyclic guanylate monophosphate (cGMP) independent pathway[73] c) reaction with oxyhemoglobin to form stable metabolite nitrosylhemoglobin[74] d) formation of nitrate[75] e) formation of peroxynitrite by reacting with superoxide[38] f) nitrosylation of proteins[76, 77]. Nitric oxide has no membrane receptor, but binds to the heme group of sGC producing a conformational change which increases its activity[78]. sGC converts guanylate triphosphate (GTP) into cGMP which activates *protein kinase G* (PKG), a cGMP dependant protein phosphorylator. PKG mediated protein phosphorylation leads to: a) inhibition of *L-type calcium channels* in the plasma membrane[79]; b) activation of $Ca^{++}$ ATPase[80] and $Ca^{++}$-$Na^{+}$ exchanger in the plasma membrane[81]; c) activation of $Ca^{++}$ ATPase at the level of the sarcoplasmic reticulum[82] and d) inhibition of *protein lipase C*[83]. Calcium levels have differential roles in the nitric oxide pathway. Intracellular free calcium levels in endothelial cells activate NOS by binding to calmodulin[84]. Nitric oxide generated by the activated NOS in the endothelium diffuses into the smooth muscle cell layer in the media, where it activates sGC which causes feedback inhibition of calcium levels through cGMP mediated mechanisms resulting in relaxation of smooth muscle cells in the medial layers of the vessel wall. One of the many proteins which are phosphorylated in response to cGMP

activation is the vasodilator-stimulated phosphoprotein (VASP). VASP is phosphorylated by cGMP dependent PKG which causes the nitric oxide mediated inhibition of smooth muscle cell proliferation[85]. cGMP also down regulates the function of some platelet receptors, including the fibrinogen receptor IIb/IIIa and P-selectin[86].

The activity of the enzymes involved in the nitric oxide pathway is altered during oxidative stress, as observed in atherosclerosis. As mentioned before, the expression and activity of eNOS is altered during atherosclerosis. In addition the formation of peroxynitrite is capable of impairing the activity of sGC[87]. Atherosclerosis is also associated with low L-arginine availability. Subsequently, the altered functional activity of the nitric oxide pathway results in vascular smooth muscle contraction and endothelial dysfunction.

### 1.4.1 Overview of NOS family

NOS (EC 1.14.13.39) catalyses the conversion of L-arginine to L-citrulline, yielding nitric oxide as a byproduct. The NOS proteins have ~60% amino acid homology and possess similar primary structures. The isoforms are expressed in different cellular compartments and function as homodimers, composed of two identical monomers. The monomers consist of a C-terminal reductase domain and a N-terminal oxygenase domain, which differ in their structure and function between isoforms. The synthesis of nitric oxide requires L-arginine as a substrate, calmodulin, molecular oxygen and four cofactors: flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin ($BH_4$) and nicotinamide adenine dinucleotide phosphate (NADPH). The reductase domain consists of the binding sites for one molecule

of NADPH, FAD and FMN, whereas the oxygenase domain binds heme, $BH_4$ and the substrate L-arginine. As shown in figure 4, between these two domains lies the calmodulin binding site, which plays an important role in both structure and function of the enzyme.

The biosynthesis of nitric oxide involves a two step oxidation reaction and consumes 1.5 mol of NADPH and 2 mol of oxygen including the formation of the intermediate product, $N^G$-hydroxy-L-arginine. The reductase domain transfers the electrons from NADPH via the flavins: FAD and FMN to the heme molecule in the oxygenase domain, where the substrate L-arginine is oxidized to L-citrulline and nitric oxide. Hence, the two domains perform catalytically distinct functions. Despite the fact that each monomer consists of both domains, dimerisation of the enzyme is essential for its catalytic activity since the electrons are transferred from the flavins in the reductase domain of one subunit to the heme centre in the oxygenase domain of the second subunit[88] (Figure 4). Heme plays a key role in dimerisation of both the subunits in all three NOS isoforms and is also required for the interaction between the reductase and oxygenase domains. Calcium dependence is the key feature that distinguishes constitutive and inducible isoforms of NOS. eNOS and nNOS are activated by elevation of intracellular calcium levels, followed by subsequent binding of calcium/ calmodulin. In contrast, iNOS contains irreversibly bound calmodulin and thus its activation is independent of intracellular calcium concentration.

Under conditions of either substrate L-arginine or cofactor $BH_4$ deficiency, all the isoforms of NOS can “uncouple”. The term “uncoupling” defines a situation during which the electrons flowing from the reductase

domain to the oxygenase domain are shifted to molecular oxygen instead of L-arginine, resulting in superoxide rather than nitric oxide production. However, the conditions and mechanisms that cause uncoupling differ between the NOS isoforms. Furthermore, NOS isoforms vary in their regulation of gene expression, catalytic activity and the cellular compartment of gene expression. These features make each isoform unique and give rise to distinct mechanistic features that are responsible for their differential function under various physiological and pathophysiological conditions.

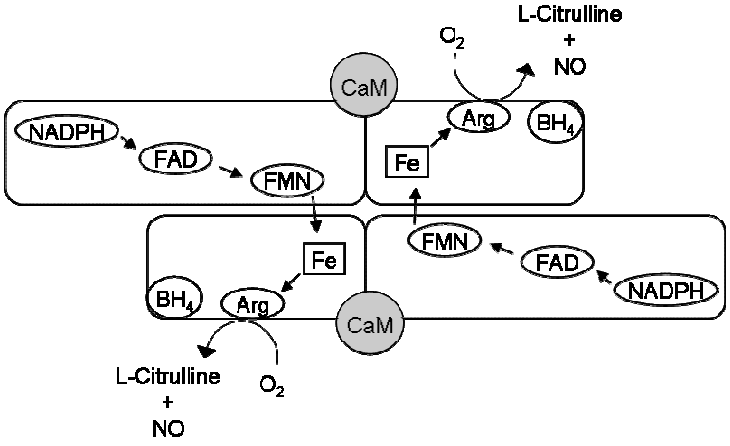


**Figure 4:** Structure of functional NOS dimers. Electrons in the NOS dimer flow via NADPH→FAD→FMN in the reductase domain (shaded region) of one monomer to the heme (Fe) in the oxygenase domain of the second monomer. Calmodulin (CaM) binding is essential for dimerisation of the enzyme. Dimerisation of NOS is required for conversion of L-arginine to L-citrulline and nitric oxide. (Picture adapted from Andrew PJ et al., Cardiovascular Research. 1999; 43: 521-31)

## 1.4.2 Unique features of NOS isoforms

The functional relevance of nitric oxide generated by each NOS isoform differs depending on the cellular compartment and the target proteins expressed in the compartment. Because of the versatile properties of nitric oxide, the expression, activity, spatial distribution and proximity of NOS

isoforms to the regulatory and target proteins are tightly regulated and vary between the isoforms.

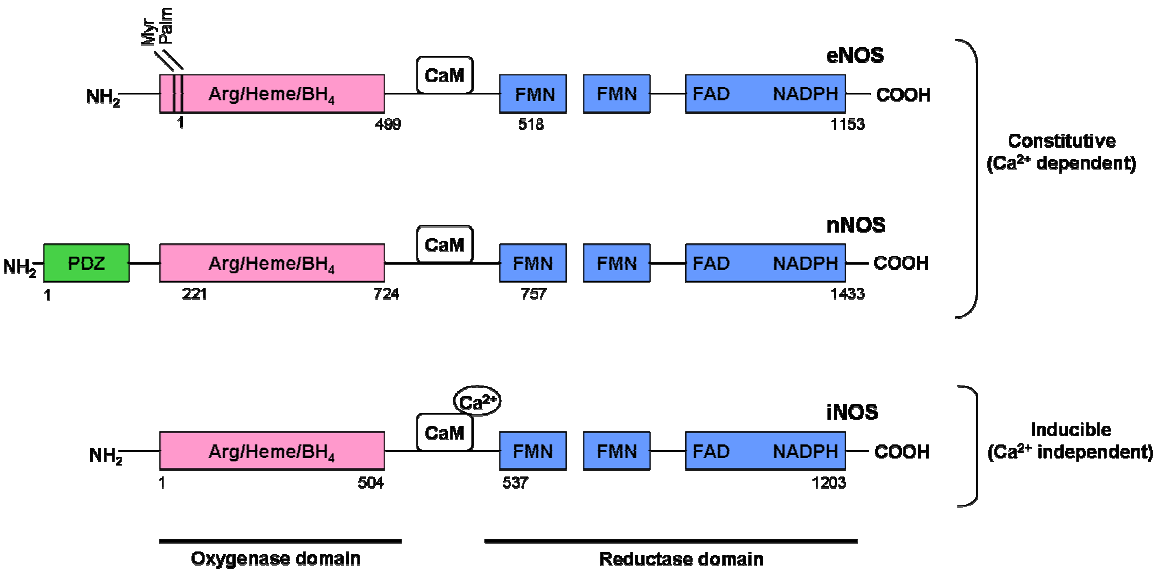


**Figure 5:** Distinct domain structure of each NOS isoform. Binding sites for L-arginine (Arg), heme, tetrahydrobiopterin ($BH_4$), calmodulin (CaM), flavins (FAD and FMN) and NADPH are indicated. The oxygenase, reductase and PDZ (nNOS) domains are indicated by solid bars. The numbers indicate the amino acid residues within in each domain. Myristoylation (Myr) and palmitoylation (Palm) sites on eNOS are shown. The irreversible binding of calcium to the calmodulin in iNOS is indicated. (Picture adapted from Alderton WK et al., Biochem J. 2001; 357: 593-615)

### 1.4.2.1 Endothelial nitric oxide synthase (eNOS)

eNOS is the main source of endothelial nitric oxide production in the vasculature. As mentioned in detail before, nitric oxide generated by eNOS plays an important role in the prevention of leukocyte/endothelial interactions and smooth muscle cell proliferation. In addition to the endothelium, eNOS is also expressed in cardiomyocytes and cardiac conduction tissue. eNOS belongs to the constitutively expressed, calcium dependant NOS isoforms.

Several physiological situations like shear stress and exercise training increase eNOS expression[89]. Transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) influence eNOS mRNA levels. While TGF-β induces eNOS mRNA and protein expression as well as enzyme activity [90], TNF-α down regulates eNOS expression[91].

The activity of eNOS is regulated by a number of mechanisms including post translational modification, mediating sub cellular localization of the enzyme[92, 93]. Hormones like estrogen, catecholamines, vasopressin and platelet derived mediators such as serotonins increase eNOS function. The activity of eNOS is also determined by signaling complexes which are composed of the enzyme and a conglomerate of adaptor proteins, structural proteins, kinases, phosphatases and potentially proteins which affect association and determine intracellular localization. The kinases and phosphatases phosphorylate and dephosphorylate eNOS at various sites resulting in activation or attenuation of the enzyme. For example, eNOS phosphorylation at Ser1177 activates eNOS whereas phosphorylation at Thr495 attenuates eNOS activity. It was shown that protein kinase C (PKC) promotes both the dephosphorylation of Ser1177 and the phosphorylation at Thr495, resulting in attenuated enzymatic activity[94]. In contrast cAMP dependent protein kinase (PKA) signaling leads to eNOS phosphorylation at Ser1177 and dephosphorylation at Thr495 resulting in activation of the enzyme[94]. In addition to the modulation by phosphorylation, protein-protein interactions also influence eNOS activity. Further, post translational modifications like N-terminal acylation, specifically myristoylation and palmitoylation determines the sub cellular localization of the enzyme. The modification targets eNOS to both the plasmalemmal vesicles, *caveolae* and the

perinuclear/golgi region within the cell[95, 96]. Within the caveolae, eNOS is bound to caveolin-1 in its inactive form[97]. Calcium influx disrupts the caveolin-1/eNOS complex and results in eNOS activation[98]. Additionally, dynamin-2 and Hsp-90 interact with eNOS and positively regulate the enzyme's activity[99, 100].

Superoxide production by eNOS "uncoupling" is believed to result from $BH_4$ deficiency rather than L-arginine deficiency[101]. The amount of superoxide generated by eNOS depends on calcium/calmodulin binding. In the absence of calcium/calmodulin, eNOS generates low amounts of superoxide and the activation by calcium/calmodulin increases superoxide production[102]. Heme blockers like cyanide or imidazole prevent eNOS mediated superoxide generation during $BH_4$ deficiency. This suggests that eNOS generates superoxide from the heme containing oxygenase domain[35]. One possible mechanism by which $BH_4$ deficiency occurs is $BH_4$ oxidation[103].

#### 1.4.2.2 Neuronal nitric oxide synthase (nNOS)

nNOS is involved in a wide variety of physiological and pathological processes, including neurotransmission, neurotoxicity, skeletal muscle contraction, body fluid homeostasis and cardiac function[104]. Though the name implies the expression of this isoform in neuronal tissues, nNOS is expressed in epithelial cells, mesanglial cells, skeletal muscle cells and cardiomyocytes. In the vasculature, nNOS is expressed in endothelial[105] as well as smooth muscle cells of rat and human origin[106]. nNOS is the largest of the NOS isoforms containing an additional 300 amino acids at the N-terminus. This domain is called the PDZ (PSD-95 discs large/ zona occludens -1 homology domain) domain or disc-large homologous region (DHR), which is essential for nNOS

binding to other proteins and sub cellular localization. In neurons, nNOS is associated with the rough endoplasmic reticulum and the synaptic membrane[107, 108] whereas in skeletal muscle, nNOS localizes to the sarcolemma[109]. Some studies have also shown the localization of nNOS protein in the cytosol[110-112]. The localization of nNOS differs depending on the cellular compartment or the pathophysiological conditions.

The gene structure and the expressional regulation of nNOS are highly complex. The expression of nNOS is tightly regulated by post-transcriptional and post-translational mechanisms. Several nNOS mRNA species are expressed in different tissues in a developmentally regulated manner. Post-transcriptional regulation of nNOS involves multiple promoter usage, alternate splicing through deletion and insertion of exons, varied sites for 3' untranslated region cleavage and polyadenylation. The alternative splicing results in the generation of nNOS proteins which differ in their structural features and catalytic activity. The full length nNOS protein, nNOS-α has high catalytic activity and is coded by multiple transcripts[113]. Two additional splice variants of nNOS, the nNOS-β and nNOS-γ lack the PDZ domain. The nNOS-β and the nNOS-γ variants have about 80% and 30% of the catalytic activity of full length nNOS-α, respectively. Because of the lack of the PDZ domain, which is responsible for targeting nNOS to synaptic membranes, nNOS-β is localized to the cytosol[114]. Another splice variant, nNOS-μ possesses an in-frame insertion of 34 amino acids between the oxygenase and the reductase domains and has similar catalytic activity compared to nNOS-α[115]. Alternative splice variants of nNOS differ in their cellular compartment of expression and serve differential roles under physiological and pathological conditions.

nNOS interacts with several proteins which determine the targeting of the enzyme or the enzymatic activity. Targeting of nNOS to appropriate sites in the cell is mediated by its PDZ domain. Some of the proteins that bind to the PDZ domain and are essential for nNOS targeting are CAPON (carboxy-terminal PDZ ligand of nNOS), NIDD (nNOS interacting DHHC domain), dystrophin family of proteins and post-synaptic density proteins (PSD) 93 and 95[100]. Proteins which negatively regulate nNOS activity are protein inhibitor of nNOS (PIN), nitric oxide synthase-interacting protein (NOSIP) and caveolin-3[100]. nNOS is also translationally regulated by phosphorylation through calmodulin-dependent kinases. Phosphorylation of nNOS by calmodulin-dependent kinase II resulted in a decrease in the enzyme activity whereas phosphorylation by PKC caused a marked increase in enzyme activity[116].

