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Elevated Ceramide Levels Contribute to the Age-Associated Decline in Vascular Endothelial Nitric Oxide. Pharmacologic Administration of Lipoic Acid Partially Restores Function.

Abstract approved:

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Tory M. Hagen

The vascular endothelium is a single cell layer that lines the lumen of the entire vasculature. It is the site of synthesis of nitric oxide (NO), a vasodilatory compound synthesized by endothelial nitric oxide synthase (eNOS). NO causes intracellular calcium sequestration of the vascular smooth muscle cells, relaxing and dilating the arteries. Age profoundly affects endothelium-dependent vasodilation, leading to specific losses of NO. We sought to determine what causes the age-specific loss of endothelial NO. This was accomplished by investigating whether there are differences in markers of eNOS post-translational regulation elements in the aortic endothelium of young (2-4 months; corresponding to an adolescent human adult) and old (32-34 months; corresponding to a 65 –75 year-old human).F 344 x Brown Norway hybrid rats.

Results show that maximal eNOS activity significantly declines with age ($n=4; p \leq 0.05$) though there was no change in eNOS protein levels in the aortic endothelium. Endothelial NOS exists in two distinct subcellular fractions. No

alterations were detected in the soluble, inactive fraction while significantly less eNOS protein is detected in the active, plasma membrane fraction of the endothelium ($n=4;p\leq0.02$). Endothelial NOS activation is also controlled by its phosphorylation state. In this work we demonstrate that free ceramides and ceramide-activated phosphatase (PP2A) activity are significantly elevated with age in the endothelium and correlate with specific alterations in eNOS phosphorylation status consistent with its inactivation. These changes were concomittent with an age-associated decline in endothelial glutathione (GSH) and increased sphingomyelinase activity which liberates ceramides from membrane sphingolipids.

In previously published reports we demonstrated that the dithiol compound *R*-α-lipoic acid (LA) increased maximal NO synthesis in cultured endothelial cells and that LA improved age-associated loss of eNOS stimulatory phosphorylation in rats. Therefore, we administered pharmacologic doses of LA (40 mg/kg, i.p. over 24 h) to old rats to determine whether it restored NO-dependent vasomotor function. Results show that LA significantly increased endothelial GSH ($p\leq0.05$ compared to saline controls), decreased sphingomyelinase activity and reversed the age-related increase in ceramide ($p\leq0.01$) in old animals. Finally, LA significantly improved endothelium-dependent vasodilation, suggesting that it might be a good therapeutic agent for age-related vascular endothelial dysfunction.

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Anthony R. Smith, Author

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Chapter 1

General Introduction

1.1 Background and significance

Endothelium and the role of nitric oxide in maintenance of vessel tone.

The vascular endothelium is a single-cell layer that lines the entire vascular lumen. The average adult human posseses approximately 10^{12} endothelial cells which occupy an area of up to 1000 m^2 [1-3]. Thus, the endothelium may represent the largest and most extensive paracrine organ in the body and may have a total mass of one kilogram or more [1, 2]. Vascular endothelial cells serve as primary barrier between the blood and systemic tissues, thus controlling the transport of nutrients, minerals and vitamins in and out of the bloodstream. The endothelium also directs angiogenesis during development and wound healing by secretion of growth factors which direct the location and proliferation of vascular smooth muscle (VSM), endothelium and fibroblasts.

One of the most important physiological functions of endothelial cells is the maintenance of vascular tone (i.e., the control of VSM contraction and relaxation). This is accomplished by the production and secretion of second messenger molecules directed at the VSM which maintain vessel tone and hence, systemic blood pressure. Several vasoconstricting factors are secreted by endothelial cells (Table 1.1). Two important endothelial factors which mediate vasoconstriction are prostaglandins and endothelin-1 (ET-1) [4, 5]. These factors are released by endothelial cells in response to arachidonic acid, imflammitory cytokines, hypoxia and rapid stretch [4, 6]. Besides its blood pressor effect, ET-1 induces vascular and myocardial hypertrophy [7].

The endothelium also synthesizes and releases vasodilator factors, which are directed at the VSM and regulate vascular relaxation (Table 1.1). The principal vasodilatory factors secreted by the endothelium are prostacyclins, endothelium-derived hyperpolarizing factor (EDHF) and nitric oxide (NO) and are produced in response to endothelial stimuli such as fluid shear stress, estrogens, bradykinin, acetylcholine, serotonin, substance P, insulin and insulin-like growth factor (IGF)

Table 1.1. Vasoactive factors secreted by the vascular endothelium.

