Regulation of Endothelial Nitric Oxide Synthase by Phosphorylation

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften

vorgelegt beim Fachbereich 15
Biologie und Informatik-Wissenschaften
der Johann Wolfgang Goethe- Universität
in Frankfurt am Main

Von
Annisuddin Mohamed
aus Bangalore, India.

Frankfurt am Main 2005

Vom Fachbereich Biologie und Informatik der Johann Wolfgang Goethe-Universität
als Dissertation angenommen.

Dekan: Prof. Dr. Osiewacz

Gutachter: Prof. Dr. I. Fleming

Datum der Disputation: 22, Dezember 2005

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation selbständig und nur mit den angegebenen Hilfsmitteln angefertigt habe.

Frankfurt am Main, den 03, August 2005

Annisuddin Mohamed

Acknowledgements

As I start to write this acknowledgement many names cross my mind, people who have helped me in their own ways towards the achievement of this milestone. Not all of them might find a mention here, but your contribution will always be remembered and appreciated.

First, I would like to express my deepest gratitude to my parents Mr. Mohamed Rafiuddin and Mrs. Azra Nasreen. They have been a constant source of encouragement and strength through out my academic and non-academic life.

I would like to express my respectful gratitude to Prof. Dr. Rudi Busse for providing me with an opportunity to spend over three fruitful years in the very stimulating environment of the Department of Cardiovascular Physiology. I am grateful for the continuous support I received from him.

My special thanks go to Prof. Karl-Dieter Entian for accepting to be my external supervisor.

I am indebted in more than one way to my supervisor Prof. Ingrid Fleming. She has been a continuous source of inspiration, an excellent guide and over all a good friend who always helped me in seeing light at the end of the tunnel, the latest being with my marriage. Words would fall short in expressing my gratitude to her for her continuous support and encouragement towards the successful completion of this thesis, not to forget the pain she took in the correction of this thesis. Her focussed and critical direction contributed to the development of my own understanding on science.

During the course of my work, many people in the laboratory have contributed in their own way to my work providing me with technical and emotional support. I would like to sincerely thank Isabel Winter, Mechtild Piepenbrock and Tanja-Maria Mareczek for their excellent technical support and friendship.

When you don’t have your family you must have good friends. When I moved to Frankfurt new friends made the distance from home and the difficulties of life much easier to deal with. Being away from India was made less traumatic by people like PD. Dr. Ralf Brandes, who took the trouble of initiating and arranging lab outings which made the difficulties of life

much easier. My thanks also go to him for introducing me to confocal microscopy and radical measurements, not to mention his help in fixing the many and varied problems with my computer.

My special thanks to Dr. Beate Fisslthaler for her kind support and critical comments during the course of this work. Not to forget Dr. Rudiger Popp, Madhu, Mariano, Voahangy, Pravir, Annemarieke, Benjamin, Elke, Anke, Timo and all the others for their friendship and the stimulating (and at times) funny discussions we had together.

I would like to acknowledge the contributions of Dr. Andrei L Kleschyov and Dr. Klaus Zwicker for introducing me to NO spin trapping and ESR spectroscopy.

Lastly, I would like to mention my fellow “KITTENS”- my siblings, Ameenuddin, Abraruddin, Tabassum, Taranum, Saba and Farha for their love and encouragement which always kept me going.

Table of Contents

1 Introduction 1

1.1 Role of endothelium in maintaining the tone of vasculature. 1

1.2 Endothelium-derived vasoactive factors 2

1.3 Importance of NO as a normal regulator of blood pressure 2

1.4 Endothelial dysfunction in cardiovascular disease 2

1.5 Nitric oxide 4

1.6 Nitric oxide synthases 5

1.6.1 Mechanism of NO biosynthesis 7

1.6.2 Substrate and cofactor requirement 9

1.7 Regulation of eNOS 10

1.7.1 Intracellular localisation 10

1.7.2 Regulation by eNOS-associated proteins 12

1.7.3 Regulation by phosphorylation 19

1.7.4 NOS reductase domain regulatory elements 26

1.8 NOS uncoupling 27

1.9 Free radicals in the cardiovascular system 28

1.9.1 Superoxide anion radical (O_2^-) 28

1.9.2 Peroxynitrite 28

1.10 Measurement of NO 30

2 Aim 32

3 Materials and methods: 33

3.1 Bacterial transformation 33

3.1.1 Preparation of competent cells by KCM method 33

3.1.2 Transformation 33

3.2 Isolation of nucleic acids 33

3.2.1 Mini plasmid DNA preparation by alkaline lysis method 33

3.2.2 Maxi plasmid DNA preparation 34

3.2.3 DNA extraction from agarose gels 34
3.2.4 RNA isolation 34
3.2.5 Purification of DNA by phenol chloroform isoamyl alcohol extraction and ethanol precipitation 35

3.3 Manipulation of nucleic acids....35
3.3.1 Restriction digestion 35
3.3.2 Dephosphorylation of linearised double-stranded DNA 35
3.3.3 Large fragment of the E.coli DNA polymerase I (Klenow fragment):....36
3.3.4 DNA ligation....36
3.3.5 Site directed mutagenesis....36
3.3.6 Sequencing of DNA....37

3.4 Detection of nucleic acids....37
3.4.1 Colony hybridisation using a ^{32}P-radiolabelled probe....37
3.4.2 Southern blot hybridisation....38
3.4.3 Northern blotting and hybridisation....38

3.5 Cell culture and adenoviral methods....39
3.5.1 Generation of eNOS adenoviruses....39
3.5.2 Culturing of cells 40
3.5.3 Transfection with calcium choloride 40
3.5.4 Adenoviral infection of COS-7 cells 41
3.5.5 Adenoviral infection of human umbilical vein endothelial cells....41
3.5.6 Ox-LDL treatment 41
3.5.7 Stimulation with bradykinin 41

3.6 Immunohistochemistry....41

3.7 Methods with protein....42
3.7.1 Isolation of protein....42
3.7.2 Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting....42
3.7.3 Low temperature- polyacrylamide gel electrophoresis (LT-PAGE) 43
3.7.4 Immunoprecipitation....43
3.7.5 Reproducing Western blots....44

3.8 Assays of eNOS activity, NO and O_2^- detection.....44
3.8.1 Citrulline assay: 44

3.9 Measurement of nitric oxide using $Fe(DETC)_2$ by electron spin resonance spectroscopy *46*

3.9.1 Measurement in cells: 46

3.10 Measurement of superoxide anion (O_2^-) *47*

3.10.1 Chemiluminescence 47

3.10.2 Electron spin resonance (ESR) spectroscopy 47

3.11 Statistical analysis *48*

4 Results **49**

4.1 Effects of mimicking phosphorylation at specific serine residues of eNOS *49*

4.1.1 Effect of Ser^{114} or Ser^{633} mutations on eNOS catalytic activity and Ca^{2+}-CaM dependence 49

4.1.2 Effect of shear stress and bradykinin on the phosphorylation of eNOS 51

4.1.3 Effect of the mutation of eNOS on NO production in intact COS-7 cells as measured by ESR spectroscopy 52

4.1.4 Effect of specific mutation on eNOS dimer formation 54

4.1.5 Effect of eNOS inhibitors, an $NO^{\cdot}$ donor and radicals on eNOS dimer stability 56

4.1.6 Effect of specific eNOS mutation on its intracellular localisation 57

4.1.7 Effect of specific mutation on superoxide anion (O_2^-) production by eNOS ... 59

4.2 Effect of ox-LDL on eNOS uncoupling *62*

4.2.1 Time course of the LDL-induced changes in eNOS phosphorylation 64

4.3 The role of tyrosine phosphorylation in the regulation of eNOS activity *70*

5 Discussion **80**

6 Summary **93**

7 Zusammenfassung **96**

Curriculum Vitae **124**

1 Introduction

Nitric oxide (NO), synthesized by the endothelial nitric oxide synthase (eNOS), is a major endothelium-derived factor that regulates the vascular tone. Its multiple roles include the regulation of vasomotion (Palmer *et al.*, 1987), cell adhesion to the endothelium (Kubes *et al.*, 1991; Lefer *et al.*, 1999), platelet aggregation (Radomski *et al.*, 1991; Wolf *et al.*, 1997) and vascular smooth muscle cell proliferation (Rudic *et al.*, 1998). At first glance, this list suggests that NO is a crucial factor in the prevention of cardiovascular damage such as that seen in atherosclerosis, ischemia/repurfusion injury, thrombosis and hypertension. Indeed, the loss of endothelium-derived NO that underlies "endothelial dysfunction" is now thought to be a major cause of such pathological conditions which emphasizes the need to understand the mechanisms that regulate eNOS expression and activity.

1.1 Role of endothelium in maintaining the tone of vasculature.

The endothelium is a monolayer of polygonal flat epithelial cells (0.2-0.3 μm thick) that extends continuously over the luminal surface of the entire vasculature and could be considered as the largest endocrine organ within the human body. In different regions, the structural features vary with specificity. In brain, endothelial cell junctions are mainly tight while an intracellular cleft is wide open in liver to facilitate protein transport. The endothelial cells of the glomerulus have small oval windows called fenestrae so that small molecules, such as glucose and urea, can be filtered readily (Ostendorf *et al.*, 1999).

The biological functions of endothelium are numerous and vary according to the size and distribution of the blood vessel that it lines. The endothelium serves and participates in highly active metabolic and regulatory function including control of primary hemostasis, platelet and leukocyte interactions with the vessel wall, clot deposition, clot lysis and selective phagocytic activity (Luscher, 2001). Also, it interacts with the lipoprotein metabolism and presentation of histocompatibility antigens. A plethora of bioactive molecules are produced by the endothelium (Garcia-Cardena *et al.*, 2001) that can be mutually antagonistic. Many protein growth factors, matrix supporting proteins and vasoactive substances are produced by the endothelium, highlighting the fact that the functions of the vascular endothelium are dynamic rather than fixed. Immune complexes, lipids, angioplasty, germs, hypertension, shear stress, hypoxia, acidosis, smoking, aging, diabetes mellitus and surgery inflict injury to the endothelium causes its activation.

1.2 Endothelium-derived vasoactive factors

In 1980 Furchgott and Zawadzki reported the existence of a non-prostanoid endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980). The term EDRF was coined to account for the finding that upon stimulation with acetylcholine the endothelium releases a relaxing factor. In 1987 pharmacological and chemical evidence indicated that EDRF is identical to, or closely related to, NO (Ignarro *et al.*, 1987). At the same time it was found that the EDRF/NO released by endothelial cells is a potent inhibitor of platelet aggregation and adhesion to the vascular wall. The effect was found to be mediated by the stimulation of soluble guanylyl cyclase in the platelets (Busse *et al.*, 1987; Palmer *et al.*, 1987). While NO is the predominant vasodilator, acting through the stimulation of the soluble guanylate cyclase in the smooth muscle cells, other substances are also important. Prostacyclin, similar to NO, is a potent vasodilator (Moncada & Vane, 1980; Moncada & Vane, 1981). The endothelium-derived hyperpolarizing factor (EDHF), another diffusible factor, was found to cause endothelium-dependent relaxation via a mechanism related to the hyperpolarization of smooth muscle cells (Busse *et al.*, 1987). On the other hand, several endothelium-derived prostanoids, such as thromboxane A2, cause vasoconstriction and promote platelet aggregation. Endothelin-1 is a peptide produced by endothelial cells with powerful vasoconstriction property. The endothelium-derived free radical superoxide (O_2^-) promotes vasoconstriction by scavenging NO (Fig. 1).

1.3 Importance of NO as a normal regulator of blood pressure

Substances of non-endothelial origin such as angiotensin and norepinephrine, were previously considered to be the major determinants of vascular tone. However, physiological studies using inhibitors of NO synthase (NOS) have indicated a primary role for endothelium-derived NO in regulating vascular tone. Intravenous administration of NOS inhibitors, such as mono-methyl arginine, to animals (Aisaka *et al.*, 1989; Rees *et al.*, 1989; Whittle *et al.*, 1989) or humans (Vallance *et al.*, 1989a; Vallance *et al.*, 1989b) provokes a rapid and marked increase in vascular resistance. In fact, NOS inhibitors cause a more notable increase in blood pressure than do drugs that influence the action of norepinephrine or angiotensin.

1.4 Endothelial dysfunction in cardiovascular disease

The endothelium contributes to cardiovascular homeostasis in health and disease (Luscher & Noll, 1995; Rubanyi, 1991). Due to its location at the interface between vessel wall and circulating blood, this cell layer is the primary target for mechanical forces and cardiovascular risk factors. Impairment of endothelial function occurs before structural changes such as

intimal hyperplasia or lipid deposition. Therefore, endothelial dysfunction is an upstream event in the pathophysiology of cardiovascular disease. Endothelial dysfunction is characterized by attenuated endothelium-dependent vascular responses, and decreased expression or activity of eNOS. Also there is an enhanced expression of a number of pro-inflammatory proteins. There are several different mechanisms contributing to endothelial dysfunction, including NO scavenging by O_2^- and eNOS uncoupling; a state of eNOS where the transfer of electrons is no longer coupled to NO formation. O_2^- is generated within atheromatous plaques by vascular smooth muscle cells, endothelial cells, neutrophils or macrophages. The potential source of O_2^- includes NADPH oxidase, xanthine oxidase and interestingly enough, eNOS that has been uncoupled. Uncoupling of eNOS occurs under conditions of substrate (L-arginine) depletion (Xia *et al.*, 1996) and limitation of the essential co-factor tetrahydrobiopterin (H_4B) (Xia *et al.*, 1998).

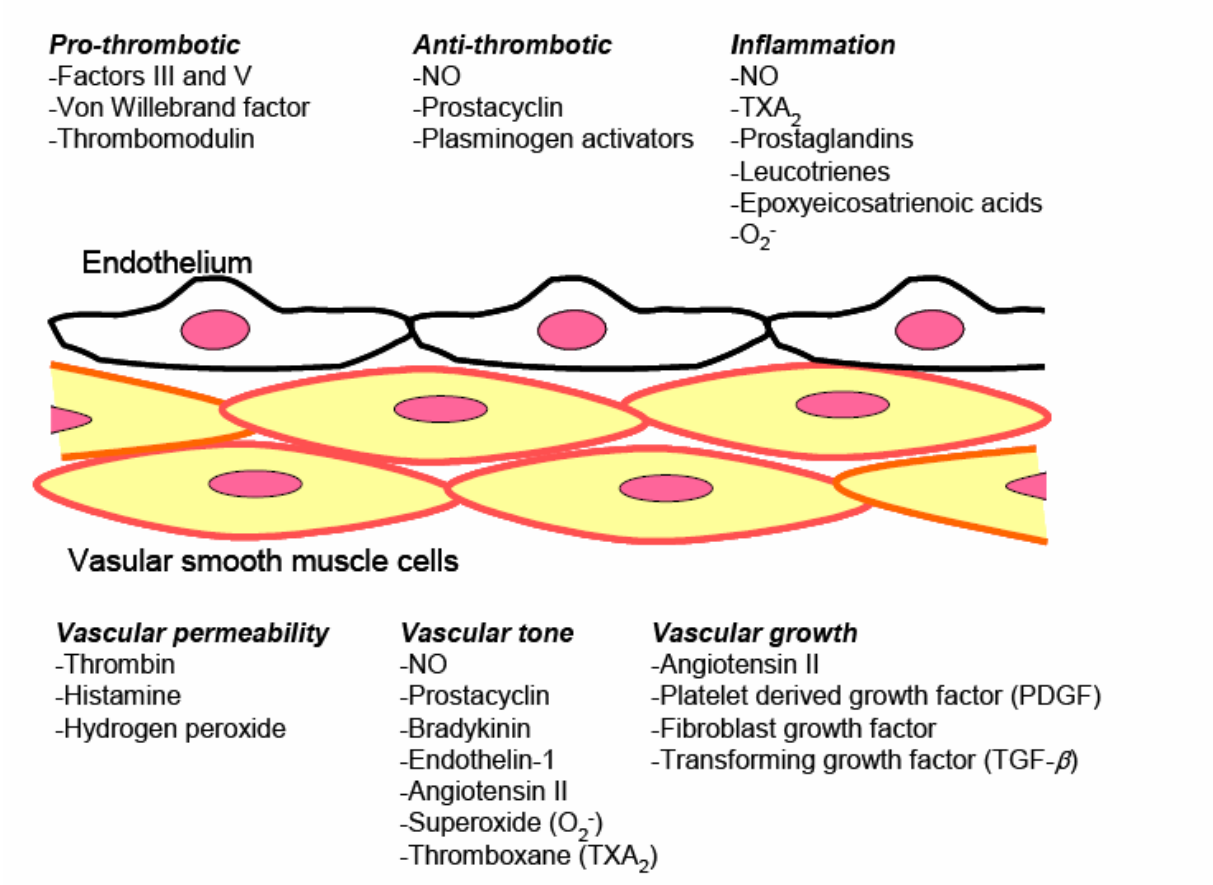

Figure 1. Products of the endothelium and their effect. *Vasoactive products from the endothelium act both in the lumen of the blood vessel and the vascular smooth muscle cells. In the lumen they are responsible for hemostasis by releasing pro- or anti-thrombotic factors and inflammation. In the vascular wall they stimulate vascular growth, influence vascular permeability and maintain vascular tone.*

Alterations in the production and/or availability of NO are accepted as a major factor in the development of cardiovascular disease (Warnholtz *et al.*, 2004), such as atherosclerosis (Kawashima, 2004), hypercholesterolemia (Stehbens, 2001), ischemia/repurfusion injury (Szocs, 2004), thrombosis (Voetsch *et al.*, 2004) and hypertension (Lassegue & Griendling, 2004). The understanding of the molecular, biochemical and cellular mechanisms that regulate eNOS activity and the chemistry behind NO and its adducts is therefore essential for identifying and developing new approaches for the treatment of endothelial dysfunction.

1.5 Nitric oxide

Nitric oxide is a small diatomic gaseous molecule with an unpaired electron, which makes it a free radical with a fairly short half-life. The hydrophobic nature of NO together with a small molecule size enables it to diffuse rapidly across cell membranes and depending upon the conditions, it is able to diffuse distances of more that several hundred microns. These properties make NO uniquely suitable as both an intra- and intercellular messenger. The unpaired electron of NO makes the molecule a potent reducing substance which reacts readily with biological targets such as heme groups, sulfhydryl groups, iron and zinc clusters, which in turn mediate its biological effects. Such a diverse range of potential targets for NO explains its importance as a regulatory molecule.

Effectors of NO

Once NO is produced by the endothelium, it can regulate several aspects of the vascular function. Its primary "receptor" is the soluble guanylyl cyclase (sGC), but it can also initiate nitrosation reactions with iron-sulphur-centred proteins or proteins with reactive thiols (S-nitrosylation). Nitrosylation of caspase-3 and caspase-8 inactivates the proteins, leading to inhibition of apoptosis (Stamler *et al.*, 2001).

In the vascular system, NO- dependent relaxation of vascular smooth muscle is predominantly sGC- and protein kinase G- (PKG) dependent, whereas the anti-proliferative actions and ion channel modulation of NO can occur via PKG or via nitrosation reactions (Feil *et al.*, 2003; Matalon *et al.*, 2003; Miranda *et al.*, 2003).

NO has the capacity to interact with a variety of enzymes, thereby altering their function and influencing inflammatory (and other) reactions. For example, NO can inhibit many iron-containing enzyme functions, including mitochondrial electron transfer (Beckman & Koppenol, 1996), which may contribute to the tumoricidal activity of macrophages. In micromolar concentrations, NO can reversibly inhibit cytochrome P-450 (Palacios-Callender *et al.*, 2004). NO has also been shown to interact with cyclooxygenase, another heme-

containing enzyme, resulting in an increase in its activity (Salvemini *et al.*, 1993). NO has been shown to inhibit inducible NOS (iNOS) expression (Cirino *et al.*, 1996), as NOS is also a heme-containing enzyme, it is possible that NO may interact with the enzyme(s) that generate it. S-nitrosylation of the eNOS is associated with enzyme monomerisation and decreased enzyme activity (Ravi *et al.*, 2004).

At micromolar concentrations, NO can inhibit ribonucleotide reductase, an enzyme critical for the synthesis of DNA precursors (Beckman & Koppenol, 1996; Moncada & Higgs, 1995). It can also inhibit transcriptional events by inhibiting the transcription factor NF-kB (Katsuyama *et al.*, 1998). This has been suggested to be an important mechanism underlying the anti-inflammatory actions of some NO-releasing drugs (Fiorucci *et al.*, 2002).

Because of these powerful functions, the production of this pivotal mediator is tightly regulated and there is ample literature to show that too little or too much NO production contributes to numerous human diseases and disorders. Decreased NO generation in the penis, for example, results in impotence. On the other hand, many other diseases and conditions such as intradialytic hypotension, hemorrhagic shock, tissue rejection, rheumatoid arthritis, and diabetes are associated with the overproduction of NO by activated immunocompetent cells.

1.6 Nitric oxide synthases

Nitric oxide is produced by a family of homodimeric NO synthases (NOS), which catalyse the conversion of L-arginine to NO and L-citrulline. To date, three genetically distinct NOS isoforms have been identified, neuronal NOS (nNOS, NOS-I) originally isolated from cerebellum (Bredt & Snyder, 1990), inducible NOS (iNOS, NOS-II) from macrophages (Xie *et al.*, 1992), and endothelial NOS (eNOS, NOS-III) from endothelial cells (Pollock *et al.*, 1991). All of the NOS isoforms share an overall 50% amino acid sequence homology and have similar co-factor requirements. Each NOS monomer contains an N-terminal oxygenase domain with a binding site for the substrate L-arginine, H_4B, Ca^{2+}/calmodulin (CaM), and Zn^{2+}, as well as a C-terminal reductase domain with one binding site each for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as electron donor. The reductase domain terminates in a C-terminal tail, which contains an autoinhibitory sequence. Both the reductase and oxygenase domain are linked by a short CaM-binding domain. In order to synthesize NO the electrons flow from the reductase domain of one monomer to the oxygenase domain of the

other (Fig. 2), a phenomenon called "domain swapping" (Panda *et al.*, 2001; Siddhanta *et al.*, 1998; Siddhanta *et al.*, 1996).

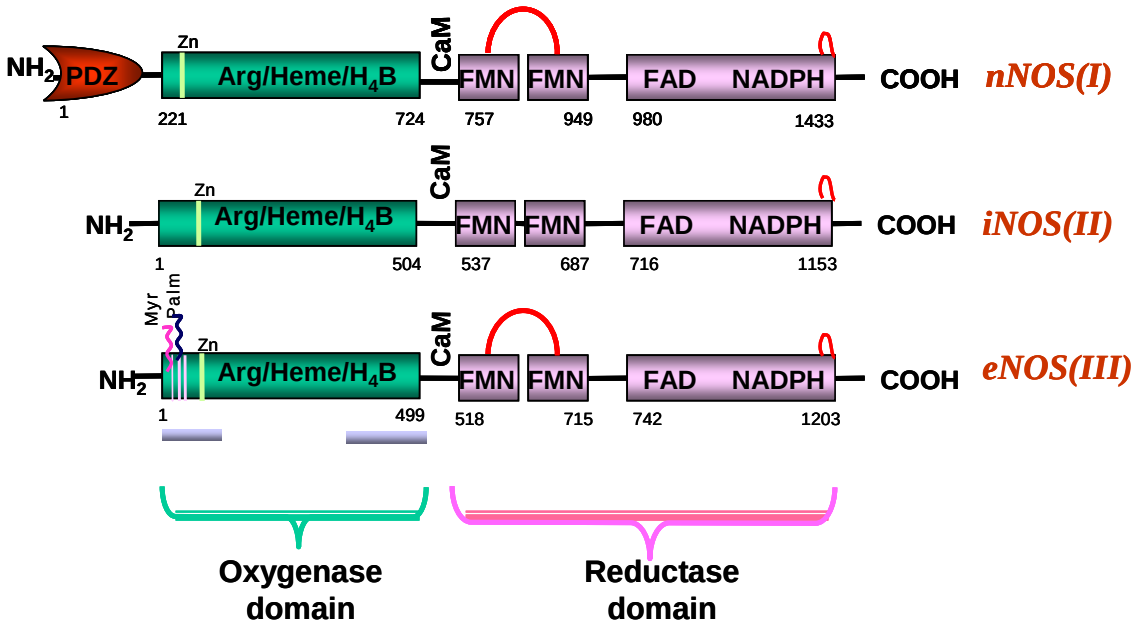

Figure 2. Domain structure of the human eNOS, nNOS and iNOS isoforms. *The PDZ, oxygenase and reductase domains are denoted by solid structures and the amino acid residue number at the start/end of each domain is shown. The myristoylation (Myr) G2 and palmitoylation (Palm) C15 and C26 sites on eNOS are shown, as is the location of the zinc-ligating cysteines (Zn). The autoinhibitory loops (red) within the FMN regions and the carboxyl end of nNOS and eNOS are also shown and the dimer interface in the oxygenase domain is indicated by grey bars. Abbreviations: PDZ, **P**ost Synaptic Density protein -95 **d**iscs large/**Z**O-1 homology domain; Arg, arginine; H4B, tetrahydrobiopterin; CaM, calmodulin; FMN, flavin mononucleotide; FAD, flavine- adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; Zn, zinc.*

Only nNOS and eNOS are constitutively expressed predominantly in neurons and skeletal muscle or endothelial cells, respectively. Both the isforms are activated by agonist-induced elevation of the intracellular free Ca^{2+} concentration with subsequent binding of Ca^{2+}/CaM to NOS (Venema *et al.*, 1996a), and hence are also referred to as calcium- dependent NOS enzymes. The constitutive isoforms also differ form iNOS in having an auto-inhibitory insert in FMN binding site in the reductase domain. In contrast, iNOS is regulated predominantly at the transcriptional level by endotoxins and cytokines in macrophages, hepatocytes, and vascular smooth muscle cells (Nathan, 1997), and binds CaM with high affinity rendering it active at the Ca^{2+} level of a resting cell (Ruan *et al.*, 1996) (Fig. 3A).

Additional regulatory mechanisms which have been proposed to regulate NOS include, protein-protein interactions, subcellular localisation of the enzymes, acylation, changes in L-arginine availability which is determined by arginase activity, phosphorylation (Fleming &

Busse, 2003; Michel & Feron, 1997), and enzyme monomerisation (Bommel *et al.*, 1998; Reif *et al.*, 1999).

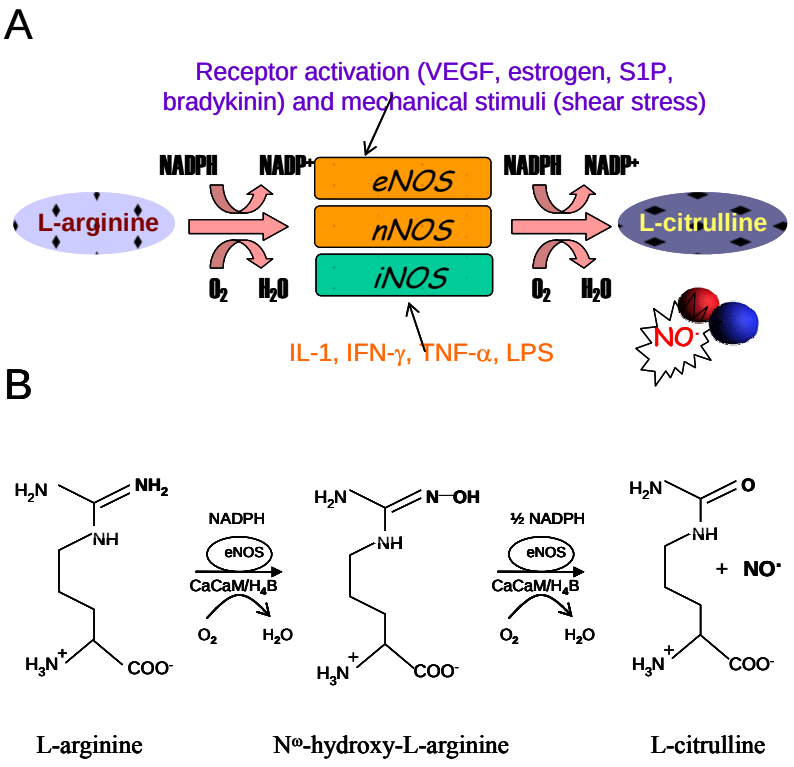

Figure 3. Activation of NOS and biosynthesis pathway of nitric oxide (NO) from L-arginine. (A) *Conversion of L-arginine to L-citrulline by the NOS isoforms eNOS, nNOS and iNOS. eNOS and nNOS are constitutively expressed, are Ca^{2+} dependent and activation is receptor-mediated, while iNOS is cytokine-inducible and is Ca^{2+} independent.* ***(B)*** *eNOS catalyzes a 5 electron oxidation of guanidino nitrogen of L-arginine to L-citrulline and NO·. N^{ω}-Hydroxy-L-arginine is formed as an intermediate that is tightly bound to the enzyme. The reaction consumes 2 mol of O2 and 1.5 mol of reduced nicotinamine adenine dinucleotide phosphate (NADPH) per mole of NO· formed and requiring calcium (Ca), calmodulin (CaM) and tetrahydrobiopterin (H_4B) as co-factors. Abbreviations: VEGF, vascular endothelial growth factor; S1P, spingosine-1-phosphate; IL-1, interleukin-1; gIFN, interferon-gamma; TNF, tumour necrosis factor; and LPS, lipopolysaccharide.*

1.6.1 Mechanism of NO biosynthesis

NOS hydroxylates a guanidino nitrogen of L-arginine and then oxidizes the N^{ω}-hydroxy-L-arginine intermediate (NOHA) to NO and L-citrulline (Fig. 3B). The NOS reductase (flavoprotein) domain first provides an electron (derived from NADPH) to the ferric heme in the oxygenase domain (Fig. 4). This is the slowest step of the biosynthetic reaction, and enables formation of a ferric heme-superoxy species (S I) in the L-arginine or NOHA reactions (Wei *et al.*, 2003c). Species I (S I) is not reactive toward L-arginine but may (Huang *et al.*, 2001) or may not (Wei *et al.*, 2003c) be reactive toward NOHA. Species I can receive an electron from H_4B (Wei *et al.*, 2003b) or from the flavoprotein domain when H_4B is absent

(Rusche *et al.*, 1998). The electron transfer from H_4B is the second, slowest step in the biosynthetic reaction and its kinetics are influenced by surrounding protein residues and by the pterin structure itself (Wang *et al.*, 2002; Wei *et al.*, 2003b). Timely electron transfer from H_4B prevents O_2^- release (Fig. 4). The heme-peroxo species (S II) has only been observed in NOS at cryogenic temperatures (Davydov *et al.*, 2002) and may become protonated and lose water to form a heme iron-oxo species (S III) (Fig. 4) that either hydroxylates L-arginine or may react directly with NOHA (Fig. 3). Importantly, the first product observed of NOS catalysis is a ferric heme-NO complex and not free NO (Negrerie *et al.*, 1999; Scheele *et al.*, 1999; Wei *et al.*, 2003c) (Fig. 4) as practically all NO binds to NOS ferric heme before exiting the enzyme. The reductase domain then reduces the ferric heme-NO complex to ferrous heme-NO species resulting in a slow release of NO.

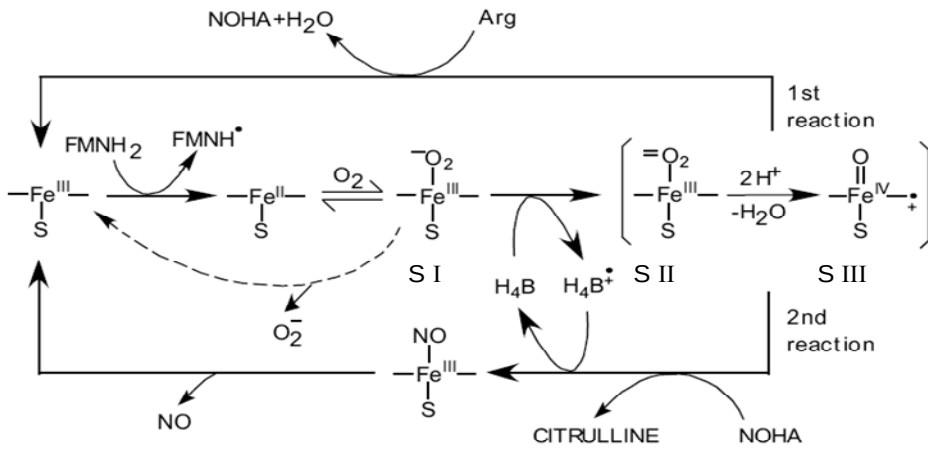

Figure 4. Mechanism for NO biosynthesis. *Mechanism of oxygen activation by the NOS heme and NO synthesis. The ferric heme first receives an electron from the FMN hydroquinone (FMNH2) that is located in the NOS flavoprotein domain. This enables dioxygen to bind, forming the ferric-superoxy species (S I). This species then receives an electron from tetrahydrobiopterin (H4B) to generate heme peroxo (S II) and perferryl (S III) species that are thought to react with L-arginine (1 reaction) or NOHA (2nd reaction) to generate NO. Modified from Stuehr et al J Biol Chem 2004; 279:36167-36170.*

After NO biosynthesis two different cycles compete: NO dissociation from the ferric heme (dissociation constant; kd) is required for a "productive cycle" that releases NO and is essential for NOS bioactivity. Conversely, reduction of the ferric heme-NO complex (reduction constant; kr') channels the enzyme into a "futile cycle" that ultimately generates nitrate in place of NO. NOS futile cycling is also influenced by the rate at which O_2 reacts with the ferrous heme-NO species (kox in Fig. 5). Together, the productive and futile cycles

create a global kinetic mechanism for NOS catalysis. A NOS must also control partitioning between both cycles by balancing heme reduction (kr, kr') and NO dissociation (kd) if it is to release the NO that it makes. For example, the specific activities of the three mammalian NOSs are of rank order iNOS > nNOS > eNOS, with nNOS activity being four times that of eNOS. But if one considers their actual rates of NO biosynthesis (the speed at which each NOS makes one NO) it is clear that nNOS is at least twice as fast as iNOS and about 30 times faster than eNOS (Stuehr *et al.*, 2004). This discrepancy is explained by the global kinetic model and the different enzyme distribution pattern of each of NOS in the steady state (Santolini *et al.*, 2001).

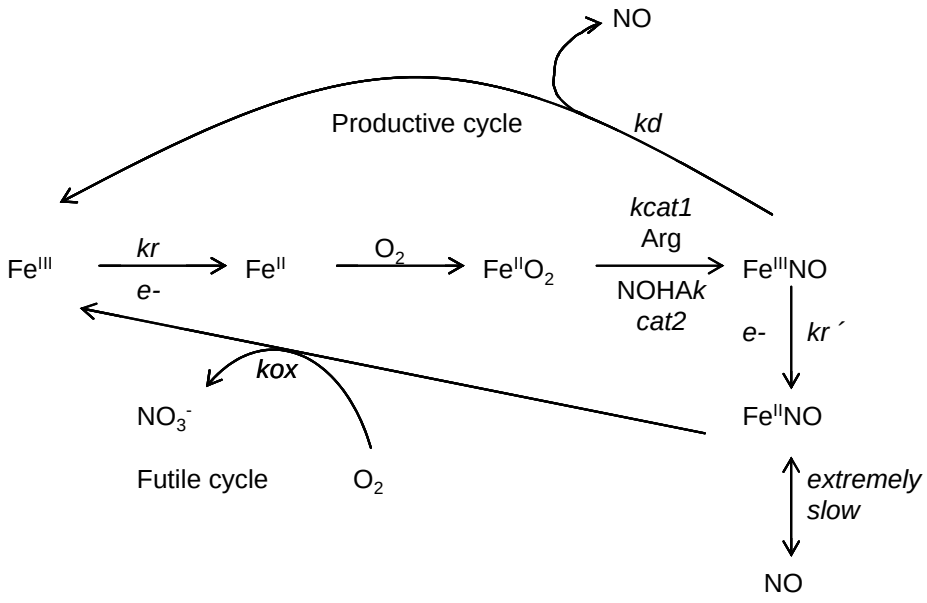

Figure.5. Global kinetic model for NOS. *Ferric enzyme reduction (kr) is rate-limiting for the biosynthetic reactions (central linear portion). kcat1 and kcat2 are the conversion rates of the FeIIO2 species to products in the L-arginine and NOHA reactions, respectively. The ferric heme-NO product complex (FeIIINO) can either release NO (kd) or become reduced (kr') to a ferrous heme-NO complex (FeIINO) which reacts with O2 (kox) to regenerate ferric enzyme. Modified from Stuehr et al J Biol Chem 2004;279:36167-36170.*

1.6.2 Substrate and cofactor requirement

All NOS enzymes require NADPH, FAD, FMN and H_4B as cofactors and L-arginine as substrate. Deficiency of L-arginine and H_4B has be reported for conditions of hypercholesterolemia, atherosclerosis and diabetes (Busse & Fleming, 1996; Stroes *et al.*, 1997).

H_4B

Tetrahydrobiopterin is a critical co-factor of NOS. It acts as a redox factor and also helps in the dimerisation of eNOS. H_4B deficiency leads to eNOS uncoupling, and H_4B supplementation restores endothelial function. During NO biosynthesis the delivery of the second electron to the heme has to be rapid enough for the enzyme to generate the heme-oxy species that will react with Arg or NOHA before auto-oxidation of ferric-superoxy species I occurs. NOS solves this dilemma by utilizing H_4B as a source of the second electron. H_4B delivers the second electron about 3 to 30 times faster than can the NOS reductase domain, and this difference is sufficient to minimise O_2^- release from the heme and so enable coupled oxygen activation (Wang *et al.*, 2002; Wei *et al.*, 2003a). H_4B exhibits a range of allosteric and structural effects, presumably resulting from binding of the pterin in the dimer interface in close proximity to the heme and substrate binding site (Crane *et al.*, 1998; Raman *et al.*, 1998).

1.7 Regulation of eNOS

Since eNOS is constitutively expressed its activity is regulated by complex post-translational modifications, protein-protein interactions, subcellular localisation, changes in substrate availability which is determined by arginase activity, phosphorylation (Fleming & Busse, 2003; Michel & Feron, 1997), and enzyme monomerisation (Bommel *et al.*, 1998; Reif *et al.*, 1999).

1.7.1 Intracellular localisation

Myristoylation and palmitoylation

Of the three NOS isoforms, only eNOS is both myristoylated and palmitoylated. eNOS is co-translationally and irreversibly myristoylated at the N-terminal glycine residue Gly^2, when the methionine corresponding to the translational initiation codon is removed by a specific aminopeptidase, exposing glycine at the N-terminus. The mutation of Gly^2 to Ala converts eNOS from a membrane- associated to a cytosolic enzyme (Pollock *et al.*, 1992; Sessa *et al.*, 1993), without affecting the activity of the enzyme. Myristoylation may not be the only mechanism responsible for the membrane attachment of eNOS. Indeed, many myristoylated proteins are cytosolic (Towler *et al.*, 1988). Purified wild-type eNOS binds to pure anionic phospholipid vesicles but not to neutral phospholipid vesicles, demonstrating that eNOS attachment to lipid bilayers requires electrostatic as well as hydrophobic interactions. Palmitoylation occurs post-translationally and reversibly at cysteine residues Cys^{15} and Cys^{26} (Prabhakar *et al.*, 2000). Dual acylation of eNOS is required for efficient localisation to the

plasmalemmal caveolae of endothelial cells (Garcia-Cardena *et al.*, 1996b). It has been suggested that palmitoylation is dynamically regulated by agonists such as bradykinin (Feron *et al.*, 1998). This is highly controversial as mutational studies to disrupt palmitoylation on eNOS did not significantly alter its membrane association. Additionally, [^{3}H] palmitic acid was not incorporated into nonmyristoylated mutant eNOS, suggesting that myristoylation is necessary for subsequent palmitoylation of the enzyme (Liu *et al.*, 1995). The available literature imply that palmitoylation does not play a major role in membrane association of eNOS.

The localisation of eNOS is well studied and described but controversy on the importance of changes of intracellular localisation still exists. eNOS is localised by virtue of its myristoylation and palmitoylation in the plasma membranes (Hecker *et al.*, 1994), caveolae (Garcia-Cardena *et al.*, 1996b; Liu *et al.*, 1996a), the perinuclear Golgi apparatus (Liu *et al.*, 1997; O'Brien *et al.*, 1995; Sessa *et al.*, 1995), and there are even some reports of eNOS in the nucleus (Feng *et al.*, 1999; Giordano *et al.*, 2002; McNaughton *et al.*, 2002). The immunostaining of native endothelial cells reveals predominant association with the plasma membrane and the Golgi apparatus (Fulton *et al.*, 2002). eNOS is also reported to shuttle between these two pools and although this was initially attributed to a rapid depalmitoylation of the enzyme (Robinson *et al.*, 1995), the exact mechanism(s) involved still remain to be clarified (Liu *et al.*, 1995). A fast translocation of eNOS from the vicinity of caveolin to other cell compartments in response to acute agonist stimulation has however been reported by several groups (Prabhakar *et al.*, 1998; Reiner *et al.*, 2001; Robinson *et al.*, 1995) and may be mediated by a dynamin-dependent process (Chatterjee *et al.*, 2003). Certainly, movement away from plasma membrane-bound signalling molecules may regulate NO output and the "mislocalisation" of eNOS has been suggested to contribute to the vascular complications associated with angiotensin II-induced hypertension (Gerzanich *et al.*, 2003). In which fraction eNOS is active in unstimulated cells and can account for the basal production of NO is also controversial, since the eNOS in caveolae is thought to be mostly inactive and disruption of the Golgi apparatus in rabbit carotid arteries failed to affect NO-mediated relaxation (Bauersachs *et al.*, 1997). Attenuating the association of eNOS with the plasma membrane by the disruption of caveolae using β-cyclodextrin, ox-LDL (Blair *et al.*, 1999; Feron *et al.*, 1999) or cyclosporin A (Lungu *et al.*, 2004) are all reported to affect the intracellular localisation of eNOS. However, these changes are generally associated with a decrease rather than an increase in NO production. It follows that any change in intracellular localisation of eNOS will be associated with changes in the eNOS signalling complex and

while caveolin-1 can associate with eNOS in caveolae, the two proteins are localised to distinct perinuclear compartments (Govers *et al.*, 2002b). Endothelial cells treated with nocodazole, a microtubule-depolymerising drug scattered the Golgi complex and caveolin-1 was found in vesicles at the periphery of the cell, while eNOS is localised at large structures near the nucleus suggesting that eNOS activity is not regulated by caveolin-1 in the Golgi complex (Govers *et al.*, 2002b). Recent studies to determine the intracellular site, which contains an active and agonist-stimulatable pool of eNOS, targeted eNOS to specific intracellular compartments. eNOS constructs that targeted to the plasma membrane were found to be constitutively active, phosphorylated and to respond to changes in Ca^{2+} but were largely unresponsive to activation by the kinase Akt, while eNOS in the Golgi complex was less sensitive to Ca^{2+} but sensitive to Akt-dependent phosphorylation (Fulton *et al.*, 2004). These and other observations suggest that the intracellular localisation of eNOS determines its sensitivity to changes in intracellular Ca^{2+} levels as well as its susceptibility to phosphorylation by different kinases (Gonzalez *et al.*, 2002).

1.7.2 Regulation by eNOS-associated proteins

It has been clear for quite a few years that eNOS is part of a protein complex with caveolin-1, CaM and heat shock protein 90 (Hsp90) and that the composition of the "eNOS signalosome" has profound effects on the intracellular localisation and activity of eNOS. Several of the eNOS associated proteins are kinases or phosphatases, which reflects the importance of phosphorylation in the regulation of eNOS activity.

Negative regulatory associated proteins

Caveolin

Caveolae are flask-shaped invaginations of the plasma membrane occupying up to 30% of cell surface in capillaries (Garcia-Cardena *et al.*, 1996b), but considerably less in cultured endothelial cells. They harbour a variety of signalling molecules such as the bradykinin 2 (B_2) receptor, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors, mitogen-activated protein (MAP) kinases, Src family non-receptor tyrosine kinases, G proteins, PKC, cationic arginine transporter-1; class B, type I scavenger receptor for high-density lipoprotein, H-Ras, Ca^{2+} ATPases and inositol 1,4,5 triphosphate-dependent Ca^{2+} channels, among others (Lisanti *et al.*, 1994; Liu *et al.*, 1996b; Parton, 1996). The chief structural components of caveolae are cholesterol and structural proteins, such as the caveolins (caveolin-1, -2 and -3). Caveolin-1 is abundant in endothelial cells and its hairpin structure can be attributed to the inclusion of a 33-residue membrane-spanning region

between the N- and C-terminal cytosolic domains. The scaffolding domain, a component of the C-terminal membrane proximal segment, (amino acids 61-101) is responsible for the presence of a number of signal proteins in caveolae. The incubation of eNOS with peptides derived from the scaffolding domains of caveolin-1 and -3 inhibits its enzymatic activity, an effect not observed following site-directed mutagenesis of the predicted caveolin binding motif (Garcia-Cardena *et al.*, 1997). A similar modulation of eNOS activity by this peptide has also been demonstrated *in vivo* and a chimeric peptide with a cellular internalisation sequence fused to the caveolin-1 scaffolding domain has been shown to selectively inhibit acetylcholine-induced, NO-mediated relaxation (Bucci *et al.*, 2000). That the association of eNOS with caveolins (Garcia-Cardena *et al.*, 1997) inhibits its activity can also account for the reports that the basal activity of eNOS is enhanced in mice deficient in either caveolin-1 or caveolin-3 (Drab *et al.*, 2001; Razani *et al.*, 2001).

G protein-coupled receptors

Multiple G protein-coupled receptors resident in caveolae have been reported to contribute to the eNOS-membrane complex and regulate eNOS activity. The intracellular domain (ID) of G-protein coupled receptors, such as ID4 of the bradykinin 2 (B_2), the angiotensin II R1 and the endothelin-1 ETB receptors can negatively regulate eNOS activity (Golser *et al.*, 2000; Ju *et al.*, 1998; Marrero *et al.*, 1999). Co-immunoprecipitation and in vitro binding assays have indicated that the bradykinin B_2 receptor, via its COOH-terminal ID4 (amino acids 310–329), interacts with eNOS in a ligand- and Ca^{2+}-dependent manner. On the basis of this evidence it was suggested that in the resting state, the receptor docks with eNOS in the caveolar membrane and participates in its inactivation. Stimulation of endothelial cells with bradykinin or Ca^{2+} ionophore would then trigger dissociation of the eNOS-B_2 receptor complex and activate eNOS (Ju *et al.*, 1998). The sites of binding to eNOS and the mechanisms by which the bradykinin B_2 receptor and caveolin-1 inhibit eNOS are suggested to be distinct (Ju *et al.*, 1998). Phosphorylation of serine or tyrosine residues in the eNOS-interacting region of the bradykinin B_2 receptor decreases the binding affinity of the receptor domain for the eNOS enzyme and relieves eNOS inhibition (Marrero *et al.*, 1999; Venema *et al.*, 1996b). Furthermore, bradykinin-induced tyrosine phosphorylation of the bradykinin B_2 receptor in cultured endothelial cells appears to promote a transient dissociation of eNOS from the receptor, accompanied by a transient increase in NO production. Mechanistically, the ID4 peptide has been shown to affect NOS catalysis by interference with the electron transfer from the flavins in the reductase domain to the heme in the oxygenase domain (Golser *et al.*, 2000; Marrero *et al.*, 1999). Indeed, G protein-coupled receptors have been suggested to participate

in the complex regulation of eNOS activity in endothelial cells and that these interactions are regulated by receptor phosphorylation (Marrero *et al.*, 1999).