Of all the three isoforms of NOS, nNOS was the first enzyme which was shown to "uncouple", to produce superoxide instead of nitric oxide[36]. In the absence of its substrate L-arginine, nNOS catalyses the generation of superoxide from the oxygenase domain[117]. In the presence of L-arginine nNOS can generate nitric oxide and superoxide. The ratio of the two radicals depends on the concentration of the substrate, $BH_4$[117, 118]. Similar to eNOS, $BH_4$ inhibited superoxide production from nNOS in a dose dependent manner. Interestingly, L-arginine alone, independent of the dose of $BH_4$ inhibited superoxide production, suggesting that substrate deficiency but not $BH_4$ deficiency determines the superoxide production by nNOS[118]. Recently studies have shown that the methyl arginines, asymmetric dimethyl arginine (ADMA) and $N^G$-monomethyl L-arginine modulate superoxide as well as nitric oxide generation from nNOS. Further this study shows that even in the presence of normal

substrate and co-factor concentration nNOS generates superoxide[119]. In addition to superoxide nNOS is capable of generating of hydrogen peroxide ($H_2O_2$) in the absence of substrate, using molecular oxygen as the terminal electron acceptor[120]. In this reaction, $BH_4$ plays a critical role in regulating the generation of superoxide and hydrogen peroxide[121]. In terms of enzymatic activity during uncoupling, nNOS differs from other NOS isoforms in its readiness to catalyze the uncoupled reaction i.e., nNOS oxidizes NADPH at a higher rate than the other NOS isoforms[122]. Supporting this concept, in the absence of substrate, nNOS produces higher amounts of superoxide than iNOS[123].

#### 1.4.2.3 Inducible nitric oxide synthase (iNOS)

Unlike eNOS and nNOS which are constitutively expressed, iNOS is expressed only when induced by external stimuli. Nitric oxide generated by iNOS mediates the cytotoxic actions of activated macrophages and neutrophils and plays an important role in the non specific immune response of the pathogenic defense mechanism. iNOS is expressed in many nucleated cells of the cardiovascular system namely vascular smooth muscle cells, endothelial cells, cardiac myocytes, inflammatory cells found in sub endothelial space such as leukocytes, fibroblasts and mast cells during various diseased conditions. In contrast to eNOS and nNOS which are regulated by intracellular calcium levels, iNOS contains irreversibly bound calmodulin and hence is independent of intracellular calcium levels. Thus, induction of iNOS results in generation of tremendous levels of nitric oxide[124].

iNOS expression is regulated transcriptionally following cytokine (tumor necrosis factor-α, interleukin-1β, interleukin-2 or interferon gamma-γ) or bacterial lipopolysaccharide stimulation. Additionally, post transcriptional regulation is implicated. The 3' untranslated region of iNOS possess an 'AUUUA' motif which potentially destabilizes iNOS mRNA[125]. LPS and IFN-γ increase mRNA stability while transforming growth factor-β (TGR-β) decreases the translation of iNOS mRNA without affecting its rate of transcription and also increases iNOS protein degradation and activity[126, 127]. Alternative splice variants of iNOS have been detected in human cells, which lack the heme domain (denoted $iNOS_{8\text{-}9\text{-}}$) or in the FMN binding region[128]. $iNOS_{8\text{-}9\text{-}}$ is functionally inactive as it is unable to form homodimers[129]. Additionally, two splice variants of iNOS were identified in normal lymphocytes and chronic lymphocytic leukemia cells[130] which regulate nitric oxide production in these cells. Further regulation of iNOS enzyme activity is achieved by phosphorylation[131] and binding of iNOS protein to caveolin-1 which results in an increased protein degradation[132]. Though iNOS does not contain specific membrane targeting sequences, it is found to be membrane associated in neutrophils and macrophages[133, 134] and localizes to both cytosol and peroxisomes in hepatocytes[135].

In contrast to eNOS and nNOS "uncoupling" of iNOS occurs in the presence of high concentration of L-arginine (5 mM)[136]. While 100 µM of L-arginine completely blocks superoxide generation from nNOS, it did not block superoxide generation by iNOS. Even in the presence of 1 mM L-arginine the superoxide production by iNOS was only partially blocked suggesting that iNOS is capable of generating superoxide even when the availability of L-arginine is

adequate[136]. While eNOS and nNOS generate superoxide from their oxygenase domains, iNOS catalyses the production of superoxide from its reductase domain[136]. Therefore, iNOS was proposed to simultaneously generate nitric oxide from L-arginine bound to its oxygenase domain, while generating superoxide from its reductase domain (Figure 6). This simultaneous generation of superoxide and nitric oxide results in iNOS mediated peroxynitrite generation, a more potent oxidant than superoxide which enhances the anti microbial activity of iNOS[37].

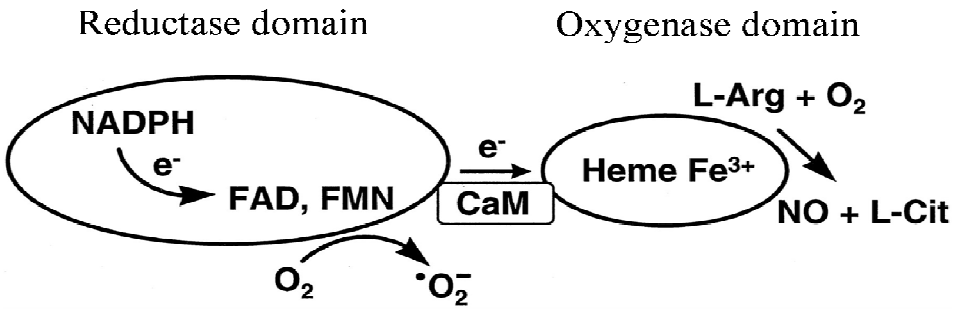


**Figure 6:** Schematic diagram depicting superoxide generation of iNOS from its reductase domain. Solid arrows indicate electron flow. In the presence of L-arginine simultaneous generation of superoxide and nitric oxide may occur at the reductase and oxygenase domains respectively. (Picture from Xia Y et al., J Biol Chem. 1998; 273: 22635-39).

## 1.5 The apoE ko model of atherosclerosis

Experimental investigation of the mechanisms and progression of atherosclerosis have been greatly facilitated by the use of mouse models. The advent of gene targeting allowed the generation of mice which lack the gene for apoE[137]. These apoE ko mice serve as a practical atherosclerosis model since they spontaneously develop complex atherosclerotic lesions closely resembling human disease. ApoE is an important component of the reverse cholesterol

transport pathway and is an essential ligand for the uptake and clearance of atherogenic lipoproteins[138]. ApoE is a constituent of chylomicrons, very low density lipoproteins (VLDL) and HDL. Genetic deletion of apoE in mice, a species normally resistant to atherosclerosis, is associated with 4-5 times increased plasma cholesterol levels. Although the pathomechanism of atherosclerosis development differs from common human disease, the apoE ko model has substantially shaped our understanding of the role of apoE in lipid transport and proved to be a valid atherosclerosis model[139].

## 1.6 Role of NOS isoforms in cardiovascular diseases

### 1.6.1 Role of eNOS in cardiovascular diseases

Endothelium derived nitric oxide plays a major role in modulating several cardiovascular functions[140]. Nitric oxide generated by eNOS serves as an endothelium derived relaxing factor, regulates vascular tone and blood pressure. Furthermore, it exerts potential anti atherosclerotic effects as it inhibits vascular smooth muscle cell proliferation, platelet aggregation and leukocyte adhesion[140],[141]. The importance of endothelium derived nitric oxide in maintaining normal endothelial function has been described in detail in section (1.3.3). Reduced bioavailability of nitric oxide has been associated with several cardiovascular diseases. One of the potential mechanisms leading to reduced nitric oxide bioavailability is the uncoupling of eNOS. Uncoupling of eNOS is observed in several cardiovascular diseases but may also serve as an important defense mechanism of the normal endothelium[142]. Furthermore, alteration in the sub cellular localization of eNOS resulting in decreased activity of the enzyme has been observed during various disease conditions[143].

Multiple lines of evidence point to an important cardioprotective effect of eNOS. For example, eNOS deficiency resulted in neoinitima proliferation in a vascular injury model[144] and over expression of eNOS decreased neointimal and medial thickening, decreased leukocyte infiltration, reduced intracellular adhesion molecule (ICAM-1) and vascular cellular adhesion molecule (VCAM-1) expression, in a *carotid artery ligation* model of vascular remodelling[145]. eNOS protects from myocardial dysfunction. Targeted over expression of eNOS within the vascular endothelium in mice attenuates cardiac and pulmonary dysfunction and dramatically improved survival in *congestive heart failure*[146]. The same authors have reported that over expression of eNOS results in attenuation of *myocardial infarction* size[147].

Atherosclerosis is associated with endothelial dysfunction, decreased eNOS activity and reduced cGMP levels[67]. Genetic deletion of eNOS resulted in increased arteriosclerosis in an aortic transplant model suggesting that eNOS protects from *transplant arteriosclerosis*[148]. We and others have shown that deletion of eNOS resulted in acceleration of plaque formation in apoE ko mice[149, 150]. Additionally, the apoE/eNOS dko mice developed vascular complications like abdominal aortic aneurysms, *aortic dissections*, distal *coronary artery disease*, as observed in human atherosclerosis[149]. Secondary to eNOS deletion, apoE/eNOS dko mice were hypertensive and showed impaired left ventricle function and *cardiac hypertrophy*, possibly a result of chronic *myocardial ischemia*, resulting from coronary artery disease[149]. However, eNOS may also increase atherosclerosis development as recently, over expression of eNOS accelerated atherosclerosis[151]. As a potential mechanism, uncoupling of eNOS with resultant superoxide production was observed in this model.

Interestingly, $BH_4$ supplementation resulted in decreased atherosclerosis, decreased superoxide and increased nitric oxide in this transgenic mice. Taken together, all these studies show that the presence of a functionally active eNOS is essential for the prevention of atherosclerosis.

### 1.6.2 Role of nNOS in cardiovascular diseases

Nitric oxide generated by nNOS functions as a *non-adrenergic non-cholinergic* neurotransmitter in the autonomous nervous system. Non-adrenergic non-cholinergic perivascular nerves (*nitrergic nerves*) found in the adventitia of cerebral and certain peripheral arteries (e.g. mesenteric, renal and femoral arteries) contain nNOS. In perivascular nitrergic nerves, nNOS derived nitric oxide causes relaxation of adjacent vascular smooth muscle cells, counterbalancing vasoconstriction mediated by the sympathetic nervous system[152]. nNOS expressed in cardiomyocytes plays an important role in regulating cardiac function[153]. In this respect, deletion of nNOS resulted in a higher heart rate and decreased heart rate variance compared to wildtype mice[154]. Genetic deficiency of nNOS resulted in increased myocardial infarction size and increased superoxide formation suggesting that nNOS serves a protective role in myocardial injury[155]. Following myocardial *reperfusion injury*, lack of nNOS resulted in a significant increase in cardiac polymorphonuclear leukocyte infiltration[156].

Recent studies have shown the expression of nNOS in normal vascular smooth muscle cells of carotid[157], coronary[158] and pulmonary[159] arteries and the aorta[160]. In the absence of functional eNOS under pathophysiological conditions, nNOS may regulate normal vascular tone[160]. Further, studies have

shown that nNOS inhibits leukocyte/endothelial cell interactions in the cremasteric microcirculation of mice, in the absence of eNOS[161]. In a mouse carotid artery ligation *model*, nNOS derived nitric oxide suppresses both neointimal formation and constrictive vascular remodelling[162]. In the same study the authors have shown that nNOS exerts an important inhibitory effect on vasoconstrictor response following balloon injury. Though there was no expression of nNOS before vascular injury, nNOS was up regulated in the neointima and medial smooth muscle cells after carotid artery ligation and balloon injury, suggesting a vasoprotective effect of nNOS in response to injury. Gene transfer of nNOS in venous bypass grafts resulted in substantial reduction of adhesion molecule expression and inflammatory cell infiltration in early venous bypass grafts (3 days after operation)[163]. In late venous bypass grafts (28 days after operation), nNOS gene transfer resulted in reduction of smooth muscle cell *hyperplasia* and reduced vascular superoxide production[163].

nNOS is detected in endothelial cells and macrophages in both early and advanced atherosclerotic lesions in humans, while it is absent in normal vessels[164]. nNOS is also expressed in the carotid artery of spontaneously hypertensive rats[157] and in the aorta of apoE ko[165] and apoE/iNOS double knockout (dko) mice[166]. Because nNOS is induced in various vascular pathologies like atherosclerosis, vascular injury and hypertension, nNOS should not be considered a “constitutive” enzyme. Rather, nNOS is subject to expressional regulation in the vascular system, while it is constitutively expressed in the nervous system[167]. We have recently shown that genetic deletion of nNOS resulted in accelerated atherosclerosis in apoE ko mice[165], suggesting that nNOS is atheroprotective. We also showed that nNOS improves

the survival rate, as apoE/nNOS dko mice had a 30% increased mortality compared to apoE ko controls. However, the exact mechanism by which nNOS acts as an anti-atherosclerotic enzyme is still not clear. It was speculated that nNOS localized towards to the lumen of the vessel might decrease leukocyte and platelet adhesion while nNOS expressed in the adventitia might inhibit smooth muscle cell proliferation[168]. Since nNOS also uncouples under conditions of substrate deficiency, the role of nNOS derived superoxide and nitric oxide in the formation of atherosclerosis still has to be defined.

### 1.6.3 Role of iNOS in cardiovascular diseases

Under normal physiological conditions, iNOS is unlikely to have any functional role in the cardiovascular system due to its low (or absent) expression. However, a large number of reports are available which provide evidence for the expression of iNOS under pathophysiological conditions, both in humans as well as in animal models. In this respect, iNOS expression is detected in atherosclerosis, following balloon injury and *restenosis*, in *cardiomyopathy*, *sepsis*, transplant rejection and a variety of disorders associated with acute and chronic inflammation. Under normal physiological conditions iNOS expression has important anti microbial and anti tumor activities since it is capable of generating high cytotoxic concentration of nitric oxide. In chronic inflammation, however, the production of high cytotoxic nitric oxide and superoxide production by the enzyme may become detrimental. LPS injection was shown to increase leukocyte rolling and adhesion to post capillary venules of iNOS knockout (iNOS ko) mice suggesting that iNOS induction can act as a negative regulator of leukocyte trafficking in the microcirculation[169].

Transient gene transfer mediated expression of iNOS decreases smooth muscle cell proliferation and prevents neointima formation following *balloon angioplasty* in rats and pigs[170]. The same authors reported that iNOS gene transfer protects aortic allografts from developing *allograft arteriosclerosis*[171]. In mice, iNOS protects from developing transplant arteriosclerosis by inhibiting neointimal smooth muscle accumulation[172]. Another recent study showed that iNOS prevents *vein graft arteriosclerosis* by inhibiting vascular smooth muscle cell proliferation[173] and neointimal hyperplasia[174].