Factor	Function	Reference
Prostaglandins (PGH_2, $PGF_{2\alpha}$)	Arachidonic acid-derived vasocontracting factors.	[6, 15-18]
Endothelin-1 (ET-1)	Peptide derived from precursor protein (preproET-1). Functions as paracrine regulator of vascular tone by causing potent contraction of VSM in response to acute hypoxia.	[4, 19-22]
Prostacyclin (PGI_2)	Arachidonic acid-derivative. Platelet anti-aggregative and potent VSM relaxant. Predominant vasorelaxant in small resistance vessels and capillaries.	[6, 17, 23]
Platelet-derived growth factor-B (PDGF-B)	Mitogenic peptide that causes VSM proliferation and vasoconstriction in response to hypoxic stress.	[19, 24, 25]
Angiotensin II (ATII)	Contracting factor via stimulation of AT receptor of VSM. Also stimulates synthesis of preproET-1 by endothelium and VSM.	[7, 15, 22, 26, 27]
Nitric Oxide (NO)	Vasodilatory factor synthesized by eNOS from arginine and oxygen. Causes cGMP-dependent relaxation of VSM. Predominant vasorelaxant in conduit arteries.	[4, 28-30]

[4, 6, 8-12]. Vasodilation of small resistance vessels and of the microvasculature is predominantly governed by production of prostacyclins [6, 13, 14]. Though the en dothelium is capable of synthesis of several different vasodilatory factors, NO is the principal factor which mediates gross vasomotor tone in the conduit arteries such as the aorta, femoral, carotid and brachial arteries [31-36]. This mode of vasomotor regulation controls overall vascular tone and ultimately systemic blood pressure. NO is synthesized in a tightly regulated manner by the enzyme endothelial nitric oxide synthase (eNOS; also known as NOS3, Type III NOS, NOS-III) which will be discussed in detail below.

NO is an uncharged gaseous radical with a half-life between 3 and 6 seconds [37]. It plays a quintessential role not solely in the regulation of systemic vasomotor tone [14, 28-31, 38] but also regulating the growth, development and homeostasis of vascular wall cells such as VSM cells and fibroblasts [14, 16, 19, 23, 33, 39-41]. In addition, NO released into the plasma acts on platelets, preventing adhesion and aggregation while also causing platelets to release ADP, ATP and serotonin—factors which further increase nitric oxide synthase activity [16, 42, 43]. Sustained low levels of NO are anti-proliferative and anti-apoptotic to VSM cells and to endothelial cells [44, 45].

The vasodilatory action of NO stems from its rapid diffusion and direct activation of soluble guanylyl cyclase in the VSM. This leads to formation of cGMP which lowers intracellular calcium, leading to relaxation of the muscle. Thus, endothelium-derived NO (EDNO) is now believed to be one of the most important agents which governs arterial homeostasis and most importantly, systemic blood pressure. This is illustrated by research which demonstrates that diseases, pharmacological agents or any other conditions which inhibit or prevent endothelial NO synthesis or interfere with NO release from endothelial cells compromises vasomotor control, vascular injury repair and angiogenesis and may ultimately cause death [7, 14, 34, 46-51]

Endothelial nitric oxide synthase.

The human eNOS protein is the source of NO in the vascular endothelium. The eNOS gene is present as a single copy in the haploid genome, localized (in humans) on chromosome 7 [52-54]. It is consists of 1203 amino acids translated from a 4052-nt mRNA, yielding a 135 kilodalton protein [52, 53, 55, 56]. Though the enzyme is generally considered to be constitutively expressed, some degree of eNOS expressional control is brought about by regulation of the transcription and translation of the enzyme, but definitive information is lacking [57-59]. For example, fluid shear stress, VEGF, insulin, and estrogens have been shown to upregulate eNOS RNA and protein expression while expression is slowed by tumor necrosis factor-α (TNF- α), hypoxia, oxidized LDL, and erythropoietin [57, 60-65]. The proximal region of the eNOS promoter displays SP1, GATA, estrogen-responsive element, cAMP-responsive element, and activator protein-1 and -2 but no TATA box which is a typical characteristic of a constitutively expressed gene [57]. The eNOS mRNA transcript has a relatively long half life in vascular endothelium, usually greater than 24-48 h, which allows the cell to rapidly translate eNOS protein when needed [63, 66-69].