Despite the claims of an interaction between eNOS and G protein-coupled receptors, perhaps the data need to be reassessed in more in vivo oriented models. Certainly, the bradykinin B_2 receptor, which has been the focus of much of this work, is not normally found in caveolae. However, the bradykinin B_2 receptor is sequestered into the caveolae in response to cell stimulation and leads to receptor desensitisation (Benzing *et al.*, 1999; Lamb *et al.*, 2001).

Moreover, there are vast differences in the characteristics of caveolae in native and primary cultures of endothelial cells. Since much of the work on the eNOS signalosome and caveolae has been obtained using multiple-passaged cells and overexpressing systems, it seems clear that some of the "physical interactions" reported in the literature actually reflect artefacts associated with in vitro models.

Positive regulatory associating proteins

Calmodulin

Calmodulin (CaM) is an ubiquitous, Ca^{2+}-binding protein and was the first protein shown to be involved in eNOS regulation (Busse & Mülsch, 1990; Forstermann *et al.*, 1991). Mechanistically, CaM binding to a 32 residue CaM binding motif can displace an adjacent autoinhibitory loop (Salerno *et al.*, 1997) located in the FMN domain on eNOS leading to conformational changes (Daff *et al.*, 2001; Siddhanta *et al.*, 1996), thus facilitating NADPH-dependent electron flux from the reductase domain of the protein through to the terminal electron acceptor heme in the oxygenase domain (Abu-Soud *et al.*, 1994). The association of CaM with the CaM-binding domain within eNOS is determined by multiple molecular interactions (Knudsen *et al.*, 2003; Salerno *et al.*, 1997) as well as by the phosphorylation/dephosphorylation of Thr^{495} (Aoyagi *et al.*, 2003; Fleming *et al.*, 2001). However, other modifications such as the binding of Hsp90 and the phosphorylation of Ser^{1177} have also been reported to affect the association of the two proteins (Gratton *et al.*, 2000). More recently the phosphorylation of CaM by the kinase CK2 was found to attenuate its ability to activate eNOS (Greif *et al.*, 2004). Deletion of CaM binding domain converts eNOS from a membrane-bound to a cytosolic protein when the enzyme is expressed in Sf9 cells (Venema *et al.*, 1995).

Heat shock protein 90 (Hsp90)

Hsp90 is a highly abundant cytosolic protein known to serve as a molecular chaperone and is responsible for the folding of proteins such as steroid receptors and cell cycle-dependent kinases (Caplan, 1999). Hsp90 is involved in the folding of nNOS and is reported to determine the insertion of heme into the immature protein (Billecke *et al.*, 2002). In addition to this function, Hsp90 can also act as an integral part of numerous signal transduction cascades by virtue of its function as a scaffolding molecule.

Hsp90 can associate with eNOS in resting endothelial cells and endothelial cell stimulation with vascular endothelial growth factor (VEGF), histamine, fluid shear stress, and estrogen all enhance the interaction between Hsp90 and eNOS at the same time as increasing NO production (Garcia-Cardena *et al.*, 1998; Russell *et al.*, 2000). The association of Hsp90 with eNOS appears to be determined by the agonist-stimulated tyrosine phosphorylation of Hsp90 (Harris *et al.*, 2000), but it is not clear whether this modification is also required for the recruitment of other proteins to the signalosome. Most of the kinases shown to phosphorylate eNOS on serine or threonine residues physically associate with the enzyme, either directly or via binding to Hsp90 (Fontana *et al.*, 2002). Hsp90 also interacts with other chaperones and the Hsp90 co-factor; carboxyl terminus of Hsp70-interacting protein (CHIP), is reported to be part of the eNOS complex and to play a role in determining its intracellular localisation (Jiang *et al.*, 2003). An enhanced association of eNOS and Hsp90 is thought to underlie adiponectin-induced protection against angiotensin II- (Lin *et al.*, 2004) and glucose-induced apoptosis (Lin *et al.*, 2005) while an impaired association of eNOS with Hsp90 has been associated with various forms of hypertension (Murata *et al.*, 2002; Shah *et al.*, 1999).

Soluble guanylyl cyclase (sGC)

The soluble guanylyl cyclase is the primary intracellular receptor for NO and was, until relatively recently, assumed to be a cytosolic enzyme. It now appears that a small proportion of the "soluble" guanylyl cyclase can become membrane-associated in a stimulus-dependent manner (Zabel *et al.*, 1999). This translocation would bring eNOS and the sGC closer together, thereby increasing the effectiveness of NO signaling and reducing the possibility of inactivation of NO by intracellular O_2^-. Not all the groups that have addressed this aspect of NO signaling have found any evidence suggesting a direct association between the eNOS and the sGC, however, the β subunit of sGC has been reported to associate with Hsp90 after agonist stimulation and therefore form part of the eNOS signalosome (Venema *et al.*, 2003) (Fig. 6).

The list of eNOS-associated proteins is continually increasing, with recent additions being polymerised actin (Su *et al.*, 2003), platelet endothelial cell adhesion molecule 1 (PECAM-1) (Govers *et al.*, 2002a) and the voltage-dependent anion channel 1, VDAC1 or porin (Sun & Liao, 2002).

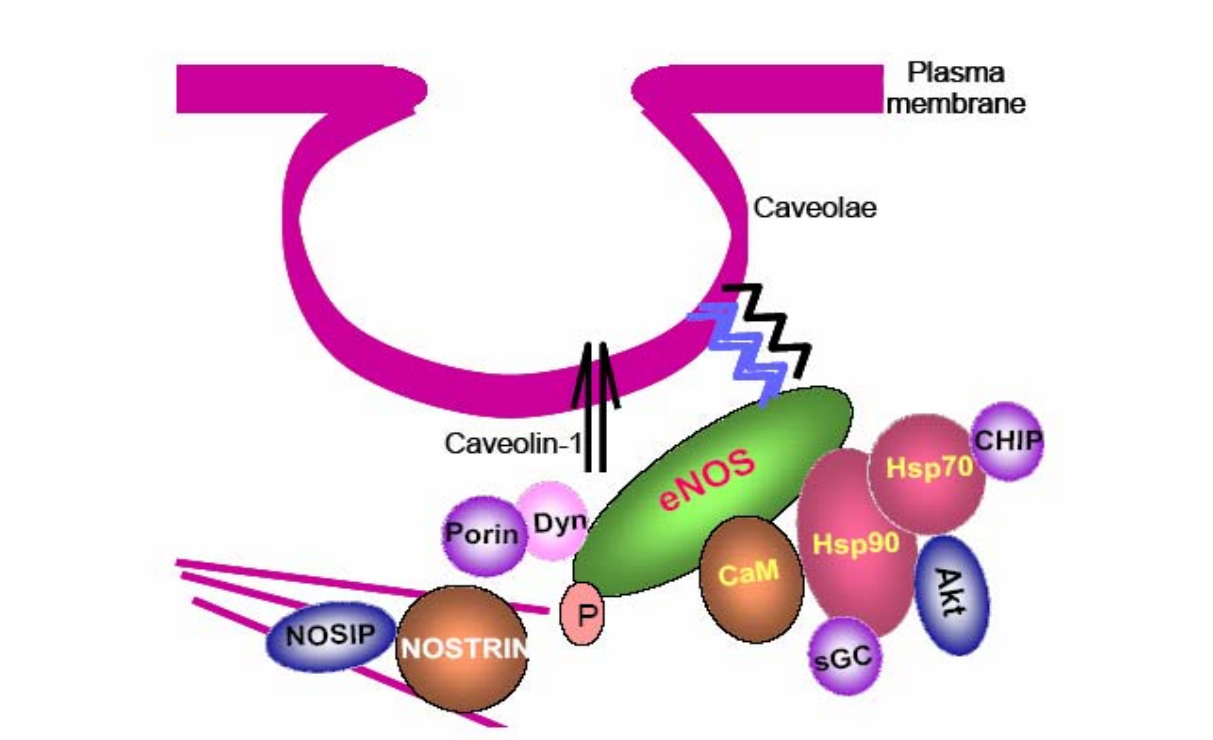

Figure 6. Protein interaction of eNOS at caveolae. *Myristoylation and palmitoylation of eNOS target it to the plasma membrane of caveolae. Interaction with caveolin inhibits eNOS activity where as stimulation promotes recruitment of Hsp90, Akt, dynamin, porin and Ca2+/CaM to eNOS increasing its activity. The interaction with CHIP, NOSTRIN and NOSIP seems to decrease the enzyme activity, while association of sGC has been reported to increase the effectiveness of NO signalling. Abbreviation: Hsp90, heat shock protein 90; Akt, protein kinase B; Dyn, dynamin; CaM, calmodulin; CHIP, carboxyl terminus of Hsp70-interacting protein; NOSTRIN, eNOS traffic inducer; NOSIP, eNOS interacting protein and sGC, soluble guanylyl cyclase.*

Interactions between caveolin, CaM, and Hsp90.

CaM has been proposed to be exclusively responsible for the dissociation of eNOS from caveolin. Immunoprecipitation studies demonstrated the presence of eNOS, caveolin-1, and Hsp90 in the same complex (Gratton *et al.*, 2000). Moreover, the addition of exogenous CaM weakly displaced caveolin from CaM. Interestingly, the binding of caveolin-1 to eNOS was displaced by the caveolin-1 scaffolding domain peptide, but not by Ca^{2+}-activated CaM, demonstrating that CaM cannot physically disrupt the eNOS-caveolin-1 complex in vitro. Reconstitution of the heterotrimeric complex in vitro showed eNOS interaction with both Hsp90 and caveolin-1, but the latter proteins did not interact with each other, demonstrating

that eNOS was the bridge holding the complex together. However, Hsp90, per se, did not influence the eNOS/caveolin-1 interaction but facilitated the ability of CaM to displace caveolin-1 from eNOS. It has been proposed that perhaps the "recruitment or activation" of Hsp90 and CaM to eNOS results in weak physical displacement of eNOS from caveolin-1, but the complex remains in caveolae; or Hsp90 and Ca^{2+}-activated CaM coexist with eNOS bound to caveolin-1, and a slight change in eNOS conformation in the absence of bulk translocation away from caveolin-1, allows for efficient stimulus-response coupling (Fulton *et al.*, 2001).

NOS trafficking

Proper cellular localisation of eNOS is critical for optimal coupling of extracellular stimulation with NO production (Sakoda *et al.*, 1995; Sessa *et al.*, 1995). It has been suggested that redistribution of eNOS is one of the events flowing its activation. However, demonstrations that NO production is a more rapid process than enzyme relocation suggest that intracellular eNOS traffic plays a role in termination rather than initiating NO release. To ensure that eNOS is at the right place at the right time, there have to be protein-protein interactions that could help in the relocation of eNOS. Some of the proteins which have come to light and which can alter the intracellular compartmentalisation of eNOS are dynamin-2, NOSIP, NOSTRIN, and CHIP.

Dynamin-2

Dynamin-2 belongs to the family of large GTPases and is believed to be involved in vesicle formation, receptor-mediated endocytosis, and the internalisation of caveolae and vesicle trafficking in and out of the Golgi. Dynamin-2 has been shown by confocal microscopy to colocalise with eNOS in the plasma membrane and Golgi membranes of endothelial cells and to bind eNOS directly, both in vivo and in vitro (Cao *et al.*, 2001). Specifically dynamin-2 associates with the reductase domain of eNOS resulting in an increased flow of electrons from FAD to FMN, thereby increasing the enzyme activity (Cao *et al.*, 2003; Cao *et al.*, 2001). Interfering with the activity of dynamin-2 appears to deplete eNOS from caveolae (Chatterjee *et al.*, 2003) in response to stimuli such as braykinin. In its function as a motor protein dynamin-2 has been hypothesised to shuttle eNOS between caveolae and the Golgi. However, it remains to be determined whether the dynamin-2-associated changes in the subcellular localisation or its direct binding to the eNOS protein exert the most pronounced effects on enzyme activity.

NOSIP

NOSIP (eNOS interacting protein) is a 34-kDa protein, and is one of the more recent additions to the eNOS signalosome. Cotransfection of NOSIP in cells expressing eNOS has two major consequences, i.e., the displacement of eNOS from the plasma membrane and its relocation to intracellular compartments, and a decrease in eNOS activity (Dedio *et al.*, 2001). The interaction between NOSIP and eNOS has been shown both in vitro and in vivo, and through deletion analysis, NOSIP was shown to bind eNOS between amino acids 366 and 486. Stimulation of cells with calcium ionophore does not change the association of NOSIP and eNOS; however, a peptide derived from the scaffolding domain of caveolin (82–101) is able to displace eNOS from NOSIP. NOSIP does not affect eNOS activity assays in vitro but, when coexpressed in CHO cells, is reported to reduce ionomycin-stimulated NO release (Nedvetsky *et al.*, 2002). NOSIP and eNOS are reported to be coexpressed in distinct cell types of the lung and the trachea, suggesting a functional role for NOSIP in regulating NO synthesis and delivery in the airway system (Konig *et al.*, 2005). However physiological evidence for the interaction between eNOS and NOSIP is lacking.

NOSTRIN

NOSTRIN (eNOS traffic inducer), overexpressed in CHO cells, binds to eNOS (also overexpressed) and results in the relocation of eNOS from the plasma membrane and Golgi membranes to vesicle-like structures distributed throughout the cytosol (Zimmermann *et al.*, 2002). The translocation of eNOS, thereby strongly attenuates eNOS-dependent NO production. Inhibition of NO production in NOSTRIN- overexpressing cells may be the consequence of this redistribution of eNOS. Together with NOSIP, NOSTRIN seems to be a promising candidate for regulating the intracellular trafficking of eNOS and perhaps other proteins in the plasma membrane.

CHIP

CHIP (carboxyl terminus of Hsp70-interacting protein), a molecular chaperone remodels the Hsp90 heterocomplex (Connell *et al.*, 2001) and causes protein degradation of some Hsp90 substrates through the ubiquitin-protein isopeptide ligase activity of CHIP. In contrast, in transiently transfected COS cells, CHIP was found to incorporate into the eNOS·Hsp90 complex and specifically decrease soluble eNOS levels. CHIP elicited the partitioning of eNOS to the detergent- insoluble cell compartment and impaired trafficking through the Golgi apparatus, which is otherwise required for trafficking of eNOS to the plasmalemma and

subsequent activation (Jiang *et al.*, 2003). Taken together, it seems that CHIP may be a negative regulator of eNOS activity.

1.7.3 Regulation by phosphorylation

Phosphorylation is one of the post-translational mechanisms employed by the cellular machinery to regulate the activity of its enzymes. As eNOS is constitutively expressed, phosphorylation plays an important role in regulating the activity of the enzyme. eNOS can be phosphorylated on serine, threonine and tyrosine residues (Boo & Jo, 2003; Fleming *et al.*, 1998; Fleming & Busse, 2003) (Fig. 7A), leading to eNOS activation or inactivation. There are numerous putative phosphorylation sites, but the most extensively studied eNOS residues; the phospho-status of which determines enzyme activity are a serine residue (human eNOS sequence: Ser^{1177}; bovine sequence Ser^{1179}) in the reductase domain, which positively regulates NO production, and a threonine residue (human eNOS sequence: Thr^{495}; bovine sequence Thr^{497}) within the CaM-binding domain (Fig. 8). The various kinases reported to be involved in the phosphorylation of eNOS following cell activation by different stimuli are shown in figure 9.

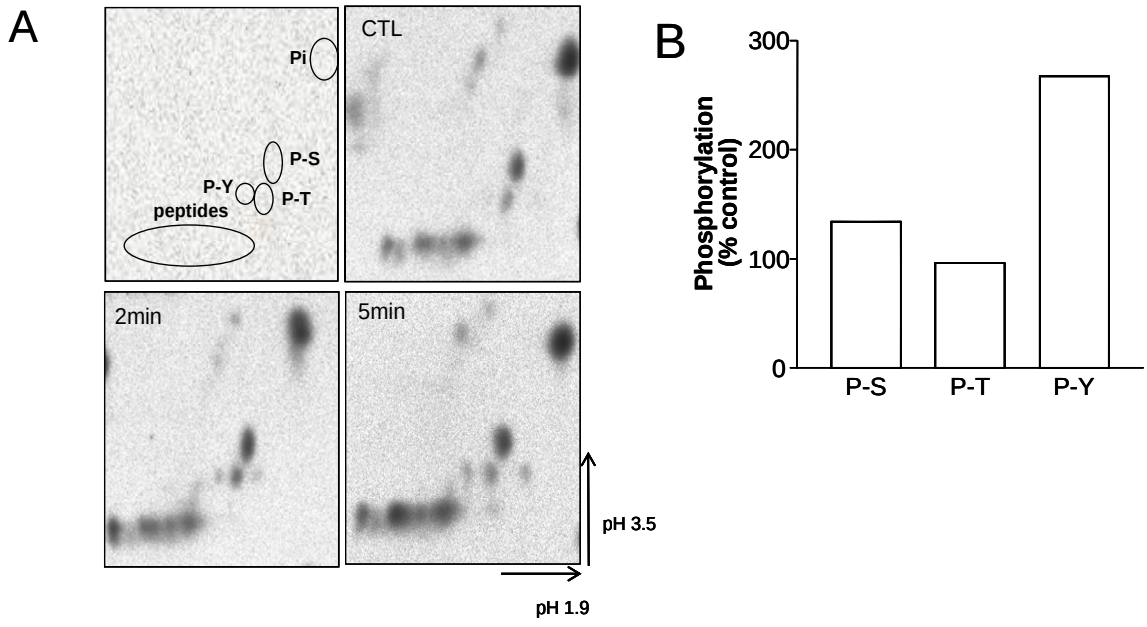

Figure 7. Effect of fluid shear stress on serine, threonine and tyrosine phosphorylation of eNOS. ***(A)*** *eNOS was immunoprecipated from ^{32}P-labelled porcine aortic endothelial cells either maintained under static condition or exposed to shear stress (12 dynes cm-², for 2 and 5 min), and subjected to two dimensional phospho amino acid analysis.* ***(B)*** *Densitometric analysis of phosphoserine, P-S; phosphothreonine, P-T; and phosphotyrosine P-Y with respect to control. Pi, inorganic phosphate (from Fisslthaler et al, Acta Physiol Scand.2000; 168: 81– 88).*

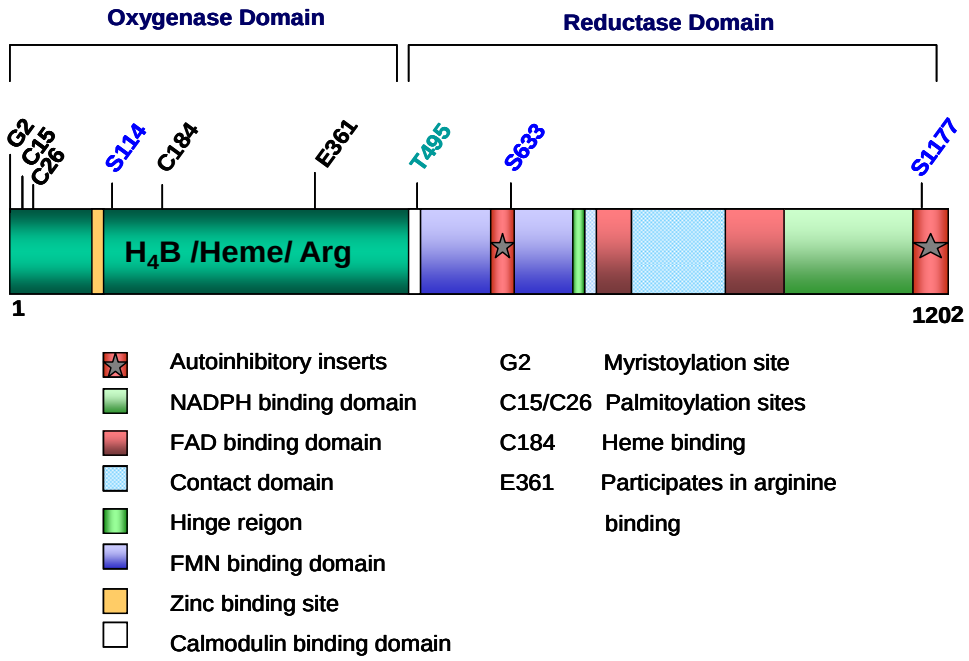

Figure 8. Scheme showing eNOS (human sequence) ligand binding domain and potentially phosphorylable amino acids. *Phosphorylation of the serine residues, Ser^{1177} and Ser^{633} in the reductase domain positively regulate enzymatic activity, while phosphorylation at threonine Thr^{495}, in the Ca/CaM binding domain and Ser^{114} near the Zn^{2+} binding domain lead to a decrease in NO production.*

Ser^{1177}

The C-terminal reductase domain of eNOS terminates in a α-helix which contains a terminal Ser^{1177}. In unstimulated, cultured endothelial cells, Ser^{1177} is not phosphorylated but is rapidly phosphorylated upon agonist stimulation with compounds such as bradykinin (Fleming *et al.*, 2001), estrogen (Haynes *et al.*, 2000), VEGF (Fulton *et al.*, 1999; Michell *et al.*, 2001) or insulin (Kim *et al.*, 2001) and mechanical stimuli such as fluid shear stress (Boo & Jo, 2003; Dimmeler *et al.*, 1999; Fissithaler *et al.*, 2000; Gallis *et al.*, 1999). The phosphorylation of eNOS Ser^{1177} enhances the activity of the enzyme and alters its Ca^{2+} sensitivity to increase NO production 2- to 3-fold above basal levels, an effect that can be attributed to an increase in the flux of electrons through the reductase domain (McCabe *et al.*, 2000). Mutation of this residue to a phosphomimetic aspartate (Dimmeler *et al.*, 1999) or deletion of 27 amino acids containing Ser^{1177} at the C-terminal tail (Lane & Gross, 2002) renders the mutant eNOS active at resting levels of intracellular Ca^{2+}.

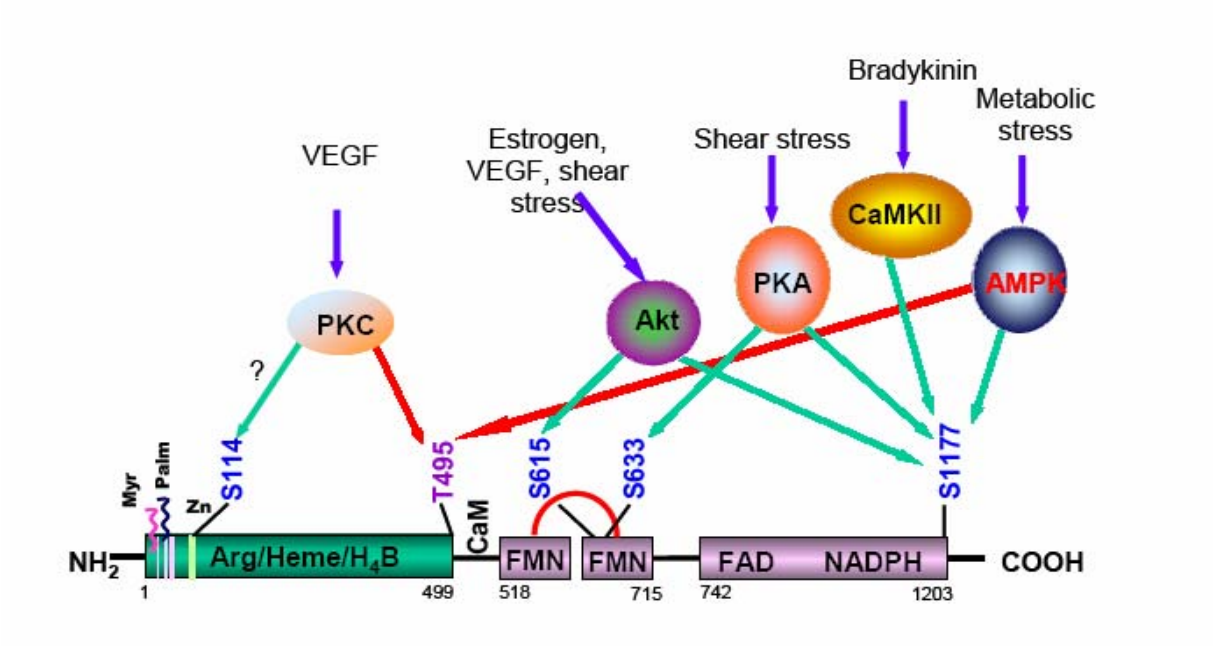

Figure 9. Kinases involved in the phosphorylation of known amino acid residues influencing eNOS activity. *Physiological stimuli such as VEGF, S1P, estrogen, bradykinin and fluid shear stress can activate various signalling pathways leading to the activation of eNOS. Ser^{1177} is known to be phosphorylated by Akt, PKA, CaMKII, ERK1/2, PKG and AMPK, Ser^{615} by Akt and Ser^{633} by PKA. Phosphorylation on Thr^{495} and Ser^{114} is mediated by PKC. Abbreviations: VEGF, vascular endothelial growth factor; S1P, sphingosine 1-phosphate; PKA, protein kinase A; Akt, protein kinase B; CaMKII, CaM kinaseII; PKC, protein kinase C; AMPK, AMP activated protein kinase; PKG, cGMP-dependent protein kinase; ERK1/2; extracellular signal-regulated kinase 1/2.*

Based on the stimuli applied, various kinases are involved in the phosphorylation of Ser^{1177}. For example, while shear stress elicits the phosphorylation of Ser^{1177} by activating Akt and PKA, insulin, estrogen and VEGF mainly phosphorylate eNOS in endothelial cells via Akt. The bradykinin-, Ca^{2+} ionophore- and thapsigargin-induced phosphorylation of Ser^{1177}, on the other hand, is mediated by CaMKII (Fleming *et al.*, 2001; Schneider *et al.*, 2003). Recent biochemical and crystallography data on the structure of the reductase domain of NOS has explained the mechanism involved in the inhibitory effect of Ser^{1177}. At the end of the C-terminal-helix, the phosphorylatable Ser^{1177} is directed toward negatively charged FMN-binding domain residues Gln^{685} (Gln in eNOS) and Asp^{587}. This structure thus suggests a mechanism for phosphorylation-induced NOS activation by electrostatically- induced conformational changes (Garcin *et al.*, 2004).

Ser^{615}

This phosphorylation site is located within the autoinhibitory loop in the FMN binding domain. It was identified by phosphopeptide mapping and is reported to be phosphorylated by both PKA and Akt. Mimicking phosphorylation at Ser^{615} significantly increases the Ca^{2+}-

calmodulin sensitivity of eNOS but is not reported to alter maximal enzyme activity (Michell *et al.*, 2002). However, Ser^{615} may be important in regulating phosphorylation at other sites as well as protein-protein interactions and the assembly of the eNOS signalosome (Bauer *et al.*, 2003).

Ser^{633}

Ser^{633} is located within the auto-inhibitory loop in the FMN binding domain. This loop is thought to interact with the CaM binding domain and to interrupt the binding of CaM, thus throttling enzyme activity. Although Ser^{633} can be phosphorylated *in vitro* by PKA and PKG (Butt *et al.*, 2000), the functional relevance of this observation was unclear and the limited experimental studies which initially compared the potential of phosphorylation on Ser^{1177} versus Ser^{633} in regulating eNOS activity, concluded that Ser^{1177} played a major role in the regulation of NO production while either no Ser^{633} phosphorylation could be detected or no consequence of phosphorylation was evident (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999). More recently it has been shown that Ser^{633} is most probably phosphorylated *in vivo* by PKA following cell stimulation by fluid shear stress, VEGF, bradykinin and 8-bromo-cAMP albeit with a slower time course of phosphorylation than that detected on Ser^{1177} and Thr^{495} (Boo *et al.*, 2002; Michell *et al.*, 2002).

Ser^{114}

This residue is located in the oxygenase domain in close vicinity to Zn (Raman *et al.*, 1998) and H_4B (Li *et al.*, 1999) binding sites. Although, bradykinin, lysophosphatidic acid (Kou *et al.*, 2001) and fluid shear stress (Gallis *et al.*, 1999) were initially reported to enhance Ser^{114} phosphorylation but the consequences of Ser^{114} phosphorylation on endothelial NO production remains to be elucidated.

Thr^{495}

The Thr^{495} was the first residue to be identified, which negatively regulates eNOS. Under basal conditions in all of the endothelial cells investigated to-date, Thr^{495} is constitutively phosphorylated resulting in a decrease in NO production (Fleming *et al.*, 2001; Harris *et al.*, 2001; Michell *et al.*, 2001). The link between phosphorylation and NO production can be explained by interference with the binding of CaM to the CaM-binding domain. Stimulation of endothelial cells with agonists, such as bradykinin, histamine or a Ca^{2+} ionophore, results in a transient dephosphorylation of Thr^{495} which facilitates the binding of CaM to eNOS (Fleming *et al.*, 2001). Crystallographic analysis of CaM bound eNOS indicates that the phosphorylation of eNOS Thr^{495} not only causes electrostatic repulsion of nearby glutamate

residues (Glu^{7} and Glu^{127}) within CaM but may also affect Glu^{498} within eNOS and thus induce a conformational change within the enzyme itself (Aoyagi *et al.*, 2003). There is a good correlation between Thr^{495} dephosphorylation, Ser^{1177} phosphorylation and NO production when bradykinin is used as an agonist for NO production, suggesting that Thr^{495} dephosphorylation is sufficient for eNOS activation(Harris *et al.*, 2001; Michell *et al.*, 2001; Michell *et al.*, 2002) (Fig. 10). Recently the dephosphorylation of Thr^{495} has been linked to the production of O_2^- by eNOS (Lin *et al.*, 2003), however it remains to be determined whether this occurs *in vivo* and whether or not the actual cause of the uncoupling is a decrease in H_4B and/or L-arginine availability as a consequence of prolonged activation of the enzyme.

The constitutively active kinase which phosphorylates eNOS Thr^{495} is most probably PKC (Fleming *et al.*, 2001; Matsubara *et al.*, 1996; Michell *et al.*, 2001), a finding which could account for the fact that protein kinase inhibitors and the down-regulation of PKC markedly increase endothelial NO production (Davda *et al.*, 1994; Hirata *et al.*, 1995). On the other hand amlodipine, which inhibits PKC activity in endothelial cells, is able to enhance NO production by attenuating eNOS Thr^{495} phosphorylation (Lenasi *et al.*, 2003). It is not entirely clear which PKC isoform phosphorylates eNOS in native endothelial cells. Clearly the PKC in question should form part of the eNOS signalosome in unstimulated cells and be constitutively active as the PKC phosphorylation site is phosphorylated in unstimulated endothelial cells. To-date, PKC-α (Fleming *et al.*, 2005), PKC-β and PKC-ε (Zhang *et al.*, 2005) have all been implicated in this process.

PKC-β has received a lot of attention as the inhibition of this isoform is reported to prevent the hyperglycemia-induced attenuation of NO production (Chu & Bohlen, 2004), and to reverse endothelial function in animal models of diabetes (Hink *et al.*, 2001) as well as in human subjects with type 2 diabetes (Beckman *et al.*, 2002; Guzik *et al.*, 2002). However, it remains to be determined whether the beneficial effects of PKC-β inhibition can be attributed to a direct effect on eNOS, to the generation of oxygen-derived free radicals or even to the generation of a vasoconstrictor compound (Cosentino *et al.*, 2003; Lagaud *et al.*, 2001).

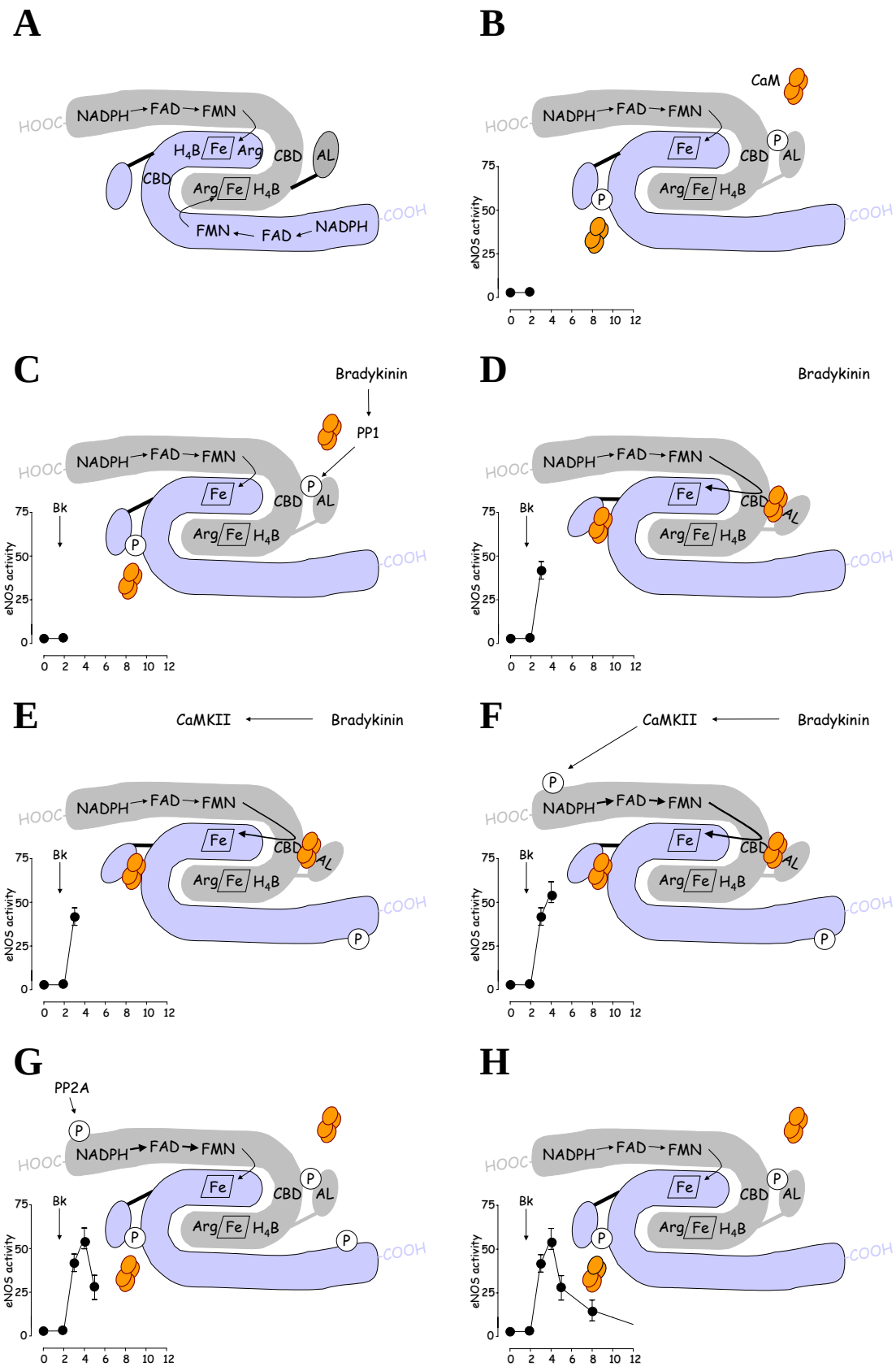
A
B
C
D
E
F
G
H
NADPH
FAD
FMN
Fe
CBD
AL
Arg
H_4B
HOOC
COOH
CaM
Bradykinin
PP1
CaMKII
PP2A
Bk
P
eNOS activity
0
25
50
75
0 2 4 6 8 10 12

Figure 10. Hypothesis for the activation of eNOS in response to the Ca^{2+}- elevating agonist bradykinin. (A) *eNOS dimer showing electron flux between the two monomers and the phenomenon of domain swapping.* ***(B)*** *Under basal (unstimulated) conditions eNOS produces low amounts of NO· (see inset) but the binding of CaM to the enzyme is prevented by phosphorylation of Thr^{495} in the CaM binding domain (CBD).* ***(C)*** *In response to cell stimulation with bradykinin (Bk) there is an increase in $[Ca^{2+}]i$ and PP1 is activated to dephosphorylate Thr^{495}.* ***(D)*** *CaM can now bind to the CBD and NO· production is increased markedly over basal levels.* ***(E)*** *At the same time, CaMKII is activated and phosphorylates Ser^{1177}, which further enhances electron flux through the reductase domain and enzyme activity reaches a peak* ***(F)***. ***(G)*** *Dephoshorylation of Thr^{495} is transient and this residue is rapidly rephosphorylated after a decrease in $[Ca^{2+}]i$, most probably by PKC. CaM dissociates and enzyme activity decreases. PP2A dephosphorylates Ser^{1177} and NO· output returns to basal levels* ***(H)***. *AL, auto-inhibitory loop, (from Fleming and Busse, Am.J.Physiol.Regul.Integr.Comp.Physiol.2003; 284: R1–R12).*

Elucidating the consequences of eNOS phosphorylation on the sites identified to-date is complicated by the fact that "cooperation between multiple phosphorylation events" has been described and the mutation of the serine phosphorylation sites 114, 615, and 1177 to alanine affects the phosphorylation state of at least one other site (Bauer *et al.*, 2003).

Tyrosine

Treatment of endothelial cells with inhibitors of tyrosine kinases as well as tyrosine phosphatases modulates the tyrosine phosphorylation of eNOS and endothelial NO production (Fleming *et al.*, 1996a; Fleming *et al.*, 1998; Takenouchi *et al.*, 2004). There is almost nothing is known about the residues which are phosphorylated or the kinases which are involved. Elaborating the functional consequences of eNOS tyrosine phosphorylation is complicated by the fact that this modification is only evident in primary cultured cells (Garcia-Cardena *et al.*, 1996a) or in cells that overexpress tyrosine kinases such as Src (Takenouchi *et al.*, 2004) but not with passaged endothelial cells (Corson *et al.*, 1996; Michel *et al.*, 1993; Venema *et al.*, 1996b). The consequences of the tyrosine phosphorylation of eNOS are unknown but are perhaps more likely to be related to the docking of associated scaffolding and regulatory proteins than to a direct effect on eNOS activity. The use of tyrosine kinase inhibitors provides only limited information on the role played by tyrosine phosphorylation in the regulation of endothelial NO production as many of these compounds directly affect Ca^{2+}-signalling processes (Fleming *et al.*, 1996b; Fleming & Busse, 1997) and/or the activity of Akt and the binding of Hsp90 (Papapetropoulos *et al.*, 2004).

1.7.4 NOS reductase domain regulatory elements

Recent structural and molecular modeling studies of the NOS reductase domain structure have provided novel insight into the molecular mechanisms that control both the intra-module electron transfer from NADPH to the flavins and the inter-module electron transfer from FMN to heme.

FMN domain auto-inhibitory insert

One of the main structural differences between the constitutive and inducible isoforms is an auto-inhibitory insert of 40-50 amino acids in the FMN-binding domain of the eNOS and nNOS which interfere with CaM binding (Daff, 2003; Salerno *et al.*, 1997). Deletion of the inserts results in mutant enzymes which bind CaM at lower Ca^{2+} concentrations and which retain activity in the absence of CaM (Daff *et al.*, 2001). Two additional roles for the auto-inhibitory insert in NOS regulation have been hypothesised: (i) that auto-inhibitory insert binds CaM and acts as a "mop" that inhibits CaM binding to the CaM- binding linker, (ii) that interaction of the auto-inhibitory insert with the FMN and the NADPH binding domains contributes to the locked electron-accepting position of the FMN domain (Garcin *et al.*, 2004). However, at elevated concentration of Ca^{2+}, CaM would bind to both the auto-inhibitory insert and CaM-binding regions and release the FMN domain for the inter-module electron transfer.

Regulatory C-terminal tail

The NOS reductase domain ends in a C-terminal tail. It has a conserved Arg^{1165} residue (eNOS), and a terminal phosphorylatable Ser^{1177} (eNOS) residue. The Ser^{1177} at the end of the C-terminal α-helix is directed towards negatively charged FMN-binding domain residues Gln^{685} and Asp^{687} and locks the FMN binding domain in an electron accepting position (Fig. 11) and prohibits intermodule electron transfer (Craig *et al.*, 2002; Garcin *et al.*, 2004). In the absence of CaM, ionic interaction between NADPH and Arg^{1165} residue (eNOS) orient the C-terminal tail in a negatively charged groove at the eNOS reductase domain, further stabilising the FMN lock (Daff, 2003). Thus, Ser^{1177} phosphorylation, or mutation to Asp to mimic phosphoserine would induce a negative charge and repel negatively charged residues thereby destabilise and displace the regulatory C-terminal tail to relieve repression of NO synthesis.

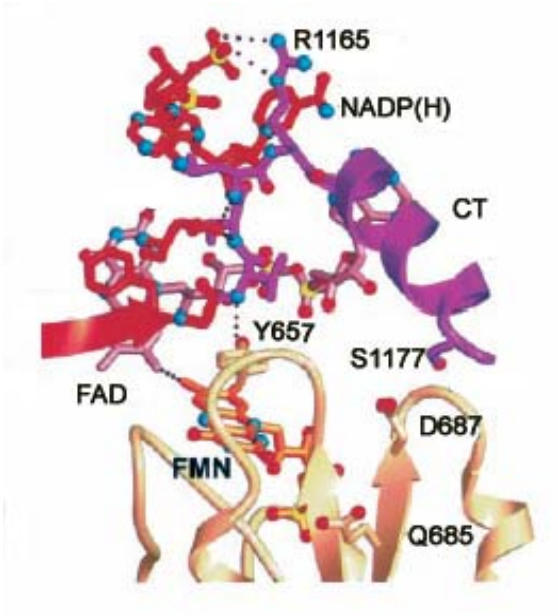

Figure 11. Regulatory action of eNOS C-terminal tail. *Structure showing the interaction of the C-terminal tail of eNOS reductase domain with other residues leading to the locking of the FMN in the electron accepting position (see text for details). Modified from Garcin et.al, J Biol Chem 2004;279:37918-37927.*

1.8 NOS uncoupling

All of the NOS isoforms generate O_2^- and hydrogen peroxide (H_2O_2) under specific conditions i.e., lower than optimal concentrations of the essential co-factor H_4B (Xia *et al.*, 1998) or the substrate L-arginine (Xia *et al.*, 1996). It is not clear yet whether H_2O_2 is a genuine product of uncoupling NOS catalysis or if H_2O_2 derives exclusively from the dismutation of O_2^- (Vasquez-Vivar *et al.*, 1999; Wei *et al.*, 2001).

The lack of H_4B results in the uncoupling of eNOS, which basically means that the transport of electrons to ferrous-heme-O_2 species generated during the stepwise activation of O_2 by NOS, does not occur fast enough to prevent their oxidative decay; the result being the generation of reactive oxygen species (ROS) (Stuehr *et al.*, 2001; Stuehr *et al.*, 2004). The enhanced generation of O_2^- is likely to result in the formation of peroxynitrite ($ONOO^-$), which may further enhance O_2^- production by oxidation of the zinc thiolate (ZnS_4) cluster within eNOS and dissociation of the functional dimer (Zou *et al.*, 2002). Although eNOS uncoupling has been successfully reversed by enhancing cellular levels of H_4B, either by using sepiapterin (Tiefenbacher *et al.*, 1996), by preventing the oxidation of H_4B (d'Uscio *et al.*, 2003), or by endothelial cell-specific overexpression of the GTP-cyclohydrolase I (Alp *et al.*, 2003; Alp *et al.*, 2004) circumstantial evidence indicates that the association of Hsp90 with eNOS,(Pritchard, Jr. *et al.*, 2001) as well as eNOS phosphorylation, in particular on eNOS Thr^{495} (Lin *et al.*, 2003), can also affect the degree of coupling and the balance of NO/O_2^- production.

1.9 Free radicals in the cardiovascular system

Reactive oxygen species (ROS), such as O_2^-, H_2O_2, $ONOO^-$, or lipid peroxides are produced by all living cells (Darley-Usmar & Halliwell, 1996). Circulating blood cells, such as neutrophils or macrophages, turn into significant sources of free radicals when activated (Beckman & Crow, 1993). The generation of free radicals is low under normal conditions and compensated by the action of antioxidative enzymes (superoxide dismutase, catalase) and biomolecules (ascorbate, glutathione). However, it has been shown that the endothelium can generate substantial amount of O_2^- (Britigan *et al.*, 1992; Rosen & Freeman, 1984), in particular following stimulation with cytokines (Bautista *et al.*, 1991; Matsubara & Ziff, 1986a), the activation of protein kinase C (PKC) (Fleming *et al.*, 2005; Matsubara & Ziff, 1986b) or an increase in $[Ca^{2+}]_i$ (Gryglewski *et al.*, 2001; Holland *et al.*, 1990). Both excessive production and diminished inactivation of O_2^-, in combination with secondary reactions, are responsible for the development of oxidative stress in vivo and in vitro. The uncompensated generation of ROS/reactive nitrogen species causes modifications at various levels, including alterations in proteins, lipids, or DNA leading to cells damage and death. Thus high levels of free radicals have been connected with a series of degenerative processes, while low levels, found under physiological conditions, play key role in a variety of signalling cascades.

1.9.1 Superoxide anion radical (O_2^-)

Superoxide is produced by the one-electron reduction of molecular oxygen. It is formed by enzymatic reactions during normal metabolism (as an intermediate of the respiratory chain), as a by-product of enzyme activity (xanthine oxidase, cyclooxygenase), or as part of the cellular defence mechanism (NADPH oxidase). Lately, eNOS has been identified as a potential source of O_2^- as a result of the uncoupling of the flow of electrons to form NO under conditions of L-arginine (Xia *et al.*, 1996) and/or H_4B defiency (Xia *et al.*, 1998). Like NO, O_2^- is a free radical with a relatively low overall reactivity (compared to $^{\cdot}OH$) however, the negative charge of O_2^- reduces its diffusion in comparison to NO. As shown for erythrocytes, the penetration of O_2^- through biological membranes requires the presence of a transport mechanism, such as anion channels.