In vitro, iNOS ko mice show an improved cardiac reserve following myocardial infarction which was thought to be necessary to the reduction in oxidative stress seen in this model[175]. Genetic deletion of iNOS gene also led to partial protection against acute cardiac mechanical dysfunction mediated by pro-inflammatory cytokines[176]. The expression of iNOS is considered to be responsible for impairment of eNOS derived nitric oxide production in vessels treated with inflammatory mediators[177]. Further studies suggest that iNOS plays an important role in the impairment of endothelium dependent vascular relaxation, which may occur in part by limiting cofactor availability ($BH_4$) and subsequent eNOS uncoupling[178]. The expression of iNOS by macrophages and smooth muscle cells in atherosclerotic lesions has been taken as evidence for its detrimental role in atherosclerosis, due to formation of peroxynitrite[179]. We and others have shown that genetic deletion of iNOS resulted in a significant reduction of lesion formation in apoE ko mice, documenting the proatherogenic potential of iNOS[180, 181]. Our results were reconfirmed by Hayashi *et al.* who showed that selective pharmacological inhibition of iNOS results in retardation of atherosclerosis in rabbits[182].

The seemingly opposing effects of iNOS under various pathological conditions may be due to differences in the cellular compartment of iNOS expression in cardiac muscle vs. vessel wall; chronic atherosclerosis vs. transplant arteriosclerosis or smooth muscle cell proliferation following balloon angioplasty. iNOS expression relevant to atherosclerosis development was detected in vascular smooth muscle cells, mononuclear cells and lymphocytes. These various cellular sources are capable of generating different amounts of iNOS and subsequently target iNOS expression to various compartments of the plaque. Moreover, the cellular source determines a specific array of genes co-expressed with iNOS, which may influence their redox status[183]. For example, leukocytes can produce substantial amounts of nitric oxide and superoxide from iNOS and NADPH oxidase, resulting in the formation of peroxynitrite[184]. Peroxynitrite can oxidize LDL and cause nitrosylation of proteins which influences protein function[71]. Moreover, under conditions of substrate (L-arginine) or cofactor ($BH_4$) deficiency NOS can "uncouple" to generate superoxide instead of nitric oxide[136]. Even more intriguing, iNOS is likely capable of producing nitric oxide and superoxide simultaneously which could directly lead to peroxynitrite formation[37]. Whether iNOS is "uncoupled" in atherosclerosis and produces substantial amounts of superoxide in addition to nitric oxide is currently unknown.

## 1.7 Aim of the study

As described in the previous section, NOS isoforms differ in their physiological regulation, cellular compartment of expression, sub cellular localization and catalytic activity suggesting a complex involvement in atherosclerosis development. Therefore, it is a considerable challenge to discern the role of each NOS isoform in atherosclerosis development, as all three NOS isoforms are expressed in the vessel wall. Unfortunately, the use of pharmacologic NOS inhibitors is limited by their inability to selectively and fully inhibit each NOS isoform. In contrast, gene knockout models allow the dissection of the distinct roles of each NOS isoform in vascular disease. In the past, we crossed eNOS ko, nNOS ko and iNOS ko mice with atherosclerotic apoE ko mice, generating apoE/eNOS dko, apoE/nNOS dko and apoE/iNOS dko mice respectively, to test the contribution of each NOS isoform in diet induced atherosclerosis.

Our previous experiments revealed that eNOS[149] and nNOS[165] are anti atherosclerotic as genetic deletion of these enzymes resulted in accelerated atherosclerosis in apoE ko mice. In contrast, genetic deletion of iNOS resulted in a significant reduction of lesion formation suggesting that iNOS is proatherogenic[180]. The contribution of the NOS isoform with regards to local superoxide and nitric oxide in atherosclerosis is currently unknown. However, detailed information about the radical production by each NOS isoform is of key importance to understand the pathomechanism involved in NOS mediated modulation of atherosclerosis development.

The purpose of the study presented here was:

1. Establishment and validation of Electron Spin Resonance (ESR) measurements of nitric oxide from vessel rings using a bench top Bruker, e-scan spectroscope.
2. Establishment and validation of ESR measurements of superoxide from the vessel rings.
3. Quantitation of the relative contribution of each NOS isoform to the total nitric oxide levels observed in atherosclerotic vessel and bioavailable nitrosyl hemoglobin (No-Hb) in the circulation.
4. Determination of superoxide production by each NOS isoform in the atherosclerotic vessel wall.

# Chapter 2

# 2.0 Materials and Methods

## 2.1 Materials

### 2.1.1 Mice

Mice were backcrossed for 10 generations to the C57Bl6 genetic background. eNOS ko[185] and nNOS ko[186], provided by Paul Huang, and apoE ko (Jackson Laboratories, Bar Harbor, ME, USA) were crossed to generate double heterozygous mice. Similarly, iNOS ko mice obtained from (Jackson Laboratories, Bar Harbor, ME, USA) were crossed with apoE ko mice to generate double heterozygous mice. Offsprings were crossed and the progenies were genotyped for eNOS and nNOS by southern blotting and for apoE and iNOS by polymerase chain reaction. apoE ko, apoE/eNOS dko, apoE/iNOS dko, apoE/nNOS dko animals were weaned at 21 days and fed a western-type diet (42% of total calories from fat; 0.15% cholesterol; Harlan Teklad, USA) for 18-20 weeks. C57Bl6 mice were obtained from Charles Rivers Laboratories (Germany). The animals were maintained at 12 hours light-dark cycle.

### 2.1.2 Chemicals and reagents

| **Reagents** | **Source** |
|---|---|
| **For Krebs Hepes Buffer (KHB)** | |
| Calcium chloride dihydrate | Sigma |
| Sodium chloride | Sigma |

| | |
|---|---|
| Magnesium sulphate, heptahydrate | Sigma |
| Potassium chloride | Sigma |
| Sodium bicarbonate | Sigma |
| Potassium dihydrogen phosphate | Sigma |
| D(+)Glucose | Sigma |
| Hepes | Sigma |

| **Reagents** | **Source** |
|---|---|
| **For Electron Spin Resonance (ESR)** | |
| Iron (II) sulphate heptahydrate | Sigma |
| Diethyldithiocarbamic acid. Sodium salt . trihydrate (DETC) | Alexis |
| 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) | Noxygen |
| 3-carboxyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (CP) | Noxygen |
| Deferoxamine mesylate salt | Sigma |
| **For High Performance Liquid Chromatography (HPLC)** | |
| HPLC water | AppliChem |
| Acetonitrile | Sigma |
| Trifluoroacetic acid | Sigma |
| Dihydroethidium | Molecular Probes |
| Methanol | J.T.Baker |

| **Reagents** | **Source** |
|---|---|
| **For Immunohistochemistry** | |
| Acetone | J.T.Baker |
| Hydrogen peroxide | Sigma |
| Blocking solution | Dako |
| ABC reagent | Vector Laboratorties |
| DAB reagent | Vector Laboratorties |
| For Mayer's Haemalaun stain | |
| Haematoxylin | Roth |
| Sodium iodate | Merck |
| Aluminium calcium sulphate | Merck |
| Chloral hydrate | Merck |
| Citric acid | Merck |
| Antibodies | |
| Anti nitrotyrosine antibody | Upstate |
| Anti rabbit IgG | Vector Laboratories |

**Buffers and Solutions**

**Krebs Hepes Buffer (KHB)**

KHB with the following composition, prepared in Milli Q water, was used for the experiments

| | |
|---|---|
| Sodium chloride | 99 mM |
| Potassium chloride | 4.69 mM |

| | |
|---|---|
| Calcium chloride | 2.5 mM |
| Magnesium sulphate | 1.20 mM |
| Sodium bicarbonate | 25 mM |
| Potassium dihydrogen phosphate | 1.03 mM |
| D (+) Glucose | 5.6 mM |
| Sodium Hepes | 20 mM |

The above reagents were dissolved in 500 ml of MilliQ water. The pH was adjusted to 7.4. The pH was checked every day before use and the buffer was filtered using a 0.22 µm filter (Schleicher&Schuell).

**Phosphate Buffered Saline (PBS)**

9.55 g of PBS were dissolved in 1 litre of distilled water to obtain a working solution of PBS.

**Mayer's Haemalaun stain**

| | |
|---|---|
| Haematoxylin | 6 g |
| Sodium iodate | 1 g |
| Aluminium calcium sulphate | 250 g |
| Chloral hydrate | 250 g |
| Citric acid | 5 g |

All these reagents were dissolved in 5 liters of distilled water.

**Others**

| **Reagents** | **Source** |
|---|---|
| Pegulated Superoxide Dismutase (PEG-SOD) | Sigma |
| Apocynin | Sigma |
| Phosphate buffered saline | Biochrom AG |
| Sodium Hydroxide | Merck |
| BCA protein assay kit | Pierce |
| Nitric oxide synthase inhibitors | |
| L-arginine methyl ester hydrochloride (L-NAME) | Sigma |
| L-NIO | Alexis |
| N-(3-aminomethyl) benzyl-acetamidine (1400W) | Alexis |
| N-[(4S)-4-amino-5-[(2-aminoethyl) amino] pentyl]-N'-nitroguanidine tris (trifluoroacetate) salt (N-AANG) | Sigma |

**Instruments and Accessories**

| | |
|---|---|
| Stereosome microscope | Leica |
| e-scan ESR spectroscope | Bruker BioSpin GmbH |
| Akta HPLC | Amersham Biosciences |
| Fluorescence Detector | Jasko |
| Image Pro-Plus software | Media Cybernetics |
| Elisa reader | Softmax (Molecular Devices) |

| | |
|---|---|
| Spectrophotometer | Pharmacia Biotech |
| Cold plate | Noxygen |
| 12 well and 96 well plates | Falcon |
| Incubator | WTC Binder |
| pH meter | InoLab |
| Centrifuge | Eppendorf |
| Water Bath | Thermomix |
| Homogenisers | Roth |
| 0.22 µm Filters | Schleicher&Schuell |
| 0.22 µm 13mm-PTFE Filters | Millipore |
| Tissue Tek | Sakura Finetek |

### 2.1.3 Preparation of reagents

KHB was filtered using a 0.22 µm filter and the pH was checked daily. KHB solution containing 25 µM Deferoxamine and 5 µM DETC was used to prepare the spin probe, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH). The solutions were prepared fresh just before use. Filtered 0.9% NaCl was used to prepare 1.6 mM $FeSO_4$ and 3.2 mM DETC solutions for nitric oxide measurements. PBS was obtained from Biochrom AG, Germany.

## 2.2 Methods

### 2.2.1 Detection of free radicals in the vasculature

The measurement of vascular free radical production is difficult for several reasons. Since radicals are very short lived, they usually do not occur at high concentrations in the biological environment. Low intracellular steady-state concentrations of superoxide result from the balance between endogenous partial reduction of oxygen to superoxide and the scavenging of superoxide by highly efficient cytoplasmic and mitochondrial SOD, resulting in intracellular superoxide concentrations which rarely exceed 1 nmol/L. Extra cellular release of small proportions of intracellularly formed superoxide may occur via anion channels. In addition, superoxide levels formed from plasma membrane bound oxidases are maintained at low local concentrations due to extra cellular fluid components, including low molecular weight oxidant scavengers and the heparin binding extracellular (EC)-SOD. Similarly, the intracellular concentrations of nitric oxide depend on the balance between the rate of formation from L-arginine and the reaction of nitric oxide with superoxide, resulting in peroxynitrite formation. Thus, the relatively short half-life (seconds) of these radicals and the efficient systems which evolved to scavenge radicals require that any detection technique must be sensitive enough to effectively compete with these intracellular and extra cellular antioxidant components for reaction with the substance in question.

Some of the currently available methods include chemiluminescence techniques, fluorescent based assays, enzymatic assays and electron spin resonance. Reduction of ferricytochrome c has been used to measure rates of formation of superoxide by numerous enzymes, tissue extracts, and whole cells.

The generation of other reactive oxygen intermediates, such as hydrogen peroxide or hydroxyl radical can also cause oxidation of ferricytochrome C and consequently result in the underestimation of superoxide production[187]. Furthermore, because of its inability to penetrate cells it can be used only to measure extra cellular superoxide. Chemiluminescent methods of superoxide and nitric oxide detection in vascular tissues have been widely used because of the ability of the method to measure intracellular radical production, the alleged specificity of the reaction, the minimal cellular toxicity and the purported increased sensitivity compared with chemical measurements. However, there is uncertainity concerning the precise mechanism of enhanced luminescence-dependent radical formation, particularly in a cell or tissue based experimental system. Therefore, chemiluminescent techniques do not reflect actual cellular radical production.

#### 2.2.1.1 Electron spin resonance (ESR)

The most commonly used methods for evaluation of superoxide and nitric production in biological systems are based on reduction of cytochrome C and chemiluminescence, respectively. But these techniques have limitations for the quantitation of superoxide and nitric oxide due to the fact that cytochrome c and luminescent probes can readily react with other products from activated cells[188]. The only analytical approach that permits a highly specific and sensitive direct detection of free radicals is ESR, also termed electron paramagnetic resonance spectroscopy.

#### 2.2.1.2 Principle of ESR

ESR spectroscopy reports on the magnetic properties of unpaired electrons and their molecular environment. Electrons possess a property called spin. The unpaired electrons exist in two orientations, either parallel or antiparallel with respect to an applied magnetic field. Electrons in the antiparallel state possess higher energy than electrons in the parallel state. Resonance is the term used to describe the total energy between the two spin states. Paired electrons possess no net spin and hence produce no ESR signal, while free radicals which contain one or more unpaired electrons, produce an ESR signal. In ESR spectroscopy a fixed frequency of microwave irradiation is used to excite the electrons at the lower energy level to the higher energy level. An external magnetic field of a specific strength is applied for the transition to occur such that the difference in energy levels is matched by the microwave frequency. A unique spectrum is obtained when a spin-trapped free radical is exposed to an applied magnetic field (Figure 7). The unpaired electrons which produce the ESR spectrum are very sensitive to their surrounding.

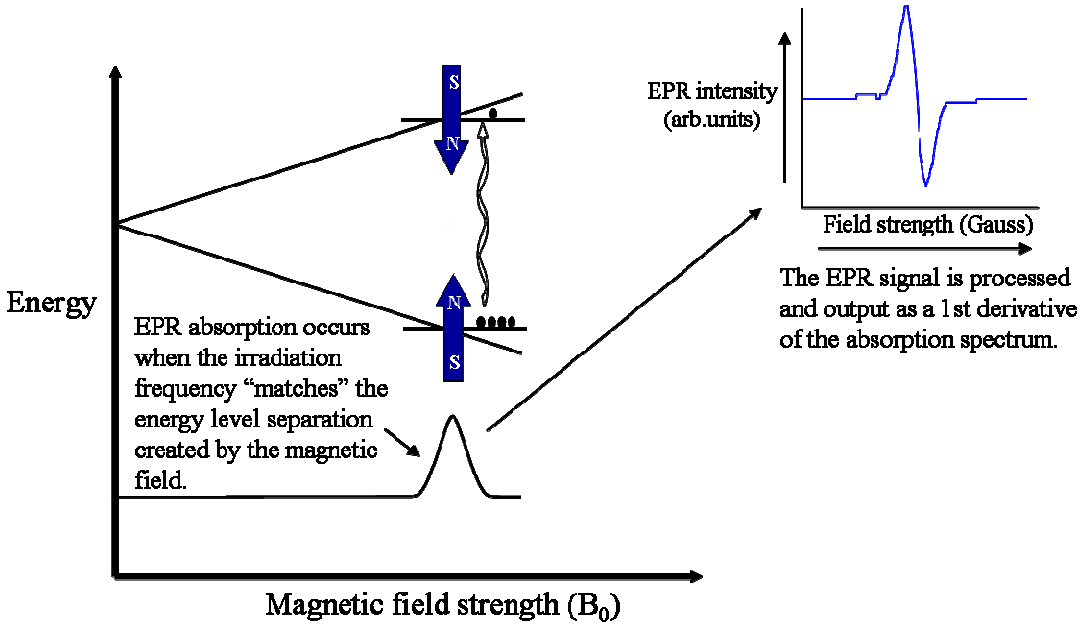


**Figure 7:** Principle of ESR spectroscopy. (Picture from Bruker Biospin GmbH, Karlsruhe, Germany).

Free radicals like nitric oxide, superoxide and peroxynitrite are too low in concentration and too short lived to be directly detectable by ESR in biological systems. This problem can be overcome by the addition of exogenous spin traps that react with free radicals to form secondary ESR detectable radicals with a higher stability. These spin traps, frequently nitroxide and nitrone derivatives, can also be used to label biomolecules and probe basal and oxidation induced events in protein and lipid microenvironments. With a sensitivity limit of ~$10^{-9}$ mol/L, ESR spectroscopy is also capable of detecting the more stable free radical species produced in the vascular compartment during oxidative stress and inflammation, including ascorbyl radical, tocopheroxyl radical, and heme-nitrosyl complexes directly[189].