Functional eNOS operates as a homodimeric, multiple-domain oxidase, reductase and heme protein which also possesses a zinc ion at its core (Figure 1.1). In addition to arginine and oxygen as substrates, the enzyme also requires NAD(P)H as a substrate and the cofactors FMN, FAD, calcium/calmodulin (Ca^{+2}/CaM), zinc and tetrahydrobiopterin for proper function (Table 1.2). Release of calcium from intracellular stores initiates the insertion of calmodulin into the eNOS apoenzyme (Fig 1.1). This activates the enzyme by promoting NAD(P)H-dependent electron flux through the eNOS dimers. Interestingly, the path of electron flux bridges the dimer interface and proceeds from the flavin mononucleotide (FMN) of one monomer to the heme-iron domain of the opposite monomer before the initial step of arginine reduction, a phenomenon known as domain swapping [70, 71]

Data indicates that eNOS activity is acutely sensitive to substrate or cofactor limitations, suggesting that regulatory physiology modulating these parameters also

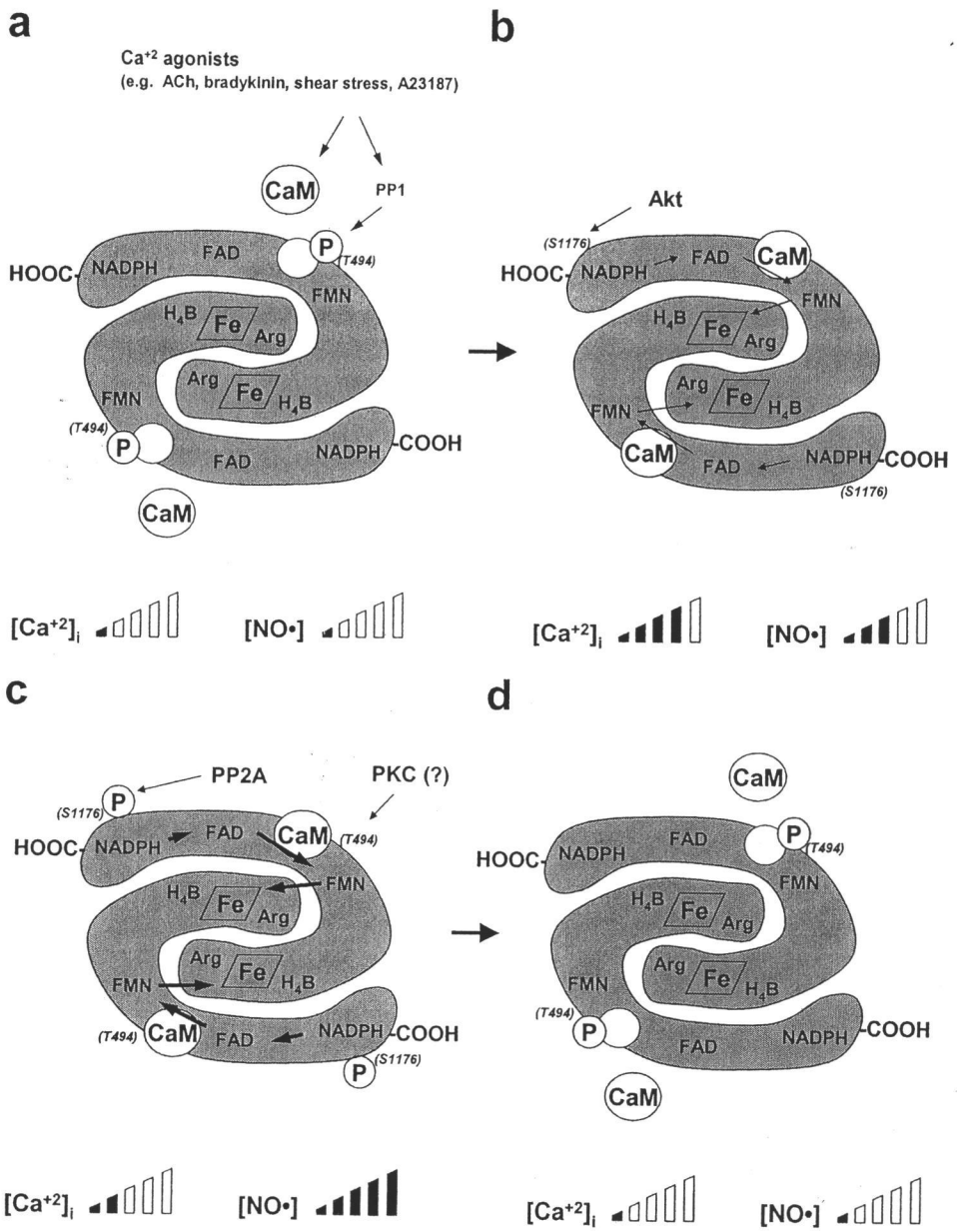


Figure 1.1. The structural and regulatory elements of eNOS which govern NO synthesis. (a) NO synthesis is inhibited in 'resting' eNOS by constitutive phosphorylation at T474. Removal of this phosphate by the phosphatase PP1 occurs upon intracellular Ca^{+2} release and allows insertion of Ca^{+2}/Calmodulin (CaM) into the reductase domain of eNOS promoting electron flux and NO synthesis. (b) The path of electron flux begins at the reductase domain of one eNOS monomer and proceeds to the heme domain of the second eNOS monomer in a phenomenon known as domain swapping. Phosphorylation of eNOS at S1176 by Akt may also occur, dramatically increasing maximal NO synthesis and prolonged NO synthesis post Ca^{+2} transient by enhancing retention of Ca^{+2}/CaM. (c) Removal of S1176 phosphorylation is mediated by the phosphatase PP2A and returns eNOS to a Ca^{+2}-sensitive state (d).