1.9.2 Peroxynitrite

Peroxynitrite ($ONOO^-$) is the product of the reaction between NO and O_2^-. It is cell-permeable, as well as being a potent and versatile oxidant that can attack a wide range of

biological molecules. The peroxynitrite anion is relatively stable under basic conditions but is protonated at physiological pH.

Biological effects of $ONOO^-$

NO reacts readily with O_2^-, at close to diffusion- limited rate to produce $ONOO^-$. It acts as a powerful oxidant, but is sufficiently stable to diffuse through a cell to react with a target. Human tissues are continually exposed to a number of damaging ROS. As a strong oxidant and nitrating agent $ONOO^-$ is more reactive and harmful than O_2^- or H_2O_2.

$ONOO^-$ is particularly efficient at oxidizing iron-sulphur clusters, zinc-fingers, and protein thiols resulting in cellular energy depletion. $ONOO^-$ also reacts with SOD to form a nitronium-like intermediate, which catalyses the 3-nitration of tyrosine residues, particularly those in cytoskeletal proteins. Although 3-nitrotyrosine can also be generated by sources other than $ONOO^-$, the accumulation of 3-nitrotyrosine-containing proteins, detected with antisera to 3-nitrotyrosine, is used as a marker to $ONOO^-$ formation (Beckman & Koppenol, 1996).

S-Nitrosylation

In a cellular environment $ONOO^-$ will react preferentially with free thiols because of the fast rate of this reaction and the high intracellular thiol concentration. Glutathione, one of the most important antioxidants is the predominant source of intracellular thiol, and protect cells against $ONOO^-$-induced cytotoxicity. S-glutathiolation by $ONOO^-$ activates the sarco/endoplasminc reticulum calcium ATPase to decrease intracellular Ca^{2+} concentration and relax cardiac, skeletal and vascular smooth muscle (Adachi *et al.*, 2004). NO- mediated S-nitrosylation of cysteine residues is reportedly linked to eNOS monomerisation and a decrease in enzyme activity (Ravi *et al.*, 2004).

Tyrosine nitration

One of the effects of $ONOO^-$ on proteins is nitration of tyrosine residues. Tyrosine phosphorylation of enzymes is a common mechanism mediating cellular signalling. In endothelial cells tyrosine nitration inhibits tyrosine phosphorylation and causes for example accelerated endothelial cell apoptosis via tyrosine nitration of the phosphatidylinositol 3-kinase (PI 3-kinase), inhibiting activity of Akt-1 kinase and increasing the activity of p38 MAP kinase (el Remessy *et al.*, 2005). $ONOO^-$ derived nitrated tyrosine has also been found in inflammatory cells in the atherosclerotic human artery, macrophages-derived foam cells, and human platelets.

Oxidation of low density lipoprotein

An increase in oxidized low- density lipoproteins (ox-LDL) is associated with coronary artery disease. Circulating ox-LDL originates from mild oxidation of LDL in the arterial wall. Oxidized LDL induces atherosclerosis by stimulating monocyte infiltration leading to their differentiation into macrophages and by stimulating smooth muscle cell migration and proliferation. It contributes to atherothrombosis by inducing endothelial cell apoptosis, and thus plaque erosion, by impairing the anticoagulant balance in endothelium, stimulating tissue factor production by smooth muscle cells and inducing apoptosis in macrophages (Mertens & Holvoet, 2001).

Peroxynitrite- mediated formation of ox-LDL was demonstrated by treating LDL with SIN-1 (which simultaneously produces NO and O_2^- resulting in $ONOO^-$ formation) which resulted in accelerated ox-LDL formation (Darley-Usmar *et al.*, 1992; Patel *et al.*, 2000). Treatment of LDL with authentic $ONOO^-$ results in the depletion of antioxidants and the formation of both lipid hydroperoxidase- and thiobarbituric acid- reactive substances, indicative of lipid peroxidation (Darley-Usmar *et al.*, 1992).

1.10 Measurement of NO

The study of NO in biological systems is complicated by the low biologically active concentrations of NO (in the nanomolar range) and its short half-life due to the fast interaction with ROS or proteins and thiols. While a number of methods have been used to assess NO concentrations, most of them are based on the detection of NO metabolites or coproducts (Archer, 1993). The consequences are both the loss of specificity and the inability to resolve temporal changes in the release of authentic NO. The most commonly used assay is the measurement of nitrite and nitrate by Griess reaction (Bender *et al.*, 1999; Ignarro *et al.*, 1987). Although measurement of nitrite and nitrate offers a simple way to indirectly reflect NO formation, the specificity of this assay has been a major concern because nitrite and nitrate may arise from a number of sources in biological systems (Archer, 1993). The detection of the NO coproduct, L-citrulline, is indeed specific, but it does not afford a direct visualization of NO generation by NOS enzymes.

Spin trapping of free radicals in combination with electron spin resonance (ESR) spectroscopy is a well-documented approach to characterize free radicals, including NO and O_2^-. The spin trapping of NO with a $Fe(DETC)_2$ complex, which has been reported to specifically trap NO (Kleschyov *et al.*, 2000; Mülsch *et al.*, 1995) results in the formation of a

paramagnetic NO-Fe^{2+} mononitrosyl iron complex (MNIC) which is detected by ESR spectroscopy. So far, ESR remains the most unambiguous technique in free radical detection.

2 Aim

The objective of this thesis was to identify eNOS residues whose phospho-status would regulate the activity of eNOS with emphasis being placed on identifying the phospo-switch that would convert eNOS from a NO generating enzyme to a O_2^- generating enzyme. Identification of the role of eNOS tyrosine phosphorylation and the residue(s) involved on the regulation of eNOS activity.

Work began by assessing serine and threonine phosphorylation and has culminated with a reassessment of the role of tyrosine phosphorylation in the regulation of eNOS.

3 Materials and methods:

3.1 Bacterial transformation

3.1.1 Preparation of competent cells by KCM method.

From a single colony, preculture of the desired *E. coli* strain(DH5α, JM109, XL-1Blue) in a 5 mL Luria-Bertani (LB) medium (10 g/L Bactotrypton, 5 g/L Yeast extract, 10g/L NaCl pH7.0) without antibiotic was obtained. 1 mL of the overnight culture was added into 100 mL of LB medium and incubated in a shaker (250 rpm) at 37°C till a OD_{600} of 0.5-0.6 was obtained. The culture was chilled on ice for 5 min and cell pellet obtained by centrifugation at 2500g for 15 minutes at 4°C. The bacteria were resuspended in 7.5 mL of TSB (5% v/v DMSO, 10 mmol/L $MgCl_2$, 10 mmoL/L $MgSO_4$, 10% w/v PEG 6000 in LB medium) and incubated on ice for 1 hour. 100 µL aliquots of competent cells were distributed into pre-cooled tubes and immediately frozen in liquid nitrogen and stored at –80°C.

3.1.2 Transformation

An aliquot of 100 µL was thawed on ice. Transformation mix was prepared with 20 µL 5X KCM (500 mmol/L KCl, 150 mmoL/L $CaCl_2$, 250 mmoL/L $MgCl_2$), 80 µL sterile water and DNA. The transformation mix was added to the competent cells and incubated on ice for 20 min, followed by 10 minutes at room temperature. 1 mL of pre warmed LB medium was added and incubated at 37°C for 1 hour. The bacteria were collected by centrifugation (2000 g, 5 minutes at room temperature). The supernatant was partially removed and the pellet was resuspended in the residual LB medium and spread on a LB agar plate with appropriate antibiotic and incubated overnight at 37°C.

3.2 Isolation of nucleic acids

3.2.1 Mini plasmid DNA preparation by alkaline lysis method

Recombinant *E. coli* were grown in a 3 mL Luria-Bertani (LB) medium (10 g/L bactotrypton, 5 g/L yeast extract, 10g/L NaCl pH 7.0) containing an appropriate antibiotic overnight (at least 16 hrs). The cells were harvested by centrifugation at 10000g for 5 minutes at room temperature. The pellet was resuspended in 250 µL of resuspension buffer (50 mmol/L Tris/HCl pH 8.0, 10 mmol/L EDTA and 100 µg/mL RNase A) for 5 minutes at room temperature followed by lysis with 250 µL freshly prepared lysis buffer (0.2 mol/L NaOH,

1%SDS), the contents of the tube were mixed gently and incubated for 5-10 minutes. 300µL of neutralization buffer (3.0 mol/L potassium acetate, pH 5.5) was added, mixed gently without vortexing and incubated on ice for 15 minutes. The tubes were centrifuged at 10000 g for 15 minutes at 4°C. The supernatant containing the plasmid was transferred to a fresh tube and the plasmid DNA was precipitated by addition of 600 µL of isopropanol and incubated at –20°C for 30 minutes. Precipitated DNA was sedimented by centrifugation at 10000 g for 15 minutes at 4°C. The DNA sediment was washed with 70% ethanol, air dried and dissolved in the 50 µL TE buffer (10 mmol/L Tris/HCl pH 8, 0.1 mmol/L EDTA).

3.2.2 Maxi plasmid DNA preparation

Cell culture experiments and sequencing reactions require DNA of high purity. To obtain large amount of highly purified plasmid DNA, commercially available kits from QIAGEN (Düsseldorf, Germany) were used. The DNA isolation was performed according to the manufacturer's instructions using a 400 mL overnight bacterial culture.

3.2.3 DNA extraction from agarose gels

After restriction digestion, the DNA fragment of interest was isolated by agarose gel extraction and purification using gel extraction kit from QIAGEN (Düsseldorf, Germany). After fractionating the digested DNA on agarose gel, the DNA was stained using ethidium bromide solution (0.5 µg/mL) for 20 min. The DNA band was visualized using a UV (315 nm) transilluminator and the fragment of interest was excised using a scalpel. The DNA was then extracted according to the manufacturer's protocol.

3.2.4 RNA isolation

For the isolation of total RNA from cultured cells expressing eNOS, a mixture of guanidine thiocyanate and phenol in a mono-phase solution (TRI reagent, Sigma, Germany) was used. Cells were lysed with TRI reagent (350 µL per 3.5 cm culture plate). After incubation of the lysate for 5 minutes at room temperature, 70 µL of chloroform was added and the mixture was vortexed thoroughly and incubated for further 15 minutes at room temperature. For the separation of the phenol and aqueous phase the extracts were centrifuged (12000 g, 15 minutes, 4°C). The upper aqueous phase containing RNA was recovered and was precipitated by the addition of 175 µL isopropanol. After vortexing and incubation for 10 minutes at room temperature the tubes were centrifuged (12000 g, 15 minutes, 4°C). The RNA sediment was washed with 600 µL of 70% ethanol air dried and dissolved in an appropriate volume of diethylpyrocarbonate (DEPC) treated water.

3.2.5 Purification of DNA by phenol chloroform isoamyl alcohol extraction and ethanol precipitation

Purification of nucleic acid following restriction digestion was performed by phenol chloroform isoamyl alcohol extraction. An equal volume of TE-saturated phenol was added to the DNA diluted in water (400 µL). The mixture was vortexed thoroughly, and then centrifuged (5 minutes, 10000 g, room temperature) for phase separation. The upper, aqueous phase containing the plasmid DNA was transferred into a new microcentrifuge tube and was subjected to two rounds of chloroform/isoamyl alcohol (24:1) extraction to remove residual phenol. The upper aqueous phase was then collected in a separate tube, and precipitated by the addition of $1/10^{th}$ volume of sodium acetate solution (3 mol/L pH5.2) and 2.5 volumes of 100% ethanol. The suspension was mixed by vortexing and then incubated overnight at -20°C or at –80°C for 3 hours. Precipitated DNA was collected by centrifugation (15 minutes, 10000 g, 4°C) and washed with 70% ethanol. The DNA sediment was air dried and dissolved in an appropriate volume (25 µL) of TE buffer.

3.3 Manipulation of nucleic acids

3.3.1 Restriction digestion

The cleavage of double-stranded DNA molecules by restriction endonucleases was performed by incubating an appropriate amount of DNA with the restriction enzyme in the specific buffer and under the conditions recommended by the supplier. A typical digestion reaction included one unit of enzyme per µg of DNA and incubation for 1-3 hours (in case of cleavage of DNA from a mini-preparation) to overnight (in case of DNA from maxi plasmid preparation) at 37°C.

3.3.2 Dephosphorylation of linearised double-stranded DNA

To inhibit the self ligation of the vector during cloning, linearised vectors were treated with CIP (calf intestinal alkaline phosphatase; Amersham Pharmacia, Freiberg, Germany) to remove the 5' phosphate of the double stranded DNA molecules. Reaction mixtures contained 1µg linearised DNA, in 50 mmol/L Tris/HCl, pH9.0, 10 mmol/L $MgCl_2$, 0.1U/reaction diluted CIP in a suitable volume. The above mentioned reaction mixture was incubated at 37°C for 1 hour, followed by heat inactivation of the alkaline phosphatase (85°C; 15 minutes) and phenol chloroform isoamyl alcohol extraction with subsequent ethanol precipitation. The DNA sediment was dissolved in sterile water.

A control ligation of the dephosphorylated and phosphorylated vectors was performed and following transformation checked for the efficiency of dephosphorylation. Few or no colonies are indicative of an efficient dephosphorylation reaction.

3.3.3 Large fragment of the E.coli DNA polymerase I (Klenow fragment):

The Klenow polymerase was used to modify 3' or 5' overhangs into blunt ends. The Klenow fragment possesses a 3' to 5' exonuclease as well as a 5' to 3' polymerase activity. A typical reaction mixture contained, 5 µg linearised DNA in 10 mmol/L Tris/HCl, 50 mmol/L NaCl, 10 mmol/L $MgCl_2$,1 mmol/L dithiothreitol pH 7.9, 0.4 mmol/L dNTPs and 3 U/reaction Klenow enzyme (New England Biolabs, Frankfurt am Main, Germany) in a final volume of 100 µL. After incubation at 37°C for 30 min the reaction was stopped by phenol chloroform isoamyl alcohol extraction and the DNA was concentrated by ethanol precipitation. The DNA pellet was dissolved in an appropriate amount of sterile water.

3.3.4 DNA ligation

Ligation of vector and inserts was performed using T4 DNA ligase. For blunt end ligation, the amount of insert was at least five times in molar excess over the vector. A typical reaction mixture for blunt end ligation contained the vector and insert in 50 mmol/L Tris/HCl (pH 7.6), 10 mmol/L $MgCl_2$, 0.5 mmol/L ATP, 0.5 mmol/L DTT, 5% (w/v) polyethylene glycol-8000 and 3 units of T4 DNA ligase enzyme (Invitrogen, Karlsruhe, Germany), in a total volume of 15 µL. The reaction mixture was incubated at room temperature for 16 to 20 hours. 5 µL of the ligation mix was used for transformation.

3.3.5 Site directed mutagenesis

The eNOS-Ser mutants were produced using polymerase chain reaction-based QuickChange XL site directed mutagenesis kit (Stratagene) and the human eNOS wild-type cDNA as template. A pair of primers; forward and reverse containing the mutation of Ser^{114} to D (GAT) or A (GCA) and $Ser^{633/634}$ (TCC/AGT) to A (GCC/GCT) were used.

Forward S114A: 5’ CTA CAG GGC CGG CCC GCA CCC GGC CCC 3’

Forward S114D: 5’CTA CAG GGC CGG CCC GAT CCC GGC CCC 3’

Reverse S114A: 5’GGG GCC GGG TGC GGG CCG GCC CTG TAG3’

Reverse S114D: 5’GGG GCC GGG ATC GGG CCG GCC CTG TAG3’

Forward S633/634A: 5’CGG AAG AGG AAG GAG GCC GCT AAC ACA GAC AGT GCA GGG 3’

Reverse S633/634A: 5'CCC TGC ACT GTC TGT GTT AGC GGC CTC CTT CCT CTT CCG 3'

The oligonucleotides were synthesized by Biospring (Frankfurt, Germany)

The PCR reaction mix contained 1X reaction buffer, dNTP mix (0.2 mmol/L), quick solution and 2.5 U Pfu turbo polymerase provided with the kit along with eNOS wild-type cDNA; 50 ng, primers; forward and reverse-125 ng each in a final volume of 50µL. The PCR was set up in a Robo- Cycler (Strategene) with the following cycle conditions.

Reaction Buffer:

1 X Reaction buffer: 10 mmol/L KCl, 10 mmol/L $(NH_4)_2SO_4$, 20 mmol/L Tris-HCl- pH 8.8, 2 mM $MgSO_4$, 0.1% Trition X-100 and 10% nuclease-free bovine serum albumine (BSA)

1 cycle : 95°C for 1 minute

18 cycles : 95°C for 1 minute, 54°C for 1 minute and 68°C 20 minutes

1 cycle : 68°C for 10 minutes

After completion of the reaction, the PCR reaction mix was subjected to Dpn I (10 U) restriction digestion at 37°C for 60 minutes, to degrade the methylated of parental plasmid DNA. 10 µL of DpnI digested DNA was transformed in XL-10 competent cells and positive clones were identified by sequencing of DNA.

3.3.6 Sequencing of DNA

To sequence a recombinant vector, the plasmid DNA was column purified and 2 µg DNA in 20 µl TE was sent to MWG Biotech (Ebersberg, Germany) for sequencing.

3.4 Detection of nucleic acids

3.4.1 Colony hybridisation using a ^{32}P-radiolabelled probe

Colony hybridisation with a ^{32}P-labelled DNA fragment was used to identify positive recombinant clones. Following transformation bacterial colonies grown on an agar plate were transferred to a Nytran N nylon transfer membrane, (Schleicher and Schuell GmbH, Germany) by spot blotting (i.e. placing the membrane directly onto the plate). The membrane was labelled and its orientation on the plate was carefully marked with a needle through the filter into the agar. The colonies were lysed by placing the nylon membrane onto filter paper soaked with 2% SDS for 2 minutes, followed by denaturation of the DNA double strands, on

a filter paper soaked with 0.5 mol/L NaOH and 1.5 mol/L NaCl for 2 minutes, followed by neutralization in 0.5 mol/L Tris-HCl pH 8.0 and 1.5 mol/L NaCl for 5 to 10 minutes. The DNA was fixed on the membrane by baking for 2 hours at 80°C. The membrane was then transferred to hybridisation vials and treated with 15 mL of prehybridization solution containing formamide (50% v/v), 5X SSPE (750 mmol/L NaCl, 0.05 mol/L NaH_2PO_4, 0.05 mol/L EDTA Ca^{2+}free); 70 µg/mL denatured herring sperm DNA (which binds to the DNA non-specifically and inhibit non-specific signal on autoradiogram after hybridisation), 5X Denhardt's solution (0.5% ficoll, 45 mmol/L polyvinylpyrrolidone, 0.5% BSA) and 0.2% SDS on a rotating wheel at 42°C for 2 hours. The prehybridization solution was replaced by the hybridisation solution containing heat denatured radiolabelled DNA probe and incubated for 16 hours at 42°C. The hybridisation solution was removed and the filters were washed twice for 15 minutes at room temperature in Buffer A (6X SSPE, 0.1% SDS) and twice in buffer B for 30 minutes at 55°C (1X SSPE, 0.1% SDS). Specifically bound radioactivity was detected by autoradiography (FUJIFILM medical X-ray film, Tokyo, Japan).

3.4.2 Southern blot hybridisation

Confirmation of the orientation of the gene of interest (insert) in the clones was performed by restriction digestion and subsequent Southern blot hydridisation with a specific radiolabelled DNA against the insert. The size fractionated double stranded DNA was denatured by soaking the gel in a denaturing solution (0.5 mol/L NaOH and 1.5 mol/L NaCl) for 15 to 30 minutes at room temperature followed by 15 minutes incubation in the neutralization solution (0.5 mol/L Tris/HCl, pH 8.0 and 1.5 mol/L NaCl). The DNA was then transferred onto a positively charged nylon membrane (Porablot, Düren, Germany) via upward capillary transfer. The gel was placed on a filter paper soaked with 10X SSC (1.5 mol/L NaCl, 0.4 mol/L sodium citrate pH 7.0 with 10N NaOH) and the ends of the filter paper were connected to a reservoir of 10X SSC. The membrane was placed on top of the gel with 2 sheets of filter paper and a stack of paper towels on top of it to facilitate the overnight (16 hours) transfer of DNA to the membrane by capillary flow. To immobilise the DNA on the membrane it was cross-linked using UV light (215 nm, 2 minutes) and incubated at 80°C for 2 hours. The membrane was then placed in the hybridisation vials and the hybridisation as well as the detection of the radioactivity was performed as described above.

3.4.3 Northern blotting and hybridisation

After isolation of total RNA, 5 µg of RNA was mixed with 5µL RNA pre mix buffer containing 20 mmol/L deionised formamide, 40 mmol/L 3-(N-morpholino) propanesulfonic

acid (pH 7.0 adjusted with acetic acid), 10 mmol/L sodium acetate and 1 mmol/L EDTA, and 0.5 μg/mL ethidium bromide. This RNA mixture was heated to 65°C for 10 minutes and separated by electrophoresis on denaturing formaldehyde agarose gel containing 1% agarose (agarose was dissolved by boiling in DEPC water, the temperature was brought down to 50-60°C and then 12.3 mol/L formaldehyde was added). Finally 1/10th volume of running buffer MOPS was added and poured in the gel plate. RNA bands were separated in RNA running buffer (40 mmol/L MOPS, 10 mmol/L Na-acetate, 1 mmol/L EDTA, pH 7.0). Under a UV-lamp (at 315nm) 28S, and 18S ribosomal RNA (the ratio of the band intensity of 28S and 18S RNA should be 2:1) were visualised. The RNA was then transferred onto a positively charged nylon membrane via upward capillary transfer, as described above but with 20X SSC (3 mol/L NaCl, 250 mmol/L sodium citrate pH 7.0 adjusted with 10 N NaOH) as transfer buffer. The hybridisation and the detection of the radioactivity were performed as described above.

3.5 Cell culture and adenoviral methods

3.5.1 Generation of eNOS adenoviruses

Construction of recombinant adenoviruses with eNOS mutants was performed as described (Fleming *et al.*, 2005).

Briefly, the full-length, myc/his-tagged eNOS wild-type and mutant cDNA inserts (~ 3.6 kb) were excised from pcDNA3.1 myc/his with sequential digestion using HindIII and PmeI in a total volume of 50 μg using 5 μg DNA. The DNA fragment of interest was purified by gel purification using a gel extraction kit from QIAGEN (Düsseldorf, Germany). The overhangs of the inserts were blunted by the large fragment of E. coli DNA polymerase I (Klenow fragment) and ligated into linearized and dephosphorylated EcoRV adenoviral shuttle vectors, pAd-Track-CMV and pAdShuttle-CMV (kindly provided by Bert Vogelstein, Howard Hughes Medical Institute, Baltimore, MD). The plasmids were recombined into the adenovirus 5 genome by homologous recombination in E. coli. Briefly, the shuttle vectors carrying the eNOS cDNAs were linearized with the restriction endonuclease PmeI, purified by phenol/chloroform extraction and ethanol precipitation. These vectors were used to transform the recombination positive E. coli strain BJ5183 carrying the plasmid pAdEasy-1. The resulting recombinant plasmids conferred resistance to kanamycin (70 μg/mL), and plasmids from antibiotic-resistant colonies were isolated and analyzed for eNOS by restriction digestion with KpnI/HindIII. Positive plasmids were transformed into E. coli JM109 for large-scale isolation. Following linearization with PacI and extraction with phenol/chloroform and

ethanol precipitation, plasmids were transfected into HEK 293 cells (American Type Culture Collection). Transfected cells were incubated for up to 2 weeks and monitored for GFP expression (for inserts in pAdTrack-CMV) and plaque appearance. Viruses were obtained from these cells and infected into fresh HEK 293 cells for amplification and to confirm expression of mRNA and protein. Constructs that expressed eNOS mRNA (detected by Northern blotting) were screened for eNOS protein. Only one orientation (sense) resulted in increased protein expression. For large scale amplification and purification, five T175 flasks of 90% confluent HEK cells were infected with the viruses. The virus particles were purified using the BD Adeno-X Virus purification kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. The resulting titre of each preparation was approximately 10^9 plaque forming unit (PFU)/mL.

3.5.2 Culturing of cells

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical vein using dispase as described (Popp *et al.*, 1996) and cultured in growth medium, a 1:1 mixture of MCDB 131 (Invitrogen, Karlsruhe, Germany) and M199 (PAA laboratory, Pasching, Austria) containing 14% fetal calf serum (FCS), epidermal growth factor (EFG; 0.05 ng/mL), basic fibroblast growth factor (bFGF; 0.5 ng/mL), endothelial cell growth supplement (1.5 mg/mL), heparin (22.5 mg/mL), penicillin (50 U/mL) and streptomycin (50 µg/mL). All experiments were performed with cells of passage one.

Porcine aortic endothelial cells (PAEC) were isolated from fresh porcine aorta using dispase and cultured in growth medium, a 1:1 mixture of MCDB 131 (Invitrogen, Karlsruhe, Germany) and M199 (PAA laboratory, Pasching, Austria) containing 14% FCS, EGF (0.05 ng/mL), bFGF(0.5 ng/mL), endothelial cell growth supplement (1.5 mg/mL), heparin (22.5 mg/mL), penicillin (50 U/mL) and streptomycin (50 µg/mL).

3.5.3 Transfection with calcium choloride

HEK 293 cells cultured in 6 cm dishs, were grown in minimal essential medium (MEM) supplemented with EARLE's salts, containing 8% FCS, 50 µg/mL gentamycin, 1% non-essential amino acids and 1 mmol/L sodium pyruvate. At 70-80% confluence cells were incubated with a transfection mix containing the desired plasmid (5µg DNA, 0.126 mol/L $CaCl_2$ and 500µL 2X HEPES buffer saline; HBS, in a total volume of 1 mL). After 4 hours the transfection mix was removed and subjected to glycerol shock for 60 seconds, with 15% glycerol in HBS to facilitate the uptake of DNA. The cells were washed with PBS and incubated in fresh growth medium for 48 hours.

3.5.4 Adenoviral infection of COS-7 cells

COS-7 cells cultured in 6 cm dishes, were grown in MEM supplemented with EARLE's salts, containing 8% FCS, 50 µg/mL gentamycin, 1% non-essential amino acids and 1mmol/L sodium pyruvate. At 70-80% confluence, cells were serum-starved (medium containing 0.1% BSA) for 4 hours. Infection with recombinant adenoviruses expressing human wild-type eNOS or the T495A, T495D, S114A, S114D, S633D or T495A/S1177D eNOS mutants (2 PFU/cell, in 1 mL medium with 0.1% BSA per 6 cm dish) was for 4 hours followed by addition of 2 mL growth medium and further incubated for 48 hours.

3.5.5 Adenoviral infection of human umbilical vein endothelial cells

For the infection of endothelial cell, the cells were serum-starved (medium containing 0.1% BSA) for 16 hours. Infection with recombinant adenoviruses expressing human eNOS wild-type or the T495A, T495D, S114A, S114D, S633D or T495A/S1177D eNOS mutants (2 PFU/cell, in 1 mL medium with 0.1% BSA per 6 cm dish) was for 4 hours followed by addition of 2 mL growth medium with 1% FCS and further incubation for 48 hours.

3.5.6 Ox-LDL treatment

Confluent cultures of human endothelial cells, were serum-starved (medium containing 0.1% BSA) for 24 hours followed by the addition of required concentration of ox-LDL (30 µg/mL, unless mentioned otherwise) and incubated for the indicated period of time.

3.5.7 Stimulation with bradykinin

Confluent cultures of porcine endothelial cells, were serum-starved (medium containing 0.1% BSA) for overnight (~ 16 hours). The cells were then stimulated with bradykinin (1 µmol/L) for different time points (10 seconds to 5 minutes). The cells were quickly washed with ice cold phosphate buffer saline (PBS) and drained and frozen in liquid nitrogen.

3.6 Immunohistochemistry

Human endothelial cells were infected with different eNOS mutants and after 48 hours of protein expression used for immunohistochemistry. The cells were fixed in 4% paraformaldehyde in PBS for 10 minutes, followed by incubation with glycine (2%) in PBS and permeabilized with Triton X-100 (0.05%) in PBS for 10 minutes each. Each step was followed by washing the cells 3 times with PBS for 3 minutes. The cells were then blocked with 3% BSA in PBS for 60 minutes and followed by incubation with a specific myc monoclonal (Santa Cruz Biotech, Heidelberg, Germany) and β-catenin polyclonal (Santa Cruz Biotech) antibodies (1:200 dilution) overnight at 4°C. The cells were washed at least 3 times

with PBS (with 0.2% Tween) and then incubated with Alexa dye-coupled secondary antibodies for 1 hour at room temperature. The cells were again washed with PBS (containing 0.2% Tween) 3 times and were mounted in antifade (Molecular probes, Karlsruhe, Germany) or Dako-Fluoromount (Dako, Hamburg, Germany). Co-localisation studies were carried out using a Zeiss laser-scanning confocal microscope LSM 510 META in multi-tracking mode to prevent interference of the dyes.

3.7 Methods with protein

3.7.1 Isolation of protein

Cells expressing the protein of interest were lysed with Triton X-100 lysis buffer containing NaF (100 mmol/L), $Na_4P_2O_7$ (15 mmol/L), Na_3VO_4 (2 mmol/L), leupeptin (2 µg/mL), pepstatin A (2 µg/mL), trypsin inhibitor (10 µg/mL), PMSF (44 µg/mL) and Triton X-100 (1% vol/vol). The sample was collected in 1.5 mL tubes and incubated on ice for 10 minutes. The lysate was centrifuged at 12,000 g (4°C for 10 minutes) and the supernatant was transferred into a fresh tube. The concentration of the proteins was determined at 570 nm using Bradford reagent.

3.7.2 Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Proteins were analysis using SDS-PAGE and Western blotting. Protein extracts (20-50 µg) per samples were denatured by heating in Laemmli buffer (7.3% w/v SDS, 29.1% v/v glycerol, 83.3 mmol/L Tris with bromophenol blue) at 95°C for 5 minutes and separated by SDS-PAGE. The proteins were then subjected to electrophoresis (running buffer: 190 mmol glycine, 0.1% SDS and 25 mM Tris-HCl) and resolved for about 2 hours with approximately 35 mA/gel.

Western blotting was performed using the tank blot technique onto nitrocellulose membranes (Bio rad, Munchen, Germany) for 90 minutes, in transfer buffer (190 mmol/L glycine, 25 mmol/L Tris-HCl and 20% methanol) with 250 mA per apparatus (two gels). The nitrocellulose membranes were stained with Ponceau S to determine the quality of transfer and equal loading of the protein. After destaining with distilled water the membranes were blocked in Tris buffer saline(TBS, 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5) containing 3% BSA and 0.03% Tween-20 for 1 hour at room temperature. The membranes were incubated with the primary antibody (routinely diluted 1:1000) in TBS containing 3%

BSA and 0.03% Tween-20 overnight at 4°C. The membranes were then washed in TBS with 0.03% Tween-20 for 30 minutes at an interval of 5 minutes. The membranes were blocked again for 1 hour. The secondary antibody coupled to horse-radish-peroxidase (HRP, 1:20000 TBS/0.03% Tween-20) was incubated with the membrane for 1 hour at room temperature and washed again. The protein were visualised on X-ray film by enhanced chemiluminescence solution (ECL, Amersham Pharmacia, Freiberg, Germany).

3.7.3 Low temperature- polyacrylamide gel electrophoresis (LT-PAGE)

SDS-resistant eNOS dimers and monomers were assayed using LT-PAGE. Protein extracts (20 µg) in Laemmli buffer with or without 2.5% β-mercaptoethanol or heating at 95°C were subjected to PAGE at 4°C and with constant current of 30 mA per apparatus. Western blotting was performed as described earlier. Fresh protein samples were used exclusively as freeze thawing disrupted the eNOS dimer.

3.7.4 Immunoprecipitation

Immunoprecipitation (IP) is employed to isolated protein of interest using a specific antibody which is subsequently pulled down using protein A/G Sepharose beads.

Endothelial cells were lysed in 1% Triton lysis buffer, and incubated on ice for 10 minutes. The lysate was centrifuged at 12,000 g (4°C for 10 min) and the supernatant was transferred into a fresh tube. All subsequent steps were performed at 4°C. Cell extract (50µl) was kept aside to check for total expression of the protein of interest and the rest volume was used for immunoprecipitation. The cell lysate (300 µg) was subjected to preclearing with 60 µl protein A/G Sepharose beads (Santa Cruz Biotechnology,Heidelberg, Germany) without a primary antibody and incubated with the sample for 1 hour at 4°C on a rotating wheel. The preclearing step, allows the clearing of the lysate of non-specific protein binding to the sepharose. The supernatant of the preclearing was used for immunoprecipitation with the specific antibodies. For this, 2 µg of the antibody of interest was added to the lysate. The mixture was agitated on a spinning wheel in tubes at 4°C, for 2 hours. Then 60 µl of protein A/G Sepharose beads were added and incubated with the sample for 1 hour on a rotating wheel at 4°C. The complex of antigen, antibody and sepharose was than isolated via multiple centrifugation at 12 000 g (4°C for, 10 minutes) and washing with 1% Triton lysis buffer. Finally, 60 µl of Laemmli buffer were added and the protein denatured at 95°C for 5 minutes. After centrifugation for 1 minute in a microfuge at 10 000g, the supernatants were separated by SDS-PAGE and Western blotting.

3.7.5 Reproducing Western blots

To strip antibodies from the bolts, stripping buffer (Tris-HCl; 67.5 mmol/L, pH 6.8,and SDS, 2%) was prewarmed (10 mL per blot) at 50°C. Membranes were added along with 70 µL β-mercaptoethanol (0.7% v/v) and incubated for 30 minutes at 50°C under gentle agitation. The buffer was decanted and membranes washed thoroughly in TBS with 0.03% Tween-20. The membranes were then blocked in TBS containing 3% BSA and 0.03% Tween-20 and incubated with specific antibody of interest and processed as described above.

3.8 Assays of eNOS activity, NO and O_2^- detection.

3.8.1 Citrulline assay:

A standard method for determining the enzymatic activity of NOS is based on determining the conversion of radiolabeled L-arginine to L-citrulline. In principle, radiolabelled L-arginine is converted by NOS to L-citrulline and separated by trapping the positively charged L-arginine on dowex, while the neutral L-citrulline (at pH 5.5), remains in the supernatant. The radioactivity in the supernatant gives a direct measure of NOS activity.

With cell lysate

The activity of the different eNOS mutants was monitored by conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. HEK 293 cells overexpressing eNOS wild-type or mutants were lysed in Triton X-100 lysis buffer and protein extracted as described above. 100 µg of total protein was used per assay reaction and was diluted in a total volume of 180 µL of activity assay buffer containing (in mmol/L: NADPH 1.0, *6R*-H_4B 0.015, CaM 0.001, FAD 0.001, HEPES 50.0 in pH 7.4, DTT 1.0, EDTA 1.0, orthovanadate 2.0, and $CaCl_2$ 2.5). All of the reaction samples were kept on ice, the reaction was initiated by the addition of 20 µL [^{3}H]L-arginine (0.5 µmol/L, 0.5 µCi) and incubation at 37 °C for 30 min. The incubations were terminated by the addition of 1000 µL ice-cold HEPES buffer (100 mmol/L, pH 5.5) containing EGTA (10 mmol/L) and 500 mg Dowex AG 50 W-X8 (counter-ion Na^+ form) cation exchange resin and incubated for 5 minutes on ice. The samples were centrifuged (4000 rpm at 4°C). To 5 mL scintillation fluid, 500 µL of supernatant was added and [^{3}H]L-citrulline in the supernatant was quantified using a liquid scintillation counting. The specific activity of eNOS was calculated as the *N*-nitro-L-arginine sensitive formation of [^{3}H]L-citrulline.

With 100000 g pellet

Transfected HEK 293 cells in 10 cm dishes overexpressing recombinant eNOS protein were washed twice with PBS and scrapped in 800 µL sterile double distilled water. The cells were subjected to 5 cycles of freeze thawing at 40°C, followed by addition of 1 mL 2X homogenization buffer I (homogenization buffer I:Tris-HCl; 50 mmol/L, pH 7.4, KCl; 1.15% w/v, EDTA; 1 mmol/L, glucose; 5 mmol/L, DTT;0.1 mmol/L, SOD; 200 U/mL, leupeptin; 2 µg/mL, pepstatin A; 2 µg/mL, trypsin inhibitor; 10 µg/mL, PMSF; 44 mg/L). The cellular debris was pelleted by centrifugation at 4000 rpm for 10 min at 4°C. The supernatant with the membrane fraction was subjected to ultra centrifugation at 35000 rpm (100000 g), for 1 hour at 4°C. The pellet was resuspended in 200 µL of 1X homogenization buffer II (2X homogenization buffer II: Tris-HCl; 50 mmol/L, pH 7.4, glycerin; 10% v/v, 0.1 mmol/L EDTA, leupeptin; 2 µg/mL, pepstatin A; 2 µg/mL, trypsin inhibitor; 10 µg/mL, PMSF; 44 mg/L) and 50 µL was used per reaction and the assay was performed as described above with varying concentration of Ca^{2+} and calmodulin.

In vivo on intact cells:

Transfected HEK 293 cells in 3.5 cm dishes overexpressing recombinant eNOS protein were washed 2X with Earle's balanced salt solution (EBSS) and incubated with 1 mL EBSS for 5 hours to starve cells of L-arginine. To determine eNOS specific activity, cells were incubated in the presence or absence of L-NA (500 µmol/L) for 1 hour before incubated with [^{14}C]L-arginine (10 µmol/L, 0.05µCi/µL) and further incubated for 15 minutes. A time interval of 3 minutes between each plate was maintained. The cells were stimulated with ionomycin (100 nmol/L) for further 5 minutes at 37°C. The reaction was quickly terminated by washing the cells twice with ice cold HT and the cells were snap frozen in liquid nitrogen. The cells were scraped in 500 µL lysis buffer (Triton X-100 0.2 %, pH 5.2, sodium acetate 20.0 mmol/L, citrulline 1.0mmol/L, EGTA 2.0 mmol/L and EDTA 2.0 mmol/L) and transferred to a 2 mL eppendorf tube. 10µL was taken to check for total incorporation of [^{14}C] as a control. 1 mL ice-cold HEPES buffer (100 mmol/L, pH 5.5) containing EGTA (10 mmol/L) and 500 mg Dowex AG 50 W-X8 (counter-ion Na^+ form) cation exchange resin was added, vortexed and incubated for 5 minutes on ice, this was repeated twice. 800 µL of the supernatant was added to 5 mL scintillation fluid and [^{14}C] L-citrulline in the supernatant was quantified by a liquid scintillation analyzer.

3.9 Measurement of nitric oxide using $Fe(DETC)_2$ by electron spin resonance spectroscopy

Give the short half life of NO and O_2^-, its very difficult to measure it in a biological system. This problem has been overcome by trapping NO and O_2^-, with other compounds, which can then be detected by ESR spectroscopy.

Preparation of $Fe(DETC)_2$ Colloide:

10ml Krebs buffer (in mmol/L: NaCl 98.93, KCl 4.69, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 2.49, $NaHCO_3$ 25.0, $K_2HPO_4.3H_2O$ 0.61, glucose 11.1 and HEPES 21.85 pH 7.4) in two 50ml falcon tubes was deoxygenated by using argon gas for at least 45 minutes. Parent solutions of ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) and sodium diethylthiocarbimate (Na DETC) were prepared by adding 2.24mg $FeSO_4.7H_2O$ and 3.6mg Na DETC to each tube and bubbled with argon. As soon as the $FeSO_4.7H_2O$ crystals were dissolved the Na DETC solution was mixed with it, giving an almost translucent solution (which is an indication of a good colloid preparation). A multi-pipette was deoxygenated by aspirating argon gas. $Fe(DETC)_2$ colloid was aspirated in the multi-pipette and use immediately.

3.9.1 Measurement in cells:

COS-7 cells (6 cm culture dish) overexpressing recombinant eNOS protein were washed twice with Krebs buffer and incubated in 1200 µL for 45 min at 37°C. 400 µL of freshly prepared $Fe(DETC)_2$ colloid,(≅ 100 µmol/L Fe^{2+}) was added and further incubated for 30 min. eNOS was inhibited by pre-incubating the cells with L-NAME (300 µmol/L, 45 minutes) and activated by stimulating the cells with ionomycin (100 nmol/L, 10 minutes) before stopping the reaction by placing the plates on ice. Buffer was aspirated and cells were scraped carefully/gently in 100µL Krebs buffer and transferred to a insulin syringe and quickly frozen in liquid nitrogen.

The formation of paramagnetic mono-nitrosyl-iron complex was determined at 77 K in a liquid nitrogen cooled dewar using a ESR EMX spectrometer (Bruker, Karlsruhe, Germany). The instrument settings were as follows: microwave frequency, 9.487 GHz; power, 20.07 mW; receiver gain, $1x10^5$; modulation amplitude, 5.000G; modulation frequency, 100 KHz; conversion time, 81.92ms; time constant, 327.680ms; sweep time, 41.94s; sweep width, 100G; resolution, 512 points and 3 scans.

3.10 Measurement of superoxide anion (O_2^-)

3.10.1 Chemiluminescence

HUVEC were cultured in 3.5 cm dishes, on attaining confluency the cells were starved of serum for 16 hours. The cells were then incubated with nLDL or ox-LDL for 24 hours. To determine eNOS-dependent O_2^- production, cells were treated with L-NAME (300 µmol/L, 1 hour). The cells were then washed twice with HEPES-Tyrode solution (in mmol/L: NaCl 137.0, KCl 2.7, $MgCl_2$ 0.5, $CaCl_2$ 1.8, NaH_2PO_4 0.36, glucose 5.0 and HEPES 10.0) scraped and gently resuspended in 500 µL. The cell suspension was then transferred to a cuvette and the reaction was initiated by the addition of freshly prepared lucigenin (5µmol/L), 15 minutes later the cells were stimulated with ionomycin (100 nmol/L), and when the signal reached its peak, SOD was added. The concentration of lucigenin used has been shown to accurately reflect levels of ambient O_2^- and is not subject to redox cycling and the artifactual production of O_2^- that is observed with higher concentrations of the reagent (Li *et al.*, 1998; Skatchkov *et al.*, 1999). Light emission was detected using a Berthold luminometer (LB 9505, Berthold, Wildbad, Germany). The mean chemiluminescence observed during a period of 10 minutes was used to estimate the production of O_2^-.

3.10.2 Electron spin resonance (ESR) spectroscopy

O_2^- generation in intact cells was assessed using the spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH, Alexis, Grunberg, Germany) (Dikalov *et al.*, 1997; Kuzkaya *et al.*, 2003).

COS-7 or HEK 293 cells in 6 cm dishes, expressing recombinant eNOS protein were washed twice with HEPES-Tyrode (ESR-HT) solution which was pre-treated with chelax-100 (5%, overnight, Sigma) and contained diethylenetriamine-pentaacetic acid (DTPA; 100 µmol/L, used to decrease the autooxidation of hydroxylamines catalysed by trace transition metal ions). To quantify eNOS-dependent formation of O_2^- , cells were incubated at 37°C with 1 mL ESR-HT in the absence or presence of L-NAME (300 µmol/L) for 1 hour prior to the addition of CMH dissolved in argon purged saline (2 mmol/L final concentraiton). After additional 5 minutes, 400 µL of the solution was withdrawn into an insulin syringe and immediately frozen in liquid nitrogen. The formation of the stable spin label 3-methoxycarbonyl-proxyl ($CM^{\bullet}$) was determined at 77 K in a liquid nitrogen cooled dewar using an EMX ESR spectrometer (Bruker, Karlsruhe, Germany). The instrument settings were as follows: microwave frequency, 9.463 GHz; power, 20.02 mW; receiver gain, $1x10^5$; modulation

amplitude, 2.010G; modulation frequency, 100 KHz; conversion time, 81.92ms; time constant, 327.680ms; sweep time, 41.943s; sweep width, 400G; resolution, 512 points and 3 scans.

3.11 Statistical analysis

Data are expressed as mean ± SEM, and statistical evaluation was performed by using the Student's *t* test for paired or unpaired data, one way ANOVA followed by a Bonferroni *t* test, or ANOVA for repeated measures, where appropriate. Values of $P<0.05$ were considered statistically significant.

4 Results

Investigation of the role of eNOS serine, threonine and tyrosine residues on the regulation of the enzyme activity provided us with the following data.

4.1 Effects of mimicking phosphorylation at specific serine residues of eNOS

4.1.1 Effect of Ser^{114} or Ser^{633} mutations on eNOS catalytic activity and Ca^{2+}-CaM dependence

Potentially phosphorylable serine residues within the eNOS sequence were identified using the NetPhos 2.0 program (http://www.cbs.dtu.dk/services/NetPhos). Based on the phosphorylation score, with a threshold score for possible phosphorylation being 0.5, two serine residues on human eNOS sequence were selected for study; Ser^{114} (probability of phosphorylation = 0.994) because of its vicinity to H_4B and Zn^{2+} binding site and Ser^{633} (probability of phosphorylation = 0.987) because of its location within the auto-inhibitory loop located with in the FMN binding domain.

The effect of serine phosphorylation on the activity of the enzyme can be mimicked by the mutation of the uncharged serine (S) to a negatively charged aspartate (D) while a non-phosphorylatable site can be generated by replacing serine with alanine (A). To assess the role of phosphorylation of eNOS on Ser^{114} and Ser^{633} we generated a series of eNOS point mutants; S114A, S114D, S633/634A and S633D. As reported previously (Dimmeler *et al.*, 1999), the activity of the S633D eNOS mutant was comparable to that of the wild-type enzyme, it was initially decided to assess the effects of mutating both serines in the $Ser^{633/634}$ doublet to alanine. The activity of eNOS was then determined by monitoring the L-NAME-sensitive conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline under V_{max} conditions using Triton X-100 soluble fractions prepared from HEK-293 cells transfected with either the wild-type eNOS or one of the eNOS mutants. There was no marked difference in the activity of the wild-type eNOS or the S114D or S633/634A mutants. However, there was a significant ~2-fold increase in the enzymatic activity of the S114A eNOS mutant (196.28 ± 11.82%, $P<0.001$, n=6) over that of the wild-type eNOS or the S114D eNOS mutant, and between the S633D eNOS mutant (193.85 ± 13.33%, $P<0.001$, n=6) and the wild-type eNOS or the S633/634A mutant (Fig. 12A). Thus under V_{max} conditions, the activity of the non-phosphorylatable S114A mutant and the phospho-mimetic S633D eNOS mutant were

significantly higher than that of the wild-type enzyme. As the S633/634A mutant exhibited similar activity as the wild-type enzyme, it was excluded from the next assays.