Reaction of nitric oxide with endogenous heme and non-heme iron proteins leads to the formation of iron-nitrosyl complexes with characteristic

spectra[190]. Addition of exogenous iron-dithiocarbamates and nitronyl nitroxides has also been used to detect nitric oxide formation[191]. Endogenous ascorbate is oxidized to ascorbate radical which can be directly detected at room temperature[192]. An emerging approach to the use of ESR in vascular biology has been the use of cyclic hydroxylamines. These molecules are not spin traps, in that they do not "trap" radicals, but they are oxidized to form very stable radicals with half-lives of several hours, permitting ESR detection. The spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine.HCl (CMH) is oxidized by superoxide and peroxynitrite to form $CM^{\cdot}$ radical. This molecule has successfully been used with intact cells and *in vivo* to detect reactive oxygen species released into the circulation. Thus, the cyclic hydroxylamines and similar compounds are very useful for the detection of superoxide and other reactive oxygen species in vascular tissues. Overall, ESR has proven to be useful as a free radical detection strategy. In this respect superoxide detection by ESR was proven to be 20 times more sensitive than the cytochrome C reduction assay.

### 2.2.2 Measurement of vascular nitric oxide production by electron paramagnetic spin trapping

Mice were injected with 100 U of heparin, anaesthetized with Avertin (10 mg/kg, i.p.) after 5 minutes and dissected on a styrofoam board. The aorta was removed rapidly and was cleaned from the perivascular fatty tissues while being maintained at 4°C in chilled KHB on a cold plate. The aorta was cut into 2mm rings and the rings were placed in each well of a 12 well plate containing 1.5 ml KHB. For the preparation of the Fe-$(DETC)_2$ spin trap, 4.5 mg of $FeSO_4$ (1.6

mM) and 7.2 mg of DETC (3.2 mM) were prepared separately in 10 ml of 0.9% filtered NaCl. The solutions were deoxygenated by bubbling $N_2$ gas for 30 minutes. Just before use, equal amounts (500 µl) of $FeSO_4$ and DETC solutions were mixed quickly in an eppendorf tube pre-filled with $N_2$ gas, to obtain a 0.8mM of Fe-$(DETC)_2$ colloidal, brown solution. The spin trap (500 µl) was then added to the vessels and incubated for 1 hour at 37°C. Subsequently, the vessel rings were frozen at the end of the column of KHB buffer in a 1 ml syringe. Nitric oxide production was quantitated using an e-scan bench top spectroscope with the following instrumental settings: Centre field: 3308 G. Sweep width: 80 G. Microwave frequency: 9.495 GHz. Microwave power: 50 mW. Modulation Amplitude: 4.6 G. Modulation frequency: 86 kHz. Time constant: 81.92 ms. Conversion Time: 20.48 ms. Number of scans: 100. The method was adapted from a previously published protocol[190]. The intensity of the ESR signal was normalized to the protein content of the sample.

### 2.2.3 Measurement of vascular oxygen radical production by ESR

ESR measurements of superoxide formation were obtained as the ESR detectable nitroxide radical $CM^{\bullet}$ formed through oxidation of the spin probe CMH by superoxide and peroxynitrite. The concentrations of reactive oxygen species in each sample were calculated from the ESR amplitude using a calibration curve of a standard solution of 3-carboxy-proxyl ($CP^{\bullet}$) radical. In order to obtain the maximum signal amplitude, the position of the finger dewar was optimized in the cavity using a standard $CP^{\bullet}$-radical solution. The vessel

rings were prepared as mentioned above. The aortas were cut into 2 mm rings and five rings were placed in 100 µl KHB containing 25 µM deferoxamine and 5 µM DETC in each well of a 98 well plate. Deferoxamine and DETC stock solutions were prepared by dissolving the compounds in KHB. The reagents were freshly prepared everyday. Samples were incubated for 1hour at 37°C with the spin probe, CMH (250 µM). Superoxide production was assessed by pre incubating aortic rings with PEG-SOD (100 U/ml) parallel to CMH for 1 hour. The experimental procedure was carried out according to a previously published protocol[193]. The instrumental settings for superoxide measurements are as follows: Centre field: 3388 G. Sweep width: 132 G. Microwave frequency: 9.497 GHz. Microwave power: 1.25 mW. Modulation Amplitude: 1.63 G. Modulation frequency: 86 kHz. Time constant: 40.96 ms. Conversion Time: 10.24 ms. Number of scans: 50. The intensity of the ESR signal was normalized to the protein content of the sample.

We chose to use the cyclic hydroxylamine CMH because of its higher stability when compared to the widely used nitrone spin traps DMPO and DEPMO[194]. Furthermore, CMH is more sensitive and reliable than the nitrone spin traps. In particular CMH has a higher scavenging efficacy for superoxide, compared to CP-H, which is another cyclic hydroxylamine.The higher stability of $CM^{\cdot}$ even in the presence of reducing agents like ascorbate or glutathione[195] and the ability of CMH to penetrate cells are additionally advantageous.

#### 2.2.3.1 Sample preparation for ESR measurements

After incubation the vessel rings were frozen at the end of a column of KHB solution. In order to do this, the needle-end of a one ml syringe was cut, 300 µl of KHB were taken up and frozen in liquid nitrogen. Subsequently the plunger was retracted an additional 5 mm. A plastic forceps (metal forceps was avoided to prevent oxidation caused by metals) were used to collect the rings from the 12 well-plate for nitric oxide measurements, without the buffer. Using a 200 µl Eppendorf tip aortic rings were collected from the 98 well-plate along with the buffer containing the spin probe, for superoxide measurements. The rings were then layered over the frozen column of KHB in a syringe and were frozen in liquid nitrogen again. The syringe plunger was used to push the frozen column directly into a liquid nitrogen containing finger dewar vacuum flask. A constant supply of dry air was provided to the cavity of the spectrometer to avoid condensation. After measurement, the samples were stored in -80°C and used for the estimation of the total protein concentration of the vessel rings.

### 2.2.4 Measurement of nitric oxide bioavailability in the bloodstream

Nitrosyl hemoglobin, a reaction product of deoxygenated hemoglobin (Hb) with nitric oxide can be used as a marker for nitric oxide bioavailability[196]. Nitrosyl hemoglobin can be detected as a characteristic triplet peak by ESR spectroscopy. Blood samples were prepared according to a published method[197]. Briefly, venous blood was drawn from the right ventricle. Following centrifugation at 2000 g the red cell cast was frozen in syringes and transferred into a liquid nitrogen containing finger dewar. Spectra were acquired using an

X-band EMX spectroscope (Bruker Biospin GmbH, Germany) with the following instrument settings: Centre field: 3340 G. Sweep width: 230 G. Microwave frequency: 9.452 GHz. Microwave power: 47.6 mW. Modulation Amplitude: 4.76 G. Modulation frequency: 86 kHz. Time constant: 40.96 ms. Conversion Time: 10.24 ms. Number of scans: 24. The amount of detected nitric oxide was determined from a calibration curve generated by incubating blood samples with known concentrations of nitrite and sodium dithionite ($Na_2S_2O_4$).

### 2.2.5 Measurement of intracellular superoxide production by HPLC detection of oxyethidium

HPLC measurements served as a second, independent method for superoxide detection. Superoxide was measured in aortic rings by detection of oxyethidium, the fluorescent reaction product of superoxide and dihydroethidium[198]. Vessel rings were prepared as mentioned before and incubated in a 12 well plate, containing KHB and 50 µM dihydroethidium, at 37°C for 15 minutes. Inhibitors (L-NIO: 100 µM, 1400W: 10 µM, N-AANG: 10 µM, L-NAME: 100 µM, apocynin: 100 µM) were added and incubated at 37°C for 30 minutes, prior to the addition of DHE. Subsequently, extracellular dihydroethidium was washed off and the rings were incubated for 1 h for intracellular accumulation of oxyethidium. The plate was covered with aluminium foil to prevent the exposure of dihydroethidium to light. Aortic rings were then homogenized in 350 µl of ice cold methanol. A 50 µl aliquot of the homogenate was stored for protein measurements. The homogenate was filtered using a syringe top filter (0.2 µm pore size) and separated by reverse phase HPLC using a C-18 column (Nucleosil 250, 4.5 mm; Sigma-Aldrich)

along with an ÄKTA HPLC system (Amersham Biosciences, GE Healthcare). The mobile phase was composed of a gradient containing 2.6 column volumes of 37- 47% acetonitrile containing 0.1% trifluoroacetic acid. Oxyethidium was quantified with a fluorescence detector (Jasco, UK) at 580 nm (emission) and 480 nm (excitation). The detected oxyethidium was normalised to the sample's protein content [199].

### 2.2.6 Tissue preparation and immunohistochemistry

Aortic arches were cleaned of adherent tissue, embedded in Tissue-Tek® (Sakura Finetek) and snap-frozen in liquid nitrogen. Serial sections (5 µm) were cut at -20°C, mounted on silane treated slides (SuperFrost®Plus, Menzel GmbH & Co KG, Braunschweig, Germany), thoroughly air-dried and fixed in acetone for 10 minutes at room temperature prior to staining. The slides were allowed to dry at room temperature for 1 hour followed by washing thrice with PBS. The sections were incubated with 0.3% hydrogen peroxide in methanol for 30 minutes and washed with PBS. A commercially available blocking reagent (Dako REAL, antibody dilutent) was used for 30 minutes and subsequently incubated along with the primary anti nitrotyrosine antibody (1:80 dilution, Upstate Biotechnology, Inc.) for 1 hour. After washing off unbound antibody, the sections were incubated for 30 minutes with the secondary biotinylated anti rabbit IgG antibody (1:200 dilution, Vector Laboratories, Inc.) at room temperature. Following three PBS washing steps sections were stained for 30 minutes with ABC reagent (Vectastain®ABC kit, Vector Laboratories, Inc.). Sections were then washed with PBS and incubated with DAB reagent for 5-20

minutes. At last sections were washed with distilled water and stained with Mayer's Haemalaun stain for 1 minute.

### 2.2.7 Histomorphometry

Photomicrographs of the vessel sections were obtained with an Olympus camera mounted on a light microscope (Zeiss, Axiophot, Germany). Pictures were digitalized with the Cell$^{B}$ Imaging software and transferred to a PC for planimetry using Image Pro Plus (Media Cybernetics). All images were analyzed at 400 fold magnification. Results were expressed as the positive staining area per total area of the plaque.

### 2.2.8 Protein Estimation

For ESR measurements vessel rings were incubated in 1N NaOH at 50℃ for 2- 3 hours. The protein concentration of the samples was determined at 562 nM using a BCA Protein Assay Kit (Pierce,USA). In order to quantify total sample protein content, a protein standard curve was generated using bovine serum albumin. Samples from vessel homogenates of HPLC samples were estimated for their protein content using the BCA Protein Assay Kit in an elisa reader (Softmax, Molecular devices).

### 2.2.9 Statistical Analyses

The data is represented as mean±SE. Statistical significance was determined by Student's *t* test for unpaired data. Two groups of data were considered to be significantly different at a p value of $< 0.05$.

# Chapter 3

# 3.0 Results

## 3.1 eNOS is a significant source of vascular wall nitric oxide production and circulating nitric oxide

Quantitation of baseline nitric oxide production in the vasculature is a challenging task, due to the radical's short half-life and very low concentrations of bioavailable nitric oxide. We used ESR, a method of highest sensitivity and specificity, to measure vascular nitric oxide production and circulating nitric oxide levels in blood samples. Spin trapping of nitric oxide with colloidal Fe-$(DETC)_2$ was used to measure baseline nitric oxide production of the vessel wall. This is considered a very specific method for detection of nitric oxide, since the Fe-$(DETC)_2$ bound to nitric oxide gives rise to a specific triplet hyperfine nitric oxide-Fe-$(DETC)_2$ peak, whereas Fe-$(DETC)_2$ alone does not give any signal (Figure 8a). Our experiments show that the baseline nitric oxide production of the vessel wall is significantly lower in apoE/eNOS dko (920±287 AU/µg protein, n=12) than in apoE ko mice (2554±195 AU/µg protein, n=14, $p<0.0001$, Figure 8a and b). In addition, the paramagnetic properties of nitrosyl hemoglobin were utilised along with the ESR method to quantify the *in vivo* concentrations of nitric oxide in whole blood[196]. The baseline nitrosyl hemoglobin levels in blood samples were significantly lower in apoE/eNOS dko (1868±100 nM, n=11), compared to apoE ko controls (3463±491 nM, n=13, $p=0.006$, Figure 8c).