Table 1.2. Endothelial NOS cofactors and substrates.

Cofactor/Substrate	Function	Reference
NAD(P)H	*Substrate*. Primary donor of electrons to reductase domain of eNOS which reduces the heme from resting state (Fe^{+3}) to Fe^{+2}.	[70, 77, 78]
Flavin adenine dinucleotide (FAD)	Redox carrier. Allows two-electron donor NAD(P)H to donate electrons singly to the heme/oxygenase domain.	[71, 78, 79]
Flavin mononucleotide (FMN)	Redox carrier. Allows two-electron donor NAD(P)H to donate electrons singly to the heme/oxygenase domain.	[71, 78, 79]
Iron protoporphyrin IX (heme)	Resides near active site of oxygenase domain. Catalyzes two subsequent monooxygenations of arginine.	[71, 78, 80]
Tetrahydrobiopterin	Bound to heme domain. Promotes and stabilizes dimerization; couples NAD(P)H oxidation to NO synthesis (inhibiting superoxide formation); Redox cycle during catalysis; may 'hold' a single electron from one cycle and donate it during subsequent cycles of catalysis	[73, 74, 81]
Zinc	Stablization of NOS dimer	[82-84]
Ca^{+2}/CaM	Enables electron flux through reductase domain	[61, 85, 86]
Arginine	*Substrate*. Terminal guanidino group serves as nitrogen source in NO product	[14, 71, 80]
Oxygen	*Substrate*. Serves as electron acceptor from the heme domain and subsequently oxygenates arginine to N-hydroxy arginine, then to citrulline and NO	[77, 78]

contribute to the physiological regulation of eNOS. For example loss of tetrahydrobiopterin has been shown to uncouple NAD(P)H oxidation from oxygen reduction leading to formation of the radical anion superoxide in place of NO [72-76]. Though this type of functional dysregulation of eNOS has been demonstrated *in vitro*, in cultured cells and in tetrahydrobiopterin-deficient transgenic mice, the physiological relevance of the observation remains unknown with respect to human health and disease states. To date only two relevant studies illuminate the subject. Stroes *et al* demonstrated in a small-scale human clinical study that the brachial artery of patients suffering from hypertension associated with familial hypercholesterolemia exhibited significant vasodilation upon tetrahydrobiopterin infusion [87]. Secondly, Hong *et al* administered tetrahydrobiopterin pharmacologically (10 mg/mg i.p.) to spontaneously hypertensive rats (SHR) and measured significant improvement in NO-dependent vasodilation of aortic rings [48]. Other studies have investigated the outcome of arginine limitation on eNOS function. In a similar outcome to that of tetrahydrobiopterin deficiency, arginine limitation also yields superoxide rather than NO [71, 88]. However, the data describing this aspect of eNOS dysfunction has been carried out on purified or recombinant eNOS using *in vitro* experiments and thus, its physiological relevance remains in question as well. Therefore, while specific pathologies caused by tetrahydrobiopterin or arginine deficiency have not been well characterized, certain data suggests that administration of these compounds may improve endothelium-dependent vasodilation [89-91].

Cellular regulation of eNOS by Ca^{+2}

Of the three known NOS isoforms (*inducible* NOS, *neuronal* NOS and *endothelial* NOS), eNOS is the most sensitive to calcium. All of the 'classical' stimulators of eNOS initially activate the enzyme by elevating intracellular Ca^{+2} levels. Intracellular calcium binds to calmodulin (CaM), activating it. A Ca^{+2}/CaM inserts itself into a binding cleft of each monomer of eNOS, displacing the inhibitory scaffold protein calmodulin. Consistently, research has show that when endothelial cells are stimulated

with agonists such as acetylcholine or bradykinin, NO production and endothelium-dependent vessel relaxation can be blocked by intracellular calcium chelation or by calmodulin antagonists [92-94]. Recently several reports have described a "calcium-independent' mode of eNOS activity [95, 96]. However, this mode of eNOS activation is confusingly misnamed. It is now widely accepted that eNOS activity can be activated by certain stimuli which do not yield a sustained increase in intracellular Ca^{+2}. These stimuli, which ultimately lead to phosphorylation of eNOS allow sustained NO synthesis for periods of time *after* the initial intracellular Ca^{+2} rise. The function of phosphorylation-dependent regulation is to increase retention Ca^{+2}/CaM active eNOS after intracellular Ca^{+2} returns to baseline levels [97, 98].