In intact HEK-293 cells over-expressing either the wild-type eNOS or one of the mutants, stimulation with ionomycin slightly increased eNOS activity but there was no significant difference between the ionomycin-induced increase in the activity of the wild-type eNOS and that of the S633D mutant. This could be explained by the fact that eNOS expressed in cultured cell, which may not retain all of the signalling pathways present in native endothelial cells is predominantly uncoupled and thus generates a lot of O_2^-. Also ionomycin stimulates other O_2^- generating enzymes. However, the ionomycin-induced increase in the activity of the S114A mutant was significantly greater than that of the S114D mutant and ~25% greater than either the wild-type enzyme or the S633D eNOS mutant (Fig. 12B).

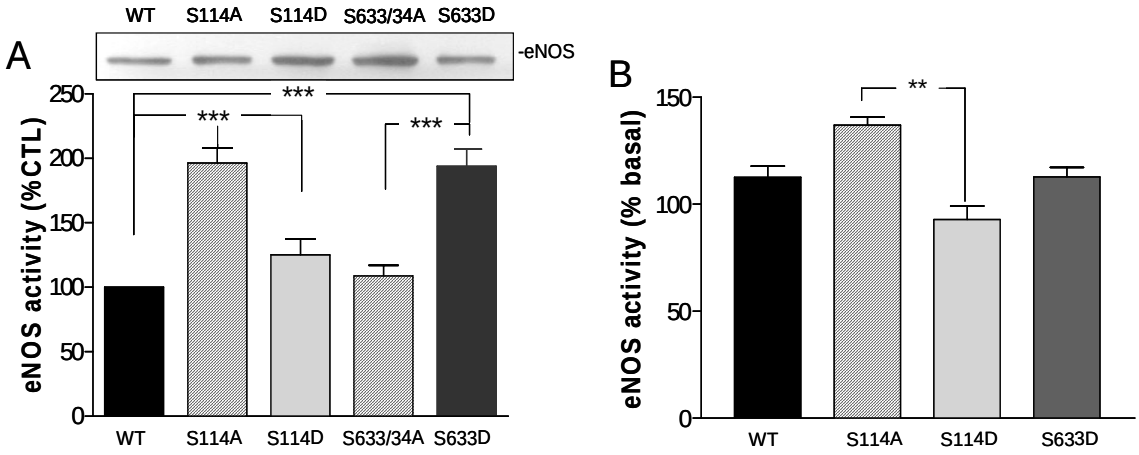

Figure 12. Comparison of the activity of wild-type eNOS and Ser^{114} and Ser^{633} eNOS mutants in vitro and in situ. (A) *eNOS activity was determined by monitoring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline under V_{max} conditions and using Triton X-100-soluble fractions prepared from HEK-293 cells transfected with either wild-type (WT) eNOS or one of the S114A, S114D, S633/634A or S633D eNOS mutants. eNOS expression was analysed by Western blotting using a specific antibody.* ***(B)*** *eNOS activity was assessed in the presence or absence of ionomycin (0.1 µmol/L, 5 minutes) in intact HEK-293 cells transfected with wild-type (WT) eNOS or the S114A, S114D and S633D eNOS mutants Data are expressed as percent eNOS activity over the activity measured in the absence of ionomycin. The bar graphs represent the mean ± SEM of data obtained in four to six independent experiments; **P<0.01, ***P<0.001.*

Next, the sensitivity of the wild-type eNOS, as well as each of the S114A, S114D and S633D eNOS mutants to activation by Ca^{2+} and CaM was determined (Fig. 13). In these experiments eNOS proteins were expressed in HEK-293 cells and eNOS activity in the 100000g cell pellet determined using the citrulline assay. The advantage of using this technique is that it facilitates the determination of changes in the sensitivity of the enzyme to Ca^{2+}/CaM.

However, all of the tested eNOS mutants (S114A, S114D and S633D) were concentration-dependently activated by increasing concentrations of Ca^{2+} and CaM. Differences in the enzymatic activity of the eNOS mutants could not be attributed to differences in protein expression, as determined by Western blotting.

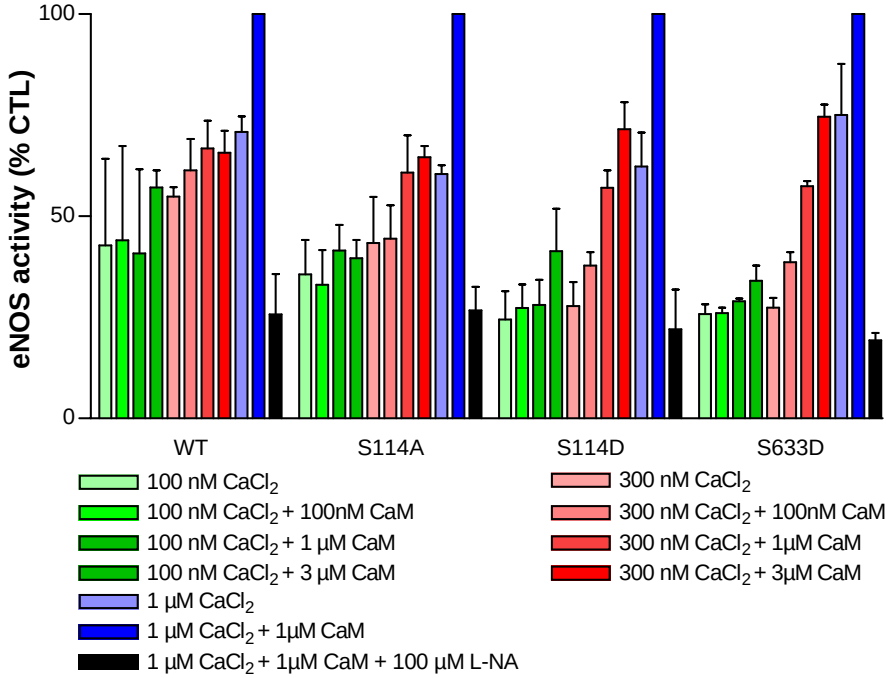

Figure 13. Effect of the mutation of eNOS Ser^{114} and Ser^{633} on the Ca^{2+} and CaM dependency of the enzyme. *eNOS activity was determined by monitoring the conversion of [³H]L-arginine to [³H]L-citrulline in samples prepared from HEK-293 cells transfected with either wild-type eNOS or the S114A, S114D and S633D eNOS mutants. The enzymatic reaction was initiated by the addition of [³H]L-arginine to a reaction mixture containing different concentrations of Ca^{2+}, CaM and N^{ω}nitro-L-arginine (L-NA; 100 µmol/L; black bar). Data are presented as percent activity with respect to the activity of the enzyme at V_{max} conditions (1µmol/L $CaCl_2$ and 1µmol/L CaM) and the bar graph represents the mean ± SEM of data obtained in three independent experiments.*

4.1.2 Effect of shear stress and bradykinin on the phosphorylation of eNOS

To assess the basal and stimulation-dependent phosphorylation of eNOS, human endothelial cells were exposed to either fluid shear stress (12 dynes cm^{-2}) or bradykinin (10 nmol/L) for up to 60 minutes. eNOS was basally phosphorylated on Thr^{495} and got dephosphorylated at one minute in response to bradykinin and back to basal levels in five minutes. Shear stress had no effect on Thr^{495} dephosphorylation. eNOS Ser^{1177} which was basally not phosphorylated showed a transient increase in phosphorylation (at 30 seconds and 1 minute) and got back to basal levels at 5 minutes. While Ser^{1177} got phosphorylated in response to shear at 10 minutes and was maintained as long as the stimuli was applied (up to 60 minutes).

eNOS was basally phosphorylated on Ser^{114} under basal conditions and there was no change in phosphorylation upon application of shear stress or bradykinin (Fig. 14). Interestingly the Triton X-100-insoluble fraction showed a decrease in the phosphorylation on Ser^{114} in response to shear, but the same was not true for bradykinin stimulation. While, under basal conditions Ser^{633} was not phosphorylated, phosphorylation was detected upon application of shear stress for 30 minutes which persisted as long as the cells were exposed to fluid shear stress i.e., for up to 60 minutes. The effect was not evident at shorter time points studied i.e. five and 10 minutes. However, there was no noticeable change in phosphorylation on Ser^{633} upon bradykinin stimulation (Fig. 14).

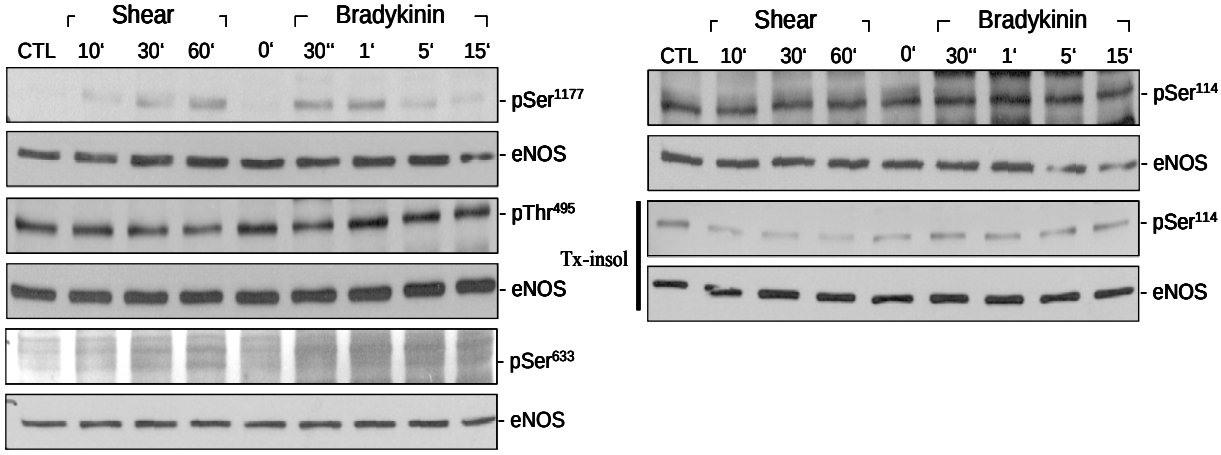

Figure 14. Effect of shear stress and bradykinin on the phosphorylation of eNOS. *Confluent primary cultures of human endothelial cells were exposed to fluid shear stress (12 dynes cm-²) or bradykinin (10 nmol/L) for the time indicated. Thereafter, the cells were harvested and Triton X-100 soluble and insoluble fractions were analyzed for eNOS phosphorylation on Ser^{1177}, Thr^{495}, Ser^{114} and Ser^{633} by Western blotting using phosphospecific antibody. The blots are representative of three independent experiments.*

4.1.3 Effect of the mutation of eNOS on NO production in intact COS-7 cells as measured by ESR spectroscopy

In vitro activity assays performed in the presence of optimal concentrations of Ca^{2+}/CaM and other essential cofactors cannot accurately reflect the production of NO in living cells since phosphorylation, sub cellular localisation and regulated protein-protein interactions can all affect eNOS activity (Ghosh *et al.*, 1998; Sessa *et al.*, 1995). Therefore, we compared the ability of wild-type eNOS to generate NO in intact COS-7 cells with that of the eNOS mutants. Recombinant adenoviruses encoding either the myc-tagged wild-type enzyme or one of the T475A/S1177D (TA/SD), S114A, S114D, T495A, T495D, or S633D eNOS mutants were used to infect COS-7 cells. The latter cells were chosen because of the need to obtain a

high level of protein expression and because the viruses used can replicate in HEK-293 cells, leading to cell lyses. In these experiments a non-phosphorylatable mutant of Thr^{495} (T495A), a phospho-mimetic mutant of Thr^{495} (T495D) and a highly active double mutant TA/SD were expressed in COS-7 cells as additional positive controls. A colloidal $Fe(DETC)_2$ complex was used to trap NO as it has been shown to be specific and appropriate for detection by ESR spectroscopy (Kleschyov *et al.*, 2000; Mülsch *et al.*, 1995). NO production was assessed under basal conditions as well as following stimulation with ionomycin (0.1 µmol/L, 15 minutes) by trapping with $Fe(DETC)_2$ at 37°C.

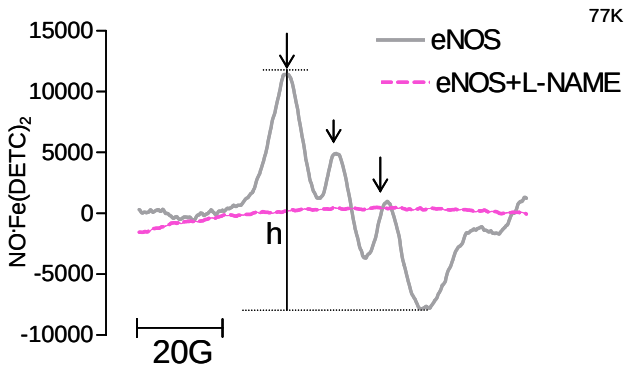

Figure 15. A typical ESR spectrum of the NO-Fe(DETC)$_2$ complex. *A typical ESR spectrum of the NO-Fe(DETC)$_2$ complex (+/- L-NAME, 300 µmol/L) obtained by incubating Fe(DETC)$_2$ for 30 min at 37°C with cells expressing eNOS. The cells were scraped, frozen and measured at 77 K in a liquid nitrogen cooled dewar using a ESR EMX spectrometer (Bruker). The arrows indicated the triplet signal that is typical for NO. The intensity of the signal marked as h in the figure gives the measure of NO detected.*

Under basal (unstimulated) conditions, COS-7 cells expressing either the TA/SD, S114A, S114D or S633D eNOS mutants generated more NO than cells expressing the wild-type eNOS enzyme, or the T495A or T495D mutants (Fig. 16A). Cell stimulation with ionomycin (0.1 µmol/L, 15 minutes) elicited a moderate increase in NO production by all of the enzymes. Differences in the enzymatic activity of the mutants were not related to differences in protein expression, as determined by Western blotting (Fig. 16A; inset).

As intracellular levels of H_4B decrease rapidly in cultured cells (d'Uscio *et al.*, 2003), and can lead to eNOS uncoupling (Landmesser *et al.*, 2003; Smith *et al.*, 2002), experiments were repeated using cells pre-treated with sepiapterin, a stable precursor of H_4B. The presence of sepiapterin resulted in a modest increases in NO production from all the samples. However, the difference in the amount of NO produced under basal condition between cells expressing the wild-type enzyme and either of TA/SD, S114A, S114D or S633D eNOS mutants was

significantly enhanced, while the activity of the T495A and T495D eNOS mutants did not deviate much from that of the wild-type eNOS (Fig. 16B). Stimulation with ionomycin caused a moderate increase in NO production in all the samples. In contrast, stimulation of endothelial cells generally results in a 10-20 fold increase in intracellular cGMP levels. This discrepancy can most likely be attributed to the fact that cultured cells do not retain all the signalling pathways that regulate eNOS in primary cells.

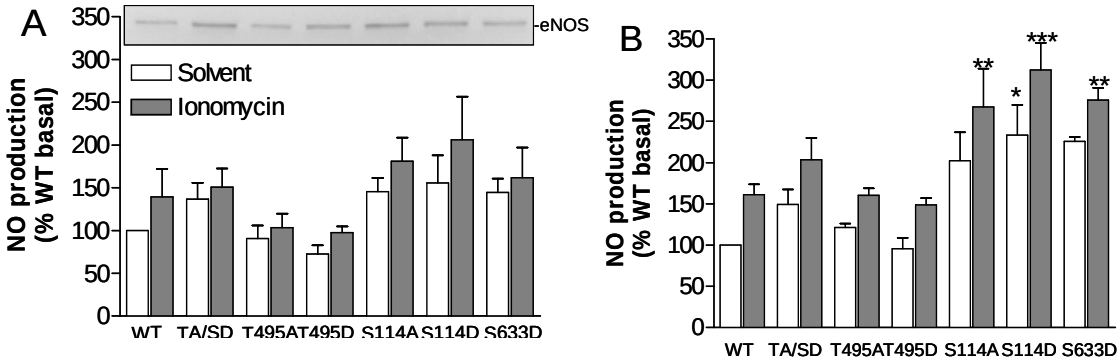

Figure 16. Effect of specific mutation of eNOS on NO production in COS-7 cells. *NO production from intact COS-7 cells infected with wild-type (WT) eNOS or the TA/SD, T495A, T495D, S114A, S114D and S633D eNOS mutants in the absence (open bars) or presence (closed bars) of ionomycin (100 nmol/L), was assessed by NO spin trapping with $Fe(DETC)_2$ and measured by electron spin resonance (ESR) spectroscopy at 77K. Experiments were performed in cells pre-treated with either* ***(A)*** *solvent or* ***(B)*** *sepiapterin (10 µmol/L, 24 hours). eNOS expression was analysed by Western blotting using an eNOS-specific antibody. The bar graphs represent the mean ± SEM of data obtained in three independent experiments; *P<0.05, **P<0.01, ***P<0.001 vs WT in the absence of ionomycin.*

4.1.4 Effect of specific mutation on eNOS dimer formation

Since the treatment of eNOS expressing cells with sepiapterin caused significant increases in the production of NO production by the wild-type (WT) eNOS as well as the TA/SD, S114A, S114D or S633D eNOS mutants and H_4B has been proposed to stabilise the eNOS dimer (Franco *et al.*, 2004; List *et al.*, 1997), we next determined whether or not the mutations studied affected the ability of eNOS to dimerise.

Protein extracts (Triton-soluble) from COS-7 cells expressing wild-type eNOS or one of the TA/SD, T495A, T495D, S114A, S114D or S633D eNOS mutants were subjected to low temperature (LT)-PAGE and Western blot analysis. The gels were run both under reducing and non-reducing conditions as the displacement of Zn^{2+} from the eNOS dimer leads to the

formation of disulphide bridges between the cysteine residues of eNOS are disrupted under reducing conditions(List *et al.*, 1997; Zou *et al.*, 2002). We found that most of the eNOS expressed in COS-7 cells was dimeric (Fig. 17) and we were unable to detect any difference between the ability of the wild-type enzyme and the eNOS mutants studied to dimerise. Any differences observed could be attributed to differences in eNOS expression levels.

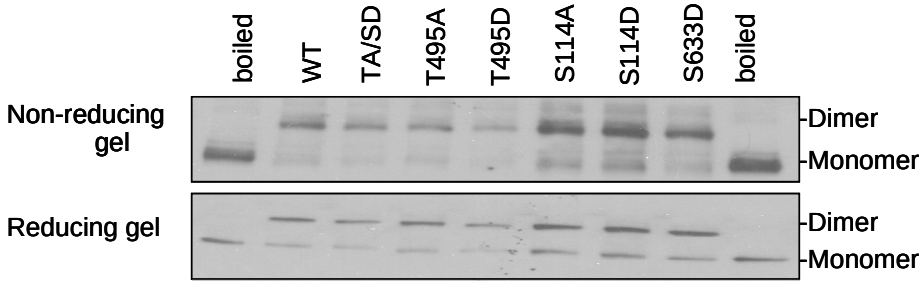

Figure 17. Effect of specific mutation on eNOS dimer formation. *The ability of eNOS to form homodimers was analysed using Triton X-100-soluble fractions prepared from COS-7 cells expressing either wild-type (WT) eNOS or one of the TA/SD, T495A, T495D, S114A, S114D and S633D eNOS mutants by low temperature PAGE (LT-PAGE) and Western blot analysis. Protein extracts (20 µg) were subjected to LT-PAGE in the presence (reducing) or absence (non-reducing) of 5% β-2 mercaptoethanol. The Western blots shown are representative of data obtained in two to three additional experiments.*

To compare the ability of eNOS to dimerise in the COS-7 over expression model versus endothelial cells as well as to determine whether or not cell stimulation was associated with any change in the dimer:monomer ratio, we assessed the effects of bradykinin (1 µmol/L, 10 seconds to 5 minutes) on eNOS dimer levels in porcine aortic endothelial cells.

In control (unstimulated) endothelial cells, eNOS existed predominantly as a dimer although significantly more monomer was detected in the endothelial cells studied than in the COS-7 cells that over-expressed eNOS. Cell stimulation with bradykinin elicited a rapid and transient dephosphorylation of Thr^{495} that was maximal at 30 seconds to 1 minute after stimulation but was rapidly rephosphorylated within 5 minutes; changes which have previously been linked to the activation of eNOS (Fleming *et al.*, 2001). However, the bradykinin-induced activation of eNOS did not affect the dimer to monomer ratio in endothelial cells (Fig. 18).

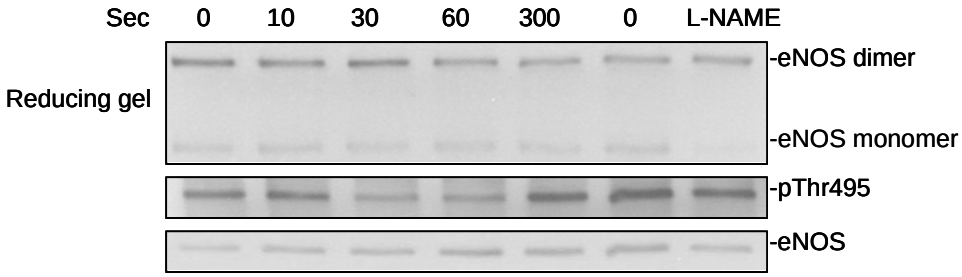

Figure 18. Effect of bradykinin stimulation on eNOS dimer formation. *To study the effect of bradykinin stimulation on eNOS activation and homodimers, porcine endothelial cells were stimulated with bradykinin (1 µmol/L) for the times indicated (10 to 300 seconds). Protein extracts (20 µg) were subjected to low temperature PAGE (LT-PAGE) and Western blot analysis. The bradykinin-induced stimulation of eNOS was evident from the dephosphorylation at Thr^{495} as determined by using phospho-specific Thr^{495} antibody The Western blot shown is representative of data obtained in two independent experiments.*

4.1.5 Effect of eNOS inhibitors, an NO· donor and radicals on eNOS dimer stability

Treatment of endothelial cells or purified eNOS with an NO donor, $ONOO^-$ and O_2^- have been reported to reduce the stability of eNOS dimer (Ravi *et al.*, 2004; Stocker *et al.*, 2004; Zou *et al.*, 2002). As dimerisation is an absolute requirement for the catalytic activity of all three NOS isoforms (Baek *et al.*, 1993; Klatt *et al.*, 1995; Lee *et al.*, 1995), we ascertained the effects of NO, $ONOO^-$ and O_2^- on the stability of eNOS dimer in porcine aortic endothelial cells using LT-PAGE. Porcine endothelial cells were used for this study as the dimer to monomer ratio was higher than that detected in human umbilical vein endothelial cells (data not shown). Under control conditions, i.e. cells treated with solvent (HEPES-Tyrode) eNOS existed predominantly as a dimer (Fig. 19). The dimer:monomer ratio was not affected by the treatment of cells with the $ONOO^-$ donor; 3-morpholino-sydnonimine (SIN-1), while a high concentration of $ONOO^-$ (3.75 mmol/L) significantly decreased the dimer levels (34.33 ± 1.2% vs CTL, $P<0.001$, n=3). The exogenous O_2^--generating system; xanthine/xanthine oxidase (X/XO) slightly but non-significantly attenuated dimerisation (Fig. 19). The NOS inhibitor L-NAME (300 µmol/L, 1hour), increased basal eNOS dimerisation and under these conditions only $ONOO^-$ was able to affect the dimer:monomer ratio (Fig. 19).

The mutation of eNOS did not interfere with the dimerisation of the enzyme and only a high concentration of $ONOO^-$ (3.75 mmol/L) was able to disrupted the eNOS dimer. This observation suggests that the eNOS dimer is too stable to be disrupted by the modification of a single amino acid.

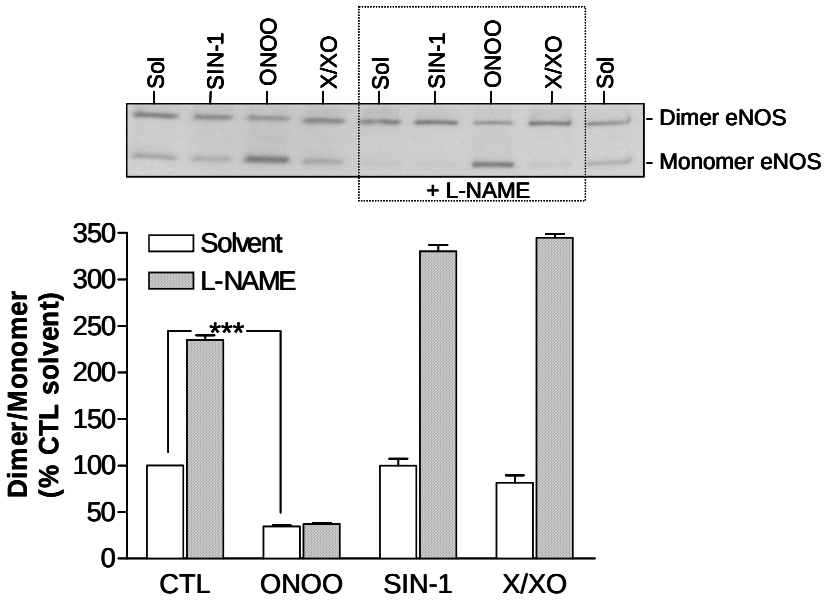

Figure 19. Effect of SIN-1, ONOO$^-$, O_2^- and a NOS inhibitor on the dimerisation of eNOS. *Porcine aortic endothelial cells were pretreated with solvent, 3-morpholino-sydnonimine (SIN-1, 1 mmol/L, 30 minutes) xanthine/xanthine oxidase (X/XO,1 mmol/0.1U/mL, 30 minutes) and peroxynitrite (ONOO$^-$, 3.75 mmol/L, 10 seconds) in the absence or presence of L-NAME (300 µmol/L). Protein (20 µg extract from each sample) was then used to determine the ratio of eNOS dimer to monomers detected by LT-PAGE. The bar graph summarizes the data from three independent experiments; ***P<0.001 vs. CTL.*

4.1.6 Effect of specific eNOS mutation on its intracellular localisation

Given that the subcellular localisation of eNOS is known to affect its activity (Jiang *et al.*, 2003; Ortiz & Garvin, 2003; Sessa *et al.*, 1995), one possible explanation for the differences in the activity of the eNOS mutants could be an alteration in its subcellular targeting. To investigate the consequence of specific mutation of eNOS on its subcellular localisation human umbilical vein endothelial cells were infected with recombinant adenovirus carrying cDNA encoding myc-tagged wild-type eNOS or one of the TA/SD, TD/SD (Fig. 20), S114A, S114D, T495A, T495D, S633/634A or S633D eNOS mutants (Fig. 21) and immunohistochemically stained with an antibody directed against myc and an appropriate secondary antibody conjugated with the dye Alexia 546. β-catenin was visualised to mark the plasma membrane. First passage endothelial cells were used as the culturing of endothelial cells results in the loss of certain cellular factors as well as signalling pathways that are important for the activation of eNOS.

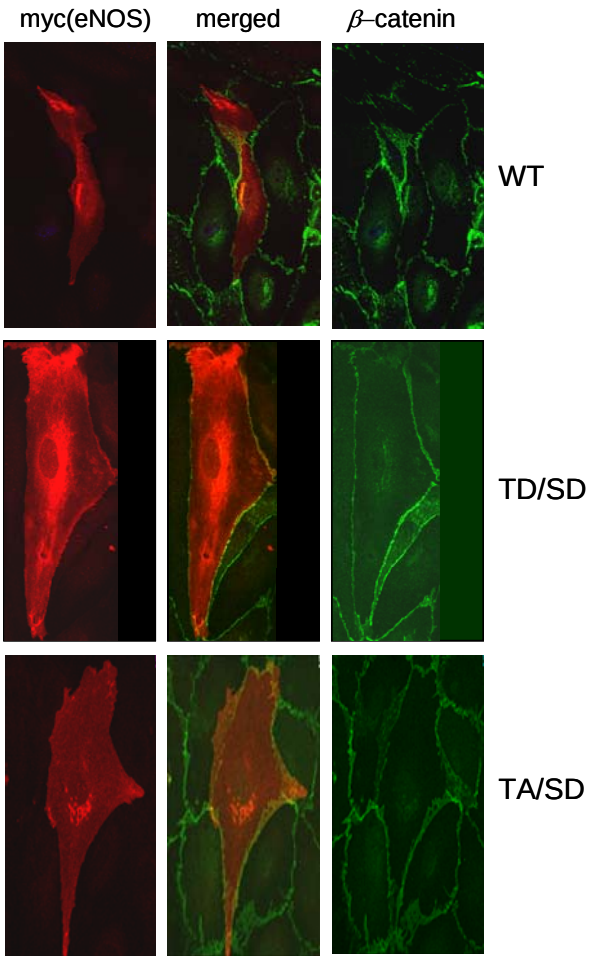

Figure 20. Effect of mutation on Thr^{495} and Ser^{1177} on the intracellular localisation of eNOS. *Confocal microscopy of human endothelial cells (first passage) infected with a myc-tagged wild-type eNOS or either of the T495D/S1177D (TD/SD) or T495A/S1177D (TA/SD) eNOS mutants and labelled with antibodies directed against c-myc (red) and β-catenin (green). The results presented are representative of data obtained in three independent experiments.*

As reported previously by others (Fulton *et al.*, 2002), the wild-type eNOS was localised discretely at the plasma membrane and in the peri-nuclear Golgi apparatus (Fig. 20). There was no difference in the localisation pattern of eNOS mutants S114A, S114D, T495A T495D and S633/634A to that of wild-type eNOS. However, the phospho-mimetic S633D mutant showed a distinct and continuous localisation of eNOS at the plasma membrane which was not observed with the wild-type enzyme (Fig. 21). The TA/SD double mutant exhibited a similar pattern of localisation to the wild-type enzyme, while the TD/SD mutant showed a greater degree of eNOS staining at the plasma membrane as well as to the peri-nuclear Golgi apparatus to that of wild-type. However, this effect could be due to the higher level of TD/SD expression (Fig. 20).

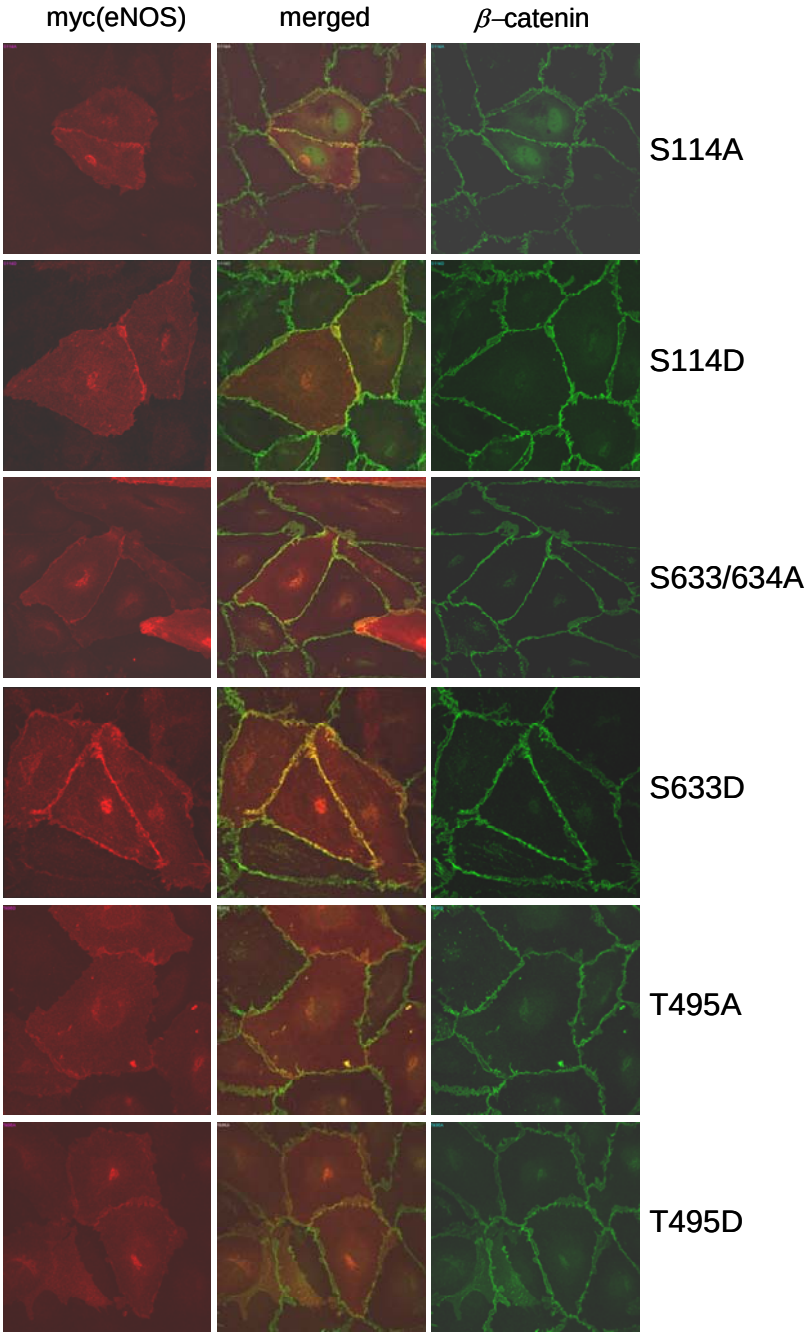

Figure 21. Effect of specific mutations on the intracellular localisation of eNOS. *Confocal microscopy of human endothelial cells (first passage) infected with a myc-tagged eNOS mutants (S114A, S114D, S633/634A, S633D, T495A and T495D) and stained with antibodies directed against myc (red) and β-catenin (green). The results presented are representative of data obtained in three independent experiments.*

4.1.7 Effect of specific mutation on superoxide anion (O_2^-) production by eNOS

eNOS has been reported to generate O_2^- under specific conditions, particularly when the concentrations of L-arginine and H_4B are limited (Xia *et al.*, 1996; Xia *et al.*, 1998) but it is

unclear whether or not phosphorylation-dependent regulatory mechanisms are involved in this process. We therefore set out to elucidate the effect of various mutations on eNOS uncoupling i.e. the ability of the enzyme to generate O_2^-. To this end, the L-NAME (300 µmol/L)-sensitive generation of O_2^- was assessed by monitoring the formation of the stable spin label 3-methoxycarbonyl-proxyl (CM•) in HEK-293 cells expressing either of the eNOS mutants S114A, S114D, TA/SD, S633D, T495A or T495D. A tracing of a typical ESR spectrum for CM• is shown in figure 22.

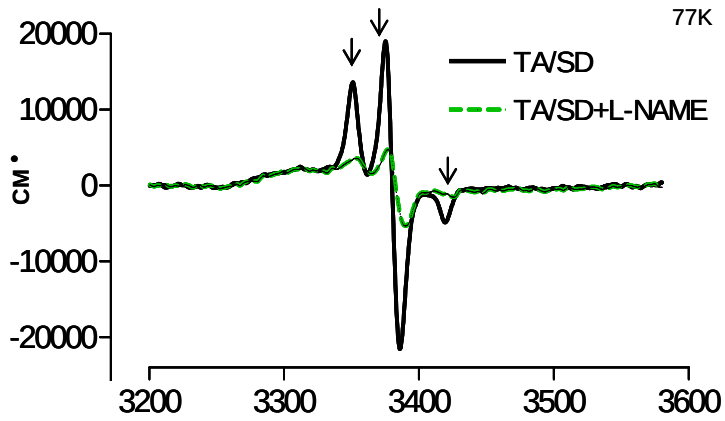

Figure 22. A typical ESR spectrum of 3-methoxycarbonyl-proxyl (CM•). *Representative tracing showing the typical ESR spectrum of 3-methoxycarbonyl-proxyl (CM•) in the presence (dotted line) or absence (continuous line) of L-NAME (300 µmol/L), obtained by incubating CMH for 5 minutes at 37°C with cells expressing eNOS. The supernatant was frozen and measured at 77 K in a liquid nitrogen cooled dewar using a EMX ESR spectrometer (Bruker). The arrows indicated the triplet signal that is typical for CM• radicals.*

Under basal conditions the TA/SD and T495A eNOS generated more O_2^- than the S633D S114D S114A or T495D mutants. An increase in O_2^- production was observed following the application of L-NAME to cells expressing either the S114A or the S114D eNOS mutants a finding which can be attributed to the loss of basal NO production which scavenges O_2^-. The NOS inhibitor did not affect O_2^- production by the S633D or the T495D eNOS mutants. However, the generation of O_2^- by the T495A mutant as well as the TA/SD double mutant was attenuated by L-NAME (Fig. 23), indicating that the phosphorylation of these residues may affect the ability of eNOS to generate O_2^- versus NO.

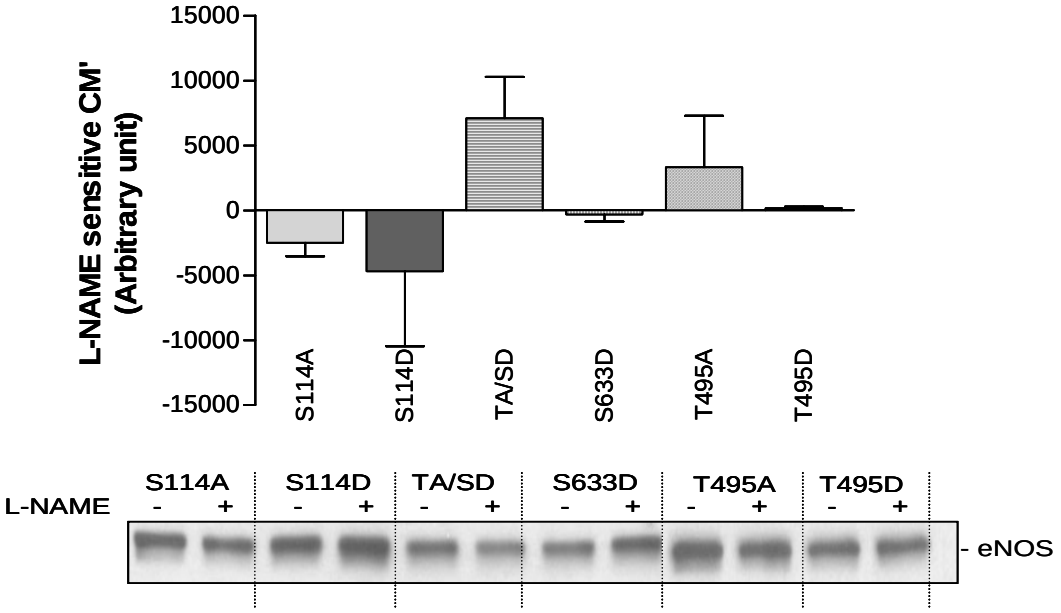

Figure 23. Effect of specific mutation on the L-NAME-sensitive production of superoxide anions (O_2^-) by eNOS. *HEK-293 cells expressing the eNOS mutants; S114A, S114D, TA/SD, S633D, T495A or T495D, were treated with either solvent or L-NAME (300 µmol/L), and O_2^- generation was assessed by monitoring the formation of the stable spin label 3-methoxycarbonyl-proxyl (CM•) using ESR spectroscopy. The bar graph summarizes the mean ± SEM of data obtained in four different experiments (each in duplicate). The inset shows a representative Western blot to demonstrate the equivalent expression of eNOS.*

To get relatively equal amounts of eNOS expressed for all the mutants studied, COS-7 cells were infected with recombinant adenoviruses. Significantly more O_2^- was generated in cells expressing the non-phosphorylatable T495A mutant than in cells expressing the phospho-mimetic T495D mutant (Fig. 24A). The additional mutation of Ser^{1177} to aspartate in order to increase electron flow through the reductase domain (McCabe *et al.*, 2000), slightly, but not significantly, enhanced O_2^- production. The L-NAME sensitive production of radicals by the T495A mutant and the wild-type enzyme were comparable; a phenomenon that can be accounted for by the fact that, unlike the situation in endothelial cells, eNOS is not basally phosphorylated on Thr^{495} in COS-7 cells (Fig. 24B).

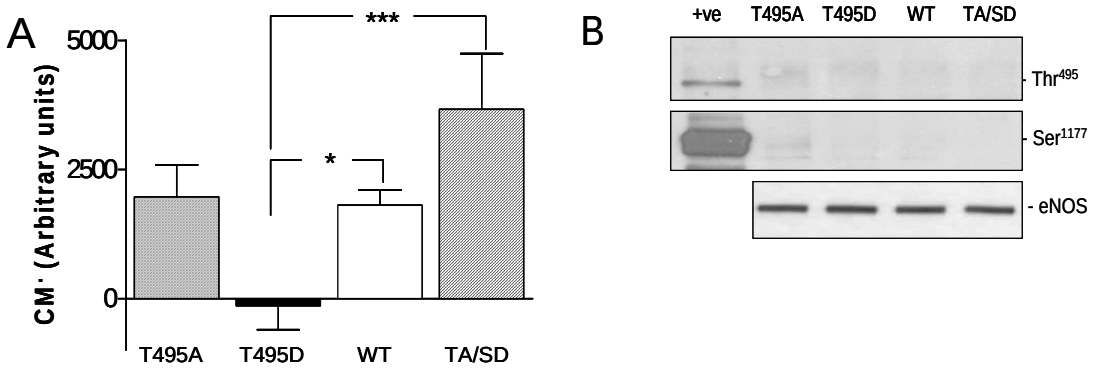

Figure 24. Superoxide anion (O_2^-) production by wild-type eNOS and eNOS mutants. *(**A**) COS-7 cells expressing either wild-type eNOS (WT) or the T495A, T495D or T495A/S114D (TA/SD) eNOS mutants were treated with either solvent (CTL) or L-NAME (300 µmol/L) and O_2^- generation was assessed by monitoring the formation of the stable spin label 3-methoxycarbonyl-proxyl ($CM^•$) using ESR spectroscopy. The bar graph summarizes the L-NAME sensitive production of O_2^- measured in six independent experiments; *P<0.05, ***P<0.001 vs. WT. (**B**) Western blots showing the phosphorylation status of wild-type eNOS (WT) and the T495A, T495D or TA/SD eNOS mutants in COS-7 cells. Positive controls (+ve; unstimulated human endothelial cells in the case of Thr^{495} and cells stimulated with bradykinin for 2 minutes in the case of Ser^{1177}) were included to demonstrate the sensitivity of the phospho-specific antibodies.*

4.2 Effect of ox-LDL on eNOS uncoupling

Oxidative stress plays a pivotal role in the pathogenesis of vascular injury and in the progression of atherosclerosis. Oxidized low-density lipoprotein (ox-LDL) is reported to increase O_2^- production in endothelial cells (Heinloth *et al.*, 2000; Rueckschloss *et al.*, 2001) and to decrease the bioavailability of NO via a process involving the lectin-like ox-LDL receptor-1 (Cominacini *et al.*, 2001). Since we have previously shown that the dephosphorylation of eNOS on Thr^{495} is associated with the enhanced production of O_2^- (Lin *et al.*, 2003) , and animal and clinical studies have revealed a strong correlation between the extent of atherosclerosis and titers of auto-antibodies to epitopes of oxLDL (Cyrus *et al.*, 1999; Palinski *et al.*, 1995; Salonen *et al.*, 1992), we set out to determine whether the ox-LDL-induced uncoupling of eNOS can be linked to changes in its phosphorylation.

The bioavailability of eNOS-derived NO from human umbilical vein endothelial cells was assessed by monitoring the basal and bradykinin-induced increase in cyclic GMP. Under basal conditions, ox-LDL attenuated cyclic GMP production by approximately 25% while the bradykinin-induced increase in cyclic GMP levels was reduced by 45% (Fig. 25A). In cells pre-treated with SOD, basal cyclic GMP levels were significantly (2.8 ± 0.1-fold, P<0.01, n=8) elevated such that there was no longer a difference between the control and ox-LDL-

treated groups. SOD however failed to completely normalize the response to bradykinin in cells exposed to ox-LDL (Fig. 25A).

A low level of O_2^- could be detected using lucigenin-enhanced chemiluminescence in human endothelial cells treated with either solvent or native LDL. Superoxide anion levels were however markedly increased in cells treated with ox-LDL for 24 hours (Fig. 25B). L-NAME attenuated O_2^- production in ox-LDL-treated cells but did not significantly affect radical production in either solvent- or native LDL-treated cells. Similar results were obtained in cells stimulated with ionomycin. Superoxide dismutase (200 U/mL) attenuated the chemiluminescence signal in all samples indicating specificity of lucigenin to O_2^- (Fig. 25C).

As H_4B is reported to influence eNOS coupling (d'Uscio *et al.*, 2003; Tiefenbacher *et al.*, 1996), we assessed cellular H_4B levels in the absence and presence of native-LDL or ox-LDL (Fig. 25D). Ox-LDL (30 µg/mL) failed to significantly attenuate H_4B levels.

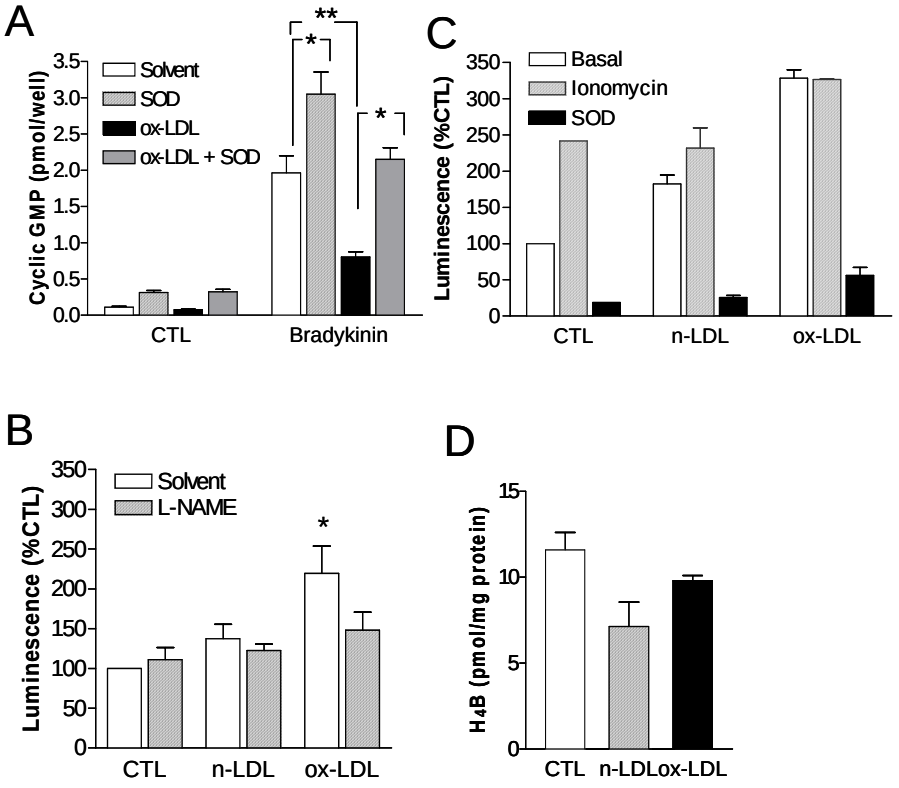

Figure 25. Effect of ox-LDL on the generation of NO and O_2^- by human endothelial cells. *Human endothelial cells were pretreated with either solvent (culture medium) native LDL (nLDL) or ox-LDL (30 µg/mL) for 24 hours. Thereafter, the production of* ***(A)*** *cyclic GMP, (**B** and **C**) O_2^- (lucigenin-enhanced chemiluminescence) were assessed and* ***(D)*** *tetrahydrobiopterin (H_4B) levels were determined. Experiments were performed in the absence and presence of bradykinin (10 nmol/L, 5 minutes), superoxide dismutase (SOD, 150 U/mL), ionomycin (100 nmol/L) and N^{ω}nitro-L-arginine methyl ester (L-NAME, 300 µmol/L). The bar graphs summarize the data obtained in three to eight independent experiments; *P<0.05, **P<0.005 vs. CTL.*

4.2.1 Time course of the LDL-induced changes in eNOS phosphorylation

In cultured human endothelial cells eNOS was phosphorylated on Thr^{495} but only weakly on Ser^{1177}, as reported previously (Fleming *et al.*, 2001). Incubation of endothelial cells with native LDL did not affect the phosphorylation of eNOS, while ox-LDL resulted in the time-dependent dephosphorylation of eNOS on Thr^{495} (Fig. 26A). Ox-LDL concentration-dependently dephosphorylated eNOS on Thr^{495}, with an approximately 50% decrease in phosphorylation being observed using concentrations of 5 and 10 µg/mL and almost complete dephosphorylation occurring after 24 hours treatment with 30 µg/mL (Fig. 26B).