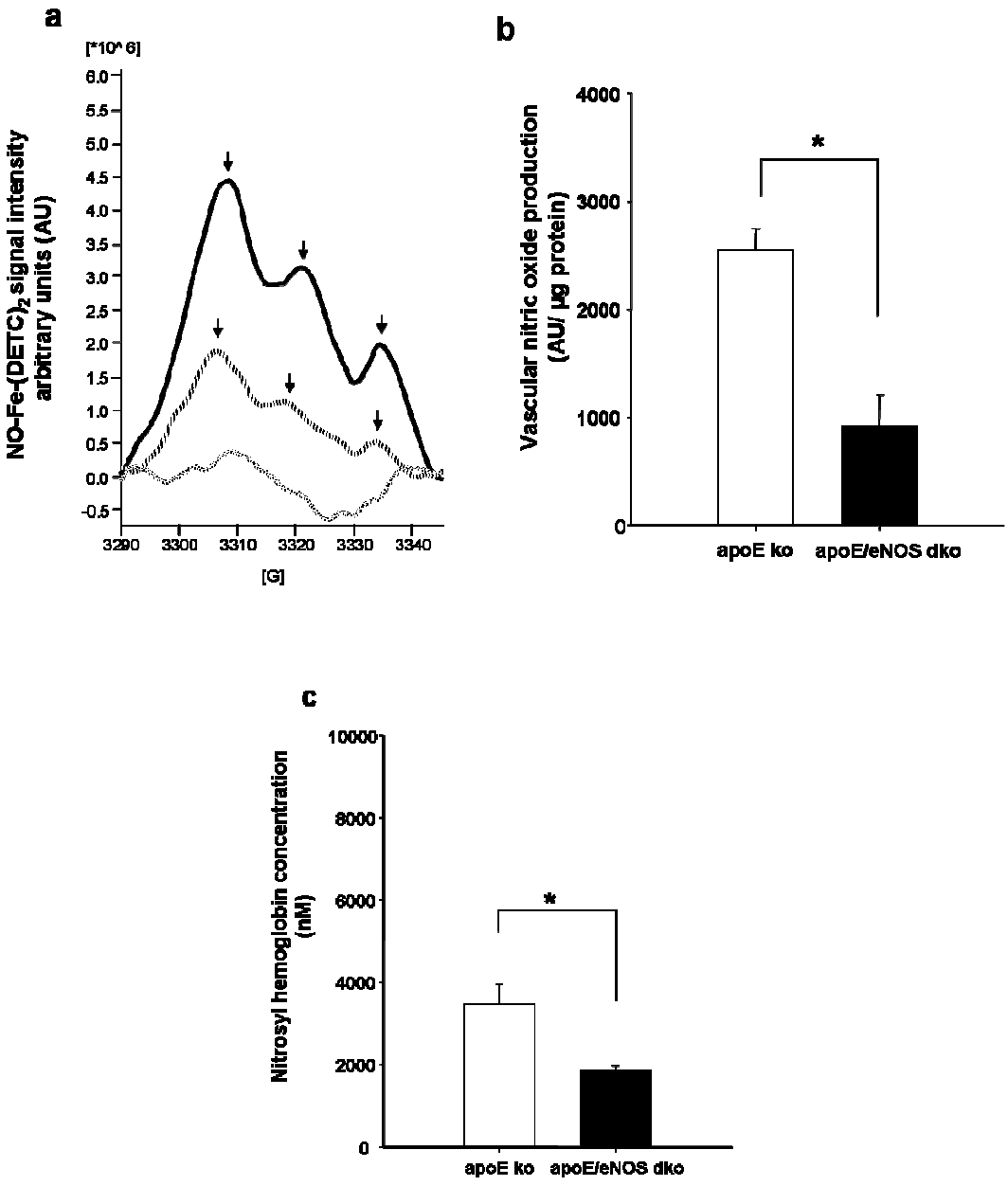


**Figure 8. Contribution of vascular and circulating nitric oxide by eNOS:** a) Representative ESR spectrum of NO-Fe-$(DETC)_2$ in aortic segments of apoE ko and apoE/eNOS dko mice. Bold lines indicate apoE ko, stripped lines indicate apoE/eNOS dko, patterned lines indicate buffer with spin trap. The arrows show the typical 3 peak signal observed for NO-Fe-$(DETC)_2$, which is absent in the buffer only sample. The vertical axis represents signal intensity in arbitrary units (AU). b) Aortic nitric oxide production of apoE /eNOS dko mice was reduced by 60%, compared to apoE ko (*$p<0.0001$). c) The nitrosyl hemoglobin concentration of blood samples from apoE/eNOS dko was reduced by 50%, compared to apoE ko controls (*$p=0.006$).

## 3.2 eNOS deletion decreases vascular production of superoxide in apoE ko vessels

To determine vascular superoxide production the formation of oxyethidium from dihydroethidium was measured by HPLC. The measurements revealed higher levels of superoxide in vessel rings of apoE ko and apoE/eNOS dko mice, compared to C57Bl6 (9.4±0.5 AU/µg protein, n=14, p=0.000001 and 6.9±0.9 AU/µg protein, n=13, p=0.04 respectively, vs. 4.4±0.32 AU/µg protein, n=9, Figure 9a). Total superoxide formation in apoE/eNOS dko mice was decreased compared to apoE ko (6.9±0.9 AU/µg protein vs. 9.4±0.5 AU/µg protein, p=0.02, Figure 9a). In addition to the experiments using chronic deletion of eNOS, acute pharmacological inhibition of NOS by L-NAME resulted in a significant reduction of superoxide formation in apoE ko vessels (9.4±0.5 AU/µg protein, n=14 vs. 4.8±0.6 AU/µg protein, n=14, p=0.00001, Figure 9b), suggesting that NOS is uncoupled and contributes to superoxide formation in apoE ko mice. Moreover, L-NIO, an eNOS specific inhibitor significantly decreased vascular superoxide production in apoE ko mice fed with the western diet for 24 weeks, supporting partial uncoupling of eNOS (9.3±0.6 AU/µg protein, n=22 vs. 11.6±0.7 AU/µg protein, n=22, p=0.02, Figure 9c). Superoxide levels in these older apoE ko mice were significantly higher than in apoE ko mice fed with the western diet for 18 weeks (11.6±0.7 AU/µg protein, n=22, p=0.05, Figure 9c) documenting increasing superoxide production with progressive atherosclerosis.

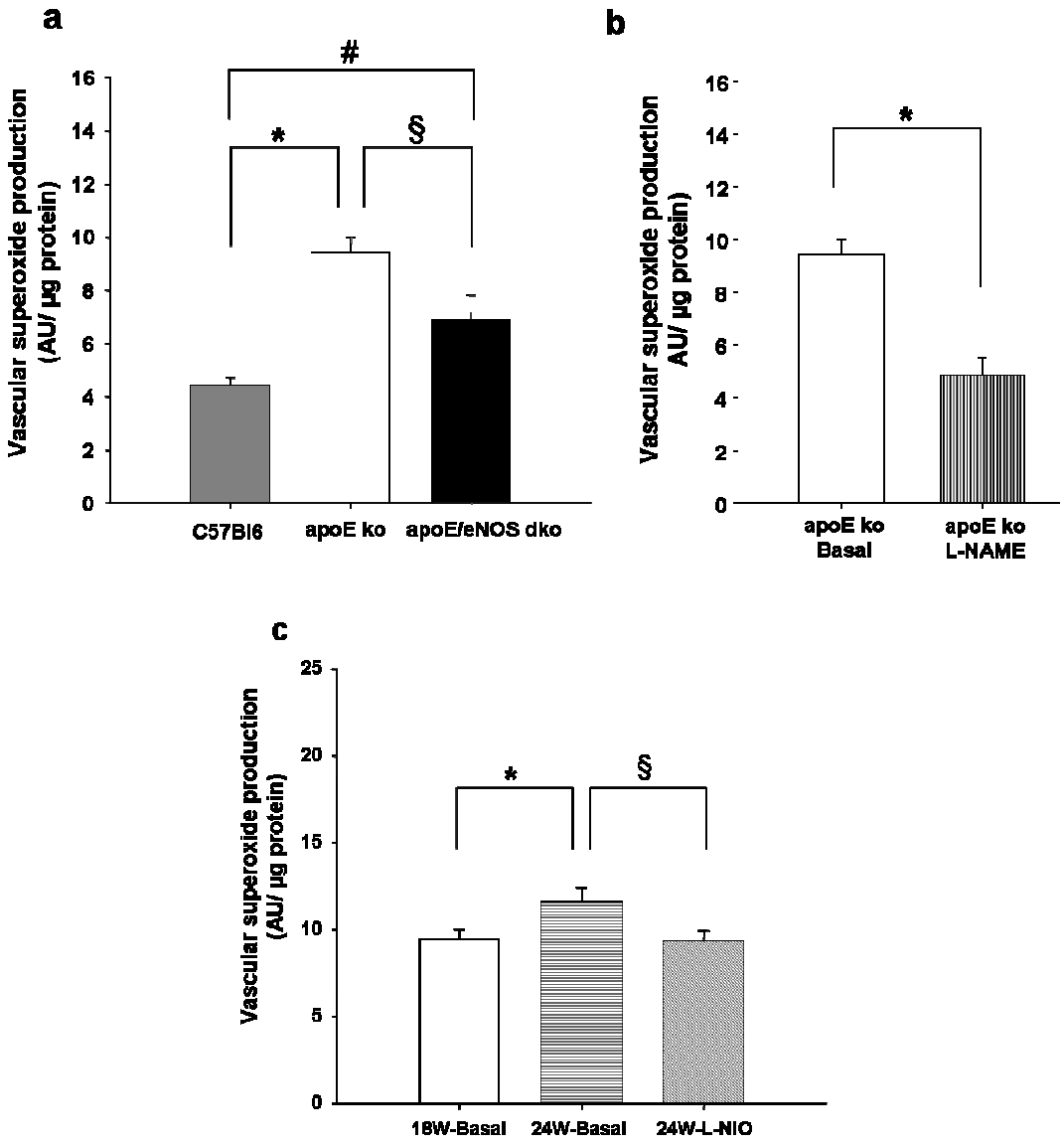


**Figure 9. Determination of eNOS derived superoxide production:** a) HPLC measurements of superoxide formation showed significantly lower levels of superoxide production in apoE/eNOS dko, compared to apoE ko mice following 18 weeks of western diet. Hyperlipidemic apoE ko and apoE/eNOS dko mice had significantly higher superoxide levels, compared to normocholesterolemic C57Bl6 animals (*p=0.000001; $^{\S}$p=0.02; $^{\#}$p=0.04). b) Unselective NOS inhibition by L-NAME resulted in inhibition of superoxide production in apoE ko mice (*p=0.00001). c) Vascular superoxide production from 24 weeks western diet fed apoE ko mice were significantly elevated, compared to the 18 weeks time point (*p=0.05). Additionally, treatment of apoE ko vessel rings with the eNOS specific inhibitor L-NIO decreased vascular superoxide formation in 24 weeks western diet fed apoE ko animals ($^{\S}$p=0.02).

## 3.3 nNOS contributes little to vascular nitric oxide production

Determination of vascular nitric oxide production using Fe-$(DETC)_2$ showed no significant difference between apoE ko and apoE/nNOS dko animals (2471±189 AU/µg protein, n=16 vs. 2293±124 AU/µg protein, n=17, p=0.43, Figure 10). However, a trend towards reduction of vascular nitric oxide production (7%) was observed in apoE/nNOS dko animals suggesting that nNOS may have a minor contribution.

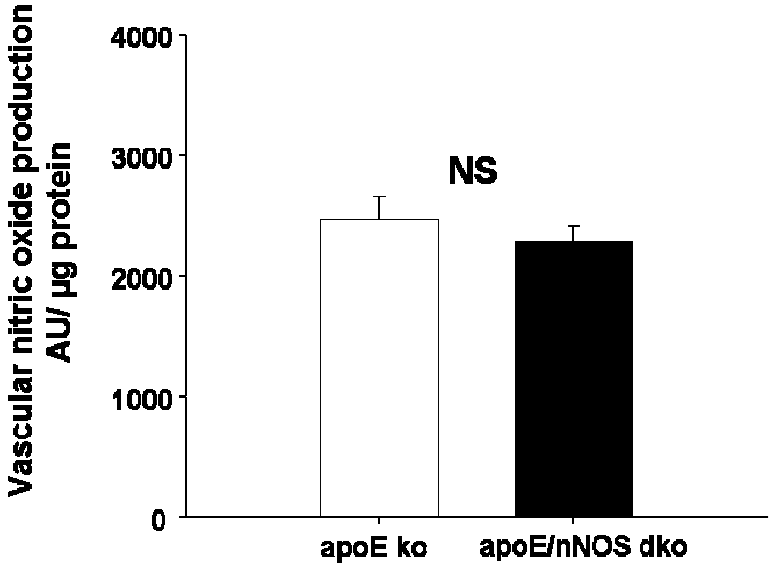


**Figure 10. Contribution of vascular nitric oxide by nNOS:** ESR measurements of vascular nitric oxide levels using Fe-$(DETC)_2$ showed no significant difference between apoE ko and apoE/nNOS dko animals (NS).

## 3.4 Contribution of nNOS to vascular superoxide production in apoE ko animals

HPLC measurements of intracellular superoxide levels, measured by DHE showed no significant difference between apoE ko (19±1.0 AU/µg protein, n=12) and apoE/nNOS dko animals (17±1.6 AU/µg protein, n=10, p=0.28, Figure 11a), suggesting that chronic nNOS deletion does not alter superoxide production in apoE ko animals. However, acute inhibition of nNOS using N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N'-nitroguanidine tris (trifluoroacetate) salt (N-AANG) showed a significant reduction of superoxide formation in apoE ko vessels compared to basal levels (19±1.0 AU/µg protein, n=12 vs. 16±1.0 AU/µg protein, n=14, $p<0.05$, Figure 11b), suggesting that vascular nNOS expression contributes to superoxide production.

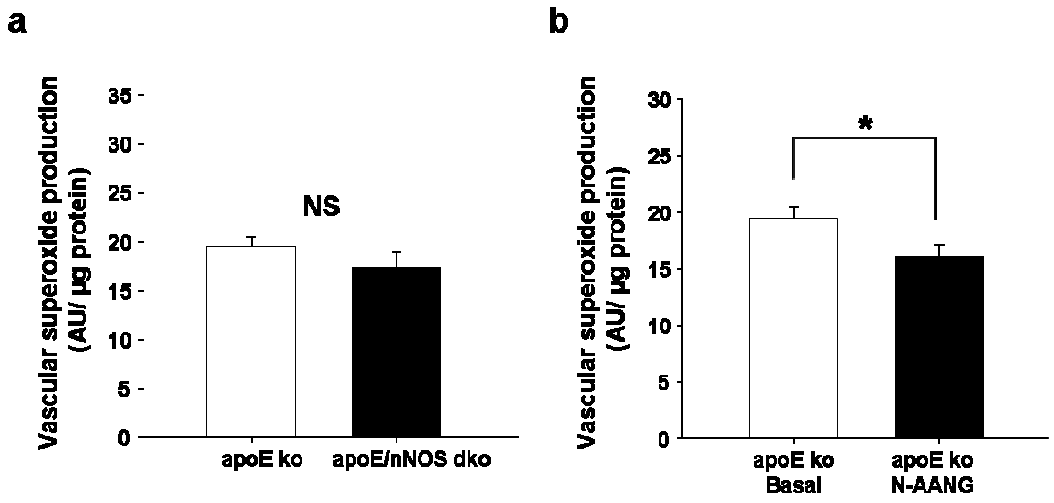


**Figure 11. Determination of nNOS mediated superoxide production:** a) HPLC measurements of superoxide did not show a significant difference between apoE ko and apoE/nNOS dko animals (NS). b) Acute inhibition of nNOS using nNOS specific inhibitor, N-AANG showed a significant reduction of superoxide production in apoE ko vessels (*$p<0.05$).

## 3.5 iNOS contributes significantly to vascular nitric oxide production

Detection of vascular wall nitric oxide production using Fe-$(DETC)_2$ spin trap showed significantly lower nitric oxide levels in apoE/iNOS dko, compared to apoE ko mice (1726±201 AU/µg protein, n=26 vs. 2554±195 AU/µg protein, n=14, p≤0.01, Figure 12). Interestingly, nitric oxide production in apoE ko mice was increased, compared to C57Bl6 animals (2554±195 AU/µg protein, n=14 vs. 1453±263 AU/µg protein, n=12, p<0.01, Figure 12).