eNOS regulation by intracellular translocation

Since eNOS is known to be regulated by direct interaction with cellular signal transduction machinery, it is not surprising that the active status of the eNOS protein is determined to a large extent by its intracellular location. There are two distinct cellular compartments that contain functional eNOS, the Golgi apparatus and plasmalemmal caveolae (specialized plasma membrane structures which organize signal transduction machinery, discussed below) [99]. Though both pools of eNOS are functional enzymes, experiments have shown that disruption of the Golgi apparatus does not adversely affect agonist-induced, endothelium-dependent vessel ring relaxation [100]. Therefore, Golgi-associated eNOS is not considered to contribute significantly to vascular NO-dependent tone *in vivo*.

The physiologically active pool of eNOS is the plasmalemmal caveolar fraction and thus it is responsible for controlling vascular tone [101-104]. Each monomer of the membrane-associated eNOS is cotranslationally myristoylated at its amino-terminus glycine residue and is an absolute requirement for membrane localization and activity [58, 61, 104]. Endothelial NOS is also palmitoylated on two cysteine residues proximal to the amino-terminus of the protein [58, 61, 104]. Palmitoylation occurs post-translationally in the Golgi apparatus and is reversible

[105, 106]. Unlike myristoylation, palmitoylation of eNOS is reversible and has thus been suggested to be a mechanism by which the cell controls turnover of the enzyme [104, 107, 108].

To date, the cellular mechanisms which regulate the intracellular transport of eNOS are almost completely unknown. Though the enzyme has been shown to shuttle between the different compartments in response to various stimuli (bradykinin, estrogen, tyrosine phosphatase inhibitors), none investigated the mechanistic control of such translocation and few have been confirmed by other laboratories and therefore remain controversial [107, 109, 110]. In general, the translocation of nascent eNOS has been described as constitutive and in response to 'cellular need' for the enzyme.

The association of eNOS with caveolae places it in the direct proximity of signal transduction proteins which form an eNOS activation complex. Association of eNOS with the plasma membrane not only facilitates the cellular regulation of eNOS, it places it in close proximity to the extracellular space (Figure 1.2). As a paracrine effector, it is important for the endothelial cell to be able to direct NO towards the medial smooth muscle layer. Caveolar eNOS is tightly bound to the scaffold protein, caveolin and in this state, eNOS is effectively inhibited by displacement of CaM from its binding site on eNOS (Fig.1.2) [95, 102, 111]. Upon stimulation via Ca^{+2}, the chaperone protein heat shock protein 90 (Hsp90) facilitates the insertion of Ca^{+2}/CaM into eNOS, displacing caveolin and initiating NO synthesis. The binding of Hsp90 to eNOS marks the initial phase of what is known as the eNOS activation complex and is an absolute requirement for association of eNOS with protein kinase B (Fig.1.2; also known as Akt) [95]. Phosphorylation of eNOS by various kinases including Akt is an important mechanism for the cell to up- and down-regulate ambient NO synthesis. This aspect of eNOS regulation will be discussed below.

eNOS control by phosphorylation

It is now well understood that eNOS control is maintained extensively by protein-protein interactions, most importantly by phorphorylation. Though intracellular rises