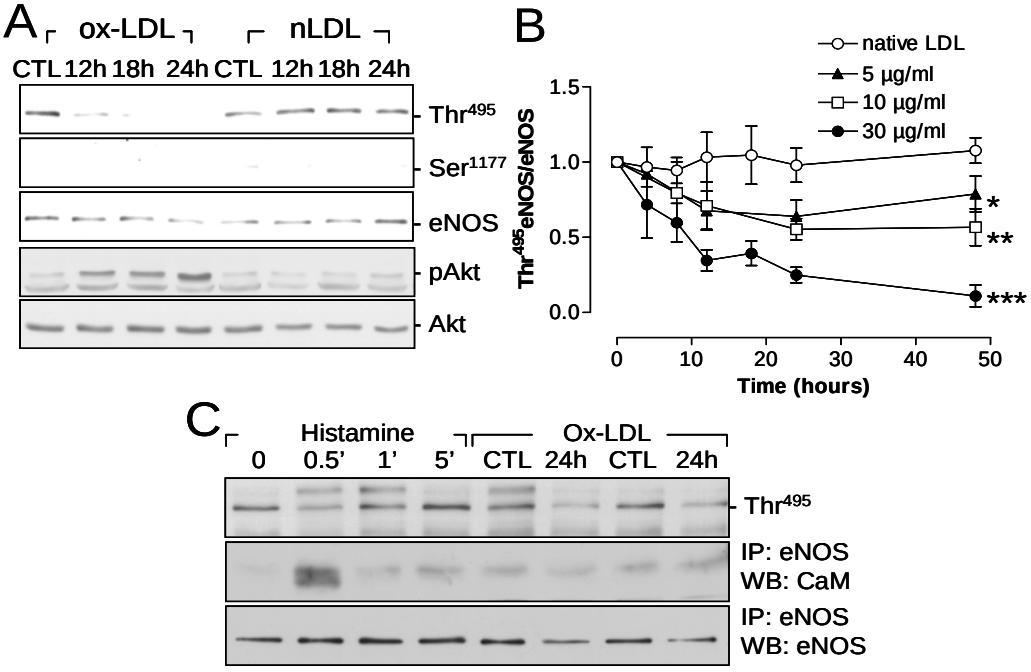

Figure 26. Time- and concentration-dependent effects of nLDL and ox-LDL on the phosphorylation of eNOS. *Human endothelial cells were incubated with native LDL (nLDL; 30 µg/mL) or ox-LDL (5 to 30 µg/mL) for 2 to 48 hours and* ***(A)*** *the phosphorylation of eNOS on Thr^{495} and Ser^{1177} and the phosphorylation of Akt were assessed by Western blotting with phospho-specific antibodies. The data were quantified relative to* ***(B)*** *total eNOS and the graph summarizes data obtained in nine to 12 independent experiments; *P<0.05, **P<0.005 vs. native LDL.* ***(C)*** *Comparison of the effects of the dephosphorylation of eNOS Thr^{495} by histamine (0.1 µmol/L; 30 seconds to 5 minutes) and by ox-LDL (30 µg/mL, 24 hours) on the association of eNOS with calmodulin (CaM). The upper Western blot (WB) shows the stimulus-induced changes in Thr^{495} phosphorylation in the same lysates used to immunoprecipitate (IP) eNOS. Identical results were obtained in three additional experiments.*

Oxidized-LDL did not alter eNOS protein expression. However there was a time-dependent increase in Akt phosphorylation, which was not associated with the phosphorylation of eNOS on Ser^{1177} (Fig. 26A).

Since dephosphorylation of eNOS on Thr^{495} lead to an increase in CaM binding, which in turn increase the turnover of NO from NOS (Fleming *et al.*, 2001), we compared the effects of histamine and long-term (24 hours) stimulation of ox-LDL on the ability of CaM to bind to eNOS by co-immunoprecipitation. In cells maintained under control conditions, histamine induced the transient dephosphorylation of eNOS Thr^{495}, which was temporally correlated with the association of CaM. Interestingly, although ox-LDL elicited the dephosphorylation of eNOS on Thr^{495}, CaM did not associate with the enzyme (Fig. 26C).

To determine whether or not ox-LDL interfered with agonist-mediated activation of eNOS, we stimulated human endothelial cells with bradykinin (100 nmol/L). In contrast to the cells treated with native LDL, stimulation of ox-LDL-treated cells with bradykinin did not further affect Thr^{495} phosphorylation (Fig. 27A), but it did increase the phosphorylation of eNOS on Ser^{1177}. Ox-LDL-treatment also attenuated the PMA (300 nmol/L)-induced phosphorylation of eNOS Thr^{495} while slightly increasing that of Ser^{1177} (Fig. 27B). The ox-LDL-induced changes in eNOS phosphorylation were not associated with the activation of PKA or the AMP-activated protein kinase (data not shown).

Loss of phosphorylation on Thr^{495}; kinase or phosphatase involved?

The loss of phosphorylation can be attributed to either inhibition of the activity or expression of a kinase or an increase in the expression or activity of a protein phosphatase. The protein phosphatase 1 (PP1) specifically dephosphorylates eNOS on Thr^{495} (Fleming *et al.*, 2001) and, the PP1 inhibitor calyculin A (300 nmol/L) increased the phosphorylation of eNOS Thr^{495} in ox-LDL-treated cells (Fig. 27C). However, the dephosphorylation of eNOS was not associated with an increase in the expression of the phosphatases PP1 or PP2A (Fig. 27D), indicating the involvement of a kinase for the loss of phosphorylation on Thr^{495}.

Role of PKC in the ox-LDL-induced dephosphorylation of eNOS

Since PKC phosphorylates Thr^{495} (Michell *et al.*, 2001), and the response to PMA was attenuated in ox-LDL-treated cells, we assessed the effects of ox-LDL on PKC phosphorylation and activity. The PKC-pan antibody used detects PKCα, βI, βII, ζ, ε and δ isoforms only when phosphorylated at a carboxy terminal residue (Tyszkiewicz *et al.*, 2004; Walker & Plows, 2003).

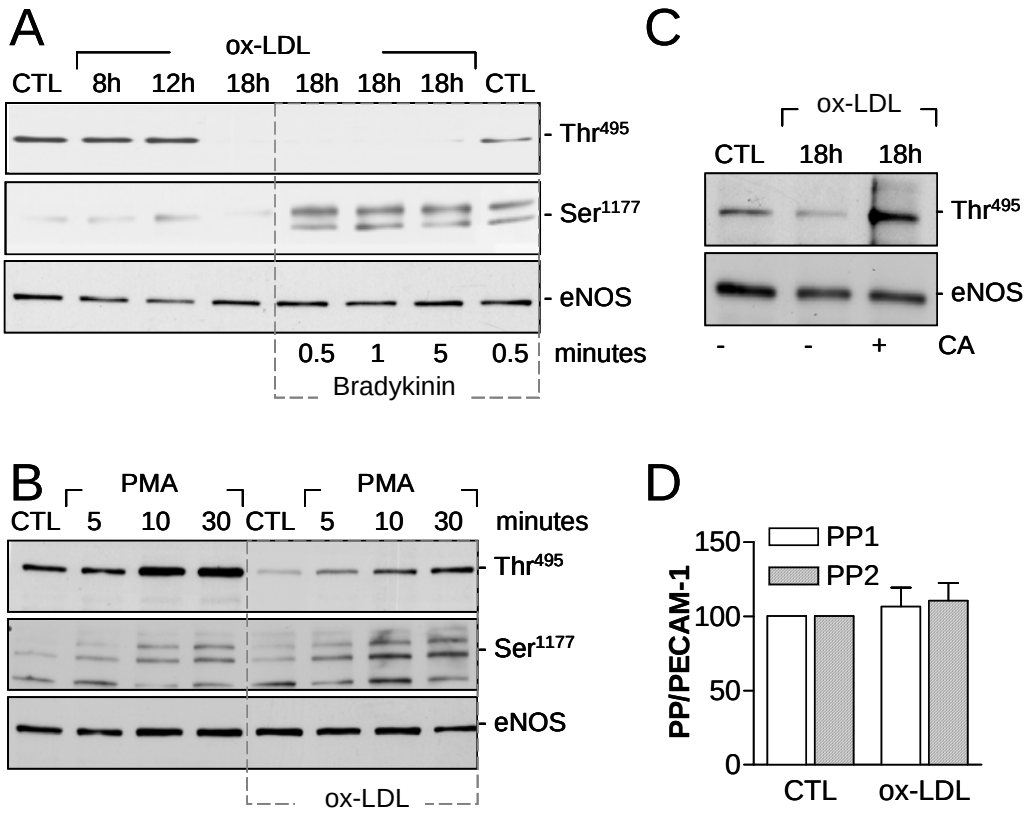

Figure 27. Effect of endothelial cell activation on the phosphorylation of eNOS in ox-LDL-treated cells. *Human endothelial cells were incubated with either solvent or ox-LDL (30 µg/mL) for the time indicated prior to stimulation with either* ***(A)*** *bradykinin (10 nmol/L, 30 seconds to 5 minutes),* ***(B)*** *PMA (300 nmol/L, 5 to 30 minutes) or* ***(C)*** *calyculin A (CA, 10 nmol/L for 10 minutes).* ***(D)*** *The effect of ox-LDL (30 µg/mL, 24 hours) on the expression of PP1 and PP2. The Western blots shown are representative of data obtained in three to five additional experiments and the bar graph summarizes data obtained in three independent experiments.*

Upon activation PKC translocates from the cytosol to the plasma membrane fraction. The basal phosphorylation of a PKC isoform (approximately 77 kDa) was detected in the Triton X-100-insoluble fraction of unstimulated endothelial cells. Native-LDL failed to affect the phosphorylation of this enzyme while ox-LDL induced a time-dependent decrease in the phospho-PKC pan signal (Fig. 28A and B). A similar phenomenon was observed using an antibody that selectively recognizes the phosphorylated forms of PKCα/β. Although a basal phosphorylation of PKC was detected in the Triton X-100-soluble cell fraction, ox-LDL affected neither the signal obtained with the PKCpan antibody nor the selective phospho PKCα/β antibody (Fig. 28C). PKCα was detected in the Triton X-100-insoluble fraction from unstimulated endothelial cells and the signal was time-dependently decreased by ox-LDL (Fig. 28A). Since the molecular mass of PKCα and the signal given by the phospho-PKC

antibodies used were identical, PKCα appears to be the isoform that phosphorylates eNOS Thr[495] and is targeted by ox-LDL.

Global PKC activity was assessed by monitoring the phosphorylation of acetylated myelin basic protein (Ac-MBP). Ox-LDL, but not nLDL, induced a time- and concentration-dependent decrease in PKC activity (Fig. 28D).

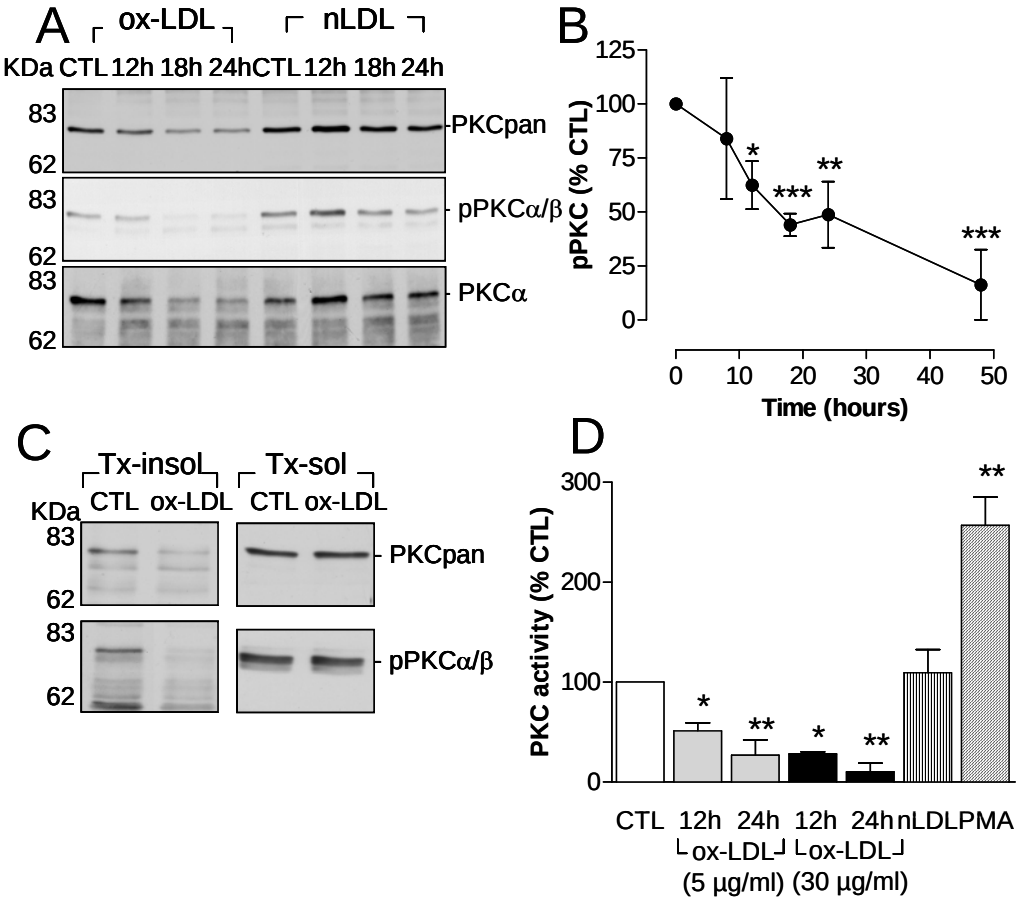

Figure 28. Effect of nLDL and ox-LDL on the phosphorylation and activity of PKC in endothelial cells. *Human endothelial cells were pretreated with either solvent (culture medium), native LDL (nLDL) or ox-LDL (30 µg/mL) for 8 to 24 hours. The phosphorylation of PKC was determined by Western blotting with phospho-specific specific antibodies* ***(A)*** *and quantified densitometrically* ***(B)****.* ***(C)*** *Comparison of the effect of ox-LDL (30 µg/mL, 18 hours) on the phosphorylation of PKC in the Triton X-100-insoluble (Tx-insol) and soluble (Tx-sol) cell fractions.* ***(D)*** *Time- and concentration-dependent effect of ox-LDL pre-treatment on the activity of PKC in endothelial cell lysates, as determined by the phosphorylation of myelin basic protein. Cells treated with PMA (1 µmol/L, 10 minutes) served as a positive control. The graphs summarize data obtained in three to 10 independent experiments; *P<0.05, **P<0.005, ***P<0.001 vs. time 0 or CTL.*

Effect of LPC on the phosphorylation of eNOS

Lysophosphatidylcholine (LPC) a major lipid constituent of ox-LDL, can mimic the effect of ox-LDL on endothelial cells (Wu *et al.*, 1998). LPC-treatment time- and concentration-dependently decreased PKC activity and led to the dephosphorylation of eNOS Thr^{495}. However, in contrast to the effects of ox-LDL, LPC also elicited the phosphorylation of Akt and eNOS Ser^{1177} (Fig. 29A). Moreover, 24 hours after application of the highest concentration of LPC used (100 µmol/L) eNOS expression was attenuated (data not shown).

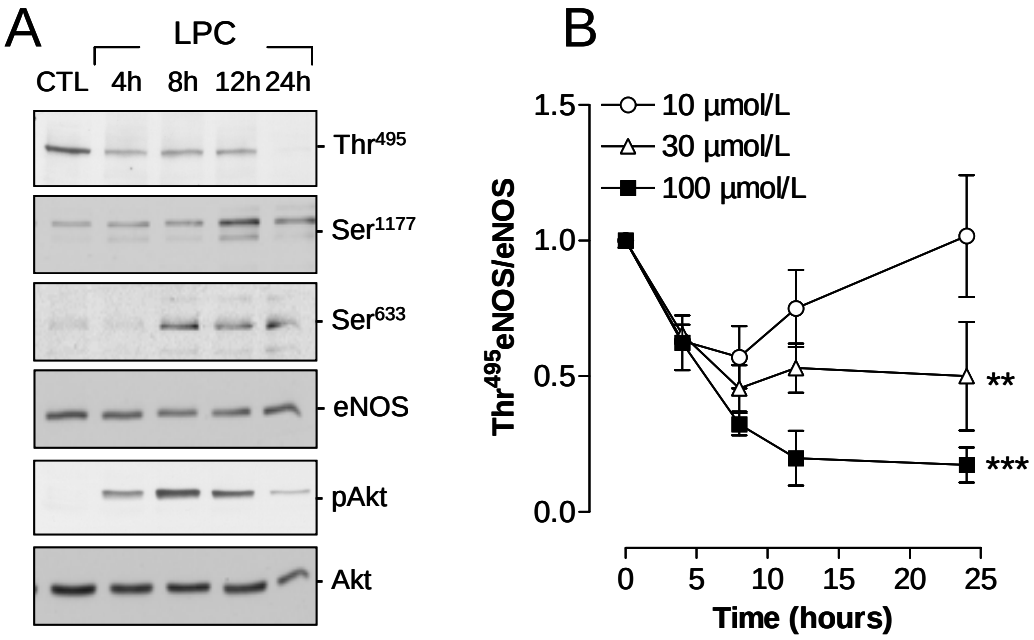

Figure 29. Time- and concentration-dependent effect of lysophosphatidyl choline (LPC) on the phosphorylation of eNOS. *Human endothelial cells were incubated with LPC (10 to 100 µmol/L) for 4 to 24 hours.* ***(A)*** *The phosphorylation of eNOS on Thr^{495}, Ser^{633} and Ser^{1177} and Akt on Ser^{473} was assessed by Western blotting with phosphospecific antibodies.* ***(B)*** *Changes in the phosphorylation of eNOS Thr^{495} were quantified relative to total eNOS levels and the graph summarizes data obtained in six independent experiments; **P<0.005, ***P<0.001 vs CTL.*

Effect of ox-LDL on the eNOS dimer

To determine whether or not the ox-LDL-induced uncoupling of eNOS was linked to an alteration in the stability of the eNOS dimer we assessed the effect of ox-LDL on the eNOS dimer to monomer ratio by LT-PAGE. Cells were pretreated for 24 hours in the absence or presence of ox-LDL and vitamin C. Treatment of endothelial cells with vitamin C increases intracellular H_4B content (Huang *et al.*, 2000) which in turn might influence the dimer to monomer ratio. Neither ox-LDL nor vitamin C treatment altered the levels of eNOS dimer. However, inhibition of eNOS with L-NAME resulted in a maximal restoration of eNOS dimer in human (Fig. 30), as well as in porcine endothelial cells (Fig. 31). The gels were run both

under reducing as well as non-reducing conditions as the displacement of Zn^{2+} from the eNOS dimer leads to the formation of disulphide bridges between the cysteine residues of eNOS which gets disrupted under reducing conditions (Zou *et al.*, 2002). However, no detectable differences were observed under the two conditions (Fig. 31).

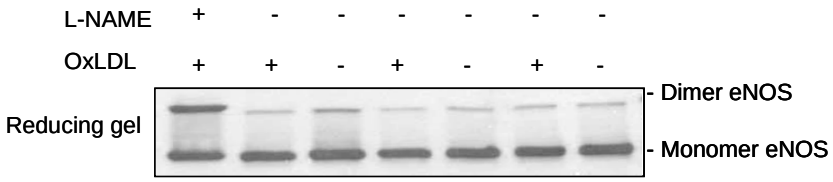

Figure 30. Effect of ox-LDL on eNOS dimerisation in endothelial cells. *Human endothelial cells were pretreated in the presence or absence of ox-LDL (30 µg/mL, 24 hours) in the presence or absence of L-NAME (300 µmol/L). Protein extracts (20 µg) from each sample were used to determine eNOS dimerisation by LT-PAGE under reducing (5% β-2 mercaptaethanol) conditions. The Western blot shown is representative of data obtained in two separate experiments performed with different batches of ox-LDL and in duplicate.*

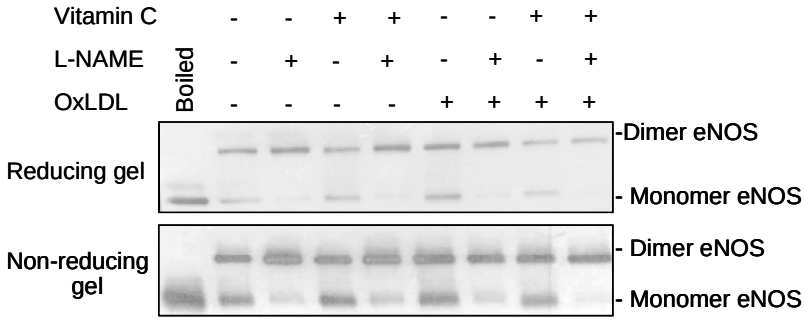

Figure 31. Effect of ox-LDL on eNOS dimerisation in endothelial cells. *Porcine aortic endothelial cells were pretreated in the presence or absence of ox-LDL (30 µg/mL) for 24 hours in the presence or absence of vitamin C (100 µmol/L) and L-NAME (300 µmol/L). Protein extracts (20 µg) from each sample were used to determine eNOS dimerisation by LT-PAGE under reducing or non-reducing conditions. The Western blots shown are representative of data obtained in three separate experiments.*

Effect of ox-LDL on the eNOS localisation

In endothelial cells treated with either solvent (CTL) or native LDL, eNOS was localised to the plasma membrane as well as to the peri-nuclear Golgi apparatus. Ox-LDL (30 µg/mL, 24 hours) resulted in the redistribution of the enzyme within the cytosol, and the Golgi disintegrated and was no longer detectable at the cell membrane (Fig. 32). These observations are consistent with previous studies, which reported the displacement of eNOS from caveolae upon ox-LDL treatment, by binding to endothelial cell CD36 receptors (Shaul, 2003)

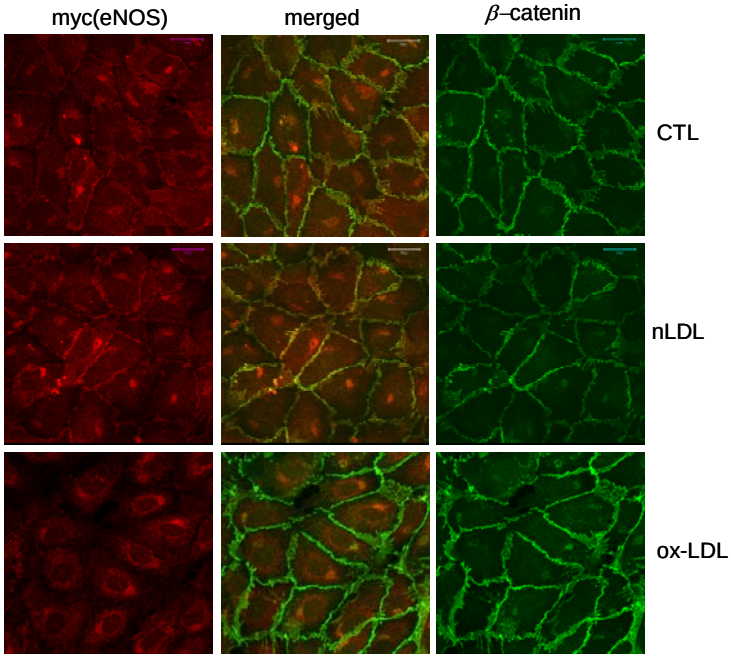

Figure 32. Effects of nLDL and ox-LDL on the intracellular localisation of eNOS. *Immunohistochemical staining of eNOS (red) and β-catenin (green) in human endothelial cells pretreated with either solvent (CTL), native LDL (n LDL; 30 µg/mL) or ox-LDL (30 µg/mL) for 24 hours. The results presented are representative of data obtained in three independent experiments.*

4.3 The role of tyrosine phosphorylation in the regulation of eNOS activity

Although eNOS can be tyrosine phosphorylated and endothelial NO production can be modulated by inhibitors of tyrosine kinases as well as tyrosine phosphatases (Fleming *et al.*, 1996a; Fleming *et al.*, 1998; Takenouchi *et al.*, 2004), almost nothing is known about the residues which are phosphorylated or the kinases which are involved. The consequences of the tyrosine phosphorylation of eNOS are unknown but are perhaps more likely to be related to the docking of associated scaffolding and regulatory proteins than to alterations in eNOS activity directly. As preliminary data indicated that both fluid shear stress (which increases eNOS activity) and insulin (which generally has no effect on NO production) elicit the tyrosine phosphorylation of eNOS (Fisslthaler, unpublished observations), we decided to determine the role of tyrosine phosphorylation on the regulation of eNOS. Since two different software programmes the NetPhos 2.0 (4 potentially phosphorylatable tyrosine residue) and DISPHOS (http://www.ist.temple. edu/DISPHOS, no phosphorylatable tyrosine residue) program gave contradicting results we decided to study tyrosine residues based on their location (Fig. 33).

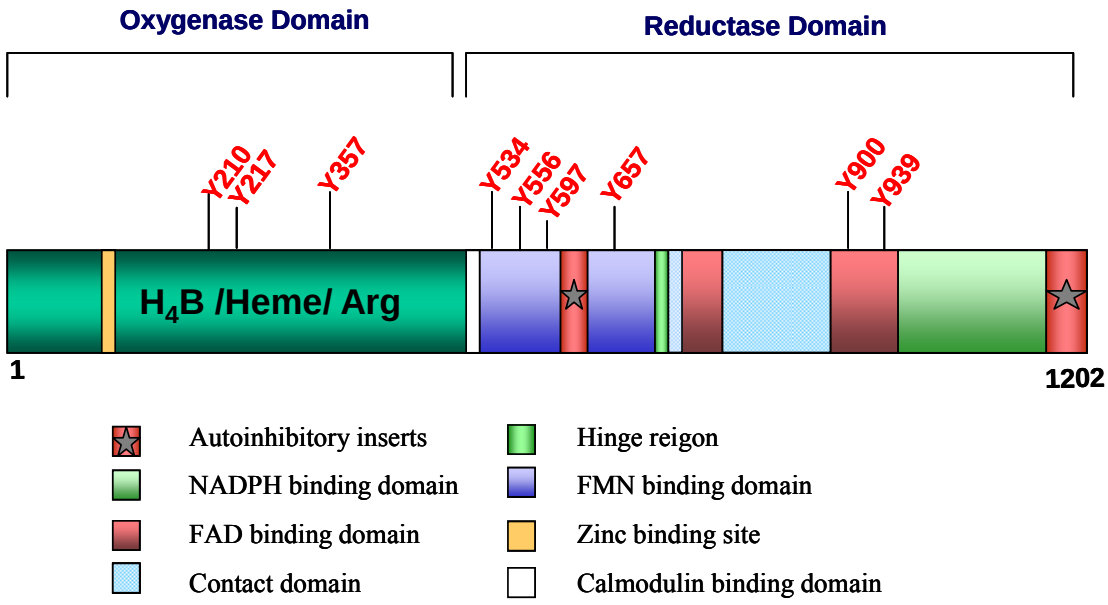

Figure 33. Scheme showing the organisation of eNOS (human sequence) and tyrosine residues. *The following tyrosine residues Tyr^{210}, Tyr^{217}, Tyr^{357}, Tyr^{467}, Tyr^{534}, Tyr^{556}, Tyr^{597}, Tyr^{657}, Tyr^{900} and Tyr^{939} based on there location in the eNOS Tyr^{357} in the arginine binding region, Tyr^{556} and Tyr^{657} in FMN binding domain and Tyr^{939} in FAD binding domain, which can potentially be phosphorylated and play a role in eNOS regulation.*

A number of tyrosine kinases e.g., Src (Cheng *et al.*, 2002; Okuda *et al.*, 1999) and Src-family kinases (Davis *et al.*, 2001; Davis *et al.*, 2004), focal adhesion kinase (FAK) (Li *et al.*, 1997; Takahashi *et al.*, 1997) and PYK2 (Cheng *et al.*, 2002; Tai *et al.*, 2002) are reported to be activated in response to the application of fluid shear stress and/or cyclic stretch to endothelial cells. Fluid shear stress is reported to induce the tyrosine phosphorylation of eNOS as well as that of PECAM and as a Src or Src-family kinase-dependent has been implicated in the tyrosine phosphorylation of PECAM (Cao *et al.*, 1998; Osawa *et al.*, 1997) we next determined the time course of Src activation in primary cultures of endothelial cells.

Effect of fluid shear stress on the tyrosine phosphorylation of Src and PYK2

The effect of shear stress on the activity of the tyrosine kinase Src, or rather on the dephosphorylation of the inhibitory tyrosine residue Tyr^{527}, was assessed in primary cultures of porcine aortic endothelial cells. We observed a time-dependent dephosphorylation of Tyr^{527} that was significant 5 minutes after the application of shear stress and increased over experimental period studied (up to 30 minutes) indicating an increase in the activity of Src Fig. 34).

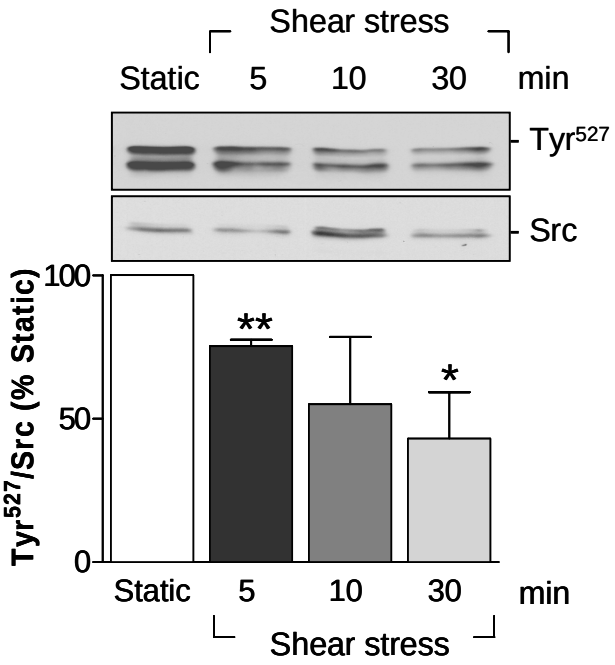

Figure 34. Effect of fluid shear stress on the tyrosine phosphorylation of Src. *Confluent primary cultures of porcine aortic endothelial cells were exposed to fluid shear stress (12 dynes cm-²) for the time indicated. Thereafter, the cells were harvested and Src phosphorylation on Tyr^{527} was analyzed by Western blotting with a specific antibody. The same blot was then stripped and reprobed for Src. The bar graph summarizes data obtained in four independent experiments; *P<0.05, **P<0.005 versus static condition.*

The application of hemodynamic stimuli to endothelial cells is reported to activate Src which in turn activates PYK2 (Cheng *et al.*, 2002), we determined the effect of shear stress on PYK2 activation. Shear stress was also associated with a slight increase in the phosphorylation of PYK2, however a significant effect was only detected after approximately 60 minutes (Fig. 35).

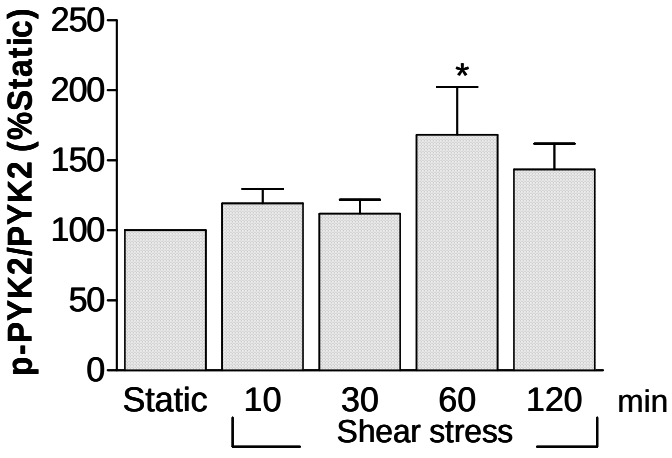

Figure 36. Effect of fluid shear stress on the phosphorylation of PYK2. *Confluent primary cultures of human umbilical vein endothelial cells were exposed to fluid shear stress (12 dynes cm-²) for up to 2 hours. Thereafter, the cells were harvested, PYK2 was immunoprecipitated and its tyrosine phosphorylation determined using a phospho-specific antibody. The bar graph summarizes the data obtained in three independent experiments; *P<0.05 versus static condition.*

In contrast to the weak effect observed on the tyrosine phosphorylation of total cellular PYK2, we observed a pronounced shear stress-induced association of PYK2 with eNOS. While only low levels of PYK2 co-precipitated with eNOS from cells maintained under static conditions there was an increase in the physical association of the two proteins that remained intact as long as cells were exposed to fluid shear stress i.e., for up to 2 hours (Fig. 36).

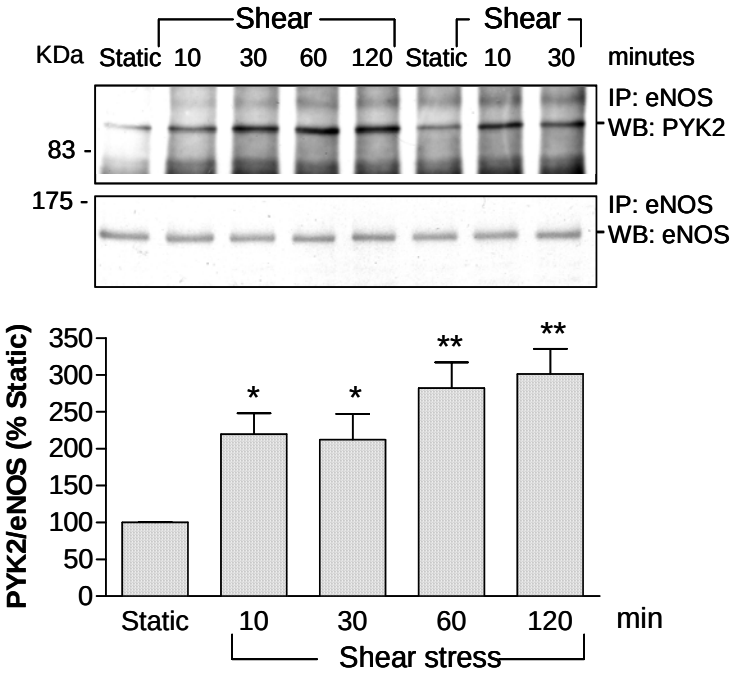

Figure 36. Effect of fluid shear stress on the association of eNOS with the tyrosine kinase PYK2. *Confluent primary cultures of porcine aortic endothelial cells were either maintained under static conditions or exposed to fluid shear stress (12 dynes cm-²) for up to 2 hours. eNOS was immunoprecipitated and the association of PYK2 determined by Western blotting. The bar graph summarizes the data obtained in four to 10 independent experiments; *P<0.05, **P<0.01 versus static condition.*

Phosphorylation of eNOS by the tyrosine kinase PYK2 and Src

To determine whether or not PYK2 and Src were able to tyrosine phosphorylate eNOS in intact cells, HEK-293 cells were co-transfected with eNOS and either PYK2 or Src. Immunoprecipitation of either eNOS (Fig. 37A) or phosphotyrosine (Fig. 37B) revealed that both kinases are able to phosphorylate eNOS in intact cells. To directly identify the tyrosine residues phosphorylated, eNOS recovered from PYK2-overexpressing HEK-293 cells, was

digested with trypsin, and subjected to MALDI-Mass spectroscopic (Toplab, Martinsried, Germany) analysis which revealed the phosphorylation of eNOS on Tyr^{657} which is located in the FMN binding region in the reductase domain of eNOS.

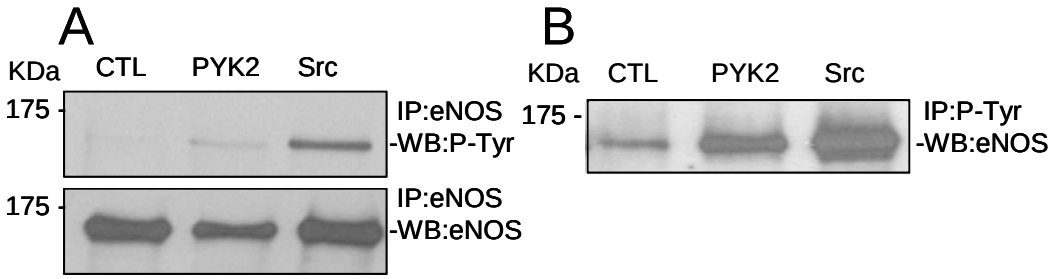

Figure 37. Phosphorylation of eNOS by the tyrosine kinases PYK2 and Src. *HEK-293 cells were transfected with eNOS alone (CTL) or in combination with either PYK2 or Src. After 48 hours the cells were lysed and either* ***(A)*** *eNOS or* ***(B)*** *tyrosine phosphorylated (P-Tyr) proteins were immunoprecipitated. Blots were then probed with either anti-P-Tyr or anti-eNOS antibodies to determine the tyrosine phosphorylation of eNOS. Similar results were obtained in three independent experiments.*

Effect of the mutation of Tyr^{657} residues on the activity of eNOS and dimer formation

The consequences of tyrosine phosphorylation on eNOS activity and subcellular localisation where then studied using eNOS mutants in which Tyr^{657} was mutated to the phosphomimetic amino acids aspartate (D) or glutamate (E) or the non-phosphorylatable phenylalanine (F). The sensitivity of the wild-type eNOS, Y657D, Y657E and Y657F eNOS to Ca^{2+}/CaM was then determined by monitoring the L-NAME sensitive conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. While the wild-type eNOS and the Y657F mutant demonstrated a similar dependency on Ca^{2+} and CaM, the Y657D and Y657E eNOS mutants were completely inactive at all concentrations (Fig. 38).

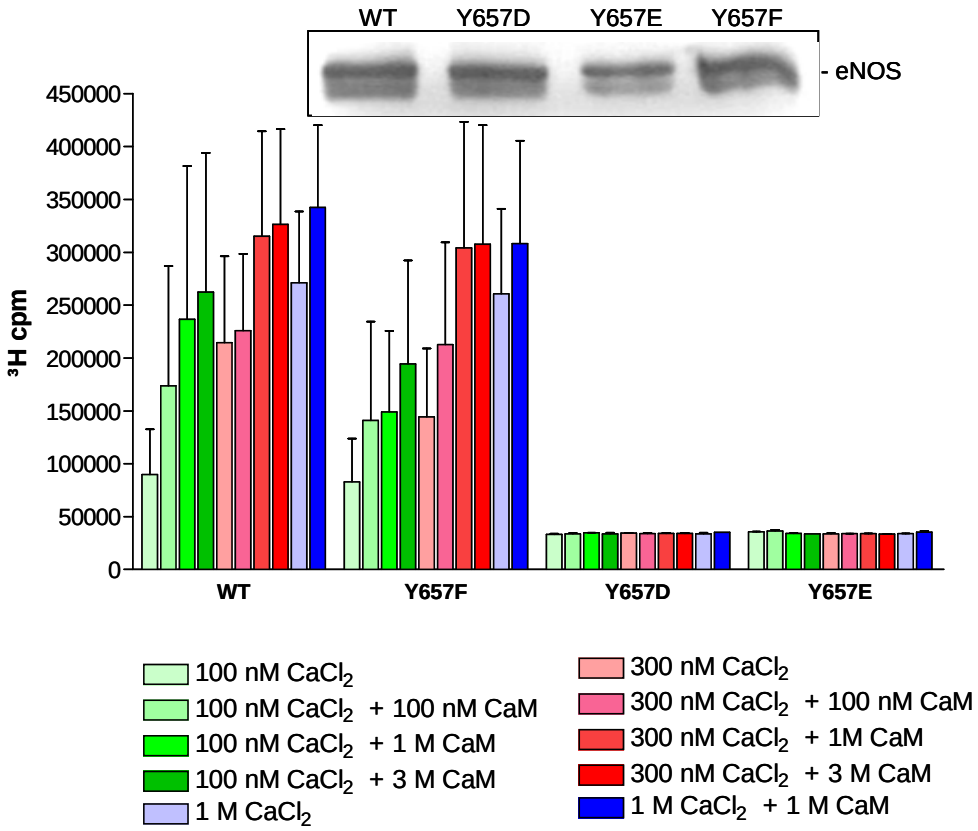

Figure 38. Effect of the mutation of Tyr^{657} on the activity of eNOS. *eNOS activity was determined by monitoring the N$^{\omega}$-nitro-L-arginine sensitive conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline in samples prepared from HEK-293 cells transfected with eNOS wild-type (WT) or the Y657D, Y657E and Y657F eNOS mutants. The bar graph summarizes data obtained in two experiments.*

Using NO spin trapping and ESR spectroscopy, the same phenomenon was observed in intact HEK-293 cells expressing wild-type eNOS, or either one of the Y657D, Y657E or Y657F eNOS mutants. A slight decrease in NO production was detected in cells expressing the Y657F mutant compared to that of wild-type eNOS which was correlated with a consistently lower recovery of the Y657F eNOS mutant in Triton X-100 lysis buffer. The Y657D and Y657E eNOS mutants were completely inactive (Fig. 39) and stimulation with ionomycin failed to affect NO production.

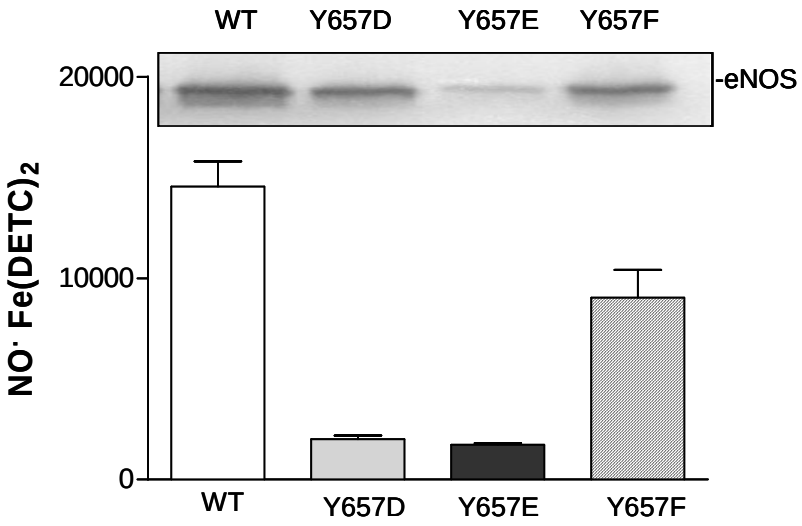

Figure 39. Effect of the mutation of Tyr ^{657} on NO production. *NO· production from intact HEK-293 cells transfected with the eNOS WT or the Y657D, Y657E and Y657F eNOS mutants was determined by NO· spin trapping with Fe(DETC)2 and measured by ESR spectroscopy at 77K. Experiments were performed in cells pretreated with sepiapterin (10 µmol/L). eNOS expression was analysed by Western blotting using an eNOS-specific antibody. The bar graph summarizes data obtained in three independent experiments.*

To determine whether the lack of activity of the Y657D and Y657E eNOS mutants was related to the inability of the enzyme to dimerise we performed LT-PAGE. No consistent difference in the ability of the wild-type eNOS and Y657D or Y657F eNOS mutants to dimerise was detected, although the Y657E eNOS mutant showed a tendency towards a greater degree of dimer disruption (Fig. 40), this effect can most probably be attributed to a difference in protein expression. The recovery of eNOS mutants in Triton X-100 lysis buffer or SDS-PAGE sample buffer showed Y657E was more resistant to Triton X-100 lysis buffer, than the wild-type or the Y657D and Y657F eNOS mutants. Upon preparing the protein extract with SDS-PAGE sample buffer relatively more protein was detected for Y657E, indicating that this eNOS mutant get localised in the Triton X-100 insoluble fraction (Fig. 41).

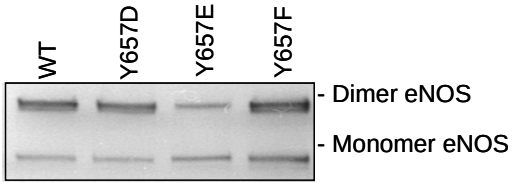

Figure 40. Effect of the mutation of Tyr^{657} on eNOS and dimer formation. *Western blot showing the effect of the Tyr^{657} mutation on eNOS dimer formation as determined by LT-PAGE. The data shown are representative of two independent experiments.*

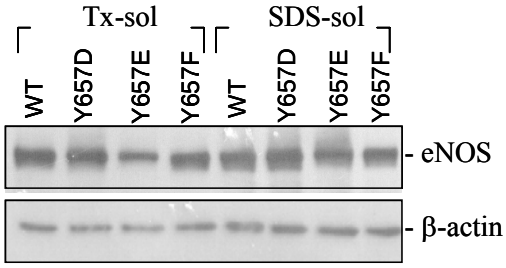

Figure 41. Effect of the mutation of Tyr [657] on the Triton solubility of eNOS. *Protein extracts (20 µg) from HEK-293 cells transfected with eNOS WT or the Y657D, Y657E and Y657F eNOS mutants, were used to determine the effect of the eNOS tyrosine mutation on the Triton X-100 solubility of the enzyme. Total eNOS expression was determined by treating the samples with Laemmli buffer (7.3% w/v SDS) and analyzed by Western blotting using eNOS specific antibody. The blot is representative of four independent experiments.*

Intracellular localisation of wild-type eNOS and the Y657D and Y657E mutants

To determine whether or not the wild-type and Tyr^{657} eNOS mutants localised to the same intracellular compartments, immunohistochemical analysis was performed. Human umbilical vein endothelial cells were infected with recombinant adenovirus carrying cDNA encoding myc-tagged wild-type eNOS or one of the Y657D, Y657E or Y657F eNOS mutants (Fig. 42) and immunohistochemically stained with an antibody directed against myc and an appropriate secondary antibody conjugated with the dye Alexia 546. β-catenin was visualized to mark the plasma membrane. The wild-type eNOS was localised discretely at the plasma membrane and in the peri-nuclear Golgi apparatus. Of the two phosphomimitic mutants Y657D and Y657E, the latter showed a greater degree of eNOS localisation in the cytoskeletal compartments. This would well explain the low levels of Y657E detection by Western blotting using Triton-X 100 soluble fraction from cells overexpressing this eNOS mutant. The Y657F mutant localised in a much diffused pattern in the Golgi apparatus and around the nucleus.