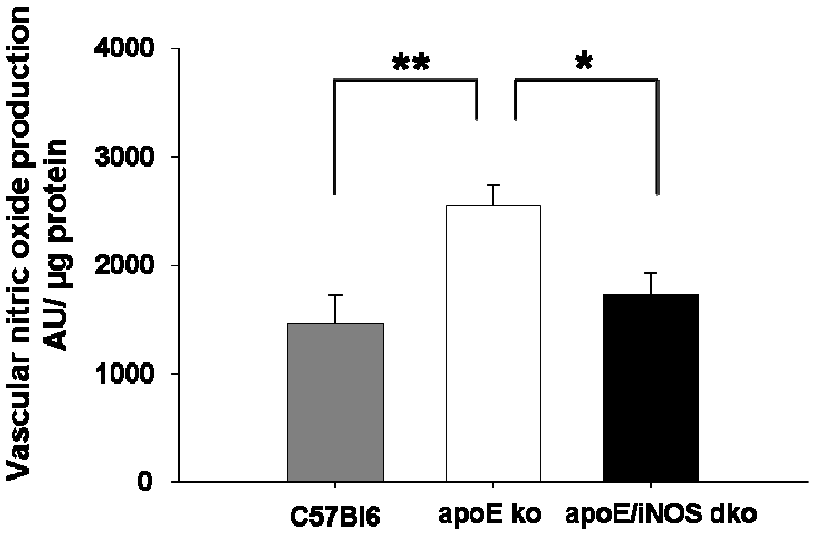


**Figure 12. Contribution of vascular nitric oxide by iNOS:** Quantitation of vascular nitric oxide levels using Fe-$(DETC)_2$ spin trap. While apoE ko vessels showed higher levels of nitric oxide formation compared to C57Bl6 animals (**p<0.01), there was no statistical difference between C57Bl6 and apoE/iNOS dko animals. Genetic deletion of iNOS in apoE ko reduced nitric oxide formation significantly (*p≤0.01).

## 3.6 iNOS plays a major role in vascular superoxide production

ESR measurements of SOD inhibitable superoxide levels showed that apoE ko aortas produce significantly higher superoxide levels than apoE/iNOS dko vessels (10.8±0.8 nM/µg protein, n=18 vs. 7.5±0.8 nM/µg protein, n=20, p≤0.01, Figure 13a). Additionally, superoxide was measured by HPLC quantification of oxyethidium, the specific reaction product of dihydroethidium and superoxide. In support of the ESR data, the HPLC assay showed that apoE ko vessels produce higher superoxide levels compared to apoE/iNOS dko mice (8.9±0.6 AU/µg protein, n=15 vs. 5.7±0.8 AU/µg protein, n=16, p≤0.01, Figure 13b). Superoxide levels of C57Bl6 animals (4.5±0.3 AU/µg protein, n=9, p<0.0001, Figure 13b) were significantly lower than the concentrations observed in apoE ko mice. The levels of superoxide in apoE/iNOS dko and C57Bl6 animals were comparable. In other words, absence of iNOS reduced superoxide production in apoE ko almost to the level of C57Bl6 animals. To determine whether acute pharmacologic inhibition of iNOS reproduces these results, we incubated apoE ko aortas with the iNOS specific inhibitor N-(3-aminomethyl) benzyl-acetamidine (1400W). Acute iNOS inhibition significantly reduced superoxide levels in apoE ko vessels, compared to basal levels (4.2±0.8 AU/µg protein, n=10 vs. 8.9±0.6 AU/µg protein, n=15, p≤0.0001, Figure 13c). Our results suggest that uncoupled iNOS itself is a significant source of superoxide formation in apoE ko vessels.

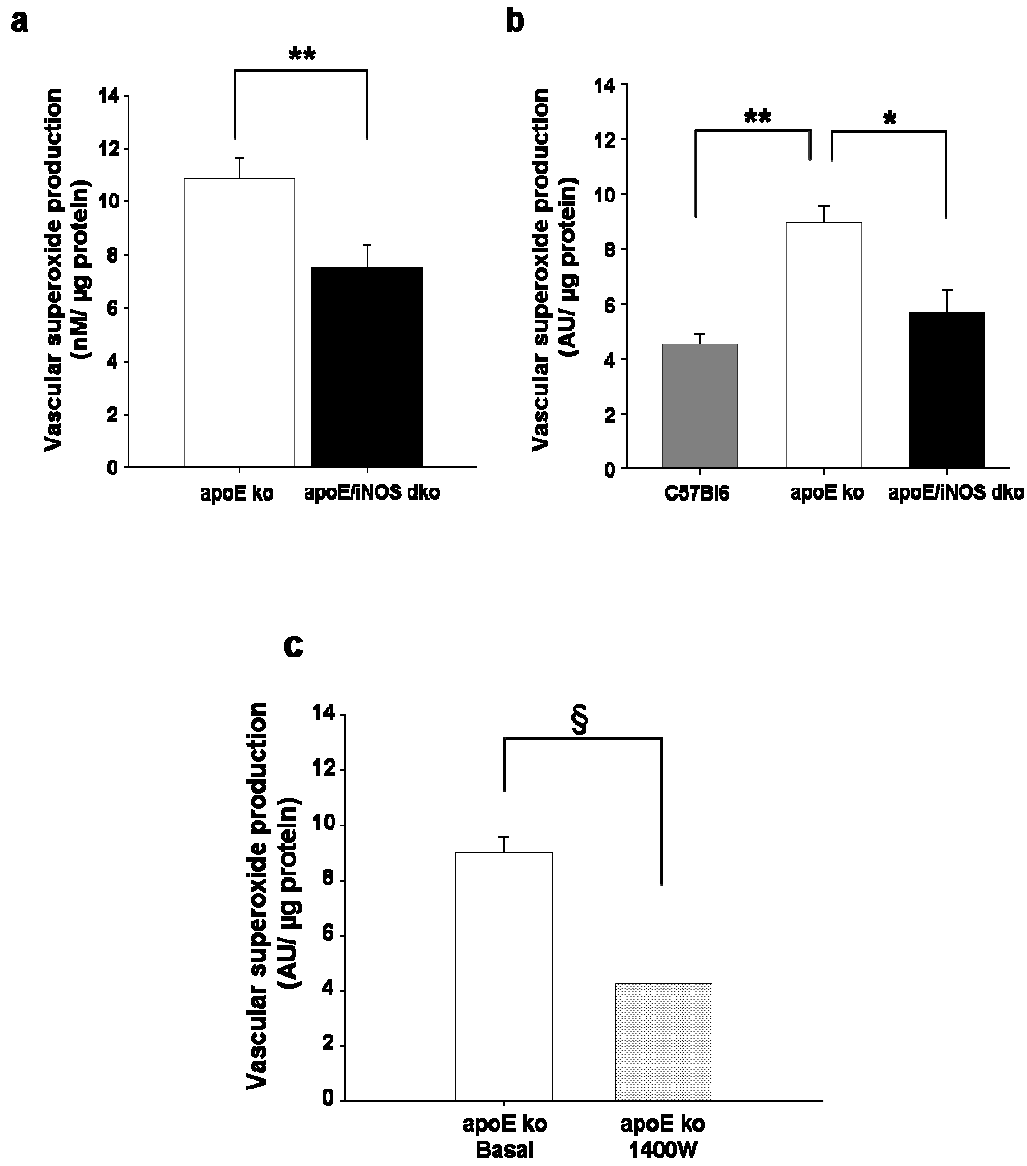


**Figure 13. Determination of iNOS derived superoxide production:** a) Superoxide levels were significantly lower in apoE/iNOS dko animals, compared to apoE ko controls (ESR measurements of SOD inhibitable $CM^{\cdot}$ signal (**p≤0.01)). b) Additionally, HPLC measurements revealed that iNOS deletion reduced vascular superoxide production in apoE ko animals. ApoE ko had significantly higher vascular superoxide levels than C57Bl6 animals (*p≤0.01, **p≤0.0001). c) Acute inhibition of iNOS in vessels of apoE ko mice, using iNOS specific inhibitor 1400W, significantly reduced superoxide production ($^{\S}$p≤0.0001).

## 3.7 Nitric oxide and superoxide generation from iNOS results in peroxynitrite formation

Peroxynitrite, a reaction product of nitric oxide and superoxide was quantified by measuring 3-nitrotyrosine positive atherosclerotic plaque areas (Figure 14a). Peroxynitrite staining was significantly reduced in apoE/iNOS dko, compared to apoE ko animals (0.4±0.1 positive staining area/total area, n=8 vs. 1.5±0.3 positive staining area/total area, n=8, $p<0.05$, Figure 14a and b).

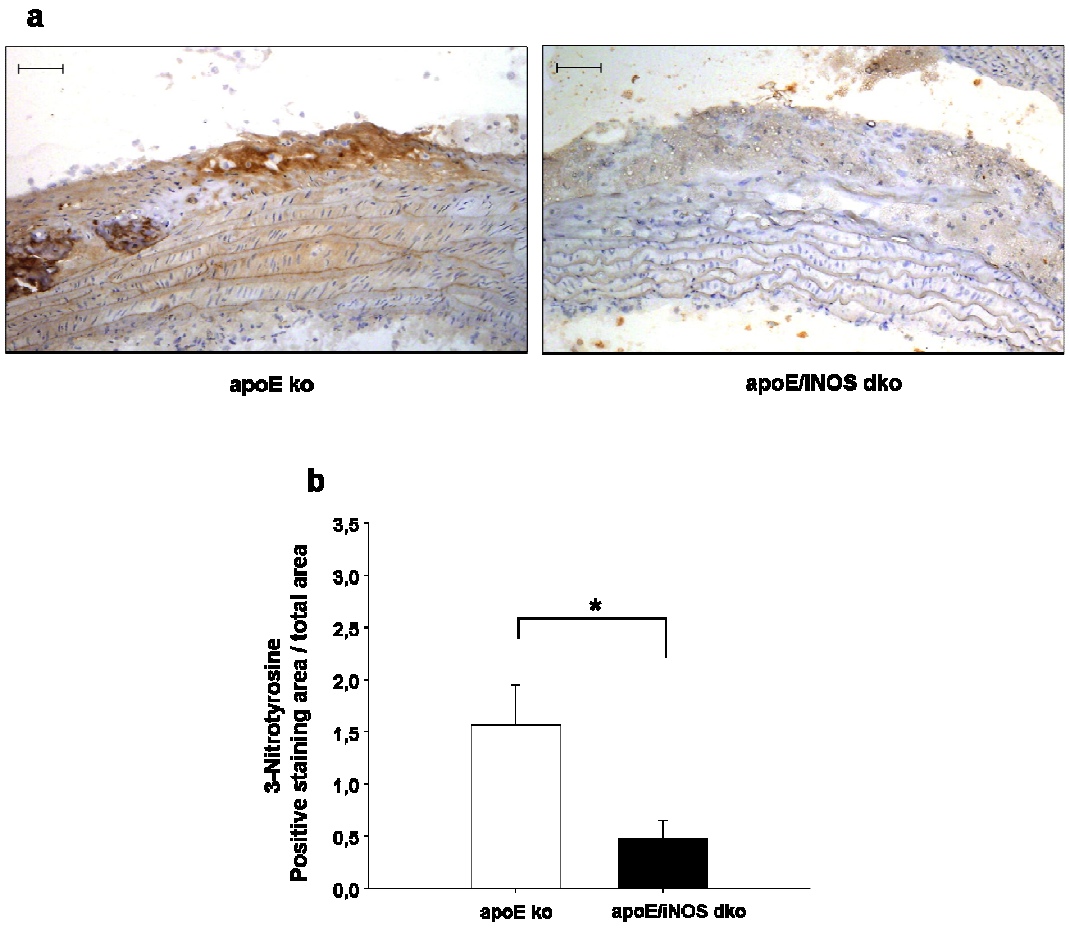


**Figure 14. Quantification of peroxynitrite mediated nitrosative stress:** a) Representative immunohistochemistry of 3-nitrotyrosine staining, a marker of peroxynitrite mediated oxidative stress in aortic plaques of apoE ko and apoE/iNOS dko animals. b) Planimetry of % positive staining plaque areas for 3-nitrotyrosine revealed decreased nitrosative stress in lesion of apoE/iNOS dko, compared to apoE ko animals (*$p<0.05$).

## 3.8 iNOS deletion influences NADPH oxidase mediated superoxide production

Since iNOS expression may regulate NADPH oxidase activity and expression we determined the contribution of NADPH oxidase to superoxide formation in apoE ko vessels. Our results show that apocynin, a NADPH oxidase inhibitor significantly reduced superoxide levels compared to basal superoxide production of apoE ko vessels (13±0.6 AU/µg protein, (n=13) vs. 19±1.0 AU/µg protein, (n=12), $p<0.001$, Figure 15). In contrast, we observed no difference in superoxide levels between basal and apocynin treated apoE/iNOS dko vessels (14±1.5 AU/µg protein, (n=11) vs. 13±1.2 AU/µg protein, (n=12), $p=0.46$, Figure 15). Therefore, our results suggest that iNOS deletion reduces the activity of the NADPH oxidase.

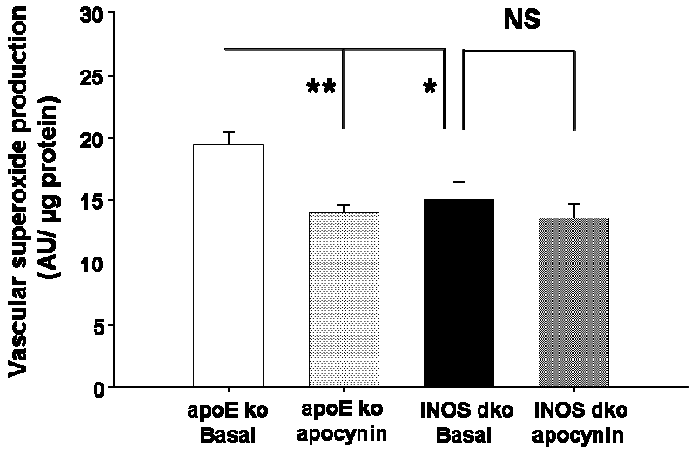


**Figure 15. Influence of iNOS in NADPH oxidase activity:** Treatment of vessel rings with the NADPH oxidase inhibitor, apocynin resulted in a significant reduction of superoxide production in apoE ko vessels (**$p<0.001$). As shown before, genetic deletion of iNOS resulted in significant reduction of superoxide (*$p<0.05$). Apocynin treatment showed a trend towards reduction of superoxide production in apoE/iNOS dko animals (NS).

# Chapter 4

# 4.0 Discussion

## 4.1 eNOS is a major contributor of nitric oxide and superoxide generation in apoE ko vessels

We have previously shown that eNOS protects from atherosclerosis, as genetic deletion of eNOS resulted in increased plaque formation in apoE ko mice. Further, we also observed increased leukocyte/endothelial cell interactions, increased macrophage infiltration and increased expression of vascular cell adhesion molecule (VCAM-1) in apoE/eNOS dko animals compared to apoE ko controls (data not shown/ unpublished data). Considering the expression of all three NOS isoforms in the atherosclerotic plaques, nitric oxide production by the vessel wall could be decreased secondary to eNOS deletion or increased since the increase in leukocyte/endothelial interactions, macrophage infiltration and plaque area observed in apoE/eNOS dko could increase cytotoxic nitric oxide formation by iNOS. Moreover, eNOS deletion could have changed the bioavailability of substrate and co-factor, thereby influencing the chemistry of iNOS and nNOS. Considering the alternative chemistry of eNOS, deletion of the enzyme could also reduce superoxide production and thereby indirectly increase nitric oxide bioavailability by nNOS and iNOS. Because of this alternative chemistry of eNOS, we measured both vascular nitric oxide and superoxide formation in aortic segments of apoE ko and apoE/eNOS dko mice using ESR and HPLC methods. Spin trapping of nitric oxide using Fe-$(DETC)_2$ is considered the utmost specific and sensitive method for detection of nitric oxide in biological systems. Unlike chemiluminescence, a frequently used method which indirectly measures nitric

oxide's metabolite nitrite, ESR directly detects nitric oxide as a specific hyperfine triplet peak[190]. Our experiments show a significant reduction of baseline nitric oxide formation in the vessel wall of apoE/eNOS dko, compared to apoE ko mice. In addition, the *in vivo* concentration of circulating nitrosyl hemoglobin, a measure for nitric oxide bioavailability in the circulation, was markedly reduced in apoE/eNOS dko mice. Our data suggest that eNOS is the major contributor of vascular and circulating nitric oxide despite all these possibilities. Since eNOS is expressed in the endothelium lining the lumen of the vessel, the nitric oxide generated by eNOS can easily diffuse into the circulation, explaining the significant contribution of eNOS to circulating levels of nitric oxide.