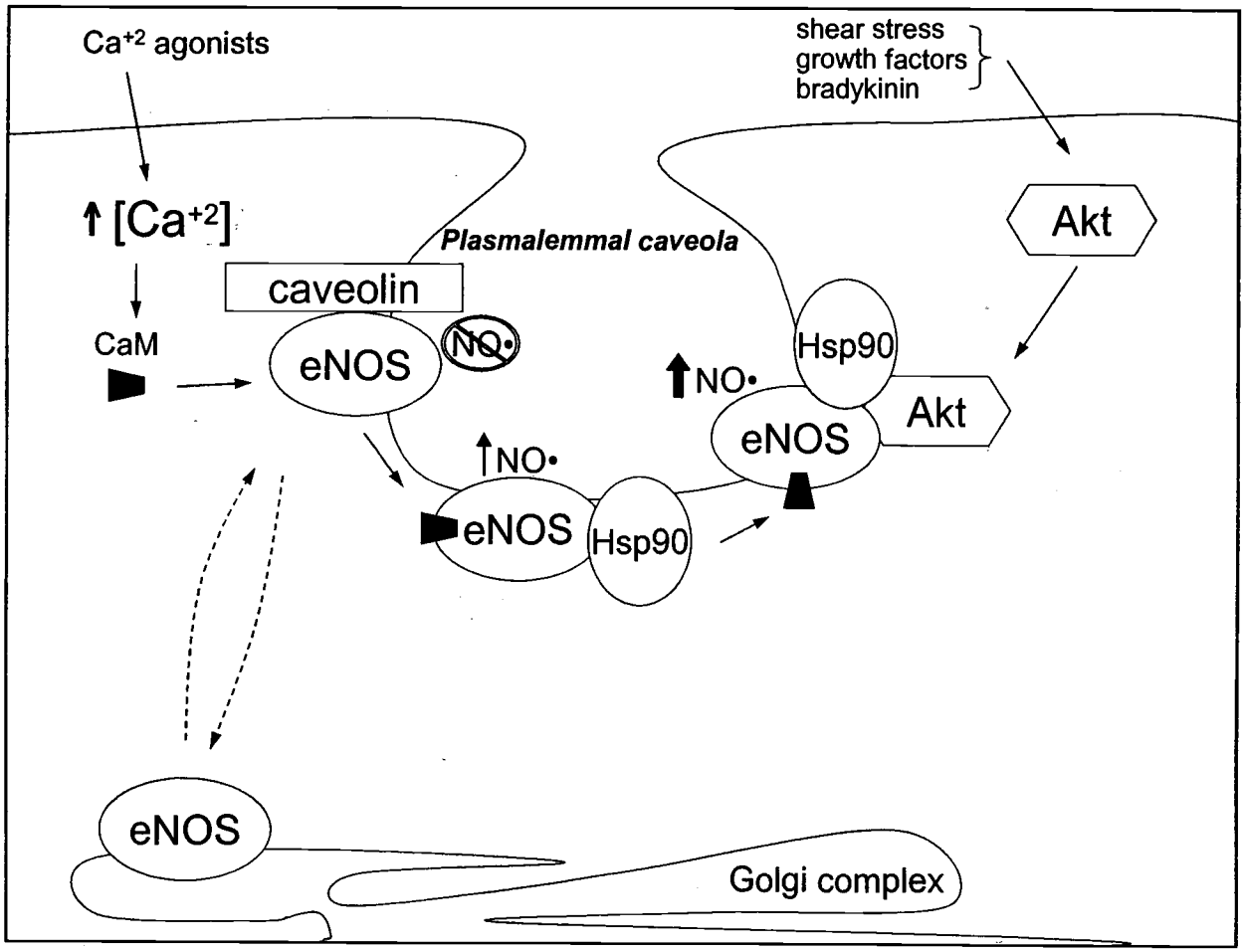


Figure 1.2. Translocation of eNOS to the plasma membrane is required for proper activation and regulation of NO synthesis. Newly translated eNOS is palmitoylated and myristoylated in the Golgi apparatus before translocation to the plasma membrane caveolae where it is tightly bound to the inhibitory scaffold protein caveolin. Upon intracellular Ca^{+2} release, Hsp90 facilitates insertion of Ca^{+2}/Calmodulin (CaM) into the reductase domain of eNOS, promoting electron flow through the enzyme and NO synthetic ability. Once active, the eNOS/Hsp90 complex may associate with the kinase Akt if stimulated by shear stress, stretch, insulin, growth factors, estrogens. The activation of Akt leads to formation of a trimeric complex of eNOS/Hsp90/Akt and S1176-phosphorylation of eNOS. Phosphorylation of eNOS by Akt allows elevated and prolonged NO synthesis after re-sequestration of intracellular Ca^{+2}.

in Ca^{+2} are an absolute requirement for initiation of NO synthesis by eNOS, the rate of synthesis and the duration (post Ca^{+2} transient) can be governed by phosphorylation at serine and threonine sites (Fig.1.1) [58, 61, 71, 97, 98, 112, 113]. Numerous phosphorylation sites have been described including serines 114, 617, 633 and 1177 (human eNOS sequence) and threonine 495 (human eNOS sequence) [61, 93, 98, 114, 115]. It has also been suggested that eNOS activity is also governed by phosphorylation at tyrosine sites [110, 116]. This assertion is made based on data describing alteration of endothelial NO by both tyrosine kinase and tyrosine phosphatase modulation. However, this work has not been repeated, nor it is generally accepted since no putative tyrosine phosphorylation site(s) have been described. The most well characterized phosphorylation sites on eNOS are the serine 1177 (human eNOS sequence; S1176 rat sequence) and threonine 495 (human eNOS sequence; rat sequence T494). The regulatory aspects of other phosphorylation sites are relatively unknown, therefore our discussion will be limited to these sites.

Laboratory work on this project was performed using eNOS isolated from *Rattus norvegicus* (rat) endothelium. Human eNOS amino acid sequence abbreviations have been introduced in order to avoid confusion and since literature as well as commercial products commonly refer to the human sequence. However, in this dissertation, rat sequence abbreviations (S1176, T494) will be used for the sake of accuracy.