Effect of the mutation on additional tyrosine residues on the activity of eNOS

As mentioned earlier, two different stimuli, shear stress (which results in an increase in eNOS activity) and insulin (which has no effect on NO production) elicit the tyrosine phosphorylation of eNOS but with opposite effects, indicating the possible involvement of more than one tyrosine residue. We screened other tyrosine residues which would be involved in the regulation of eNOS. Additional tyrosine residues, which could regulate eNOS activity, were determined by citrulline assay. Mutation of either of theTyr^{357}, Tyr^{534}, Tyr^{556} and Tyr^{939} tyrosine residue of eNOS to Ala proved to be lethal to the activity of the enzyme, while eNOS

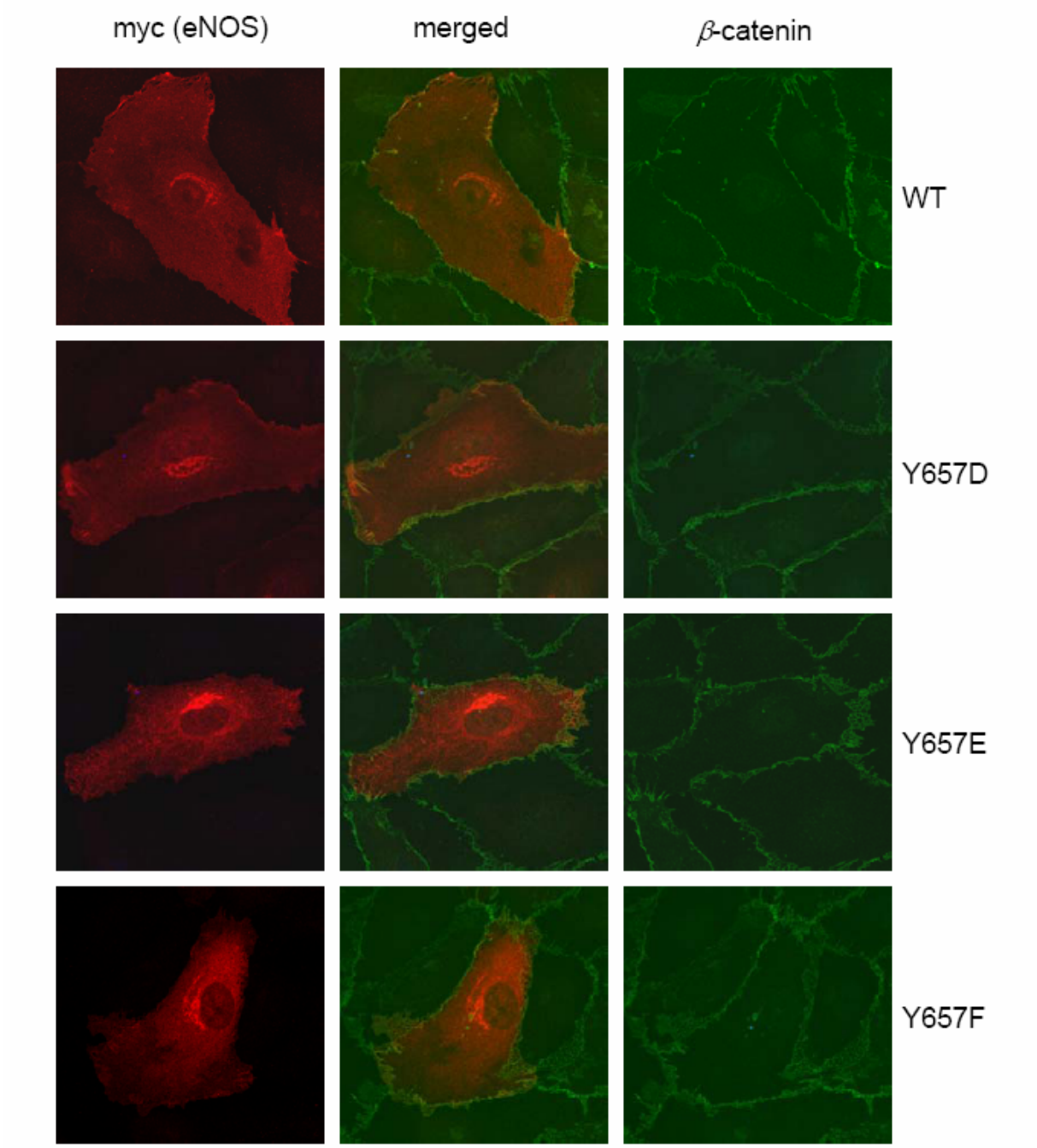

Figure 42. Comparision of the intracellular localisation of wild-type eNOS and the Y657D, Y657E and Y657F mutants. *Immunohistochemical staining of myc (red) and β-catenin (green) in human endothelial cells (first passage) infected with a myc-tagged wild-type eNOS (WT) or the Y657D, Y657E and Y657F eNOS mutants for 48 hours. The results presented are representative of data obtained in three independent experiments.*tyrosine residue Tyr^{210}, Tyr^{217}, Tyr^{597} and Tyr^{900} mutated to Ala were at least 100% more active than the wild-type, with Tyr^{900} Ala mutants showing a maximum increase of approximately 400% (Fig. 43).

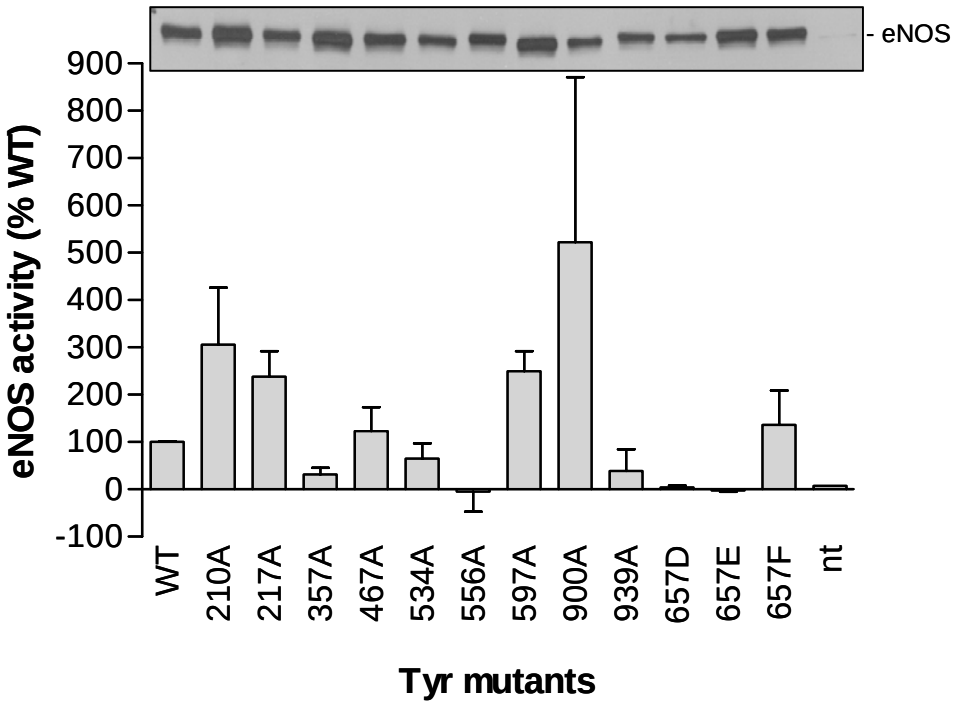

Figure 43. Effect of the mutation of specific tyrosine residues on eNOS activity. *HEK-293 cells were transfected with wild-type eNOS (WT) or one of the Y210A, 217A, Y357A, Y467A, Y534A, Y556A, Y597A, Y900A and Y939A tyrosine to alanine mutants. Thereafter eNOS activity was assessed by monitoring the conversion of [³H]L-arginine to [³H]L- citrulline under Vmax conditions (1 µmol/L $CaCl_2$ and 1 µmol/L CaM. The bar graph summarizes data obtained in three experiments and corrected to the protein expressed. nt: non-transfected control.*

The work done with the eNOS tyrosine residues (40% of the total tyrosine residues were studied) have provided evidence for the involvement of the putative tyrosine residues whose phosphostatus could modulate the activity of eNOS. Since some of the alanine mutants completely inhibited the activity of the enzyme, and while some others increased the activity by at least 100%, it is possible that more than two tyrosine residues might regulate the activity of eNOS with opposing effects. Clearly much further work needs to be done to establish the physiological relevance of these tyrosine residues, and the signalling pathway involved for their phosphorylation and/or dephosphorylation.

Conclusion: The data with tyrosine mutants clearly indicate the potential importance of Tyr^{657} for the regulation of eNOS activity, as Tyr^{657} mutated to a phosphomimetic aspartate or glutamate completely inactivated the enzyme. Recent published data (Garcin *et al.*, 2004) on the structure of reductase domain of eNOS goes a long way to explaining why this residue is critical, given its involvement in the correct positioning or "stacking" of the FMN on FAD so that electron transfer can take place.

5 Discussion

The findings of this study highlight the central role of phosphorylation in the regulation of the function of eNOS. We have demonstrated the consequences of phosphorylation on the activity of eNOS and identified the amino acid residues which can modulate eNOS activity to either produce NO, or lead to eNOS uncoupling i.e. the flow of electrons in the eNOS dimer does not result in the formation of NO but reactive oxygen species (ROS) which reacts with NO to generate RNS.

In unstimulated endothelial cells eNOS is phosphorylated on Thr^{495} and no phosphorylation is detected on Ser^{1177}. The role of Ser^{1177} and Thr^{495} in the regulation of eNOS has been well documented. Upon activation by various stimuli such as bradykinin, VEGF etc, Thr^{495} is rapidly dephosphorylated while there is a concomitant increase in Ser^{1177} phosphorylation. Not much is known about the other phosphoryatable residues on eNOS which can regulate the enzyme activity. We have identified two phosphorylation sites; Ser^{114}, within the vicinity of Zn^{2+} and H_4B binding sites and Ser^{633} in the putative CaM autoinhibitory sequence (586–641) within the FMN binding domain of eNOS, whose phospho-status influenced the enzyme activity.

Previous reports suggest that eNOS stimulation with fluid shear stress resulted in an increase in serine and tyrosine phosphorylation as evident from 2D gel analysis (Dimmeler *et al.*, 1999) (Fissithaler *et al.*, 2000) as well as mass spectroscopic analysis, which revealed the existence of at least two serine residues which gets phosphorylated (Gallis *et al.*, 1999). Based on the location and the probability of phosphorylation as predicted by NetPhos 2.0 programme, we identified serine residues Ser^{114} and Ser^{633} for our study.

Gallis et al (Gallis *et al.*, 1999) reported that fluid shear stress stimulates the phosphorylation of Ser^{1177}as well as that at Ser^{114} but that these events were mediated by two different kinases, Akt/PKB and a proline- directed protein kinase respectively. The regulatory consequences of Ser^{114} phosphorylation are almost entirely unknown. Our preliminary experimental data on the enzyme activity using the citrulline assay revealed that the activity of wild-type eNOS and the S114D mutant was almost the same, while the S114A mutant showed a two-fold increase in its activity, compared to the S114D mutant or wild-type enzymes. This gave us the first indication of the involvement of this site in enzyme activity. Since it is proposed that multiple sites of phosphorylation contribute to NO formation and activity assays performed with protein extracts may be misleading until the mutants are examined in an appropriate cellular environment, we performed the activity assay and direct NO measurement by ESR

spectroscopy at either basal or elevated $[Ca^{2+}]_i$ concentrations in intact HEK-293 cells and COS-7 cells overexpressing the protein in study. Under basal conditions there was no difference in the activity of wild-type eNOS, S114A and the S114D mutants, but upon stimulation with ionomycin, the S114A mutant exhibited a higher activity than either the wild-type eNOS or the S114D mutant as measured with citrulline assay. Surprisingly, cells expressing the S114A and S114D eNOS mutants released almost equal amounts of NO and were twice as active as the wild-type enzyme when NO was assessed by ESR spectroscopy. These findings regarding the S114A eNOS mutant were independently confirmed by Kou et al (Kou *et al.*, 2002), who reported that the activity of wild-type eNOS was similar to that of the S114A mutant under basal conditions, and was significantly enhanced upon stimulation with a calcium ionophore. However, their study lacked the S114D mutants, which would have been ideal in studying the consequence of phosphorylation on this serine residue. On the basis of the data obtained with this mutant on direct NO production, surprisingly both S114A and S114D made similar amounts of NO. Also both the mutants produce higher amounts of NO than the wild-type enzyme or an active double mutant TA/SD. The inhibition of the S114A and S114D mutants with L-NAME resulted in an increase in O_2^- production as measured by spin labelling with CMH, due to the loss of the scavenging effect of NO on O_2^-. Also no changes in phosphorylation on Ser^{114} were observed on stimulating the cells with shear stress or bradykinin.

It is presently not clear whether the importance of Ser^{114} is actually conferred by changes in its phosphorylation status as evident from the same amount of NO released from both the S114A and S114D mutants. It may be possible that Ser^{114} will determine the phospho-status of other amino acids. Recent reports have suggested that S114A promoted a greater interaction with Hsp-90 and Akt and an enhanced Ser^{1177} phosphorylation (Bauer *et al.*, 2003).

The mechanisms whereby Ser^{114} modulates eNOS enzyme activity remain to be determined. Some clues may be derived from inspecting the crystal structure of eNOS in the vicinity of Ser^{114}; which is the region that includes sites for Zn^{2+} ligation and H_4B binding, both of which stabilizes the dimer. The Zn^{2+} is tetrahedrally coordinated by four cysteine residues, two from each subunit (Cys94-Cys99) forming a zinc tetrathiolate (ZnS_4) cluster, (Fischmann *et al.*, 1999; Li *et al.*, 1999; Raman *et al.*, 1998) (Fig. 43). The residue Ser^{102} helps in the binding of H_4B (Li *et al.*, 1999). Disruption of ZnS_4 cluster by S-nitrosylation (Ravi *et al.*, 2004) or by $ONOO^-$ (Zou *et al.*, 2002), and also depletion of H_4B (List *et al.*, 1997) result in an unstable eNOS dimer . This eNOS dimer monomerises under reducing condition due to the disruption

of the disulphide bridges. However, Ser^{114} mutated to Ala or Asp did not interfere with the dimerisation of eNOS, suggesting that Ser^{114} may not critically interfere with Zn^{2+} or H_4B binding.

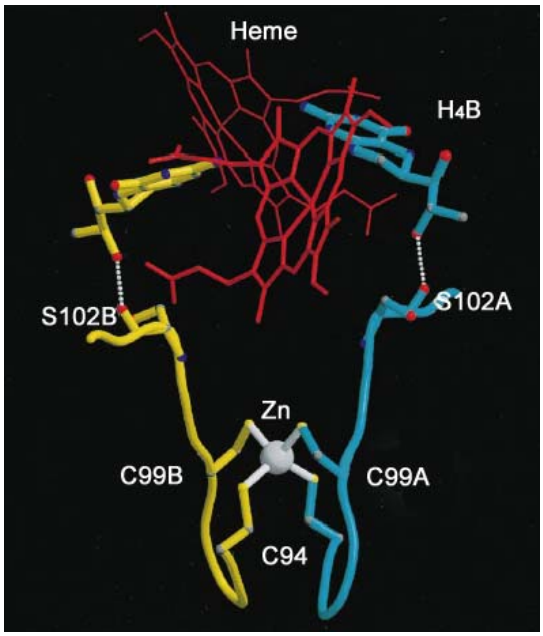

Figure 43. Zinc thiolate cluster of eNOS. *Crystal strcuture of eNOS showing the zinc thiolate cluster (C94 and C99) and the interaction with heme and tetrahydrobiopterin (H4B). Modified from Raman et al, Cell 1998; 95: 939-950.*

Our findings on Ser^{114} contradict findings by other groups (Kou *et al.*, 2002) attributing a role for Ser^{114} phosphorylation in the regulation of eNOS which we dispute and conclude that this residue might not be directly relevant in the regulation of eNOS by phosphorylation.

Ser^{633} is situated in the autoinhibitory loop which impart part of the Ca^{2+} dependency of the constitutively expressed NOS isoforms. The putative auto-inhibitory loop which has been speculated to interfere with binding of CaM with the CaM binding peptide is present only in nNOS and eNOS and, interestingly, Ser^{633} is conserved only in human, mouse, and bovine eNOS, but not in nNOS (Butt *et al.*, 2000; Garcin *et al.*, 2004; Salerno *et al.*, 1997). Deletion of the auto-inhibitory loop increased the activity of the Ca^{2+}-dependent enzyme. In previous studies it was reported that the activity of S633D eNOS mutant was similar to that of wild-type enzyme when determined with the citrulline assay in lysates from transfected cells as well as by assessing nitrate accumulation (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999). Ser^{633} has been reported to be phosphorylated in vitro by PKA and PKG (Butt *et al.*, 2000), but the functional relevance of Ser^{633} in regulating eNOS activity in intact cell system has not been addressed so far. Keeping this in mind, we decided to reassess the role of this residue in regulating eNOS activity in intact cell system.

Using three different methods we examined the effects of the mutation of Ser^{633} to aspartate (S633D) on eNOS activity by performing citrulline assays using cell lysates, in intact cells overexpressing the wild-type and mutant eNOS enzymes and by directly measuring NO production from intact cells with the aid of ESR spectroscopy. Studies using cell lysates obtained from transfected cells showed that the abilities of the S633D mutant and wild-type eNOS to convert L-arginine to L-citrulline were similar (Bauer *et al.*, 2003; Dimmeler *et al.*, 1999). In contrast, with the aid of the citrulline assay and ESR spectroscopy we observed that in intact cells under basal conditions the S633D eNOS mutant was twice as active as the wild-type enzyme. These apparent differences can most probably be attributed to the experimental conditions employed. The other groups employed nitrate accumulation over a period of 24 hours to determine the enzymatic activity and nitrate in the cells cannot be solely attributed to eNOS activity. However, we were careful in selecting NO spin trapping using Fe-DETC for determining NO release from the cells, which gives a direct measure of NO release. In intact cells or in broken cell lysates, eNOS interacts with many regulatory proteins such as caveolin, Hsp90, and numerous other factors (Boo & Jo, 2003; Fleming *et al.*, 2001; Fulton *et al.*, 2001) which could play a role in the activity of the enzyme. We did not see any change in the Ca^{2+} sensitivity for S633D mutant using 100,000 g pellet and citrulline assay but Boo et al have reported Ser^{633} to be Ca^{2+} insensitive (Boo *et al.*, 2002; Boo *et al.*, 2003) which they observed by determining the total nitrate content from intact cells. It could also be due to the fact that phosphorylation of Ser^{633} by PKA and PKG enhanced the V_{max} of eNOS activity in the presence of Ca^{2+}/CaM, which would explain our result as we determined the enzyme activity over a period of 30 minutes, which would have been too long to detect small differences between the eNOS wild-type and the S633D mutant.

Stimulation of human endothelial cells with shear stress resulted in an increase in the phosphorylation on Ser^{633} at 30 minutes and remained phosphorylated as long as shear was applied up to 60 minutes which suggest the physiological relevance of this residue in the regulation of the eNOS enzyme.

Based on our finding and other work reported during our study, the physiological implication of eNOS phosphorylated at Ser^{633} can be as follows. Shear stress elicits the biphasic production of NO so that responses generally consist of an initial short NO burst phase followed by a maintained phase where the levels of NO are maintained at levels two to three times higher than the basal levels (Fleming *et al.*, 1997; Kuchan & Frangos, 1994). The initial NO burst phase is characterised by a transient increase in the levels of intracellular Ca^{2+} (Ayajiki *et al.*, 1996; Corson *et al.*, 1996) leading to a Ca/CaM dependent activation of eNOS,

which is associated with the transient dephosphorylation of Thr^{495} and phosphorylation of Ser^{1177} by CaMKII (Fleming *et al.*, 2001) or Akt (Dimmeler *et al.*, 1998; Fulton *et al.*, 1999; Michell *et al.*, 1999). As a result the levels of NO are maintained at levels much higher than basal over a period of time. Ser^{633} has been reported to become phosphorylated in a PKA-dependent manner upon exposing endothelial cells to shear stress for 30 minutes (Boo *et al.*, 2002), roughly corresponding to the beginning of the second phase of NO production in response to shear (Frangos *et al.*, 1996). Therefore it can be suggested that the Ser^{633} phosphorylation may be responsible for the long-term potentiation of eNOS activation that persists at basal Ca^{2+} levels beyond peak activation, resulting in a low level of NO production which may play a critical role in the atheroprotective role of chronic shear stress.

Many studies have demonstrated a link between the localisation and activity of eNOS. We studied the influence of eNOS mutants on this link. There was no marked difference between the mutants studied, wild-type eNOS and TA/SD, TD/SD, S114A, S114D, or S633/634A, all of them localising at the plasma membrane and at the perinuclear Golgi apparatus. However, S633D showed a greater localisation at the plasma membrane.

With respect to the possible molecular mechanism underlying Ser^{633} phosphorylation-dependent activation of eNOS, studies by Salerno et al (Salerno *et al.*, 1997) presented evidence for an auto-inhibitory control element, a 45-amino acid insert located near the CaM-binding region of only constitutive NOS forms (eNOS and nNOS), not iNOS. The autoinhibitory region is postulated to stabilise an inhibited NOS conformation. Binding of CaM could cause displacement of the auto-inhibitory element, thereby evoking a conformational change of eNOS to support activation. The auto-inhibitory domain is notably rich in positively charged amino acid residues, especially in the case of eNOS which contains the sequence RRKRK (Lane & Gross, 2000) immediately before Ser^{633}. Phosphorylation of Ser^{633} would increase the negative charge in the autoinhibitory domain, and could thereby facilitate displacement of this domain from the site with which it interacts, thus partially activating NOS in the absence of Ca^{2+}/CaM, as well as facilitating Ca^{2+}/CaM binding and enhancing maximal NOS activity. Based on CaM binding and displacement of auto-inhibitory element its tempting to propose a cooperation between Ser^{633} and Thr^{495}. This scenario is however unlikely as the dephosphorylation on Thr^{495} is transient and rapid which is evident within the first 3 minutes of stimuli application, the phosphorylation on Ser^{633} is evident only at 30 minutes. Although eNOS uncoupling has received a lot of interest, the mechanisms involved are not entirely clear and very little is known about the role of phosphorylation in regulating eNOS uncoupling. Here we report the identification of Thr^{495}, as a potential

"phospho switch" for eNOS uncoupling. Oxidized-LDL was identified as a pathophysiological stimuli and PKCα as the kinase responsible for the phosphorylation of Thr^{495}. Stimulation of eNOS with ox-LDL led to a decrease in the basal phosphorylation on Thr^{495} with a consequential increase in O_2^- production as a result of eNOS uncoupling. The importance of this residue in the ability of eNOS to generate O_2^- was evident from the fact that a non-phosphorylatable T495A eNOS mutant generated more O_2^- than the phospho-mimetic T495D eNOS mutant does.

As eNOS has been reported to uncouple and produce O_2^- during endothelial dysfunction, we were interested in elucidating if there was any eNOS residue involved whose phosphostatus determined whether eNOS makes NO or O_2^-. Lin el al (Lin *et al.*, 2003) had previously reported that dephosphorylation of eNOS on The^{495} is associated with the enhanced production of O_2^-. It has also been shown by other investigators that exposure of endothelial cells to ox-LDL results in the formation of ROS, although the source of these species remains an area of uncertainty (Cominacini *et al.*, 2001). Our study with the eNOS mutants expressed in COS-7 cells indicated the involvement of Thr^{495} in O_2^- production.

Based on previous reports and on our findings it is tempting to postulate that the dephosphorylation of Thr^{495} leads to eNOS uncoupling. We initially observed that the dephosphorylation of Thr^{495} elicited by down regulating of PKC by long-term treatment with PMA was previously found to enhance rather than attenuate NO production by eNOS (Fleming *et al.*, 2001). This gave us a hint for the involvement of other factors involved in the regulation of eNOS activity. While dephosphorylation of Thr^{495} is associated with a concomitant increase in the binding of CaM to eNOS (Fleming *et al.*, 2001), surprisingly eNOS dephosphorylated by ox-LDL at Thr^{495} failed to associate CaM, but histamine stimulation of eNOS led to transient binding of CaM to eNOS which was very short-lived as the level of CaM returned to levels as observed in ox-LDL treated eNOS with in 1 minute. However, this would make a good argument for eNOS-dependent O_2^- generation. As CaM binding results in an increased flow of electrons from the reductase domain to the oxygenase domain(Abu-Soud & Stuehr, 1993; Matsuda & Iyanagi, 1999), this loss in the ability of eNOS to bind to CaM could possible result in a state where the electrons are flowing through the reductase domain but are not able to cross over to the oxygenase domain for the effective generation of NO. Under such conditions the electron is accepted by O_2 leading to radical formation (Stuehr *et al.*, 2001), which was exactly what was observed with ox-LDL treated endothelial cells.

There has been a lot of discrepancy in the reports on the effect of ox-LDL on Ser^{1177} phosphorylation as well as on the kinases involved. This can be attributed to the diverse concentration of ox-LDL employed in such studies, ranging from 5 to 250 μg/ml and the duration of the stimulation. In our study we used a maximal concentration of 30 μg/mL. A transient enhanced phosphorylation of Ser^{1177} has been reported in endothelial cells exposed to higher concentration of ox-LDL (150 μg/mL), we failed to see the same with 30 μg/mL, while similar levels of ox-LDL levels were found to attenuate eNOS Ser^{1177} phosphorylation in VEGF-stimulated endothelial cells and, thus, to inhibit cell migration (Chavakis *et al.*, 2001). However, we found no significant attenuation of the bradykinin-induced phosphorylation of Ser^{1177} in cells pre-treated with ox-LDL. This latter discrepancy may be explained by the fact that the kinases activated by the two stimuli that mediate the phosphorylation of eNOS on Ser^{1177} are distinct; VEGF-induced phosphorylation is Akt-dependent, while the phosphorylation induced by bradykinin is CaM kinase II-dependent (Fleming *et al.*, 2001). While such observations indicate that ox-LDL differentially affects Akt-mediated cell signalling, we found that ox-LDL treatment resulted in modest, but significant, time- and concentration-dependent activation of this kinase without a concomitant increase in eNOS Ser1177 phosphorylation, rather than the dephosphorylation and inactivation of Akt reported previously (Chavakis *et al.*, 2001).

The loss of phosphorylation of eNOS Thr^{495} as a result of ox-LDL stimulation can be the consequence of either the down-regulation/inhibition of a kinase or an up-regulation/activation of a phosphatase. The phosphatase involved in the dephosphorylation of Thr^{495} is thought to be PP1 (Fleming *et al.*, 2001; Michell *et al.*, 2001), but although the PP1 inhibitor, calyculin A , was able to increase Thr^{495} phosphorylation in ox-LDL treated cells, we found no evidence to suggest that its activity or expression was increased by ox-LDL. Harris et al have reported that the serine/threonine phosphatase, calcineurin (PP2B) can also dephosphorylate eNOS Thr^{495} (Harris *et al.*, 2001), but this is not a universally observed phenomenon (Fleming *et al.*, 2001; Michell *et al.*, 2001). Moreover, since this phosphatase is sensitive to inhibition by O_2^- (Namgaladze *et al.*, 2002), ox-LDL would be expected to result in a decrease rather than an increase in its activity. Therefore, our data indicate that ox-LDL-induced dephosphorylation of eNOS Thr^{495} can be attributed to the down-regulation/inactivation of a kinase.

eNOS is reported to be basally phosphorylated on Thr^{495} by a constitutively active kinase most probably PKC (Fleming *et al.*, 2001; Matsubara *et al.*, 1996; Michell *et al.*, 2001), a finding that can account for the fact that protein kinase inhibitors and the down-regulation of

PKC attenuate the phosphorylation of this residue. The inhibition and down-regulation of PKC were also associated with a marked increase in endothelial NO production, which is consistent with a negative regulatory role of Thr^{495} on CaM binding and eNOS activity (Davda *et al.*, 1994; Hirata *et al.*, 1995). Mukherjee et al (Mukherjee *et al.*, 2001) have reported that ox-LDL stimulates PKC activity, in contrast, our data clearly demonstrate that a constitutively active isoform of PKC is targeted by ox-LDL and that this effect underlies the ox-LDL-stimulated Thr^{495} dephosphorylation of eNOS. Indeed, the dephosphorylation of Thr^{495} was temporally correlated with a decrease in the phosphorylation and activity of PKC in ox-LDL-treated endothelial cells. The PKC isoform targeted by ox-LDL was tentatively identified as PKCα on the basis of its molecular mass and the fact that the same signal was obtained using three different antibodies. So far there has not been any report on the PKC isoform involved, ours is the first study which has identified the PKC isoform involved in Thr^{495} phosphorylation. Whether PKCα forms part of the eNOS signalosome remains to be determined, as this analysis is hampered by the fact that the activated PKC is detergent-insoluble. However, eNOS can also be detected in the Triton-insoluble cell fraction and the bradykinin- as well as the fluid shear stress- induced increase in NO production has been linked to a change in the solubility of eNOS (McCabe *et al.*, 2000; Venema *et al.*, 1996b). Oxidized-LDL has previously been reported to acutely increase (within 15 minutes) the activity of PKCα in coronary artery smooth muscle cells (Giardina *et al.*, 2001) and to increase global PKC activity (over 24 hours) in coronary endothelial cells by activating the LOX-1 receptor (Li *et al.*, 2003). Clearly, our results are in direct contrast with the results of this study. The reasons for this apparent contradiction can most likely be attributed to the differences in the concentration and time of incubation with ox-LDL, as well as the cells used. However, it is important to note that, in the present study, we were careful to use cells after only one passage to avoid artefacts related to the gradual loss of cell signalling pathways in multi-passaged cultured cells.

Intracellular localisation of eNOS plays an important role in the activity of the enzyme (Sakoda *et al.*, 1995; Sessa *et al.*, 1995). Oxidized-LDL and hypochlorite-modified LDL have been previously reported to alter the intracellular localisation of eNOS (Blair *et al.*, 1999; Nuszkowski *et al.*, 2001). Oxidized-LDL causes displacement of eNOS from caveolae by binding to endothelial cell CD36 receptors and by depleting caveolae cholesterol content (Shaul, 2003; Uittenbogaard *et al.*, 2000), resulting in the disruption of eNOS signalling complex and activation. Oxidized-LDL is also reported to interfere with the association of eNOS with Hsp90 (Stepp *et al.*, 2002). The association of eNOS with Hsp90 is necessary to

mediate the balance of NO and O_2^- release from eNOS (Pritchard, Jr. *et al.*, 2001). Thus, it appears that ox-LDL has diverse effects on eNOS regulatory mechanisms which includes inactivation of a kinase leading to dephosphorylation and loss of interaction with regulatory protein and intracellular localisation of the enzyme.

It has been suggested that oxidative stress results in the disruption of the eNOS dimer by disrupting the ZnS_4 cluster (Zou *et al.*, 2002) and oxidizing H_4B (List *et al.*, 1997), while our study has showed that eNOS dimer is relatively stable. These conflicting results can be due to difference in the methodologies and experimental conditions employed. In the previous study, the experiments were carried out using purified eNOS, where the eNOS dimer is in direct contact with the oxidants without any interference in their action on the eNOS dimer. While we were not able to detect the same in endothelial cells, where $ONOO^-$ can be rapidly degraded, we did not detect any difference in levels of eNOS dimer upon treatment with ox-LDL, or SIN-1 or X/XO. Only when cells were stimulated with high concentration of $ONOO^-$ (3.75 mmol/L) was the dimer completely disrupted. However, recent reports indicate that the NOS dimer once formed is relatively stable, indicating that the uncoupling of eNOS has not necessarily to be associated, if at all, with the disruption of the dimer.

There are numerous reports of the restoration of endothelial dysfunction by treating the cells with H_4B (Tiefenbacher *et al.*, 1996) or vitamin C (d'Uscio *et al.*, 2003) which is know to restore the H_4B content (Baker *et al.*, 2001; Heller *et al.*, 2001). Levels of this essential cofactor are reported to be decreased by oxidative stimuli, in particular, by $ONOO^-$ (Kuzkaya *et al.*, 2003), which oxidizes H_4B (Milstien & Katusic, 1999), as well as by ox-LDL (Dulak *et al.*, 1997; Frank *et al.*, 1998). The H_4B oxidation hypothesis also implies that eNOS uncoupling is preceded by the activation of other endothelial O_2^- generating enzymes. Arguing against such a central role of H_4B in the uncoupling of eNOS, in the present study, ox-LDL did not significantly affect endothelial H_4B levels, and the mutation of eNOS Thr^{495} to alanine was sufficient to increase O_2^- production. Since Thr^{495} is upstream of H_4B, it deserves attention in itself as a key player in eNOS uncoupling or which act as a phospho-switch, determining whether the eNOS makes NO or O_2^- upon experiencing pathophysiological stimuli.

Experiments performed on endothelial cells using inhibitors of tyrosine kinases as well as tyrosine phosphatases (Fleming *et al.*, 1996a; Fleming *et al.*, 1998; Takenouchi *et al.*, 2004) have been reported to modulate the NO production. But there is nothing much known about the residues which are phosphorylated or the kinases which are involved. Elaborating the

functional consequences of eNOS tyrosine phosphorylation is complicated by the fact that this modification is only evident in primary cultured cells (Fleming *et al.*, 1998; Garcia-Cardena *et al.*, 1996a) or in cells that overexpress tyrosine kinases such as Src (Takenouchi *et al.*, 2004). The consequences of the tyrosine phosphorylation of eNOS are unknown but are perhaps more likely to be related to the docking of associated scaffolding and regulatory proteins than to affect eNOS activity directly.

Studies using tyrosine kinase inhibitors provide only limited information on the role played by tyrosine phosphorylation in the regulation of endothelial NO production as many of these compounds directly affect Ca^{2+}-signalling processes (Fleming *et al.*, 1996b; Fleming & Busse, 1997) and/or the activity of Akt and the binding of Hsp90 (Fleming *et al.*, 1996b). Since preliminary data from our group indicated that both fluid shear stress (which increases eNOS activity) and insulin (which generally has no effect on NO production) elicit the tyrosine phosphorylation of eNOS (Fisslthaler, unpublished observations) we decided to determine the role of tyrosine phosphorylation in the regulation of eNOS.

Shear stress has been previously reported to activate Src in endothelial cells (Jalali *et al.*, 1998). We found that application of shear stress on endothelial cells resulted in the activation of Src by the displacement of phospho Tyr^{527} from the Src SH2 domain and mediated its dephosphorylation. The dephosphorylation was significant within 5 minutes of shear stress application, which increased over a period of 30 minutes, indicating an increase in the activity of Src. The activation time followed by Src correlates with the activity pattern exhibited by eNOS in response to shear stress. This clearly indicated the possible involvement of a Src tyrosine kinase in the shear mediated activation of eNOS (Fig 35).

Proline-rich tyrosine kinase 2 (PYK2) is a non-receptor tyrosine kinase, structurally related to focal adhesion kinase, and has been shown to play a role in signaling cascades (Litvak *et al.*, 2000; Nakamura *et al.*, 2001). Src has been reported to associate and activate PYK2 by tyrosine phosphorylation in response to cyclic stress (Cheng *et al.*, 2002). So we looked for the effect of shear stress on PYK2 activation. We found that upon application of shear stress on endothelial cells there was a time-dependent increase in the phosphorylation of PYK2 which results in the activation of the kinase. The increase in phosphorylation was evident in 10 minutes and increased over the experimental period studied (up to 120 minutes) which reached significantly highest levels at 60 minutes. So having witnessed the activation of Src and PYK2 upon shear stress we determined whether or not a tyrosine kinase is associated with eNOS.

In contrast to the slow tyrosine phosphorylation of PYK2 which was maximum at 60 minutes of shear, the association of PYK2 with eNOS in response to shear was rapid. While only low levels of PYK2 co-precipitated with eNOS from cells maintained under static conditions there was a time-dependent increase in the association of the two proteins. The increase in association was at least 2-fold more within the first 10 minutes of shear, and remained intact as long as cells were exposed to fluid shear stress i.e., for up to 2 hours.

We next determined the tyrosine phosphorylation of eNOS overexpressed in HEK-293 cells along with either PYK2 or Src. Immunoprecipitation of eNOS and Western blotting using phosphotyrosine antibody revealed both PYK2 and Src were able to tyrosine phosphorylate eNOS. However the level of phosphorylation in PYK2 cotransfected cells was lower than that observed in Src expressing cells. This probably could be due to the fact that PYK2 is activated by Src, which HEK-293 lack. The phosphorylation of eNOS tyrosine residue(s) by Src and PYK2 was also confirmed by immunoprecipitating phosphotyrosine and Western blotting using eNOS antibody. This data helps us to suggest that both kinases are able to phosphorylate eNOS in intact cells.

Garcia-Cardena et al (Garcia-Cardena *et al.*, 1996a) had reported that tyrosine phosphorylation of eNOS observed following the stimulation of endothelial cells with H_2O_2 resulted in the loss of eNOS activity by 50% compared to non-treated cells. However the residues involved were not determined in the latter study. Mass spectroscopic analysis for tyrosine residues on the eNOS recovered from PYK2-overexpressing HEK-293 cells revealed the phosphorylation of eNOS on Tyr^{657} which is located in the FMN binding region in the reductase domain of eNOS. We mutated this residue to a phosphomimetic aspartate (D) or glutamate (E) or the non-phosphorylatable phenylalanine (F). The data obtained using the citrulline assay performed on the membrane fraction of the HEK-293 cells overexpressing eNOS mutants and NO measurement by ESR spectroscopy clearly indicated that the eNOS mutants Y657D and Y657E were completely inactive, while the Y657F mutant was slightly less active compared to wild-type eNOS. The slight decrease in the activity of Y657F can be accounted for by a consistently lower level of transfection rather than an effect on enzyme activity. Recent biochemical and crystallographic study has revealed the importance of this residue, which is situated in the FMN binding domain, and is suggested to stack the ring plane of the FMN almost parallel to the ring plane of another aromatic residue Phe^{574} (Garcin *et al.*, 2004) (Fig. 44). This conformation is necessary for the transfer of electrons from FAD to FMN. This data clearly demonstrate the significance Tyr^{657} can play in eNOS regulation.

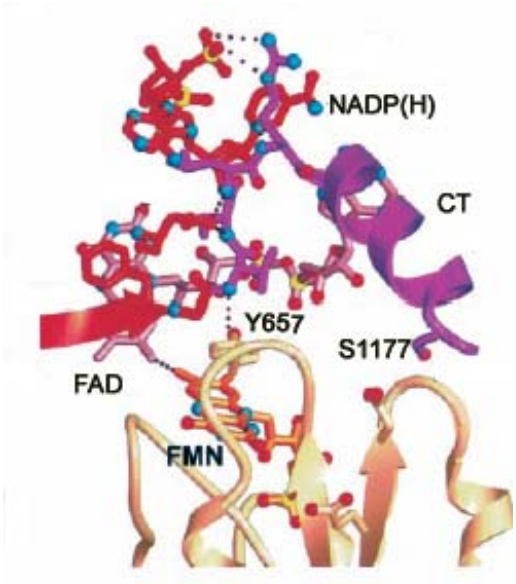

Figure 44. Stacking of FMN by Tyr657 residue. *Structure showing the interaction of tyrosin residue Y657 and FMN with FAD and NADPH respectively. Tyr657 is proposed to stack FMN in the conformation required to accept electron from the FAD. Modified from Garcin et al, JBC 2004; 279: 37918-37927.*

Further, to determine whether the lack of activity of the phosphomimetic eNOS mutants Y657D and Y657E was related to the inability of the enzyme to dimerise we performed LT-PAGE and did not find any consistent difference in the ability of the wild-type eNOS and Y657D, Y657E or Y657F eNOS mutants to dimerise, which further suggests that the inactivation of the enzyme is due to the disruption of the flow of electrons at the FMN binding domain.

Bradykinin and H_2O_2 stimulation of endothelial cells resulted in an enhanced tyrosine phosphorylation of eNOS, an effect which has been implicated to the localisation of eNOS to the cytoskeleton (Venema *et al.*, 1996b) or caveolae (Garcia-Cardena *et al.*, 1996a). Also the intact cytoskeleton is required for LPC-stimulated PYK2 phosphorylation (Rikitake *et al.*, 2001). The tyrosine mutants studied gave a distint localisation pattern. The wild-type eNOS is localised mainly in the perinuclear Golgi apparatus and at the plasma membrane. The phosphomimetic mutants Y657D and Y657E localised markedly in the cytoskeleton, with Y657E showing a greater degree of localisation in the cytoskeleton, while the Y657F exhibited a very diffused distribution of eNOS in the cytosol, giving an impression that the enzyme is stuck in the endoplasmic reticulum. Further experiments need to be performed with specific endoplasmic reticulum markers to confirm this claim.

However, previous studies have shown that enhanced tyrosine phosphorylation resulting in an increased NO production (Fleming *et al.*, 1996a; Takahashi *et al.*, 1997; Takenouchi *et al.*, 2004) and studies in our group, have observed that two different stimuli, shear stress (activated eNOS) and insulin (which does not have any effect on NO production) culminated

in the tyrosine phosphorylation of eNOS but with opposing effects, indicating the possible involvement of more than one tyrosine residue which can be phosphorylated. Additional eNOS tyrosine residues Y210, Y357, Y534, and Y556 mutated to Ala proved to be lethal to the activity of the enzyme, while eNOS mutants Y217A, Y467A, Y597A and Y900A showed an enhanced activity of at least 2-fold more than the wild-type enzyme, with Y900 showing a maximum increase of 4.5 fold.

So far no post-translation modification has been know to knock out the activity of eNOS completely, it is still premature to speculate the physiological effect of these tyrosine residues on the enzyme activity. However, preliminary data suggest a role of Tyr^{657} in the translocation of the enzyme into the cytoskeletal fraction. However it would be interesting to study the effect of phosphorylation of Tyr^{657} on eNOS uncoupling, and if it generates O_2^-, then it could be the strongest candidate in contention for the phospho-switch which uncouples eNOS. The determination of the phospho-switch of eNOS would help us in developing strategies by targeting the kinase or phosphatase involved to treat endothelial dysfunction.

6 Summary

Since its recognition as an endothelium-derived relaxing factor, the control and consequences of nitric oxide (NO) production have been investigated intensely. We know now that NO is not simply a vasodilator or regulator of smooth muscle tone but is a potent anti-platelet agent, neuromodulator and regulator of gene expression. NO is synthesized from the amino acid L-arginine by a family of enzymes termed NO synthases (NOS). The 'endothelial' (eNOS or NOS III) and 'neuronal' (nNOS, NOS I or bNOS) NOS isoforms, which were named after the tissues in which they were first identified, are expressed constitutively and are generally regulated by Ca^{2+}/calmodulin (CaM). Endothelium-derived NO is thought to be responsible for maintaining the vasculature in an anti-atherosclerotic state and a decrease in the bioavailability of NO (a state generally referred to as endothelial dysfunction) results in "pro-atherosclerotic" alterations in vascular gene expression. Recently it has become clear that the activity of eNOS is largely determined by its association with regulatory proteins as well as by the phosphorylation of the enzyme on serine, threonine and possibly tyrosine residues. Moreover, the enzyme can be "uncoupled" i.e. transformed from a NO generating to a superoxide (O_2^-)-generating enzyme, which would be expected to attenuate vasodilator responses and enhance vascular inflammation. The aim of this thesis was to study the consequences of phosphorylation on specific serine, threonine and tyrosine residues on the activity and intracellular localisation of eNOS and in particular to determine whether a phospho-switch for eNOS uncoupling exists.

eNOS is phosphorylated under basal conditions and its serine phosphorylation can be enhanced following cell stimulation with hemodynamic stimuli such as cyclic stretch and fluid shear stress as well as by hormonal stimuli such as histamine and bradykinin. Our group has previously demonstrated the importance of Ser^{1177} in the activation of eNOS and here I set out to determine the relative importance of phosphorylation on Ser^{633} and Ser^{114}. By generating point mutants in which serine was replaced by either alanine (non-phosphorylatable mutants) or aspartate (phosphomimetic mutants) it was observed that the activity of the S633D and S114A eNOS mutants exhibited an 2-fold increase over the activity of the wild-type enzyme or either of the S633/634A or S114D eNOS mutants as determined by monitoring the conversion of L-arginine to L-citrulline.

eNOS is basally phosphorylated on Thr^{495} and stimulation of endothelial cells with Ca^{2+}-elevating agonists generally results in the transient dephosphorylation of this residue. The latter is essential to allow the binding of calmodulin to the enzyme and is the actually initiating step in the generation of NO. Correspondingly, the T495A eNOS mutant can be activated at lower Ca^{2+} and calmodulin concentrations than the T495D mutant. However, some eNOS mutants (T494A/S1177D and T495A) showed an enhanced ability to generate O_2^- in a NOS inhibitor-sensitive manner suggesting that the phosphorylation of the enzyme may also play a role in the uncoupling process. To determine the physiological relevance of eNOS dephosphorylation on Thr^{495} we assessed the consequences of treating cells with oxidised low-density lipoprotein (ox-LDL) on eNOS phosphorylation as well as on the eNOS-dependent generation of NO and O_2^-. Oxidised LDL concentration- and time-dependently decreased phosphorylation of eNOS on Thr^{495} and led to a concomitant decrease in cellular levels of cyclic GMP and an enhanced production of O_2^- compared to cells treated with native LDL. Alterations in the activity of protein kinase C (PKC) were related to the change in eNOS Thr^{495} phosphorylation. There was not only the basal activity of PKCα inhibited by ox-LDL but the PKC activator phorbol-12-myristate-13-acetate also failed to elicit the phosphorylation of Thr^{495} in ox-LDL-treated endothelial cells. The dephosphorylation of eNOS on Thr^{495} in response to the addition of ox-LDL was not associated with an increase in the binding of calmodulin to eNOS, an association usually necessary for the activation of eNOS. Moreover, following treatment with ox-LDL for 24 hours eNOS was no longer detected at the plasma membrane but was redistributed to the cytosol indicating that ox-LDL may disrupt the eNOS signalling complex or signalosome.