To assess vascular superoxide formation we used a HPLC assay which measures the formation of oxyethidium, a specific reaction product of dihydroethidium with superoxide[199]. These measurements revealed a significant reduction in superoxide production following genetic deletion of the enzyme (chronic) in apoE/eNOS dko, and acute pharmacological inhibition of eNOS in apoE ko. The reduction of superoxide generation observed in apoE/eNOS dko reflects the specific contribution of eNOS derived superoxide production in endothelial cells. Since both, significant nitric oxide and superoxide production by eNOS is detectable in apoE ko mice, we conclude that eNOS is partially uncoupled during atherosclerosis development in this model. We speculate that eNOS produces nitric oxide in the healthy regions of the vessel, whereas in the diseased regions, it is uncoupled and contributes to superoxide production.

As recently shown in eNOS transgenic apoE ko mice, reduced nitric oxide and increased superoxide generation by uncoupled eNOS can accelerate atherosclerosis[151]. However, despite decreased vascular superoxide formation

in our apoE/eNOS dko model, these animals develop increased atherosclerosis, suggesting that the atheroprotective effect of decreased superoxide production does not outweigh the proatherogenic effects of nitric oxide deficiency. Ample evidence implicates increased superoxide production in atherosclerosis[43, 70]. Our data is consistent with this hypothesis since we observed increased superoxide production in apoE ko and apoE/eNOS dko compared to C57Bl6 animals. In fact our results suggests that uncoupled eNOS is a significant source of superoxide production in apoE ko. It appears that the decrease in superoxide production from eNOS is not able to counterbalance the proatherogenic effects that resulted from the inhibition of eNOS dependant nitric oxide production.

## 4.2 Vascular nNOS generates low amounts of nitric oxide and superoxide

We had previously shown that nNOS has an anti atherogenic role, as genetic deletion of nNOS resulted in accelerated atherosclerosis in apoE ko mice[165]. Since nNOS is expressed in endothelial cell, smooth muscle cells and macrophages[164] we determined to estimate the contribution by nNOS to total vascular nitric oxide production in atherosclerosis. Our ESR experiments show that nNOS only contributes little to total vascular nitric oxide production (7% reduction of total nitric oxide production, $p>0.05$). However, atheroprotection by nNOS may be mediated through several direct or indirect mechanisms. nNOS is expressed outside the cardiovascular system, for example in nerves innervating endocrine organs such as pituitary and adrenal medulla[104] and hence may regulate hormones which potentially modulate atherosclerosis development.

Further, since nNOS is expressed in smooth muscle cells and macrophages of the atherosclerotic plaques[164, 165] nitric oxide generated by nNOS may directly inhibit smooth muscle cell proliferation. And indeed, we observed increased smooth muscle cell proliferation in apoE/nNOS dko animals. Within the smooth muscle cells and macrophages nNOS can potentially co-localize with other PDZ domain containing proteins which in turn might target nNOS towards intracellular components which regulate migration and proliferation. Furthermore, it is plausible that nNOS might decrease leukocyte and platelet adhesion to the endothelium when localized toward the vessel lumen, whereas nNOS localized toward the adventitia might inhibit smooth muscle cell proliferation. In the small intestine of rats nNOS negatively regulates the expression of iNOS[200]. However, this regulation does not seem to occur in apoE ko mice, since we observed equal amounts of iNOS expression in apoE ko and apoE/nNOS dko animals[165].

Studies in nNOS ko mice have shown that deficiency of nNOS resulted in increased xanthine oxidase mediated superoxide formation[201] suggesting that nNOS mediated nitric oxide decreases superoxide production. In order to check the influence of nNOS on superoxide production in apoE ko vessels, we performed HPLC measurements of superoxide. Our results show that genetic deletion of nNOS did not alter vascular superoxide levels as the enzyme produces only low levels of nitric oxide. However, acute pharmacological inhibition of nNOS resulted in a significant reduction in superoxide formation, suggesting that nNOS might be uncoupled in atherosclerosis. Our data raises the possibility, that chronic nNOS deletion initiates compensatory mechanisms, like increased SOD or glutathione activity. *In vitro* studies have shown that

under conditions of substrate or co-factor deficiency, nNOS uncouples to generate superoxide[117, 121]. It was recently shown that induced expression of nNOS in neuroepithelial cells[202] and over expression of nNOS in *neuroblastoma* cells resulted in increased nNOS mediated superoxide formation[203]. These results are consistent with our observation in atherosclerotic vessel where the expression of nNOS is induced during atherosclerosis[164].

## 4.3 iNOS generates nitric oxide and superoxide simultaneously in apoE ko vessels

Our ESR measurements show that iNOS significantly contributes to nitric oxide production in the vasculature of atherosclerotic apoE ko mice. Interestingly, total aortic nitric oxide formation is increased in apoE ko, compared to healthy wild type C57Bl6 mice. Vascular nitric oxide production in apoE/iNOS dko mice was reduced to the levels of C57Bl6 mice, suggesting that the source of increased nitric oxide production in apoE ko mice is iNOS. Since iNOS is expressed in smooth muscle cells and leukocytes in the plaque, these cell types mediate the additional aortic nitric oxide production[164]. iNOS is regulated transcriptionally and catalyses the production of high concentrations of nitric oxide, once the enzyme is expressed. In contrast, eNOS and nNOS produce low amounts of nitric oxide in a highly regulated way.

Indirect measurements of nitric oxide like the L-arginine to L-citrulline conversion assay and cGMP levels suggest that there is reduced eNOS activity in atherosclerotic vessels[67]. The impaired endothelial nitric oxide production results in impaired endothelial vasodilator function also termed endothelial dysfunction. Therefore, atherosclerosis is generally considered a

state of decreased nitric oxide bioavailability. In contrast, our data shows that iNOS increases total nitric oxide production of the vessel wall in the apoE ko model. Since iNOS activity is not regulated by calcium influx, the iNOS mediated nitric oxide production does not locally mediate endothelium derived vasorelaxation. Instead, the iNOS mediated increased nitric oxide production observed during bacterial sepsis can result in life threatening hypotension due to systemic vasorelaxation[204]. Our study is consistent with the previously published report by Minor et al., who showed increased nitrogen oxides in the aorta of atherosclerotic rabbits[205]. This study employed an indirect method for nitric oxide detection and didn't specify the source of nitric oxide production. Thus, the induction of iNOS expression in atherosclerosis results in increased vascular nitric oxide production, compared to wild type mice.

Increased superoxide production can decrease nitric oxide bioavailability and result in proatherogenic oxidative stress. Aside from other enzymatic sources of superoxide, iNOS itself may contribute directly to superoxide production. Therefore, we evaluated superoxide production in apoE ko and apoE/iNOS dko mice. Employing two independent direct methods, we found a significant decrease in vascular wall superoxide production in apoE/iNOS dko, compared to apoE ko. In addition, total vascular reactive oxygen species (ROS) production, assessed by measuring the conversion of CMH to nitroxide $CM^{\cdot}$ was significantly reduced in apoE/iNOS dko, compared to apoE ko animals (data not shown). Since decreased oxidative stress in chronically iNOS deficient mice could also result from secondary changes, i.e. iNOS mediated regulation of gene expression; we tested whether acute pharmacological iNOS inhibition reduces vascular superoxide production. And indeed, pretreatment of the

vessels with the iNOS specific inhibitor 1400W significantly reduced superoxide levels, indicating that iNOS itself contributes to superoxide production in atherosclerosis. To the best of our knowledge this is the first evidence supporting superoxide generation by iNOS in atherosclerosis. In contrast, ample evidence supports eNOS "uncoupling" and subsequent superoxide, instead of nitric oxide production[151, 206]. The mechanism of superoxide generation by iNOS differs from eNOS and nNOS. The latter two enzymes produce superoxide from the heme centers of their oxygenase domains[35, 117], while iNOS produces superoxide from the reductase domain[136]. While 100 µM of L-arginine was sufficient to block superoxide generation from nNOS[117], there was no effect of 100 µM L-arginine on iNOS mediated superoxide generation[136]. Higher concentrations of L-arginine (1mM–5mM) only reduced, but did not abolish superoxide production, indicating that superoxide generation by iNOS may even occur when substrate is not limited[136]. Xia et al. reported that induction of iNOS in macrophages reduced L-arginine concentrations, indicating high substrate demand[37]. These authors hypothesized that iNOS may catalyze nitric oxide from the oxygenase- and superoxide from the reductase domain simultaneously, which could directly lead to peroxynitrite formation.

Quantification of tissue 3-nitrotyrosine, considered a footprint of peroxynitrite mediated protein damage showed reduced levels of 3-nitrotyrosine in apoE/iNOS dko vessels, compared to apoE ko vessels. Peroxynitrite oxidizes $BH_4$, a cofactor of NOS[207] which in its oxidized form causes uncoupling of the constitutive NOS enzymes[103]. Further, peroxynitrite can directly cause irreversible uncoupling and inactivation of nNOS[208]. iNOS mediated peroxynitrite formation and its high substrate demand may thus cause

uncoupling of constitutive NOS isoforms, resulting in further superoxide production. The residual peroxynitrite detected in apoE/iNOS dko might be due to the reaction of nitric oxide from constitutive NOS with superoxide generated from myeloperoxidase, NADPH oxidase and xanthine oxidase.

iNOS and NADPH oxidase are pro inflammatory enzymes and known to be expressed in atherosclerotic vessels[43, 164] and the expression of both enzymes coincides with localized oxidative stress in the lesions. NADPH oxidase is proposed to negatively regulate the expression of iNOS in smooth muscle cells[209]. While increased nitric oxide generated by iNOS is suppresses the expression and activity of NADPH oxidase under certain inflammatory conditions[210] interleukin-1 induced nitric oxide (i.e iNOS) increases NADPH oxidase activity in smooth muscle cells of coronary arteries[211]. Since complex interactions exists between iNOS and NADPH oxidase we determined the contribution of NADPH oxidase to superoxide production in apoE ko and apoE/iNOS dko atherosclerosis. Consistent with the previous observation that NADPH oxidase is one of the major sources of superoxide generation in atherosclerosis[44], we found that NADPH oxidase significantly contributes to superoxide production in atherosclerosis. The reduction of superoxide production mediated by the NADPH oxidase inhibitor apocynin was similar to that observed in apoE/iNOS dko animals and inhibition of NADPH oxidase by apocynin did not further reduce the superoxide levels in apoE/iNOS dko vessels. These results suggest that the decrease of superoxide production observed in apoE/iNOS dko aorta is largely due to decreased NADPH oxidase activity in this genotype.

## Summary and Hypothesis

The principle product of each NOS is nitric oxide. However, under conditions of substrate and cofactor deficiency the enzymes directly catalyze superoxide formation. Considering this alternative chemistry of each NOS, the effects of each single enzyme on key events of atherosclerosis are difficult to predict. Here, we evaluate nitric oxide and superoxide production by all three NOS isoforms in atherosclerosis.

ESR measurements of circulating and vascular wall nitric oxide production showed significantly reduced nitric oxide levels in apoE/eNOS double knockout (dko) and apoE/iNOS dko animals but not in apoE/nNOS dko animals suggesting that eNOS and iNOS majorly contribute to vascular nitric oxide production in atherosclerosis. Pharmacological inhibition and genetic deletion of eNOS and iNOS reduced vascular superoxide production suggesting that eNOS and iNOS are uncoupled in atherosclerotic vessels. Though genetic deletion of nNOS did not alter superoxide production, acute inhibition of nNOS showed that nNOS contributes significantly to superoxide production.

In conclusion, uncoupling of eNOS occurs in apoE ko atherosclerosis but eNOS mediated superoxide production does not outweigh the protective effects of eNOS mediated nitric oxide production. We show that although nNOS is not a major contributor of the vascular nitric oxide formation, it prevents atherosclerosis development. Acute inhibition of nNOS showed a significant reduction of superoxide formation suggesting that nNOS is uncoupled. The exact mechanism of action of nNOS in atheroprotection is yet to be elucidated. Genetic deletion of iNOS reduced NADPH oxidase activity. Thus, iNOS has both direct and indirect proatherosclerotic effects, as it directly generates both

nitric oxide and superoxide simultaneously resulting in peroxynitrite formation and indirectly modulates NADPH oxidase activity.

We hypothesize that eNOS is coupled in the disease free regions of the vessel and contributes to nitric oxide generation whereas in the diseased region of the vessel it is uncoupled to produce superoxide (Figure 16). nNOS expressed in the smooth muscle cells of the plaque contributes to the local superoxide generation. iNOS expressed in smooth muscle cells and leukocytes of the plaque generates superoxide and nitric oxide simultaneously to produce the strong oxidant peroxynitrite.

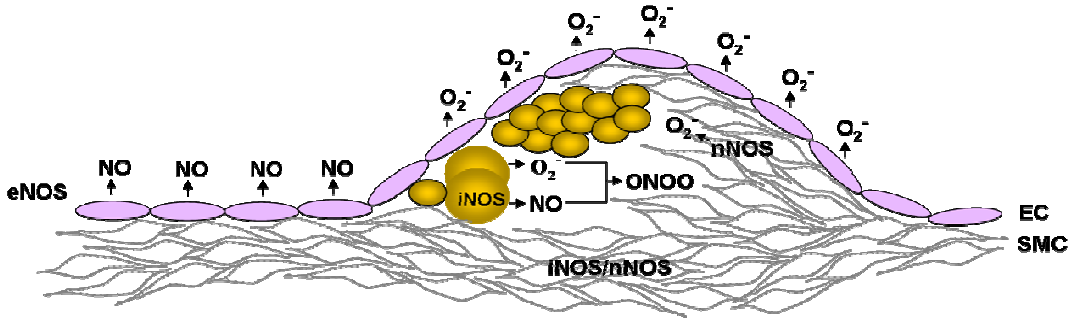


**Figure 16.** Proposed hypothesis.

# Zusammenfassung und Hypothese

Stickstoffmonoxid (NO) ist das prinzipielle Produkt aller Stickstoffmonoxid-Synthasen (NOS). Im Falle eines Mangels an Substrat (L-arginin) und Kofaktoren (Tetrahydrobiopterin, $BH_4$) katalysieren die NOS-Enzyme direkt Superoxid ($O_2^-$). Diese Veränderung in der Radikalproduktion wird auch als Entkopplung der NOS bezeichnet. Die alternative Produktion von NO oder $O_2^-$ durch die NOS bedingen, dass eine Voraussage über die Schlüsselfunktion der einzelnen Enzyme in der Entstehung der Atherosklerose schwierig ist. In unserer Studie evaluieren wir die Produktion von NO sowie $O_2^-$ in atherosklerotischen Läsionen von apoE ko Mäusen und apoE/NOS doppel knockout (dko) Mäusen denen jeweils eine NOS-Isoform fehlt.