Serine 1176 (S1176). Phosphorylation of eNOS S1176 occurs in response to numerous stimuli which are known to activate endothelial NO synthesis and NO-dependent vasodilation including shear stress, estrogen, vascular endothelial growth factor (VEGF), insulin and bradykinin (Fig 1.1). The predominant kinase which mediates phosphorylation at S1176 is the protein kinase B (PKB, known as Akt). Other kinases have been reported to also phosphorylate eNOS at this site and include PKA, AMP-activated kinase (AMPK) and calmodulin-dependent kinase (CaMK), in response to many of the same stimuli [93, 117]. Shear stress, VEGF and insulin also are known to activate Akt [118]. Inhibitors of phosphatidylinositol 3-kinase

(LY294002 and wortmannin), an upstream activator of Akt, prevent both eNOS phosphorylation at S1176 and endothelial NO-dependent vasodilation in response to shear stress, VEGF and insulin [25, 98, 113, 119, 120]. Phosphorylation at S1176 more than doubles electron flux through the eNOS reductase domain and yields similar increases in NO production compared to unphosphorylated eNOS (Fig 1.1) [94, 97, 120]. This is coincident with increased stability of the eNOS-Ca^{+2}/CaM association and results in sustained NO production at very low Ca^{+2}/CaM [97, 120]. Mutation of S1176 to alanine (S1176A) completely blocks Akt-dependent NO production [97, 98]. The ceramide-activated protein phosphatase 2A (PP2A) removes phosphate from S1176 (Fig 1.1; as well as other phosphorylation substrates of Akt) and PP2A inhibitors such as okadaic acid result in increased eNOS S1176 phosphorylation and NO synthesis [115, 121-123].

Threonine 494 (T494). At resting conditions, eNOS T494 is constitutively phosphorylated and is a negative regulator of NO synthesis [93, 114]. Changes in T494 phosphorylation are classically downregulated by phosphate removal by the phosphatase PP1 in response to stimuli which elevate intracellular Ca^{+2}, such as bradykinin, histamine, acetylcholine and Ca^{+2} ionophores (Fig. 1.1). Phosphorylation at this site interferes with the binding of Ca^{+2}/CaM to the CaM binding domain of eNOS. However, T494 phosphorylation control has been suggested to regulate eNOS in a far more complex manner, by coordinating electron flux to favor arginine consumption over oxygen reduction to superoxide [114] The putative kinase responsible for phosphorylating T494 is protein kinase C (PKC) [93, 115, 124] This point is best illustrated by research showing that eNOS is phosphorylated at T494 in the CaM binding domain by PKC in response to phorbol-myristate acetate in bovine aortic endothelium [124]. However, the involvement of PKC in eNOS-related signaling is not completely clear. This is due particularly to research showing that PKC inhibition can enhance eNOS NO production [125], a finding that may be explained by the diversity of the PKC family which demonstrate, among other differences, altered sensitivity to diacylglycerol, phorbol-myristate acetate and calcium.

Since regulation of eNOS is mediated both positively and negatively by phosphorylation, it has been suggested that in the endothelium, eNOS activity is controlled by systematic coordination of the phosphorylation and dephosphorylation of both the S1176 and T494 sites. Several independent laboratories have effectively demonstrated that reciprocal phosphorylation of S1176 and dephosphorylation occur in response to the same stimuli [98, 126-128]. Furthermore, though S1176 phosphorylation increases NO production over baseline levels, T494 dephosphorylation is a requisite for NO production by eNOS [114].

Caveolae in the endothelium

Caveolae are structurally specialized plasmalemmal vesicles. They are bottle-shaped invaginations of the plasma membrane approximately 50 - 100 nm in diameter. Though a thorough biochemical understanding of caveolar function is still lacking, it has become increasingly clear that they play a critical role in endothelial cell physiology. Electron microscopy studies have demonstrated that caveolae are the major plasmalemmal vesicular structure in endothelium as opposed to clathrin-coated pits (membrane structures which are specialized for endocytosis). In vivo, endothelial caveolae function as signal transduction microdomains and are clustered on the luminal and abluminal surfaces. One underappreciated observation is the comparison of caveolar density in cultured endothelial cells versus endothelium in vivo; Cultured endothelium *do* express ample caveolin-1, however, they typically display 0.1-9 caveolae per μm^2 plasma membrane area, where in vivo endothelium commonly display 80-100 per μm^2 [129-131]. Demonstrating their importance in cellular regulation, caveolae in vascular endothelium typically concentrate a large number of important signal transduction components including: Tyrosine kinase receptors (VEGF-R, PDGF-R and EGF-R), G-protein coupled receptors (endothelin-R, muscarinic-R, bradykinin-R, angiotensin-R and β-adrenergic-R), accessory/adapter proteins (Ras, Raf, G-proteins, Jak, Erk) and lipid-modified proteins (eNOS, Akt, neutral sphingomyelinase and other Src-kinases) [129, 132, 133]

Caveolae are formed from a distinct lipid composition consisting of cholesterol, sphingolipids (sphingomyelin and glycosphingolipids) and phosphatitylinositols [134]. One prominent mechanism utilized in caveolar signal transduction is the hydrolysis of sphingolipids to yield the second messenger lipid ceramide. This reaction is catalyzed (in the pH-neutral environment of the plasma membrane) by the neutral-isoform of sphingomyelinase (nSMase) in response to shear stresses on the endothelial luminal surface and serves as a method of mechanotransduction to activate caveolar constituents [134, 135].