To date the role played by the tyrosine phosphorylation of eNOS in the regulation of its activity or intracellular association is controversial. However, during the preparation of this thesis we have been able to demonstrate a link between the tyrosine phosphorylation of eNOS and the activation of the tyrosine kinases Src and PYK2. The application of fluid shear stress to endothelial cells resulted in the activation of Src and PYK2 as well as in the association of PYK2 with eNOS. Co-expression of eNOS and PYK2 led to the putative identification of Tyr^{657} as a potential modulatory site. Mutating eNOS at Tyr^{657} to Asp or Glu resulted in the localisation of the mutant eNOS predominantly in the cytoskeleton and also in a complete inactivation of the enzyme. The Y657F mutants, on the other hand, did not demonstrate any marked alteration in the activity when compared with the wild-type eNOS. However, the

intracellular localisation pattern was very diffuse in the cytoplasm, giving the impression that this mutant may be stuck in the endoplasmic reticulum.

In conclusion, the results describe in this thesis indicate that eNOS is regulated by phosphorylation at multiple sites. Depending on the phosphorylation site involved phosphorylation can inhibit or activate NO production or even uncouple the enzyme so that it generates O_2^-. While the phosphor-status of eNOS on Ser^{114} and Ser^{633} influenced NO release they did not contribute to O_2^- production and the dephosphorylation of Thr^{495} seems sufficient to uncouple eNOS. Cell treatment with ox-LDL, which is known to increase eNOS-derived O_2^- output was correlated with a dephosphorylation of Thr^{495} as well as a decrease in the activity of the kinase that phosphorylates this site i.e., PKCα. The phosphorylation status of all the eNOS serine and threonine residues studied however did not influence the ability of the enzyme to dimerise, indicating that contrary to previously published reports the eNOS dimer is highly stable in endothelial cells. The tyrosine phosphorylation of eNOS was not initially expected to play a determinant role in the regulation but rather to facilitate the docking of associated regulatory proteins. However, Tyr^{657} seems to play a critical role in the generation of NO as its mutation resulted in the generation of a completely inactive enzyme as well as in an apparent intracellular mislocalisation of the protein. The physiological relevance of these findings remain to be further elucidated.

7 Zusammenfassung

Seit der Identifizierung des endothel-abhängigen, relaxierenden Faktors als Stickstoffmonoxid (NO) durch R. F. Furchgott und L. Ignarro im Jahre 1986 sind die Regulation und die funktionellen Konsequenzen der endothelialen NO-Produktion Gegenstand intensiver Forschung. Heute ist NO nicht nur als relaxierender Faktor für Gefäße bekannt, sondern auch als potenter Hemmer der Thrombozytenaggregation, als Neuromodulator sowie Regulator der Genexpression. Die Enzyme der Familie der NO-Synthasen bilden aus der Aminosäure L-Arginin, NO und L-Citrullin. Die endotheliale und neuronale NO-Synthase, die nach den Geweben, in welchen sie zuerst gefunden wurden, benannt sind, sind konstitutiv exprimiert und werden vor allem über Ca^{2+}/Calmodulin aktiviert. Als wichtigste Funktion von endothelial gebildetem NO wird derzeit angesehen, dass es eine protektive, anti-atherosklerotische Wirkung auf die Gefäßwand ausübt. Eine Verminderung der Bioverfügbarkeit von NO (allgemein als endotheliale Dysfunktion bezeichnet) führt zur Expression von pro-atherosklerotischen Genen. Es wurde gezeigt, dass die Aktivität der NO-Synthasen von der Interaktion mit regulatorischen Proteinen und von der Phosphorylierung des Enzyms an Serin-, Threonin- und möglicherweise auch Tyrosinresten abhängt. Außerdem kann das Enzym entkoppelt werden, was bedeutet, dass nicht NO sondern Superoxidanionen (O_2^-) gebildet werden, welche dann die vasodilatierende Wirkung von NO einschränken und inflammatorische Antworten verstärken.

Das Ziel dieser Dissertation war es, die Konsequenzen der Phosphorylierung der endothelialen NOS (eNOS) an Serin-, Threonin- und auch Tyrosinresten auf die Aktivität und intrazelluläre Lokalisation zu untersuchen, vor allem mit der Frage, inwieweit eine Phosphorylierung für die Entkopplung des Enzyms mitverantwortlich ist.

Die schon unter basalen Bedingungen beobachtbare Phosphorylierung der eNOS an Serinresten kann durch hämodynamische Stimuli wie Schubspannung oder rhythmische Dehnung der Gefäßwand bzw. der Endothelzellen sowie durch agonisten-vermittelte Stimulation mit Histamin oder Bradykinin erhöht werden. Unsere Gruppe hat vor kurzem die Bedeutung der Phosphorylierung von Ser^{1177} für die Ca^{2+}-unabhängige Aktivierung der eNOS gezeigt. In der vorliegenden Arbeit wurde die Relevanz der Phosphorylierungen von Ser^{633} und Ser^{114} untersucht. Durch gerichtete Punktmutagenese („site-directed mutagenesis“) wurden Mutanten erstellt, in denen die entsprechenden Serinreste zu Alanin (nicht

phosphorylierbare Mutante, A) bzw. zu Aspartat (phosphomimetische Mutante, D) mutiert sind. Die Aktivität der überexprimierten eNOS-Mutanten wurde in-vivo und in-vitro durch die Bildung von L-Citrullin aus L-Arginin bestimmt, die Menge an gebildetem NO oder O_2^- durch Elektron-Spin Resonanz (ESR) Analysen ermittelt. Hierbei wurde gefunden, dass die Aktivität der S114A- bzw. der S633D-Mutanten um das zweifache gegenüber der Aktivität des Wildtyps oder der S114D- bzw. der S633/634A-Mutanten erhöht waren, die Bildung von O_2^- dieser Mutanten jedoch gleich war wie beim Wildtype

In nicht-stimulierten Zellen ist die eNOS auch am Thr^{495} phosphoryliert und die Stimulation mit Ca^{2+}-erhöhenden Agonisten führt zu einer transienten Dephosphorylierung dieses Aminosäurerestes. Dies ermöglicht erst die Bindung von Calmodulin beim initialen Schritt der NO-Bildung. Dementsprechend kann die T495A-Mutante im Vergleich zur T495D-Mutante bei niedrigeren Ca^{2+}-Konzentrationen aktiviert werden. Bei den Mutanten, bei denen Thr^{495} nicht phosphorylierbar ist (T495A und T495A/S1177D), wurde eine Erhöhung der O_2^--Bildung beobachtet, die sensitiv gegenüber NOS-Inhibitoren war, was auf eine Funktion der Thr^{495}-Phosphorylierung auch für die Entkopplung der eNOS deutet. Die Stimulation von Endothelzellen mit oxidiertem „low-density lipoprotein“ (ox-LDL) führt bekanntermaßen zur eNOS-vermittelten Radikalbildung. Die Bedeutung der Thr^{495}-Phosphorylierung für diese Beobachtung wurde mittels Western-Blot-Analysen sowie Bestimmung der NO- und O_2^--Bildung ermittelt. Die Behandlung von Endothelzellen mit ox-LDL führte zu einer konzentrations- und zeitabhängigen Erniedrigung der Thr^{495}-Phosphorylierung, die mit einer verringerten intrazellulären cGMP-Konzentration einherging. Zugleich war die O_2^--Produktion gesteigert im Vergleich zu Zellen, die mit nativem LDL inkubiert wurden. Vor allem die Proteinkinase C (PKC) ist für die Thr^{495}-Phosphorylierung verantwortlich und die Verringerung der eNOS Phosphorylierung am Thr^{495} korrelierte nicht nur mit der Hemmung der basalen PKCα Aktivität durch ox-LDL, sondern auch der PKC-Aktivator 12-Myristat-13-Acetat konnte in ox-LDL-behandelten Endothelzellen keine Phosphorylierung am Thr^{495} vermitteln. Allerdings führte die Dephosphorylierung des Thr^{495}-Restes nicht zur erhöhten Bindung von Calmodulin an die eNOS, was bei Agonisten-Stimulation zu beobachten ist und hier den initialen Schritt zur NO-Bildung darstellt. Außerdem konnte mittels Immunhistochemie gezeigt werden, dass in Endothelzellen, die über 24 Stunden mit ox-LDL inkubiert worden waren, die eNOS nicht mehr an der Plasmamembran lokalisiert war, sondern ins Zytosol translozierte. Dies deutet auf eine Zerstörung des „eNOS Signalosoms“ durch ox-LDL hin.

Derzeit wird die Rolle der Tyrosinphosphorylierung der eNOS für die Aktivätsregulation und intrazellulären Lokalisation kontrovers diskutiert. Während der Arbeit an dieser Dissertation konnte ein Zusammenhang zwischen der eNOS-Tyrosinphosphorylierung und der Aktivierung der Tyrosinkinasen PYK2 und Src gefunden werden. In Endothelzellen führt die Stimulation mit Schubspannung zur Aktivierung von PYK2 und Src sowie zur Assoziation von PYK2 mit der eNOS. Die Überexpression der eNOS und PYK2 in COS-7 Zellen führte zur Identifizierung des Tyr^{657}-Restes als phosphoryliertes Tyrosin der eNOS. In Endothelzellen, welche die phosphomimetischen eNOS-Mutanten Y657D bzw. Y657E (Y = Tyrosin; E = Glutamat) überexprimierten, zeigte die eNOS in immunhistochemischen Untersuchungen eine Assoziation mit dem Zytoskelett und sowohl in ESR-Untersuchungen als auch in Citrullin-Assays war keinerlei NO-Bildung nachzuweisen. Die nicht-phosphorylierbare Y657F-Mutante (F = Phenylalanin) zeigte keine Veränderung der Aktivität im Vergleich zum Wildtyp. Die intrazelluläre Lokalisation dieses Proteins war allerdings sehr diffus über das gesamte Cytoplasma und vermittelte den Eindruck, dass eine Hemmung des Transports vom endoplasmatischen Retikulum zum Golgi-Apparat vorliegt.

Zusammenfassend beschreiben die Ergebnisse dieser Arbeit, dass die eNOS an verschiedensten Aminosäureresten phosphoryliert werden kann. Abhängig von der Lokalisation dieser Phosphorylierung kann die NO-Produktion gesteigert oder gehemmt werden bzw. das Enzym kann entkoppelt werden und O_2^- bilden. Während der Phosphorylierungszustand von Ser^{114} und Ser^{633} die NO-Synthese moduliert, haben diese Aminosäurereste keinen Einfluss auf die O_2^--Bildung. Die Dephosphospoylierung von Thr^{495} scheint für eine Entkopplung des Enzyms ausreichend zu sein. Die Inkubation von Endothelzellen mit ox-LDL führte zu einer Dephosphorylierung der eNOS am Thr^{495} sowie zu einer Inaktivierung der PKCα, die diesen Aminosäurerest phosphoryliert. Der Phosphorylierungsstatus keiner der untersuchten eNOS-, Serin- und Threonin-Reste hatte einen Einfluss auf die Dimerisierung des Enzyms, was darauf hindeutet, dass im Gegensatz zu bislang publizierten Untersuchungen das Dimer der eNOS in Endothelzellen sehr stabil ist. Funktionell wird von der eNOS-Tyrosinphosphorylierung zunächst eine veränderte Interaktion mit regulatorischen Proteinen erwartet, mit einer möglichen Modulation der Aktivität. In dieser Arbeit wurde gezeigt, dass die Phosphorylierung von Tyr^{657} eine kritische Rolle sowohl für die Bildung von NO als auch für die Interaktion mit anderen Proteinen hat, da eine phosphomimetische Tyrosin-Mutante vollständig inaktiv ist und außerdem keine

korrekte intrazelluläre Lokalisation zeigt. Die physiologische Relevanz dieser Tyrosinphosphorylierung muss noch weiter untersucht werden.

References

ABU-SOUD, H. M., FELDMAN, P. L., CLARK, P., & STUEHR, D. J. (1994). Electron transfer in the nitric-oxide synthases. Characterization of L- arginine analogs that block heme iron reduction. *J.Biol.Chem.*, **269,** 32318-32326.

ABU-SOUD, H. M. & STUEHR, D. J. (1993). Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. *Proc.Natl.Acad.Sci.U.S.A*, **90,** 10769-10772.

ADACHI, T., WEISBROD, R. M., PIMENTEL, D. R., YING, J., SHAROV, V. S., SCHONEICH, C., & COHEN, R. A. (2004). S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat.Med.*, **10,** 1200-1207.

AISAKA, K., GROSS, S. S., GRIFFITH, O. W., & LEVI, R. (1989). NG-methylarginine, an inhibitor of endothelium-derived nitric oxide synthesis, is a potent pressor agent in the guinea pig: does nitric oxide regulate blood pressure in vivo? *Biochem.Biophys.Res.Commun.*, **160,** 881-886.

ALP, N. J., MCATEER, M. A., KHOO, J., CHOUDHURY, R. P., & CHANNON, K. M. (2004). Increased endothelial tetrahydrobiopterin synthesis by targeted transgenic GTP-cyclohydrolase I overexpression reduces endothelial dysfunction and atherosclerosis in ApoE-knockout mice. *Arterioscler.Thromb.Vasc.Biol.*, **24,** 445-450.

ALP, N. J., MUSSA, S., KHOO, J., CAI, S., GUZIK, T., JEFFERSON, A., GOH, N., ROCKETT, K. A., & CHANNON, K. M. (2003). Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. *J.Clin.Invest*, **112,** 725-735.

AOYAGI, M., ARVAI, A. S., TAINER, J. A., & GETZOFF, E. D. (2003). Structural basis for endothelial nitric oxide synthase binding to calmodulin. *EMBO J.*, **22,** 766-775.

ARCHER, S. (1993). Measurement of nitric oxide in biological models. *FASEB J.*, **7,** 349-360.

AYAJIKI, K., KINDERMANN, M., HECKER, M., FLEMING, I., & BUSSE, R. (1996). Intracellular pH and tyrosine phosphorylation but not calcium determine shear stress-induced nitric oxide production in native endothelial cells. *Circ Res*, **78,** 750-758.

BAEK, K. J., THIEL, B. A., LUCAS, S., & STUEHR, D. J. (1993). Macrophage nitric oxide synthase subunits. Purification, characterization, and role of prosthetic groups and substrate in regulating their association into a dimeric enzyme. *J.Biol.Chem.*, **268,** 21120-21129.

BAKER, T. A., MILSTIEN, S., & KATUSIC, Z. S. (2001). Effect of vitamin C on the availability of tetrahydrobiopterin in human endothelial cells. *J Cardiovasc.Pharmacol.*, **37,** 333-338.

BAUER, P. M., FULTON, D., BOO, Y. C., SORESCU, G. P., KEMP, B. E., JO, H., & SESSA, W. C. (2003). Compensatory phosphorylation and protein-protein interactions revealed by loss of function and gain of function mutants of multiple serine phosphorylation sites in endothelial nitric-oxide synthase. *J.Biol.Chem.*, **278,** 14841-14849.

BAUERSACHS, J., FLEMING, I., SCHOLZ, D., POPP, R., & BUSSE, R. (1997). Endothelium-derived hyperpolarizing factor, but not nitric oxide, is reversibly inhibited by brefeldin A. *Hypertension*, **30,** 1598-1605.

BAUTISTA, A. P., SCHULER, A., SPOLARICS, Z., & SPITZER, J. J. (1991). Tumor necrosis factor-alpha stimulates superoxide anion generation by perfused rat liver and Kupffer cells. *Am.J.Physiol*, **261,** G891-G895.

BECKMAN, J. A., GOLDFINE, A. B., GORDON, M. B., GARRETT, L. A., & CREAGER, M. A. (2002). Inhibition of protein kinase C beta prevents impaired endothelium-dependent vasodilation caused by hyperglycemia in humans. *Circ.Res.*, **90,** 107-111.

BECKMAN, J. S. & CROW, J. P. (1993). Pathological implications of nitric oxide, superoxide and peroxynitrite formation. *Biochem.Soc.Trans.*, **21,** 330-334.

BECKMAN, J. S. & KOPPENOL, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am.J.Physiol*, **271,** C1424-C1437.

BENDER, A. T., SILVERSTEIN, A. M., DEMADY, D. R., KANELAKIS, K. C., NOGUCHI, S., PRATT, W. B., & OSAWA, Y. (1999). Neuronal nitric-oxide synthase is regulated by the hsp90-based chaperone system in vivo. *J.Biol.Chem.*, **274,** 1472-1478.

BENZING, T., FLEMING, I., BLAUKAT, A., MULLER-ESTERL, W., & BUSSE, R. (1999). Angiotensin-converting enzyme inhibitor ramiprilat interferes with the sequestration of the B-2 kinin receptor within the plasma membrane of native endothelial cells. *Circulation*, **99,** 2034-2040.

BILLECKE, S. S., BENDER, A. T., KANELAKIS, K. C., MURPHY, P. J., LOWE, E. R., KAMADA, Y., PRATT, W. B., & OSAWA, Y. (2002). Hsp90 is required for heme binding and activation of apo-neuronal nitric-oxide synthase: geldanamycin-mediated oxidant generation is unrelated to any action of Hsp90. *J.Biol Chem.*, **277,** 20504-20509.

BLAIR, A., SHAUL, P. W., YUHANNA, I. S., CONRAD, P. A., & SMART, E. J. (1999). Oxidized low density lipoprotein displaces endothelial nitric-oxide synthase (eNOS) from plasmalemmal caveolae and impairs eNOS activation. *J.Biol.Chem.*, **274,** 32512-32519.

BOMMEL, H. M., REIF, A., FROHLICH, L. G., FREY, A., HOFMANN, H., MARECAK, D. M., GROEHN, V., KOTSONIS, P., LA, M., KOSTER, S., MEINECKE, M., BERNHARDT, M., WEEGER, M., GHISLA, S., PRESTWICH, G. D., PFLEIDERER, W., & SCHMIDT, H. H. (1998). Anti-pterins as tools to characterize the function of tetrahydrobiopterin in NO synthase. *J.Biol.Chem.*, **273,** 33142-33149.

BOO, Y. C., HWANG, J., SYKES, M., MICHELL, B. J., KEMP, B. E., LUM, H., & JO, H. (2002). Shear stress stimulates phosphorylation of eNOS at Ser(635) by a protein kinase A-dependent mechanism. *Am.J.Physiol Heart Circ.Physiol*, **283,** H1819-H1828.

BOO, Y. C. & JO, H. (2003). Flow-dependent regulation of endothelial nitric oxide synthase: role of protein kinases. *Am.J.Physiol Cell Physiol*, **285,** C499-C508.

BOO, Y. C., SORESCU, G. P., BAUER, P. M., FULTON, D., KEMP, B. E., HARRISON, D. G., SESSA, W. C., & JO, H. (2003). Endothelial NO synthase phosphorylated at Ser635 produces NO without requiring intracellular calcium increase. *Free Radic.Biol.Med.*, **35,** 729-741.

BREDT, D. S. & SNYDER, S. H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc.Natl.Acad.Sci.U.S.A*, **87,** 682-685.

BRITIGAN, B. E., ROEDER, T. L., & SHASBY, D. M. (1992). Insight into the nature and site of oxygen-centered free radical generation by endothelial cell monolayers using a novel spin trapping technique. *Blood*, **79,** 699-707.

BUCCI, M., GRATTON, J. P., RUDIC, R. D., ACEVEDO, L., ROVIEZZO, F., CIRINO, G., & SESSA, W. C. (2000). In vivo delivery of the caveolin-1 scaffolding domain inhibits nitric oxide synthesis and reduces inflammation. *Nat.Med.*, **6,** 1362-1367.

BUSSE, R. & FLEMING, I. (1996). Endothelial dysfunction in atherosclerosis. *J.Vasc.Res.*, **33,** 181-194.

BUSSE, R., LUCKHOFF, A., & BASSENGE, E. (1987). Endothelium-derived relaxant factor inhibits platelet activation. *Naunyn Schmiedebergs Arch.Pharmacol.*, **336,** 566-571.

BUSSE, R. & MÜLSCH, A. (1990). Calcium-dependent nitric oxide synthesis in endothelial cytosol is mediated by calmodulin. *FEBS Lett.*, **265,** 133-136.

BUTT, E., BERNHARDT, M., SMOLENSKI, A., KOTSONIS, P., FROHLICH, L. G., SICKMANN, A., MEYER, H. E., LOHMANN, S. M., & SCHMIDT, H. H. (2000). Endothelial nitric-oxide synthase (type III) is activated and becomes calcium independent upon phosphorylation by cyclic nucleotide-dependent protein kinases. *J.Biol.Chem.*, **275,** 5179-5187.

CAO, M. Y., HUBER, M., BEAUCHEMIN, N., FAMIGLIETTI, J., ALBELDA, S. M., & VEILLETTE, A. (1998). Regulation of mouse PECAM-1 tyrosine phosphorylation by the Src and Csk families of protein-tyrosine kinases. *J.Biol.Chem.*, **273,** 15765-15772.

CAO, S., YAO, J., & SHAH, V. (2003). The proline-rich domain of dynamin-2 is responsible for dynamin-dependent in vitro potentiation of endothelial nitric-oxide synthase activity via selective effects on reductase domain function. *J.Biol.Chem.*, **278,** 5894-5901.

CAO, S., YAO, J., MCCABE, T. J., YAO, Q., KATUSIC, Z. S., SESSA, W. C., & SHAH, V. (2001). Direct interaction between endothelial nitric-oxide synthase and dynamin-2. Implication of nitric-oxide synathase function. *J Biol.Chem.*, **276,** 14249-14256.

CAPLAN, A. J. (1999). Hsp90's secrets unfold: new insights from structural and functional studies. *Trends Cell Biol*, **9,** 262-268.

CHATTERJEE, S., CAO, S., PETERSON, T. E., SIMARI, R. D., & SHAH, V. (2003). Inhibition of GTP-dependent vesicle trafficking impairs internalization of plasmalemmal eNOS and cellular nitric oxide production. *J.Cell Sci.*, **116,** 3645-3655.

CHAVAKIS, E., DERNBACH, E., HERMANN, C., MONDORF, U. F., ZEIHER, A. M., & DIMMELER, S. (2001). Oxidized LDL inhibits vascular endothelial growth factor-induced endothelial cell migration by an inhibitory effect on the Akt/endothelial nitric oxide synthase pathway. *Circulation*, **103,** 2102-2107.

CHENG, J. J., CHAO, Y. J., & WANG, D. L. (2002). Cyclic strain activates redox-sensitive proline-rich tyrosine kinase 2 (PYK2) in endothelial cells. *J.Biol.Chem.*, **277,** 48152-48157.

CHU, S. & BOHLEN, H. G. (2004). High concentration of glucose inhibits glomerular endothelial eNOS through a PKC mechanism. *Am.J.Physiol Renal Physiol*, **287,** F384-F392.

CIRINO, G., WHEELERJONES, C. P. D., WALLACE, J. L., DELSOLDATO, P., & BAYDOUN, A. R. (1996). Inhibition of inducible nitric oxide synthase expression by novel nonsteroidal anti-inflammatory derivatives with gastrointestinal-sparing properties. *Br.J.Pharmacol.*, **117,** 1421-1426.

COMINACINI, L., RIGONI, A., PASINI, A. F., GARBIN, U., DAVOLI, A., CAMPAGNOLA, M., PASTORINO, A. M., LO CASCIO, V., & SAWAMURA, T. (2001). The binding of oxidized low density lipoprotein (ox-LDL) to ox-LDL receptor-1 reduces the intracellular concentration of nitric oxide in endothelial cells through an increased production of superoxide. *J.Biol.Chem.*, **276,** 13750-13755.

CONNELL, P., BALLINGER, C. A., JIANG, J., WU, Y., THOMPSON, L. J., HOHFELD, J., & PATTERSON, C. (2001). The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nat.Cell Biol.*, **3,** 93-96.

CORSON, M. A., JAMES, N. L., LATTA, S. E., NEREM, R. M., BERK, B. C., & HARRISON, D. G. (1996). Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ.Res.*, **79,** 984-991.

COSENTINO, F., ETO, M., DE PAOLIS, P., VAN DER, L. B., BACHSCHMID, M., ULLRICH, V., KOUROEDOV, A., DELLI, G. C., JOCH, H., VOLPE, M., & LUSCHER, T. F. (2003). High glucose causes upregulation of cyclooxygenase-2 and alters prostanoid profile in human endothelial cells: role of protein kinase C and reactive oxygen species. *Circulation*, **107,** 1017-1023.

CRAIG, D. H., CHAPMAN, S. K., & DAFF, S. (2002). Calmodulin activates electron transfer through neuronal nitric-oxide synthase reductase domain by releasing an NADPH-dependent conformational lock. *J.Biol.Chem.*, **277,** 33987-33994.

CRANE, B. R., ARVAI, A. S., GHOSH, D. K., WU, C., GETZOFF, E. D., STUEHR, D. J., & TAINER, J. A. (1998). Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. *Science*, **279,** 2121-2126.

CYRUS, T., WITZTUM, J. L., RADER, D. J., TANGIRALA, R., FAZIO, S., LINTON, M. F., & FUNK, C. D. (1999). Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo Eûdeficient mice. *J.Clin.Invest.*, **103,** 1597-1604.

D'USCIO, L. V., MILSTIEN, S., RICHARDSON, D., SMITH, L., & KATUSIC, Z. S. (2003). Long-term vitamin C treatment increases vascular tetrahydrobiopterin levels and nitric oxide synthase activity. *Circ.Res.*, **92,** 88-95.

DAFF, S. (2003). Calmodulin-dependent regulation of mammalian nitric oxide synthase. *Biochem.Soc.Trans.*, **31,** 502-505.

DAFF, S., NOBLE, M. A., CRAIG, D. H., RIVERS, S. L., CHAPMAN, S. K., MUNRO, A. W., FUJIWARA, S., ROZHKOVA, E., SAGAMI, I., & SHIMIZU, T. (2001). Control of electron transfer in neuronal NO synthase. *Biochem.Soc.Trans.*, **29,** 147-152.

DARLEY-USMAR, V. & HALLIWELL, B. (1996). Blood radicals: reactive nitrogen species, reactive oxygen species, transition metal ions, and the vascular system. *Pharm.Res.*, **13,** 649-662.

DARLEY-USMAR, V. M., HOGG, N., O'LEARY, V. J., WILSON, M. T., & MONCADA, S. (1992). The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. *Free Radic.Res.Commun.*, **17,** 9-20.

DAVDA, R. K., CHANDLER, L. J., & GUZMAN, N. J. (1994). Protein kinase C modulates receptor-independent activation of endothelial nitric oxide synthase. *Eur.J.Pharmacol.*, **266,** 237-244.

DAVIS, M. E., CAI, H., DRUMMOND, G. R., & HARRISON, D. G. (2001). Shear stress regulates endothelial nitric oxide synthase expression through c-Src by divergent signaling pathways. *Circ.Res.*, **89,** 1073-1080.

DAVIS, M. E., GRUMBACH, I. M., FUKAI, T., CUTCHINS, A., & HARRISON, D. G. (2004). Shear stress regulates endothelial nitric-oxide synthase promoter activity through nuclear factor kappaB binding. *J.Biol.Chem.*, **279,** 163-168.

DAVYDOV, R., LEDBETTER-ROGERS, A., MARTASEK, P., LARUKHIN, M., SONO, M., DAWSON, J. H., MASTERS, B. S., & HOFFMAN, B. M. (2002). EPR and ENDOR characterization of intermediates in the cryoreduced oxy-nitric oxide synthase heme domain with bound L-arginine or N(G)-hydroxyarginine. *Biochemistry*, **20;41,** 10375-10381.

DEDIO, J., KONIG, P., WOHLFART, P., SCHROEDER, C., KUMMER, W., & MULLER-ESTERL, W. (2001). NOSIP, a novel modulator of endothelial nitric oxide synthase activity. *FASEB J.*, **15,** 79-89.

DIKALOV, S., SKATCHKOV, M., & BASSENGE, E. (1997). Spin trapping of superoxide radicals and peroxynitrite by 1-hydroxy-3-carboxy-pyrrolidine and 1-hydroxy-2,2,6, 6-tetramethyl-4-oxo-piperidine and the stability of corresponding nitroxyl radicals towards biological reductants. *Biochem.Biophys.Res.Commun.*, **231,** 701-704.

DIMMELER, S., FLEMING, I., FISSLTHALER, B., HERMANN, C., BUSSE, R., & ZEIHER, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*, **399,** 601-605.

DIMMELER, S., FISSTHALER, B., FLEMING, I., ASSMUS, B., HERMANN, C., & ZEIHER, A. M. (1998). Shear stress stimulates the protein kinase Akt - involvement in regulation of the endothelial nitric oxide synthase. *Circulation*, **98,** 312.

DRAB, M., VERKADE, P., ELGER, M., KASPER, M., LOHN, M., LAUTERBACH, B., MENNE, J., LINDSCHAU, C., MENDE, F., LUFT, F. C., SCHEDL, A., HALLER, H., & KURZCHALIA, T. V. (2001). Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science*, **293,** 2449-2452.

DULAK, J., POLUS, M., GUEVARA, I., POLUS, A., HARTWICH, J., & DEMBINSKA-KIEC, A. (1997). Regulation of inducible nitric oxide synthase (iNOS) and GTP cyclohydrolase I (GTP-CH I) gene expression by ox-LDL in rat vascular smooth muscle cells. *J.Physiol.Pharmacol.*, **48,** 689-697.

EL REMESSY, A. B., BARTOLI, M., PLATT, D. H., FULTON, D., & CALDWELL, R. B. (2005). Oxidative stress inactivates VEGF survival signaling in retinal endothelial cells via PI 3-kinase tyrosine nitration. *J.Cell Sci.*, **118,** 243-252.

FEIL, R., LOHMANN, S. M., DE JONGE, H., WALTER, U., & HOFMANN, F. (2003). Cyclic GMP-dependent protein kinases and the cardiovascular system: insights from genetically modified mice. *Circ.Res.*, **93,** 907-916.

FENG, Y., VENEMA, V. J., VENEMA, R. C., TSAI, N., & CALDWELL, R. B. (1999). VEGF induces nuclear translocation of Flk-1/KDR, endothleial nitric oxide synthase, and caveolin-1 in vascular endothleial cells. *Biochem.Biophys.Res.Commun.*, **256,** 192-197.

FERON, O., MICHEL, J. B., SASE, K., & MICHEL, T. (1998). Dynamic regulation of endothelial nitric oxide synthase: complementary roles of dual acylation and caveolin interactions. *Biochemistry*, **37,** 193-200.

FERON, O., DESSY, C., MONIOTTE, S., DESAGER, J. P., & BALLIGAND, J. L. (1999). Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. *J.Clin.Invest.*, **103,** 897-905.

FIORUCCI, S., ANTONELLI, E., DISTRUTTI, E., DEL SOLDATO, P., FLOWER, R. J., CLARK, M. J. P., MORELLI, A., PERRETTI, M., & IGNARRO, L. J. (2002). NCX-1015, a nitric-oxide derivative of prednisolone, enhances regulatory T cells in the lamina propria and protects against 2,4,6-trinitrobenzene sulfonic acid-induced colitis in mice. *Proc.Natl.Acad.Sci.U.S.A*, **99,** 15770-15775.

FISCHMANN, T. O., HRUZA, A., NIU, X. D., FOSSETTA, J. D., LUNN, C. A., DOLPHIN, E., PRONGAY, A. J., REICHERT, P., LUNDELL, D. J., NARULA, S. K., & WEBER, P. C. (1999). Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. *Nat.Struct.Biol.*, **6,** 233-242.

FISSITHALER, B., DIMMELER, S., HERMANN, C., BUSSE, R., & FLEMING, I. (2000). Phosphorylation and activation of the endothelial nitric oxide synthase by fluid shear stress. *Acta Physiol Scand.*, **168,** 81-88.

FLEMING, I., BARA, A. T., & BUSSE, R. (1996a). Calcium signalling and autacoid production in endothelial cells are modulated by changes in tyrosine kinase and phosphatase activity. *J.Vasc.Res.*, **33,** 225-234.

FLEMING, I., BAUERSACHS, J., & BUSSE, R. (1997). Calcium-dependent and calcium-independent activation of the endothelial NO synthase. *J.Vasc.Res.*, **34,** 165-174.

FLEMING, I., BAUERSACHS, J., FISSLTHALER, B., & BUSSE, R. (1998). Ca^{2+}-independent activation of the endothelial nitric oxide synthase in response to tyrosine phosphatase inhibitors and fluid shear stress. *Circ.Res.*, **82,** 686-695.

FLEMING, I. & BUSSE, R. (1997). Tyrosine phosphorylation and bradykinin-induced signaling in endothelial cells. *Am.J.Cardiol.*, **80,** A102-A109.

FLEMING, I., FISSLTHALER, B., & BUSSE, R. (1996b). Interdependence of calcium signaling and protein tyrosine phosphorylation in human endothelial cells. *J.Biol.Chem.*, **271,** 11009-11015.

FLEMING, I., MOHAMED, A., GALLE, J., TURCHANOWA, L., BRANDES, R. P., FISSLTHALER, B., & BUSSE, R. (2005). Oxidized low-density lipoprotein increases superoxide production by endothelial nitric oxide synthase by inhibiting PKCα. *Cardiovasc.Res.*, **65,** 897-906.

FLEMING, I. & BUSSE, R. (2003). Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am J Physiol Regul Integr Comp Physiol*, **284,** R1-12.

FLEMING, I., FISSLTHALER, B., DIMMELER, S., KEMP, B. E., & BUSSE, R. (2001). Phosphorylation of Thr495 regulates Ca2+/calmodulin-dependent endothelial nitric oxide synthase activity. *Circ.Res.*, **88,** 68e-75.

FONTANA, J., FULTON, D., CHEN, Y., FAIRCHILD, T. A., MCCABE, T. J., FUJITA, N., TSURUO, T., & SESSA, W. C. (2002). Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circ.Res.*, **90,** 866-873.

FORSTERMANN, U., POLLOCK, J. S., SCHMIDT, H. H., HELLER, M., & MURAD, F. (1991). Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proc.Natl.Acad.Sci.U.S.A*, **88,** 1788-1792.

FRANCO, M. C., FORTES, Z. B., AKAMINE, E. H., KAWAMOTO, E. M., SCAVONE, C., DE BRITTO, L. R., MUSCARA, M. N., TEIXEIRA, S. A., TOSTES, R. C., CARVALHO, M. H., & NIGRO, D. (2004). Tetrahydrobiopterin improves endothelial dysfunction and vascular oxidative stress in microvessels of intrauterine undernourished rats. *J.Physiol.*, **558,** 239-248.

FRANGOS, J. A., HUANG, T. Y., & CLARK, C. B. (1996). Steady shear and step changes in shear stimulate endothelium via independent mechanisms--superposition of transient and sustained nitric oxide production. *Biochem.Biophys.Res.Commun.*, **224,** 660-665.

FRANK, S., MADLENER, M., PFEILSCHIFTER, J., & WERNER, S. (1998). Induction of inducible nitric oxide synthase and its corresponding tetrahydrobiopterin-cofactor-synthesizing enzyme GTP-cyclohydrolase I during cutaneous wound repair. *J.Invest Dermatol.*, **111,** 1058-1064.

FULTON, D., GRATTON, J. P., MCCABE, T. J., FONTANA, J., FUJIO, Y., WALSH, K., FRANKE, T. F., PAPAPETROPOULOS, A., & SESSA, W. C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*, **399,** 597-601.

FULTON, D., BABBITT, R., ZOELLNER, S., FONTANA, J., ACEVEDO, L., MCCABE, T. J., IWAKIRI, Y., & SESSA, W. C. (2004). Targeting of endothelial nitric-oxide synthase to the cytoplasmic face of the golgi complex or plasma membrane regulates Akt- versus calcium-dependent mechanisms for nitric oxide release. *J.Biol.Chem.*, **279,** 30349-30357.

FULTON, D., FONTANA, J., SOWA, G., GRATTON, J. P., LIN, M., LI, K. X., MICHELL, B., KEMP, B. E., RODMAN, D., & SESSA, W. C. (2002). Localization of endothelial nitric-oxide synthase phosphorylated on serine 1179 and nitric oxide in golgi and plasma membrane defines the existence of two pools of active enzyme. *J.Biol.Chem.*, **277,** 4277-4284.

FULTON, D., GRATTON, J. P., & SESSA, W. C. (2001). Post-translational control of endothelial nitric oxide synthase: Why isn't calcium/calmodulin enough? *J.Pharmacol.Exp.Ther.*, **299,** 818-824.

FURCHGOTT, R. F. & ZAWADZKI, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288,** 373-376.

GALLIS, B., CORTHALS, G. L., GOODLETT, D. R., UEBA, H., KIM, F., PRESNELL, S. R., FIGEYS, D., HARRISON, D. G., BERK, B. C., AEBERSOLD, R., & CORSON, M. A. (1999). Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. *J.Biol.Chem.*, **274,** 30101-30108.

GARCIA-CARDENA, G., COMANDER, J., ANDERSON, K. R., BLACKMAN, B. R., & GIMBRONE, M. A., JR. (2001). Biomechanical activation of vascular endothelium as a determinant of its functional phenotype. *Proc.Natl.Acad.Sci.U.S.A*, **98,** 4478-4485.

GARCIA-CARDENA, G., FAN, R., SHAH, V., SORRENTINO, R., CIRINO, G., PAPAPETROPOULOS, A., & SESSA, W. C. (1998). Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature*, **392,** 821-824.

GARCIA-CARDENA, G., FAN, R., STERN, D. F., LIU, J., & SESSA, W. C. (1996a). Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J.Biol.Chem.*, **271,** 27237-27240.

GARCIA-CARDENA, G., OH, P., LIU, J., SCHNITZER, J. E., & SESSA, W. C. (1996b). Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. *Proc.Natl.Acad.Sci.U.S.A*, **93,** 6448-6453.

GARCIA-CARDENA, G., MARTASEK, P., MASTERS, B. S. S., SKIDD, P. M., COUET, J., LI, S., LISANTI, M. P., & SESSA, W. C. (1997). Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the NOS caveolin binding domain in vivo. *J.Biol.Chem.*, **272,** 25437-25440.

GARCIN, E. D., BRUNS, C. M., LLOYD, S. J., HOSFIELD, D. J., TISO, M., GACHHUI, R., STUEHR, D. J., TAINER, J. A., & GETZOFF, E. D. (2004). Structural basis for isozyme-specific regulation of electron transfer in nitric-oxide synthase. *J.Biol.Chem.*, **279,** 37918-37927.

GERZANICH, V., IVANOVA, S., ZHOU, H., & SIMARD, J. M. (2003). Mislocalization of eNOS and upregulation of cerebral vascular Ca^{2+} channel activity in angiotensin-hypertension. *Hypertension*, **41,** 1124-1130.

GHOSH, S., GACHHUI, R., CROOKS, C., WU, C., LISANTI, M. P., & STUEHR, D. J. (1998). Interaction between caveolin-1 and the reductase domain of endothelial nitric-oxide synthase. Consequences for catalysis. *J.Biol.Chem.*, **273,** 22267-22271.

GIARDINA, J. B., TANNER, D. J., & KHALIL, R. A. (2001). Oxidized-LDL enhances coronary vasoconstriction by increasing the activity of protein kinase C isoforms alpha and epsilon. *Hypertension*, **37,** 561-568.

GIORDANO, A., TONELLO, C., BULBARELLI, A., COZZI, V., CINTI, S., CARRUBA, M. O., & NISOLI, E. (2002). Evidence for a functional nitric oxide synthase system in brown adipocyte nucleus. *FEBS Lett.*, **514,** 135-140.

GOLSER, R., GORREN, A. C., LEBER, A., ANDREW, P., HABISCH, H. J., WERNER, E. R., SCHMIDT, K., VENEMA, R. C., & MAYER, B. (2000). Interaction of endothelial and neuronal nitric-oxide synthases with the bradykinin B2 receptor. Binding of an inhibitory peptide to the oxygenase domain blocks uncoupled NADPH oxidation. *J.Biol.Chem.*, **275,** 5291-5296.

GONZALEZ, E., KOU, R., LIN, A. J., GOLAN, D. E., & MICHEL, T. (2002). Subcellular targeting and agonist-induced site-specific phosphorylation of eNOS. *J.Biol.Chem.*

GOVERS, R., BEVERS, L., DE BREE, P., & RABELINK, T. J. (2002a). Endothelial nitric oxide synthase activity is linked to its presence at cell-cell contacts. *Biochem.J.*, **361,** 193-201.

GOVERS, R., VAN DER SLUIJS, P., VAN DONSELAAR, E., SLOT, J. W., & RABELINK, T. J. (2002b). Endothelial nitric oxide synthase and its negative regulator caveolin-1 localize to distinct perinuclear organelles. *J.Histochem.Cytochem.*, **50,** 779-788.

GRATTON, J. P., FONTANA, J., O'CONNOR, D. S., GARCIA-CARDENA, G., MCCABE, T. J., & SESSA, W. C. (2000). Reconstitution of an endothelial nitric-oxide synthase (eNOS), Hsp90, and caveolin-1 complex in vitro.Evidencec that Hsp90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1. *J.Biol.Chem.*, **275,** 22268-22272.

GREIF, D. M., SACKS, D. B., & MICHEL, T. (2004). Calmodulin phosphorylation and modulation of endothelial nitric oxide synthase catalysis. *Proc.Natl.Acad.Sci.U.S.A*, **101,** 1165-1170.

GRYGLEWSKI, R. J., CHLOPICKI, S., URACZ, W., & MARCINKIEWICZ, E. (2001). Significance of endothelial prostacyclin and nitric oxide in peripheral and pulmonary circulation. *Med.Sci.Monit.*, **7,** 1-16.

GUZIK, T. J., MUSSA, S., GASTALDI, D., SADOWSKI, J., RATNATUNGA, C., PILLAI, R., & CHANNON, K. M. (2002). Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation*, **105,** 1656-1662.

HARRIS, M. B., JU, H., VENEMA, V. J., BLACKSTONE, M., & VENEMA, R. C. (2000). Role of heat shock protein 90 in bradykinin-stimulated endothelial nitric oxide release. *Gen.Pharmacol.*, **35,** 165-170.

HARRIS, M. B., JU, H., VENEMA, V. J., LIANG, H., ZOU, R., MICHELL, B. J., CHEN, Z. P., KEMP, B. E., & VENEMA, R. C. (2001). Reciprocal phosphorylation and regulation of endothelial nitric-oxide synthase in response to bradykinin stimulation. *J.Biol.Chem.*, **276,** 16587-16591.

HAYNES, M. P., SINHA, D., RUSSELL, K. S., COLLINGE, M., FULTON, D., MORALES-RUIZ, M., SESSA, W. C., & BENDER, J. R. (2000). Membrane estrogen

receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. *Circ.Res.*, **87,** 677-682.

HECKER, M., MÜLSCH, A., BASSENGE, E., FÖRSTERMANN, U., & BUSSE, R. (1994). Subcellular localization and characterization of nitric oxide synthase(s) in endothelial cells: physiological implications. *Biochem J*, **299,** 247-252.

HEINLOTH, A., HEERMEIER, K., RAFF, U., WANNER, C., & GALLE, J. (2000). Stimulation of NADPH oxidase by oxidized low-density lipoprotein induces proliferation of human vascular endothelial cells. *J.Am.Soc.Nephrol.*, **11,** 1819-1825.

HELLER, R., UNBEHAUN, A., SCHELLENBERG, B., MAYER, B., WERNER-FELMAYER, G., & WERNER, E. R. (2001). L-ascorbic acid potentiates endothelial nitric oxide synthesis via a chemical stabilization of tetrahydrobiopterin. *J.Biol.Chem.*, **276,** 40-47.

HINK, U., LI, H., MOLLNAU, H., OELZE, M., MATHEIS, E., HARTMANN, M., SKATCHKOV, M., THAISS, F., STAHL, R. A., WARNHOLTZ, A., MEINERTZ, T., GRIENDLING, K., HARRISON, D. G., FORSTERMANN, U., & MUNZEL, T. (2001). Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ.Res.*, **88,** E14-E22.

HIRATA, K. I., KURODA, R., SAKODA, T., KATAYAMA, M., INOUE, N., SUEMATSU, M., KAWASHIMA, S., & YOKOYAMA, M. (1995). Inhibition of endothelial nitric oxide synthase activity by protein kinase C. *Hypertension*, **25,** 180-185.

HOLLAND, J. A., PRITCHARD, K. A., PAPPOLLA, M. A., WOLIN, M. S., ROGERS, N. J., & STEMERMAN, M. B. (1990). Bradykinin induces superoxide anion release from human endothelial cells. *J.Cell Physiol.*, **143,** 21-25.

HUANG, A., VITA, J. A., VENEMA, R. C., & KEANEY, J. F., JR. (2000). Ascorbic acid enhances endothelial nitric-oxide synthase activity by increasing intracellular tetrahydrobiopterin. *J.Biol.Chem.*, **275,** 17399-17406.

HUANG, H., HAH, J. M., & SILVERMAN, R. B. (2001). Mechanism of nitric oxide synthase. Evidence that direct hydrogen atom abstraction from the O-H bond of NG-hydroxyarginine is not relevant to the mechanism. *J.Am.Chem.Soc.*, **123,** 2674-2676.

IGNARRO, L. J., BUGA, G. M., WOOD, K. S., BYRNS, R. E., & CHAUDHURI, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc.Natl.Acad.Sci.U.S.A*, **84,** 9265-9269.

JALALI, S., LI, Y. S., SOTOUDEH, M., YUAN, S., LI, S., CHIEN, S., & SHYY, J. Y. J. (1998). Shear stress activates p60Src-Ras-MAPK signaling pathways in vascular endothelial cells. *Arterioscler.Thromb.Vasc.Biol.*, **18,** 227-234.

JIANG, J., CYR, D., BABBITT, R. W., SESSA, W. C., & PATTERSON, C. (2003). Chaperone-dependent regulation of endothelial nitric-oxide synthase intracellular trafficking by the co-chaperone/ubiquitin ligase CHIP. *J.Biol.Chem.*, **278,** 49332-49341.

JU, H., VENEMA, V. J., MARRERO, M. B., & VENEMA, R. C. (1998). Inhibitory interactions of the bradykinin B2 receptor with endothelial nitric-oxide synthase. *J.Biol.Chem.*, **273,** 24025-24029.

KATSUYAMA, K., SHICHIRI, M., MARUMO, F., & HIRATA, Y. (1998). NO inhibits cytokine-induced iNOS expression and NF-kappa B activation by interfering with phosphorylation and degradation of I kappa B-alpha. *Arterioscler.Thromb.Vasc.Biol.*, **18,** 1796-1802.