Elektronen Spin Resonanz (ESR) Messungen konnten eine signifikante Reduktion sowohl des zirkulierenden, als auch der Gefäßwand eigenen Produktion von NO in apoE/eNOS dko und apoE/iNOS dko Mäusen zeigen, nicht jedoch in apoE/nNOS dko Mäusen. Dies lässt darauf schließen, dass eNOS und iNOS den hauptsächlichen Anteil der vaskulären NO-Produktion in atherosklerotischen Läsionen bewerkstelligen.

Die pharmakologische Inhibierung wie auch die genetische Deletion von eNOS und iNOS führten ebenfalls zu einer reduzierten vaskulären $O_2^-$ produktion, was die partielle Entkopplung beider Enzyme in atherosklerotisch veränderten Gefäßen nahe legt. Obwohl die chronische genetische Deletion von nNOS in apoE/nNOS dko die $O_2^-$ Produktion nicht verändert, zeigte sich bei der akuten pharmakologischen Inhibierung von nNOS (durch L-NAANG) eine maßgebliche Beteiligung von nNOS an der $O_2^-$ produktion in apoE ko Mäusen.

Schlussfolgernd lässt sich sagen, dass in atherosklerotischen Gefäßen von apoE ko Tieren eine Entkopplung von eNOS statt findet, diese jedoch zu keinem Ausgleich der protektiven Effekte der eNOS vermittelten NO-Produktion führt. Unsere Ergebnisse in apoE/nNOS dko Mäusen zeigen eine atheroprotektive Rolle der nNOS, die sich nicht allein durch eine lokale, vaskuläre NO-Produktion durch das Enzym erklären lässt. Wir postulieren weitere systemisch atheroprotektive Eigenschaften der nNOS. Die signifikante Reduktion der Superoxidproduktion durch eine akute Inhibierung der nNOS weist auf eine Entkopplung der nNOS hin. Der exakte Wirkungsmechansimus von nNOS in der Atheroskleroseprävention ist weiterhin noch zu eruieren. Die genetische Deletion von iNOS führt zu einer reduzierten Aktivität der NADPH-Oxidase. Demnach sind für iNOS direkte sowie indirekte atherosklerosefördernde Effekte anzunehmen, da sie auf direktem Wege gleichzeitig NO und $O_2^-$ produziert, was in einer Peroxynitritbildung resultiert.

Wir stellen die Hypothese auf, dass eNOS in den läsionsfreien Gefäßregionen gekoppelt ist und dort seine atheroprotektiven Effekte durch die NO-Produktion vermittelt, während die eNOS in atherosklerotischen Läsionen entkoppelt vorliegt und hier $O_2^-$ produziert (Fig. 16). iNOS, welches vor allem in den Plaques, in glatten Muskelzellen und Leukozyten zu finden ist, produziert gleichzeitig hohe Konzentrationen von $O_2^-$ und NO, die als gemeinsames Endprodukt das stark oxidierende Peroxynitrit ergeben und die von uns dokumentierte proatherosklerotische Wirkung der iNOS vermittelt.

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# Abbreviations

| | |
|---|---|
| Apo E | : Apolipoprotein E |
| $BH_4$ | : Tetrahydrobiopterin |
| CAD | : Coronary artery disease |
| DETC | : Diethyldithiocarbamic acid |
| ESR | : Electron spin resonance |
| KHB | : Krebs Hepes Buffer |
| L-NAME | : L-arginine methyl ester |
| LDL | : Low density lipoprotein |
| HDL | : High density lipoprotein |
| HPLC | : High Performance Liquid Chromatography |
| N-AANG | :N-[(4S)-4-amino-5-[(2-aminoethyl) amino] pentyl]-N'-nitroguanidine tris (trifluoroacetate) |
| NADPH | : Nicotinamide adenine dinucleotide phosphate |
| NHIS 95 | : National Health Interview Survey 1995 |
| NO | : Nitric oxide |
| NOS | : Nitric oxide synthase |
| Ox-LDL | : Oxidized LDL |
| TNF-α | : Tumor necrosis factor- α |
| 1400W | : N-(3-aminomethyl) benzyl-acetamidine |

# Thesaurus

## Definitions

- **Allograft / vein graft arteriosclerosis** refers to arteriosclerosis developed following tissue or vein graft transplantation. An **allograft** or **homograft** is a transplant in which transplanted cells, tissues, or organs are sourced from a genetically non-identical member of the same species. Most human tissue and organ transplants are allografts.

- **Angina pectoris**, commonly known as **angina**, is chest pain due to ischemia of the heart muscle, generally due to obstruction or spasm of the coronary arteries (the heart's blood vessels). Coronary artery disease, the main cause of angina, is due to atherosclerosis of the cardiac arteries.

- **Aortic aneurysm** is a general term for any swelling of the aorta, usually representing an underlying weakness in the wall of the aorta. While the stretched vessel may occasionally cause discomfort, a greater concern is the risk of rupture which causes severe pain; massive internal bleeding and without prompt treatment results in quick death.

- **Aortic dissection** is a tear in the wall of the aorta that causes blood to flow between the layers of the wall of the aorta and force the layers apart. Aortic dissection is a medical emergency and can quickly lead to death, even with optimal treatment.

- **Balloon angioplasty** is a widely used catheter-based technique for opening blocked arteries and treating coronary artery disease (CAD) and is one of the standard treatments for CAD. To restore normal blood flow, a balloon-tipped catheter is guided into one of the clogged arteries, and the balloon is rapidly inflated. This action helps blood flow more freely through the vessel by pushing the plaque back against the artery wall, creating small cracks or fissures called plaque fractures within the brittle, fatty deposit and by stretching the artery.

- **Cardiac hypertrophy** is a thickening of the heart muscle (myocardium) which results in a decrease in size of the chamber of the heart, including the left and right ventricles.

- **Cardiomyopathy** is the deterioration of the function of the myocardium (i.e., the heart muscle) for any reason. People with cardiomyopathy are often at risk of sudden cardiac death.

- **Carotid artery ligation model** is an animal model that is used to study arterial injuries. The carotid artery is ligated using a wire, to cause denudation of the endothelium, representing vascular injury during angioplasty.

- **Caveolae** (Latin for *little caves*) are small (50–100 nanometer) invaginations of the plasma membrane in many vertebrate cell types, especially in endothelial cells and adipocytes.

- **Claudication** refers to leg pain brought on by exertion and relieved with rest. Claudication is also known as intermittent claudication, peripheral vascular disease, or "poor circulation." The primary cause of claudication is atherosclerosis, or narrowing of the arteries that feed the leg muscles.

- **Congestive heart failure** or heart failure is a condition in which the heart can't pump enough blood to the body's other organs.

- **Coronary artery disease (CAD)**- a disease in which the blood flow to the heart is restricted due to hardened arteries (atherosclerosis) that are clogged with plaque deposits.

- **Endothelial dysfunction** is the physiological dysfunction of normal biochemical processes carried out by the endothelium.

- **Hypercholesterolemia** is the presence of high levels of cholesterol in the blood. It is not a disease but a metabolic derangement that can be secondary to many diseases and can contribute to many forms of cardiovascular disease.

- **Hyperplasia** is a general term referring to the proliferation of cells within an organ or tissue beyond that which is ordinarily seen in constantly dividing cells. Hyperplasia may result in the gross enlargement of an organ, the formation of a benign tumor, or may be visible only under a microscope.

- **Ischemia** is a restriction in blood supply, generally due to factors in the blood vessels, with resultant damage or dysfunction of tissue.

- **L type calcium channels** are a type of voltage-dependent calcium channel. Voltage-dependent calcium channels are a group of voltage-gated ion channels found in excitable cells (*e.g.*, muscle, glial cells, neurons, etc.) with a permeability to the ion $Ca^{2+}$. At physiologic or resting membrane potential, voltage-dependent calcium channels are normally closed. They are activated (*i.e.*, opened) at depolarized membrane potentials and this is the source of the "voltage-dependent" epithet. Activation of particular voltage-dependent calcium channels allows $Ca^{2+}$ entry into the cell, which depending on the cell type, results in muscular contraction, excitation of neurons, up-regulation of gene expression, or release of hormones or neurotransmitters.

- **Myocardial infarction** more commonly known as a **heart attack** is a medical condition that occurs when the blood supply to a part of the heart is interrupted, most commonly due to rupture of a vulnerable plaque.

- **Myocardial ischemia** is a condition in which oxygen deprivation to the heart muscle is accompanied by inadequate removal of metabolites because of reduced blood flow or perfusion.

- **Neuroblastoma i**s the most common extracranial solid cancer in childhood and the most common cancer in infancy, with an annual incidence of about 650 new

cases per year in the US. It is a neuroendocrine tumor, arising from any neural crest element of the sympathetic nervous system.

- **Nitrergic nerves:** Nerve where transmission is mediated by nitric oxide.

- **Non-adrenergic non-cholinergic** neuron usually refers to autonomic efferent neuron whose transmission is not blocked by blocking adrenergic (catecholamines) and cholinergic (acetylcholine) transmission. Nitric oxide may be the transmitter in some cases.

- **Protein kinase G (PKG)** is a serine/threonine-specific protein kinase that is activated by cGMP. It phosphorylates a number of biologically important targets and is implicated in the regulation of smooth muscle relaxation, platelet function, sperm metabolism, cell division and nucleic acid synthesis.

- **Reperfusion injury** refers to damage to tissue caused when blood supply returns to the tissue after a period of ischemia. The absence of oxygen and nutrients from blood creates a condition in which the restoration of circulation results in inflammation and oxidative damage through the induction of oxidative stress rather than restoration of normal function.

- **Restenosis** literally means the reoccurrence of stenosis. This is usually restenosis of an artery, or other blood vessel that has been "unblocked" by mechanically widening a narrowed or totally obstructed blood vessel (as a result of atherosclerosis) using interventional techniques like angioplasty.

- **Sepsis** is a serious medical condition characterized by a whole-body inflammatory state caused by infection. Sepsis is broadly defined as the presence of various pus-forming and other pathogenic organisms, or their toxins, in the blood or tissues.

- **Soluble guanylate cyclase** (sGC, (EC 4.6.1.2), also known as **guanylyl cyclase** or **GC**) is a lyase enzyme. It catalyzes the conversion of guanosine triphosphate (GTP) to 3',5'-cyclic guanosine monophosphate (cGMP) and pyrophosphate. sGC is a receptor for NO (thus also called NO receptor). It is soluble i.e. completely intracellular. It is most notably involved in vasodilation.

- **Transplant arteriosclerosis** develops following organ transplantation.

- **Vasodilator** is a drug or chemical that relaxes the smooth muscle in blood vessels, which causes them to dilate.

# Curriculum vitae


| | |
|---|---|
| Name | : P. Padmapriya |
| Sex | : Female |
| Date of Birth | : 03-03-1980 |
| Nationality | : Indian |
| Present address | : 114, Schwestern Wohnheim-2<br>Straubmülweg No. Nr<br>Würzburg, D97078<br>Germany<br>Tel : 0049 17661118717<br>Fax : 0049 931 201 36664<br>Email: pppris@yahoo.com |
| Permanent address | : 61/23, Sowthamalar layout<br>Ramaswamy nagar<br>Udumalpet-642126<br>Coimbatore District, Tamilnadu<br>INDIA |


## Educational Qualification

| | |
|---|---|
| 1. Bachelor of Science | : B.Sc Biotechnology (2000)<br>Bharathiyar University, Coimbatore<br>Tamilnadu, India<br>Division: First class with distinction |
| 2. Master of Science | : M.Sc Biotechnology (2002)<br>Bharathidasan University, Tiruchirapalli<br>Tamilnadu, India<br>Division: First class |

## Awards

- Proficiency Prize holder in graduation.
- Secured *Gold Medal* and University 3rd rank in graduation.
- Won prizes in inter-department quiz competitions in college.
- Won prize in state level essay writing competition.

## Oral and poster presentations

- **Padmapriya Ponnuswamy,** E. Ostermeier, P. Kuhlencordt . *Inducible nitric oxide synthase (iNOS) modulates oxidative stress in vessels of atherosclerotic mice*; Oral presentation at the 72nd Annual conference of the German society for cardiology (DGK), 20-22nd April, 2006.

- **Padmapriya Ponnuswamy,** E. Ostermeier, A.Schröttle, G. Ertl and P. Kuhlencordt. *Alternative chemistry of inducible nitric oxide synthase (iNOS) in atherosclerosis*; Oral presentation at the 36th Annual conference of the German society for angiology (DGA) and the 14th Conference of the German, Austrian and Swiss societies for angiology, 9-12th September, 2007.

- **Padmapriya Ponnuswamy,** Angelika Schröttle, Eva Ostermeier, Sabine Grüner, David Varga-Szabo, Paul Huang, Georg Ertl, Bernhard Nieswandt, Peter Kuhlencordt. *Endothelial nitric oxide production inhibits leukocyte endothelial cell- but not platelet endothelial cell interactions*; Poster presentation at the Scientific sessions meeting of American Heart Association, 4-7th November, 2007.

- **Padmapriya Ponnuswamy,** Angelika Schröttle, Sabine Grüner, Andreas Schäfer, Paul Huang , Johann Bauersachs, Bernhard Nieswandt, Georg Ertl and Peter Kuhlencordt. *Role of nNOS in endothelial relaxation and leukocyte/endothelial cell (L/E-) interactions in atherosclerosis;* Oral

presentation at the 37th Annual conference of the German society for angiology (DGA), 24th-27th September, 2008.

## List of publications

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- Peter J. Kuhlencordt*, **P. Padmapriya***, S. Rützel, J. Schödel, K. Hu, A. Schäfer, P.L. Huang, G. Ertl, J. Bauersachs. Ezetimibe potently reduces vascular inflammation and arteriosclerosis in eNOS-deficient ApoE ko mice. Atherosclerosis 2008 Apr 6 [Epub ahead of print].

- **P. Padmapriya**, A. Schröttle, E. Ostermeier, S. Grüner, D. Varga-Szabo, P.L. Huang, G. Ertl, B. Nieswandt and P.J. Kuhlencordt. Endothelial nitric oxide inhibits leukocyte endothelial cell- but not platelet endothelial cell-interactions in atherosclerosis. *(In revision Arter. Thromb. Vas. Biol.)*

- **P. Padmapriya**, Eva Ostermeier, Angelika Schröttle, Jiqiu Chen, Paul L. Huang, Georg Ertl, Bernhard Nieswandt, Peter J. Kuhlencordt. Oxidative stress and compartment of gene expression determine proatherosclerotic effects of inducible nitric oxide synthase. *(In revision Am J of Pathology).*

- Johannes Schödel, **P. Padmapriya**, Alexander Marx, Paul L. Huang, Georg Ertl, Peter J. Kuhlencordt. Expression of neuronal nitric oxide synthase splice variants in atherosclerotic plaques of apoE knockout mice. *(In revision Atherosclerosis)*

## Declaration

I hereby declare that all the above information provided is true to the best of my knowledge.

Date :

Place : (P.Padmapriya)