KAWASHIMA, S. (2004). Malfunction of vascular control in lifestyle-related diseases: endothelial nitric oxide (NO) synthase/NO system in atherosclerosis. *J.Pharmacol.Sci.*, **96,** 411-419.

KIM, F., GALLIS, B., & CORSON, M. A. (2001). TNF-alpha inhibits flow and insulin signaling leading to NO production in aortic endothelial cells. *Am.J.Physiol Cell Physiol*, **280,** C1057-C1065.

KLATT, P., SCHMIDT, K., LEHNER, D., GLATTER, O., BACHINGER, H. P., & MAYER, B. (1995). Structural analysis of porcine brain nitric oxide synthase reveals a role for tetrahydrobiopterin and L-arginine in the formation of an SDS-resistant dimer. *EMBO J.*, **14,** 3687-3695.

KLESCHYOV, A. L., MOLLNAU, H., OELZE, M., MEINERTZ, T., HUANG, Y., HARRISON, D. G., & MUNZEL, T. (2000). Spin trapping of vascular nitric oxide using colloid Fe(II)-diethyldithiocarbamate. *Biochem.Biophys.Res.Commun.*, **275,** 672-677.

KNUDSEN, G. M., NISHIDA, C. R., MOONEY, S. D., & DE MONTELLANO, P. R. O. (2003). Nitric-oxide synthase (NOS) reductase domain models suggest a new control element in endothelial NOS that attenuates calmodulin-dependent activity. *J.Biol.Chem.*, **278,** 31814-31824.

KONIG, P., DEDIO, J., OESS, S., PAPADAKIS, T., FISCHER, A., MULLER-ESTERL, W., & KUMMER, W. (2005). NOSIP and its interacting protein, eNOS, in the rat trachea and lung. *J.Histochem.Cytochem.*, **53,** 155-164.

KOU, R., PRABHAKAR, P., & MICHEL, T. (2001). Phosphorylation of the endothelial isoform of nitric oxide synthase at serine 116: Identification of a novel path for eNOS regulation by lysophosphatidic acid. *Circulation*, **104,** 509.

KOU, R., GREIF, D., & MICHEL, T. (2002). Dephosphorylation of endothelial nitric-oxide synthase by vascular endothelial growth factor. Implication for the vascular responses to cyclosporin A. *J.Biol.Chem.*, **277,** 29669-29673.

KUBES, P., SUZUKI, M., & GRANGER, D. N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc.Natl.Acad.Sci.U.S.A*, **88,** 4651-4655.

KUCHAN, M. J. & FRANGOS, J. A. (1994). Role of calcium and calmodulin in flow-induced nitric oxide production in endothelial cells. *Am.J.Physiol*, **266,** C628-C636.

KUZKAYA, N., WEISSMANN, N., HARRISON, D. G., & DIKALOV, S. (2003). Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: Implications for uncoupling endothelal nitric-oxide synathse. *J.Biol.Chem.*, **278,** 22546-22554.

LAGAUD, G. J., MASIH-KHAN, E., KAI, S., VAN BREEMEN, C., & DUBE, G. P. (2001). Influence of type II diabetes on arterial tone and endothelial function in murine mesenteric resistance arteries. *J.Vasc.Res.*, **38,** 578-589.

LAMB, M. E., DE WEERD, W. F., & LEEB-LUNDBERG, L. M. (2001). Agonist-promoted trafficking of human bradykinin receptors: arrestin- and dynamin-independent sequestration of the B2 receptor and bradykinin in HEK293 cells. *Biochem.J.*, **355,** 741-750.

LANDMESSER, U., DIKALOV, S., PRICE, S. R., MCCANN, L., FUKAI, T., HOLLAND, S. M., MITCH, W. E., & HARRISON, D. G. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J.Clin.Invest*, **111,** 1201-1209.

LANE, P. & GROSS, S. S. (2000). The autoinhibitory control element and calmodulin conspire to provide physiological modulation of endothelial and neuronal nitric oxide synthase activity. *Acta Physiol Scand.*, **168,** 53-63.

LANE, P. & GROSS, S. S. (2002). Disabling a C-terminal autoinhibitory control element in endothelial nitric-oxide synthase by phosphorylation provides a molecular explanation for activation of vascular NO synthesis by diverse physiological stimuli. *J.Biol.Chem.*, **277,** 19087-19094.

LASSEGUE, B. & GRIENDLING, K. K. (2004). Reactive oxygen species in hypertension; An update. *Am.J.Hypertens.*, **17,** 852-860.

LEE, C. M., ROBINSON, L. J., & MICHEL, T. (1995). Oligomerization of endothelial nitric oxide synthase. Evidence for a dominant negative effect of truncation mutants. *J.Biol.Chem.*, **270,** 27403-27406.

LEFER, D. J., JONES, S. P., GIROD, W. G., BAINES, A., GRISHAM, M. B., COCKRELL, A. S., HUANG, P. L., & SCALIA, R. (1999). Leukocyte-endothelial cell interactions in nitric oxide synthase-deficient mice. *Am.J.Physiol*, **276,** H1943-H1950.

LENASI, H., KOHLSTEDT, K., FICHTLSCHERER, B., MULSCH, A., BUSSE, R., & FLEMING, I. (2003). Amlodipine activates the endothelial nitric oxide synthase by altering phosphorylation on Ser(1177) and Thr(495). *Cardiovasc.Res.*, **59,** 844-853.

LI, D., LIU, L., CHEN, H., SAWAMURA, T., & MEHTA, J. L. (2003). LOX-1, an oxidized LDL endothelial receptor, induces CD40/CD40L signaling in human coronary artery endothelial cells. *Arterioscler.Thromb.Vasc.Biol.*, **23,** 816-821.

LI, H., RAMAN, C. S., GLASER, C. B., BLASKO, E., YOUNG, T. A., PARKINSON, J. F., WHITLOW, M., & POULOS, T. L. (1999). Crystal structures of zinc-free and -bound heme domain of human inducible nitric oxide synthase. Implications for dimer stability and comparison with endothelial nitric oxide synthase. *J.Biol.Chem.*, **274,** 21276-21284.

LI, S., KIM, M., HU, Y. L., JALALI, S., SCHLAEPFER, D. D., HUNTER, T., CHIEN, S., & SHYY, J. Y. (1997). Fluid shear stress activation of focal adhesion kinase. Linking to mitogen-activated protein kinases. *J.Biol.Chem.*, **272,** 30455-30462.

LI, Y., ZHU, H., KUPPUSAMY, P., ROUBAUD, V., ZWEIER, J. L., & TRUSH, M. A. (1998). Validation of lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J.Biol.Chem.*, **273,** 2015-2023.

LIN, L. Y., LIN, C. Y., HO, F. M., & LIAU, C. S. (2005). Up-regulation of the association between heat shock protein 90 and endothelial nitric oxide synthase prevents high glucose-induced apoptosis in human endothelial cells. *J.Cell Biochem.*, **94,** 194-201.

LIN, L. Y., LIN, C. Y., SU, T. C., & LIAU, C. S. (2004). Angiotensin II-induced apoptosis in human endothelial cells is inhibited by adiponectin through restoration of the association between endothelial nitric oxide synthase and heat shock protein 90. *FEBS Lett.*, **574,** 106-110.

LIN, M. I., FULTON, D., BABBITT, R., FLEMING, I., BUSSE, R., PRITCHARD, K. A., & SESSA, W. C. (2003). Phosphorylation of threonine 497 in endothelial nitric-oxide synthase coordinates the coupling of L-arginine metabolism to efficient nitric oxide production. *J.Biol.Chem.*, **278,** 44719-44726.

LISANTI, M. P., SCHERER, P. E., TANG, Z., & SARGIACOMO, M. (1994). Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. *Trends Cell Biol.*, **4,** 231-235.

LIST, B. M., KLOSCH, B., VOLKER, C., GORREN, A. C., SESSA, W. C., WERNER, E. R., KUKOVETZ, W. R., SCHMIDT, K., & MAYER, B. (1997). Characterization of bovine endothelial nitric oxide synthase as a homodimer with down-regulated uncoupled NADPH oxidase activity: tetrahydrobiopterin binding kinetics and role of haem in dimerization. *Biochem.J.*, **323,** 159-165.

LITVAK, V., TIAN, D., SHAUL, Y. D., & LEV, S. (2000). Targeting of PYK2 to focal adhesions as a cellular mechanism for convergence between integrins and G protein-coupled receptor signaling cascades. *J.Biol.Chem.*, **275,** 32736-32746.

LIU, J., GARCIA-CARDENA, G., & SESSA, W. C. (1995). Biosynthesis and palmitoylation of endothelial nitric oxide synthase: mutagenesis of palmitoylation sites, cysteines-15 and/or -26, argues against depalmitoylation-induced translocation of the enzyme. *Biochemistry*, **34,** 12333-12340.

LIU, J., GARCIA-CARDENA, G., & SESSA, W. C. (1996a). Palmitoylation of endothelial nitric oxide synthase is necessary for optimal stimulated release of nitric oxide: implications for caveolae localization. *Biochemistry*, **35,** 13277-13281.

LIU, J., HUGHES, T. E., & SESSA, W. C. (1997). The first 35 amino acids and fatty acylation sites determine the molecular targeting of endothelial nitric oxide synthase into the Golgi region of cells: a green fluorescent protein study. *J.Cell Biol.*, **137,** 1525-1535.

LIU, P., YING, Y., KO, Y. G., & ANDERSON, R. G. (1996b). Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. *J.Biol.Chem.*, **271,** 10299-10303.

LUNGU, A. O., JIN, Z. G., YAMAWAKI, H., TANIMOTO, T., WONG, C., & BERK, B. C. (2004). Cyclosporin A inhibits flow-mediated activation of endothelial nitric oxide synthase by altering cholesterol content in caveolae. *J.Biol.Chem.*, **279,** 48794-48800.

LUSCHER, T. F. (2001). Vascular protection: current possibilities and future perspectives. *Int.J.Clin.Pract.Suppl* 3-6.

LUSCHER, T. F. & NOLL, G. (1995). The pathogenesis of cardiovascular disease: role of the endothelium as a target and mediator. *Atherosclerosis*, **118,** S81-S90.

MARRERO, M. B., VENEMA, V. J., JU, H., HE, H., LIANG, H., CALDWELL, R. B., & VENEMA, R. C. (1999). Endothelial nitric oxide synthase interactions with G-protein-coupled receptors. *Biochem.J.*, **343 Pt 2:335-40.,** 335-340.

MATALON, S., HARDIMAN, K. M., JAIN, L., EATON, D. C., KOTLIKOFF, M., EU, J. P., SUN, J., MEISSNER, G., & STAMLER, J. S. (2003). Regulation of ion channel structure and function by reactive oxygen-nitrogen species. *Am.J.Physiol.Lung Cell Mol.Physiol.*, **285,** L1184-L1189.

MATSUBARA, M., TITANI, K., & TANIGUCHI, H. (1996). Interaction of calmodulin-binding domain peptides of nitric oxide synthase with membrane phospholipids: regulation by protein phosphorylation and Ca(2+)-calmodulin. *Biochemistry*, **35,** 14651-14658.

MATSUBARA, T. & ZIFF, M. (1986a). Increased superoxide anion release from human endothelial cells in response to cytokines. *J.Immunol.*, **137,** 3295-3298.

MATSUBARA, T. & ZIFF, M. (1986b). Superoxide anion release by human endothelial cells: synergism between a phorbol ester and a calcium ionophore. *J.Cell Physiol.*, **127,** 207-210.

MATSUDA, H. & IYANAGI, T. (1999). Calmodulin activates intramolecular electron transfer between the two flavins of neuronal nitric oxide synthase flavin domain. *Biochim.Biophys.Acta.*, **1473,** 345-355.

MCCABE, T. J., FULTON, D., ROMAN, L. J., & SESSA, W. C. (2000). Enhanced electron flux and reduced calmodulin dissociation may explain "calcium-independent" eNOS activation by phosphorylation. *J.Biol.Chem.*, **275,** 6123-6128.

MCNAUGHTON, L., PUTTAGUNTA, L., MARTINEZ-CUESTA, M. A., KNETEMAN, N., MAYERS, I., MOQBEL, R., HAMID, Q., & RADOMSKI, M. W. (2002). Distribution of nitric oxide synthase in normal and cirrhotic human liver. *Proc.Natl.Acad.Sci U.S.A*, **99,** 17161-17166.

MERTENS, A. & HOLVOET, P. (2001). Oxidized LDL and HDL: antagonists in atherothrombosis. *FASEB J.*, **15,** 2073-2084.

MICHEL, T. & FERON, O. (1997). Nitric oxide synthases: which, where, how, and why? *J.Clin.Invest*, **100,** 2146-2152.

MICHEL, T., LI, G. K., & BUSCONI, L. (1993). Phosphorylation and subcellular translocation of endothelial nitric oxide synthase. *Proc.Natl.Acad.Sci.U.S.A*, **90,** 6252-6256.

MICHELL, B. J., GRIFFITHS, J. E., MITCHELHILL, K. I., RODRIGUEZ-CRESPO, I., TIGANIS, T., BOZINOVSKI, S., DE MONTELLANO, P. R., KEMP, B. E., & PEARSON, R. B. (1999). The Akt kinase signals directly to endothelial nitric oxide synthase. *Curr.Biol.*, **9,** 845-848.

MICHELL, B. J., CHEN, Z. P., TIGANIS, T., STAPLETON, D., KATSIS, F., POWER, D. A., SIM, A. T., & KEMP, B. E. (2001). Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *J.Biol.Chem.*, **276,** 17625-17628.

MICHELL, B. J., HARRIS, M. B., CHEN, Z. P., JU, H., VENEMA, V. J., BLACKSTONE, M. A., HUANG, W., VENEMA, R. C., & KEMP, B. E. (2002). Identification of regulatory sites of phosphorylation of the bovine endothelial nitric-oxide synthase at serine 617 and serine 635. *J.Biol.Chem.*, **277,** 42344-42351.

MILSTIEN, S. & KATUSIC, Z. (1999). Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem.Biophys.Res.Commun.*, **263,** 681-684.

MIRANDA, K. M., NIMS, R. W., THOMAS, D. D., ESPEY, M. G., CITRIN, D., BARTBERGER, M. D., PAOLOCCI, N., FUKUTO, J. M., FEELISCH, M., & WINK, D. A. (2003). Comparison of the reactivity of nitric oxide and nitroxyl with heme proteins. A chemical discussion of the differential biological effects of these redox related products of NOS. *J.Inorg.Biochem.*, **93,** 52-60.

MONCADA, S. & HIGGS, E. A. (1995). Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.*, **9,** 1319-1330.

MONCADA, S. & VANE, J. R. (1980). Biological significance and therapeutic potential of prostacyclin. *J.Med.Chem.*, **23,** 591-593.

MONCADA, S. & VANE, J. R. (1981). Prostacyclin and the vascular endothelium. *Bull.Eur.Physiopathol.Respir.*, **17,** 687-701.

MUKHERJEE, S., COAXUM, S. D., MALEQUE, M., & DAS, S. K. (2001). Effects of oxidized low density lipoprotein on nitric oxide synthetase and protein kinase C activities in bovine endothelial cells. *Cell Mol.Biol.(Noisy.-le-grand)*, **47,** 1051-1058.

MÜLSCH, A., MORDVINTCEV, P., BASSENGE, E., JUNG, F., CLEMENT, B., & BUSSE, R. (1995). In vivo spin trapping of glyceryl trinitrate-derived nitric oxide in rabbit blood vessels and organs. *Circulation*, **92,** 1876-1882.

MURATA, T., SATO, K., HORI, M., OZAKI, H., & KARAKI, H. (2002). Decreased endothelial nitric-oxide synthase (eNOS) activity resulting from abnormal interaction between eNOS and its regulatory proteins in hypoxia-induced pulmonary hypertension. *J.Biol.Chem.*, **277,** 44085-44092.

NAKAMURA, I., LIPFERT, L., RODAN, G. A., & LE, T. D. (2001). Convergence of α v β3 integrin- and macrophage colony stimulating factor-mediated signals on phospholipase Cγ in prefusion osteoclasts . *J.Cell Biol.*, **152,** 361-374.

NAMGALADZE, D., HOFER, H. W., & ULLRICH, V. (2002). Redox control of calcineurin by targeting the binuclear Fe^{2+}-Zn^{2+} center at the enzyme active site. *J.Biol.Chem.*, **277,** 5962-5969.

NATHAN, C. (1997). Inducible nitric oxide synthase: what difference does it make? *J.Clin.Invest*, **100,** 2417-2423.

NEDVETSKY, P. I., SESSA, W. C., & SCHMIDT, H. H. (2002). There's NO binding like NOS binding: protein-protein interactions in NO/cGMP signaling. *Proc.Natl.Acad.Sci.U.S.A*, **99,** 16510-16512.

NEGRERIE, M., BERKA, V., VOS, M. H., LIEBL, U., LAMBRY, J. C., TSAI, A. L., & MARTIN, J. L. (1999). Geminate recombination of nitric oxide to endothelial nitric-oxide synthase and mechanistic implications. *J.Biol.Chem.*, **274,** 24694-24702.

NUSZKOWSKI, A., GRABNER, R., MARSCHE, G., UNBEHAUN, A., MALLE, E., & HELLER, R. (2001). Hypochlorite-modified low density lipoprotein inhibits nitric oxide synthesis in endothelial cells via an intracellular dislocalization of endothelial nitric-oxide synthase. *J.Biol.Chem.*, **276,** 14212-14221.

O'BRIEN, A. J., YOUNG, H. M., POVEY, J. M., & FURNESS, J. B. (1995). Nitric oxide synthase is localized predominantly in the Golgi apparatus and cytoplasmic vesicles of vascular endothelial cells. *Histochemistry*, **103,** 221-225.

OKUDA, M., TAKAHASHI, M., SUERO, J., MURRY, C. E., TRAUB, O., KAWAKATSU, H., & BERK, B. C. (1999). Shear stress stimulation of p130(cas) tyrosine phosphorylation requires calcium-dependent c-Src activation. *J.Biol.Chem.*, **274,** 26803-26809.

ORTIZ, P. A. & GARVIN, J. L. (2003). Trafficking and activation of eNOS in epithelial cells. *Acta.Physiol.Scand.*, **179,** 107-114.

OSAWA, M., MASUDA, M., HARADA, N., LOPES, R. B., & FUJIWARA, K. (1997). Tyrosine phosphorylation of platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) in mechanically stimulated vascular endothelial cells. *Eur.J.Cell Biol.*, **72,** 229-237.

OSTENDORF, T., KUNTER, U., EITNER, F., LOOS, A., REGELE, H., KERJASCHKI, D., HENNINGER, D. D., JANJIC, N., & FLOEGE, J. (1999). VEGF165 mediates glomerular endothelial repair. *J.Clin.Invest.*, **104,** 913-923.

PALACIOS-CALLENDER, M., QUINTERO, M., HOLLIS, V. S., SPRINGETT, R. J., & MONCADA, S. (2004). Endogenous NO regulates superoxide production at low oxygen concentrations by modifying the redox state of cytochrome c oxidase. *Proc.Natl.Acad.Sci.U.S.A*, **101,** 7630-7635.

PALINSKI, W., TANGIRALA, R. K., MILLER, E., YOUNG, S. G., & WITZTUM, J. L. (1995). Increased autoantibody titers against epitopes of oxidized LDL in LDL receptor deficient mice with increased atherosclerosis. *Arterioscler.Thromb.Vasc.Biol.*, **15,** 1569-1576.

PALMER, R. M., FERRIGE, A. G., & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327,** 524-526.

PANDA, K., GHOSH, S., & STUEHR, D. J. (2001). Calmodulin activates intersubunit electron transfer in the neuronal nitric-oxide synthase dimer. *J.Biol.Chem.*, **276,** 23349-23356.

PAPAPETROPOULOS, A., FULTON, D., LIN, M. I., FONTANA, J., MCCABE, T. J., ZOELLNER, S., GARCIA-CARDENA, G., ZHOU, Z., GRATTON, J. P., & SESSA, W. C. (2004). Vanadate is a potent activator of endothelial nitric-oxide synthase: evidence for the role of the serine/threonine kinase Akt and the 90-kDa heat shock protein. *Mol.Pharmacol.*, **65,** 407-415.

PARTON, R. G. (1996). Caveolae and caveolins. *Curr.Opin.Cell Biol*, **8,** 542-548.

PATEL, R. P., LEVONEN, A., CRAWFORD, J. H., & DARLEY-USMAR, V. M. (2000). Mechanisms of the pro- and anti-oxidant actions of nitric oxide in atherosclerosis. *Cardiovasc.Res.*, **47,** 465-474.

POLLOCK, J. S., FORSTERMANN, U., MITCHELL, J. A., WARNER, T. D., SCHMIDT, H. H., NAKANE, M., & MURAD, F. (1991). Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc.Natl.Acad.Sci.U.S.A*, **88,** 10480-10484.

POLLOCK, J. S., KLINGHOFER, V., FORSTERMANN, U., & MURAD, F. (1992). Endothelial nitric oxide synthase is myristylated. *FEBS Lett.*, **309,** 402-404.

POPP, R., BAUERSACHS, J., HECKER, M., FLEMING, I., & BUSSE, R. (1996). A transferable, β-naphthoflavone-inducible, hyperpolarizing factor is synthesized by native and cultured porcine coronary endothelial cells. *J.Physiol.(Lond)*, **497,** 699-709.

PRABHAKAR, P., CHENG, V., & MICHEL, T. (2000). A chimeric transmembrane domain directs endothelial nitric-oxide synthase palmitoylation and targeting to plasmalemmal caveolae. *J.Biol.Chem.*, **275,** 19416-19421.

PRABHAKAR, P., THATTE, H. S., GOETZ, R. M., CHO, M. R., GOLAN, D. E., & MICHEL, T. (1998). Receptor-regulated translocation of endothelial nitric oxide synthase. *J.Biol.Chem.*, **273,** 27383-27388.

PRITCHARD, K. A., JR., ACKERMAN, A. W., GROSS, E. R., STEPP, D. W., SHI, Y., FONTANA, J. T., BAKER, J. E., & SESSA, W. C. (2001). Heat shock protein 90 mediates the balance of nitric oxide and superoxide anion from endothelial nitric-oxide synthase. *J.Biol.Chem.*, **276,** 17621-17624.

RADOMSKI, M. W., PALMER, R. M., & MONCADA, S. (1991). Modulation of platelet aggregation by an L-arginine-nitric oxide pathway. *Trends Pharmacol.Sci.*, **12,** 87-88.

RAMAN, C. S., LI, H., MARTASEK, P., KRAL, V., MASTERS, B. S., & POULOS, T. L. (1998). Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. *Cell*, **95,** 939-950.

RAVI, K., BRENNAN, L. A., LEVIC, S., ROSS, P. A., & BLACK, S. M. (2004). S-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *Proc.Natl.Acad.Sci.U.S.A*, **101,** 2619-2624.

RAZANI, B., ENGELMAN, J. A., WANG, X. B., SCHUBERT, W., ZHANG, X. L., MARKS, C. B., MACALUSO, F., RUSSELL, R. G., LI, M., PESTELL, R. G., DI VIZIO, D.,

HOU, H., JR., KNEITZ, B., LAGAUD, G., CHRIST, G. J., EDELMANN, W., & LISANTI, M. P. (2001). Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *J.Biol.Chem.*, **276,** 38121-38138.

REES, D. D., PALMER, R. M., & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc.Natl.Acad.Sci.U.S.A*, **86,** 3375-3378.

REIF, A., FROHLICH, L. G., KOTSONIS, P., FREY, A., BOMMEL, H. M., WINK, D. A., PFLEIDERER, W., & SCHMIDT, H. H. (1999). Tetrahydrobiopterin inhibits monomerization and is consumed during catalysis in neuronal NO synthase. *J.Biol.Chem.*, **274,** 24921-24929.

REINER, M., BLOCH, W., & ADDICKS, K. (2001). Functional Interaction of Caveolin-1 and eNOS in Myocardial Capillary Endothelium Revealed by Immunoelectron Microscopy. *J.Histochem.Cytochem.*, **49,** 1605-1610.

RIKITAKE, Y., KAWASHIMA, S., TAKAHASHI, T., UEYAMA, T., ISHIDO, S., INOUE, N., HIRATA, K., & YOKOYAMA, M. (2001). Regulation of tyrosine phosphorylation of PYK2 in vascular endothelial cells by lysophosphatidylcholine. *Am.J.Physiol Heart Circ.Physiol.*, **281,** H266-H274.

ROBINSON, L. J., BUSCONI, L., & MICHEL, T. (1995). Agonist-modulated palmitoylation of endothelial nitric oxide synthase. *J.Biol.Chem.*, **270,** 995-998.

ROSEN, G. M. & FREEMAN, B. A. (1984). Detection of superoxide generated by endothelial cells. *Proc.Natl.Acad.Sci.U.S.A*, **81,** 7269-7273.

RUAN, J., XIE, Q., HUTCHINSON, N., CHO, H., WOLFE, G. C., & NATHAN, C. (1996). Inducible nitric oxide synthase requires both the canonical calmodulin-binding domain and additional sequences in order to bind calmodulin and produce nitric oxide in the absence of free Ca^{2+}. *J.Biol.Chem.*, **271,** 22679-22686.

RUBANYI, G. M. (1991). Endothelium-derived relaxing and contracting factors. *J.Cell Biochem.*, **46,** 27-36.

RUDIC, R. D., SHESELY, E. G., MAEDA, N., SMITHIES, O., SEGAL, S. S., & SESSA, W. C. (1998). Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J.Clin.Invest.*, **101,** 731-736.

RUECKSCHLOSS, U., GALLE, J., HOLTZ, J., ZERKOWSKI, H. R., & MORAWIETZ, H. (2001). Induction of NAD(P)H oxidase by oxidized low-density lipoprotein in human endothelial cells: antioxidative potential of hydroxymethylglutaryl coenzyme A reductase inhibitor therapy. *Circulation*, **104,** 1767-1772.

RUSCHE, K. M., SPIERING, M. M., & MARLETTA, M. A. (1998). Reactions catalyzed by tetrahydrobiopterin-free nitric oxide synthase. *Biochemistry*, **37,** 15503-15512.

RUSSELL, K. S., HAYNES, M. P., CAULIN-GLASER, T., ROSNECK, J., SESSA, W. C., & BENDER, J. R. (2000). Estrogen stimulates heat shock protein 90 binding to endothelial nitric oxide synthase in human vascular endothelial cells. Effects on calcium sensitivity and NO release. *J.Biol.Chem.*, **275,** 5026-5030.

SAKODA, T., HIRATA, K., KURODA, R., MIKI, N., SUEMATSU, M., KAWASHIMA, S., & YOKOYAMA, M. (1995). Myristoylation of endothelial cell nitric oxide synthase is important for extracellular release of nitric oxide. *Mol.Cell Biochem.*, **152,** 143-148.

SALERNO, J. C., HARRIS, D. E., IRIZARRY, K., PATEL, B., MORALES, A. J., SMITH, S. M., MARTASEK, P., ROMAN, L. J., MASTERS, B. S., JONES, C. L., WEISSMAN, B.

A., LANE, P., LIU, Q., & GROSS, S. S. (1997). An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase. *J.Biol.Chem.*, **272,** 29769-29777.

SALONEN, J. T., YLA-HERTTUALA, S., YAMAMOTO, R., BUTLER, S., KORPELA, H., SALONEN, R., NYYSSONEN, K., PALINSKI, W., & WITZTUM, J. L. (1992). Autoantibody against oxidised LDL and progression of carotid atherosclerosis. *Lancet*, **339,** 883-887.

SALVEMINI, D., MISKO, T. P., MASFERRER, J. L., SEIBERT, K., CURRIE, M. G., & NEEDLEMAN, P. (1993). Nitric oxide activates cyclooxygenase enzymes. *Proc.Natl.Acad.Sci.U.S.A*, **90,** 7240-7244.

SANTOLINI, J., MEADE, A. L., & STUEHR, D. J. (2001). Differences in three kinetic parameters underpin the unique catalytic profiles of nitric-oxide synthases I, II, and III. *J.Biol.Chem.*, **276,** 48887-48898.

SCHEELE, J. S., BRUNER, E., KHARITONOV, V. G., MARTASEK, P., ROMAN, L. J., MASTERS, B. S., SHARMA, V. S., & MAGDE, D. (1999). Kinetics of NO ligation with nitric-oxide synthase by flash photolysis and stopped-flow spectrophotometry. *J.Biol.Chem.*, **274,** 13105-13110.

SCHNEIDER, J. C., EL KEBIR, D., CHEREAU, C., LANONE, S., HUANG, X. L., BUYS ROESSINGH, A. S., MERCIER, J. C., DALL'AVA-SANTUCCI, J., & DINH-XUAN, A. T. (2003). Involvement of Ca2+/calmodulin-dependent protein kinase II in endothelial NO production and endothelium-dependent relaxation. *Am.J.Physiol.Heart Circ.Physiol*, **284,** H2311-H2319.

SESSA, W. C., BARBER, C. M., & LYNCH, K. R. (1993). Mutation of N-myristoylation site converts endothelial cell nitric oxide synthase from a membrane to a cytosolic protein. *Circ.Res.*, **72,** 921-924.

SESSA, W. C., GARCA-CARDEA, G., LIU, J., KEH, A., POLLOCK, J. S., BRADLEY, J., THIRU, S., BRAVERMAN, I. M., & DESAI, K. M. (1995). The Golgi association of endothelial nitric oxide synthase is necessary for the efficient synthesis of nitric oxide. *J.Biol.Chem.*, **270,** 17641-17644.

SHAH, V., WIEST, R., GARCIA-CARDENA, G., CADELINA, G., GROSZMANN, R. J., & SESSA, W. C. (1999). Hsp90 regulation of endothelial nitric oxide synthase contributes to vascular control in portal hypertension. *Am.J.Physiol.*, **277,** G463-G468.

SHAUL, P. W. (2003). Endothelial nitric oxide synthase, caveolae and the development of atherosclerosis. *J.Physiol.(Lond)*, **547,** 21-33.

SIDDHANTA, U., PRESTA, A., FAN, B., WOLAN, D., ROUSSEAU, D. L., & STUEHR, D. J. (1998). Domain swapping in inducible nitric-oxide synthase. Electron transfer occurs between flavin and heme groups located on adjacent subunits in the dimer. *J.Biol.Chem.*, **273,** 18950-18958.

SIDDHANTA, U., WU, C., ABU-SOUD, H. M., ZHANG, J., GHOSH, D. K., & STUEHR, D. J. (1996). Heme iron reduction and catalysis by a nitric oxide synthase heterodimer containing one reductase and two oxygenase domains. *J.Biol.Chem.*, **271,** 7309-7312.

SKATCHKOV, M. P., SPERLING, D., HINK, U., MULSCH, A., HARRISON, D. G., SINDERMANN, I., MEINERTZ, T., & MUNZEL, T. (1999). Validation of lucigenin as a chemiluminescent probe to monitor vascular superoxide as well as basal vascular nitric oxide production. *Biochem.Biophys.Res.Commun.*, **19;254,** 319-324.

SMITH, A. R., VISIOLI, F., & HAGEN, T. M. (2002). Vitamin C matters: increased oxidative stress in cultured human aortic endothelial cells without supplemental ascorbic acid. *FASEB J.*, **16,** 1102-1104.

STAMLER, J. S., LAMAS, S., & FANG, F. C. (2001). Nitrosylation. the prototypic redox-based signaling mechanism. *Cell*, **106,** 675-683.

STEHBENS, W. E. (2001). Coronary heart disease, hypercholesterolemia, and atherosclerosis. I. False premises. *Exp.Mol.Pathol.*, **70,** 103-119.

STEPP, D. W., OU, J., ACKERMAN, A. W., WELAK, S., KLICK, D., & PRITCHARD, K. A., JR. (2002). Native LDL and minimally oxidized LDL differentially regulate superoxide anion in vascular endothelium in situ. *Am.J.Physiol.Heart Circ.Physiol.*, **283,** H750-H759.

STOCKER, R., HUANG, A., JERANIAN, E., HOU, J. Y., WU, T. T., THOMAS, S. R., & KEANEY, J. F., JR. (2004). Hypochlorous acid impairs endothelium-derived nitric oxide bioactivity through a superoxide-dependent mechanism. *Arterioscler.Thromb.Vasc.Biol.*, **24,** 2028-2033.

STROES, E., KASTELEIN, J., COSENTINO, F., ERKELENS, W., WEVER, R., KOOMANS, H., LUSCHER, T., & RABELINK, T. (1997). Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J.Clin.Invest.*, **99,** 41-46.

STUEHR, D., POU, S., & ROSEN, G. M. (2001). Oxygen reduction by nitric-oxide synthases. *J.Biol.Chem.*, **276,** 14533-14536.

STUEHR, D. J., SANTOLINI, J., WANG, Z. Q., WEI, C. C., & ADAK, S. (2004). Update on mechanism and catalytic regulation in the NO synthases. *J.Biol.Chem.*, **279,** 36167-36170.

SU, Y., EDWARDS-BENNETT, S., BUBB, M. R., & BLOCK, E. R. (2003). Regulation of endothelial nitric oxide synthase by the actin cytoskeleton. *Am.J.Physiol.Cell Physiol.*, **284,** C1542-C1549.

SUN, J. & LIAO, J. K. (2002). Functional interaction of endothelial nitric oxide synthase with a voltage-dependent anion channel. *Proc.Natl.Acad.Sci.U.S.A*, **99,** 13108-13113.

SZOCS, K. (2004). Endothelial dysfunction and reactive oxygen species production in ischemia/reperfusion and nitrate tolerance. *Gen.Physiol.Biophys.*, **23,** 265-295.

TAI, L. K., OKUDA, M., ABE, J., YAN, C., & BERK, B. C. (2002). Fluid shear stress activates proline-rich tyrosine kinase via reactive oxygen species-dependent pathway. *Arterioscler.Thromb.Vasc.Biol.*, **22,** 1790-1796.

TAKAHASHI, M., ISHIDA, T., TRAUB, O., CORSON, M. A., & BERK, B. C. (1997). Mechanotransduction in endothelial cells: temporal signaling events in response to shear stress. *J.Vasc.Res.*, **34,** 212-219.

TAKENOUCHI, Y., OO, M. L., SENGA, T., WATANABE, Y., MACHIDA, K., MIYAZAKI, K., NIMURA, Y., & HAMAGUCHI, M. (2004). Tyrosine phosphorylation of NOS3 in a breast cancer cell line and Src-transformed cells. *Oncol.Rep.*, **11,** 1059-1062.

TIEFENBACHER, C. P., CHILIAN, W. M., MITCHELL, M., & DEFILY, D. V. (1996). Restoration of endothelium-dependent vasodilation after reperfusion injury by tetrahydrobiopterin. *Circulation*, **94,** 1423-1429.

TOWLER, D. A., GORDON, J. I., ADAMS, S. P., & GLASER, L. (1988). The biology and enzymology of eukaryotic protein acylation. *Annu.Rev.Biochem.*, **57:69-99.,** 69-99.

TYSZKIEWICZ, J. P., GU, Z., WANG, X., CAI, X., & YAN, Z. (2004). Group II metabotropic glutamate receptors enhance NMDA receptor currents via a protein kinase C-

dependent mechanism in pyramidal neurones of rat prefrontal cortex. *J.Physiol.(Lond)*, **554,** 765-777.

UITTENBOGAARD, A., SHAUL, P. W., YUHANNA, I. S., BLAIR, A., & SMART, E. J. (2000). High density lipoprotein prevents oxidized low density lipoprotein-induced inhibition of endothelial nitric-oxide synthase localization and activation in caveolae. *J.Biol.Chem.*, **275,** 11278-11283.

VALLANCE, P., COLLIER, J., & MONCADA, S. (1989a). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet*, **2,** 997-1000.

VALLANCE, P., COLLIER, J., & MONCADA, S. (1989b). Nitric oxide synthesised from L-arginine mediates endothelium dependent dilatation in human veins in vivo. *Cardiovasc.Res.*, **23,** 1053-1057.

VASQUEZ-VIVAR, J., HOGG, N., MARTASEK, P., KAROUI, H., PRITCHARD, K. A., JR., & KALYANARAMAN, B. (1999). Tetrahydrobiopterin-dependent inhibition of superoxide generation from neuronal nitric oxide synthase. *J.Biol.Chem.*, **274,** 26736-26742.

VENEMA, R. C., SAYEGH, H. S., ARNAL, J. F., & HARRISON, D. G. (1995). Role of the enzyme calmodulin-binding domain in membrane association and phospholipid inhibition of endothelial nitric oxide synthase. *J.Biol.Chem.*, **270,** 14705-14711.

VENEMA, R. C., SAYEGH, H. S., KENT, J. D., & HARRISON, D. G. (1996a). Identification, characterization, and comparison of the calmodulin-binding domains of the endothelial and inducible nitric oxide synthases. *J.Biol.Chem.*, **271,** 6435-6440.

VENEMA, R. C., VENEMA, V. J., JU, H., HARRIS, M. B., SNEAD, C., JILLING, T., DIMITROPOULOU, C., MARAGOUDAKIS, M. E., & CATRAVAS, J. D. (2003). Novel complexes of guanylate cyclase with heat shock protein 90 and nitric oxide synthase. *Am.J.Physiol.Heart Circ.Physiol.*, **285,** H669-H678.

VENEMA, V. J., MARRERO, M. B., & VENEMA, R. C. (1996b). Bradykinin-stimulated protein tyrosine phosphorylation promotes endothelial nitric oxide synthase translocation to the cytoskeleton. *Biochem.Biophys.Res.Commun.*, **226,** 703-710.

VOETSCH, B., JIN, R. C., & LOSCALZO, J. (2004). Nitric oxide insufficiency and atherothrombosis. *Histochem.Cell Biol.*, **122,** 353-367.

WALKER, A. J. & PLOWS, L. D. (2003). Bacterial lipopolysaccharide modulates protein kinase C signalling in Lymnaea stagnalis haemocytes. *Biol.Cell.*, **95,** 527-533.

WANG, Z. Q., WEI, C. C., & STUEHR, D. J. (2002). A conserved tryptophan 457 modulates the kinetics and extent of N-hydroxy-L-arginine oxidation by inducible nitric-oxide synthase. *J.Biol.Chem.*, **277,** 12830-12837.

WARNHOLTZ, A., WENDT, M., AUGUST, M., & MUNZEL, T. (2004). Clinical aspects of reactive oxygen and nitrogen species. *Biochem.Soc.Symp.* 121-133.

WEI, C. C., CRANE, B. R., & STUEHR, D. J. (2003a). Tetrahydrobiopterin radical enzymology. *Chem.Rev.*, **103,** 2365-2383.

WEI, C. C., WANG, Z. Q., ARVAI, A. S., HEMANN, C., HILLE, R., GETZOFF, E. D., & STUEHR, D. J. (2003b). Structure of tetrahydrobiopterin tunes its electron transfer to the heme-dioxy intermediate in nitric oxide synthase. *Biochemistry*, **42,** 1969-1977.

WEI, C. C., WANG, Z. Q., HEMANN, C., HILLE, R., & STUEHR, D. J. (2003c). A tetrahydrobiopterin radical forms and then becomes reduced during Nomega-hydroxyarginine oxidation by nitric-oxide synthase. *J.Biol.Chem.*, **278,** 46668-46673.

WEI, C. C., WANG, Z. Q., WANG, Q., MEADE, A. L., HEMANN, C., HILLE, R., & STUEHR, D. J. (2001). Rapid kinetic studies link tetrahydrobiopterin radical formation to heme-dioxy reduction and arginine hydroxylation in inducible nitric-oxide synthase. *J.Biol.Chem.*, **276,** 315-319.

WHITTLE, B. J., LOPEZ-BELMONTE, J., & REES, D. D. (1989). Modulation of the vasodepressor actions of acetylcholine, bradykinin, substance P and endothelin in the rat by a specific inhibitor of nitric oxide formation. *Br.J.Pharmacol.*, **98,** 646-652.

WOLF, A., ZALPOUR, C., THEILMEIER, G., WANG, B. Y., MA, A., ANDERSON, B., TSAO, P. S., & COOKE, J. P. (1997). Dietary L-arginine supplementation normalizes platelet aggregation in hypercholesterolemic humans. *J.Am.Coll.Cardiol.*, **29,** 479-485.

WU, R., HUANG, Y. H., ELINDER, L. S., & FROSTEGARD, J. (1998). Lysophosphatidylcholine is involved in the antigenicity of oxidized LDL. *Arterioscler.Thromb.Vasc.Biol.*, **18,** 626-630.

XIA, Y., DAWSON, V. L., DAWSON, T. M., SNYDER, S. H., & ZWEIER, J. L. (1996). Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc.Natl.Acad.Sci.U.S.A*, **93,** 6770-6774.

XIA, Y., TSAI, A. L., BERKA, V., & ZWEIER, J. L. (1998). Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J.Biol.Chem.*, **273,** 25804-25808.

XIE, Q. W., CHO, H. J., CALAYCAY, J., MUMFORD, R. A., SWIDEREK, K. M., LEE, T. D., DING, A., TROSO, T., & NATHAN, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science*, **256,** 225-228.

ZABEL, U., HAUSLER, C., WEEGER, M., & SCHMIDT, H. H. (1999). Homodimerization of soluble guanylyl cyclase subunits. Dimerization analysis using a glutathione s-transferase affinity tag. *J.Biol.Chem.*, **274,** 18149-18152.

ZHANG, J., BAINES, C. P., ZONG, C., CARDWELL, E. M., WANG, G., VONDRISKA, T. M., & PING, P. (2005). Functional proteomic analysis of a three-tier PKCepsilon-Akt-eNOS signaling module in cardiac protection. *Am.J.Physiol Heart Circ.Physiol*, **288,** H954-H961.

ZIMMERMANN, K., OPITZ, N., DEDIO, J., RENNE, C., MULLER-ESTERL, W., & OESS, S. (2002). NOSTRIN: a protein modulating nitric oxide release and subcellular distribution of endothelial nitric oxide synthase. *Proc.Natl.Acad.Sci.U.S.A*, **99,** 17167-17172.

ZOU, M. H., SHI, C., & COHEN, R. A. (2002). Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J.Clin.Invest.*, **109,** 817-826.

List of abbreviations

Ab	Antibody
ATP	Adenosine triphosphate
a.u.	Arbitrary units
Akt	Protein kinase B
H_4B	Tetrahydrobiopterin
BK	Bradykinin
BSA	Bovine serum albumin
CMH	1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine HCl
CM^-	3-methoxycarbonyl-proxyl
CaM	Calmodulin
Ca^{2+}	Calcium
cpm	Counts per minute
°C	Degrees centigrade
cAK and cGK	cAMP- and cGMP-dependent protein kinases
eNOS/ NOS III	Endothelial nitric oxide synthase
nNOS/ NOS I	Neuronal nitric oxide synthase
i NOS/ NOS II	Inducible nitric oxide synthase
E. coli	*Escherichia coli*
ESR	Electron spin resonance spectroscopy
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylene diamine tetraacetic acid
FAD	Flavin adenine dinucleotide
FCS	Fetal calf serum
FMN	Flavine mononucleotinde
sGC	Soluble guanylyl cyclase
Hsp90	Heat shock protein 90
HT	HEPES Tyrode
H_2O_2	Hydrogen peroxide
D/Asp	Aspartate
A/Ala	Alanine
Y/Tyr	Tyrosine

S/Ser	Serine
T/Thr	Threonine
KD	kiloDalton
L-NAME	N^{ω}-Nitro-L-arginine methyl ester
L-NA	Nitro L-arginine
LOX-1	Lectin-like oxidized LDL receptor-1
LPC	Lysophosphatidylcholine
mL	milli Liter
mol	molar
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOHA	N^{ω}-hydroxy-L-arginine
$ONOO^-$	Peroxynitrite
O_2^-	Superoxide
Ox-LDL	Oxidized low density lipoprotein
PBS	Phosphate buffer saline
PKA	Protein kinase A
PKG	Protein Kinase G
PKC	Protein kinase C
PYK2	Proline-rich tyrosine kinase 2
PMA	Phorbol 12-myristate 13-acetate
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
rpm	Revolutions per minute
SEM	Standard error of mean
SOD	Superoxide dismutase
TEMED	N, N, N.,N.,-tetramethylethylenediamine
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
U	Unit
VEGF	Vascular endothelial growth factor
g	gram
μ	micro
n	nano

ZnS_4	Zinc thiolate cluster

List of publications

Keller, A., **Mohamed, A.,** Drose, S., Brandt, U., Fleming, I., Brandes, R.P. (2004). Analysis of dichlorodihydrofluorescein and dihydrocalcein as probes for the detection of intracellular reactive oxygen species**. Free Radic Res**. 38:1257-67.

Fleming, I., **Mohamed, A.,** Galle, J., Turchanowa, L., Brandes, R.P., Fisslthaler, B., Busse, R. (2005). Oxidized low-density lipoprotein increases superoxide production by endothelial nitric oxide synthase by inhibiting PKC α. **Cardiovasc Res.** 65:897-906.

Dixit, M., Loot, A.E., **Mohamed, A.,** Fisslthaler B., Boulanger, C.M., Ceacareanu, B., Hassid, A., Busse, R., Fleming, I. (2005). Gab1, SHP2 and protein kinase A are crucial for the activation of the endothelial nitric oxide synthase by fluid shear stress. **Circ Res.** 97(12):1236-44.

Sasaki, K., Heeschen, C., Aicher, A., Urbich, C., Rossig, L., **Mohamed, A.,** Brandes, R.P., Rütten, H., Zeiher, A.M., Dimmeler, S. (2005). Ex vivo pre-treatment of bone marrow stem and progenitor cells with the eNOS enhancer AVE9488 to enhance their functional activity for cell therapy. **Submitted**

Curriculum Vitae

Personal Information:

Date of birth:	11 August, 1975
Place of birth:	Bangalore
Citizenship:	Indian

Education and work experience:

2001-2005	Ph.D. student, Institute for Cardiovascular Physiology Johann Wolfgang Goethe-Universität Frankfurt am Main, Germany Supervisor: Prof. Ingrid Fleming
2001- (Aug-Oct)	Ph.D. student, Department of Molecular Neurooncology Martin-Luther-Universität Halle-Wittenberg Supervisor: PD. Dr. Ariane Soling
2000-2001	Junior Research fellow Indian Institute of Science (IISc), Bangalore Supervisor: Dr. Saumitra Das
1997-1999	M.Sc Microbiology Al-Ameen college; Bangalore University Bangalore, India
1994-1996	B.Sc (Chemistry, Zoology, Microbiology) Al-Ameen college; Bangalore University Bangalore, India
1992-1994	Pre-university Al-Ameen college; Bangalore University Bangalore, India
1989-1992	Secondary School Leaving Certificate (SSLC) Karnatake State Board Bangalore, India