Sphingomyelinases and ceramides in endothelial signal transduction.

Ceramide is an N-acylated sphingosine lipid second messenger utilized in nearly al eukaryotic cells. In the cardiovascular system, it may be found in endothelial cells, VSM, monocytes, macrophages neutrophils and platelets and functions as a mediator of apoptosis and a second messenger molecule operating in numerous cellular signaling pathways. Endogenous ceramides are a class of molecules and vary in their composition depending upon the type of fatty acid attached to it. Ceramide molecules derive from two enzymatic pathways. They are generated *de novo*, by coordinated CO_2-condensation from serine + palmitoyl-CoA to form the amino alcohol sphingosine, then N-acylation of sphingosine with a fatty acyl CoA to form ceramide. Ceramide is also generated by the hydrolytic cleavage of phosphorylcholine from the membrane structural lipid sphingomyelin by the action of the enzyme sphingomyelinase. The mechanism of ceramide activity is thought to arise from the formation of ceramide-rich microdomains in the membrane bilayer which reorganize and coordinate membrane and caveolar components. The steady-state level of cellular free ceramide is thought to be dynamic and the control of ceramide crucial to the proper activation and regulation of signal transduction enzymes. Though low steady-state levels of ceramide production are critical to proper signal transduction and cell function (including mechanotransduction activation of eNOS), increases in free

ceramides due to either *de novo* synthesis or by abberant over-activation of sphingomyelin hydrolysis can lead to serious cellular dysfunction.

Ceramide-mediated cellular dysfunction may occur when an imbalance of ceramide production (by sphingymyelin hydrolysis + de novo synthesis) and degradation (by ceramidases) occurs. This condition may occur if excess long chain fatty acids and acyl-CoAs accumulate due to alterations in β-oxidation. The overload of acids and acyl-CoAs may be remediated by non-oxidizing pathways such as ceramide synthesis. Though there is evidence that aging, diabetes or chronic inflammation compromises lipid oxidation in the heart, liver and β-cells of the pancreas [136, 137], suggesting that *de novo* synthesis of ceramide may contribute to pathology, there is little or no evidence relating to alterations of *de novo* synthesis in the vascular endothelium. However, there is a growing body of research characterizing the role of SMases and the role in the production of ceramides in endothelial cells.

Both acidic and neutral SMase isoforms are expressed in the endothelium, though the neutral isoform predominates [134, 135, 138, 139]. Neutral SMase (nSMase) is acutely regulated by cellular redox environment and by oxidative stress. Glutathione, the predominant low-molecular weight antioxidant in nearly all cells, is typically maintained at millimolar concentrations intracellularly. Thus, it plays a major role in cellular redox environment and homeostasis of the endothelium. Furthermore, it has been shown to be one of the most potent regulators of nSMase activity *in vitro* and *in vivo* [140-143]. In fact depletion of GSH pharmacologically, or by TNF-α stimulation initiates rapid sphingomyelin hydrolysis by nSMase leading to apoptotic cell death [138, 142]. Furthermore, Hannun's group has demonstrated that inhibition of nSMase or application of exogenous GSH (by GSH-ethyl ester repletion) prevents this TNF-α dependent apoptosis [142]. The observation that GSH inhibits nSMase is of upmost importance to our understanding of ceramide-mediated cellular dysfunction, since we have observed significant losses of intracellular GSH in the aged vascular endothelium of rats.

Though a thorough understanding of ceramide-dependent signaling is lacking, certain aspects of this important second messenger have been characterized. One of the most important of these its potent action on a class of ceramide-activated serine/threonine protein phosphatases [144-146]. These are the protein phosphatase 1 (PP1) and PP2A. Ceramide also inactivates certain members of the protein kinase C family [147, 148]. In fact recent work suggests that the mechanism of ceramide-activated apoptosis occurs by phosphatase-dependent deactivation of the pro-survival proteins Bcl2 and Bad [146]. The phosphatase PP2A is responsible for removal of the activational phosphorylation on Akt (at S473) and eNOS (at S1176) [115, 122, 123, 145]

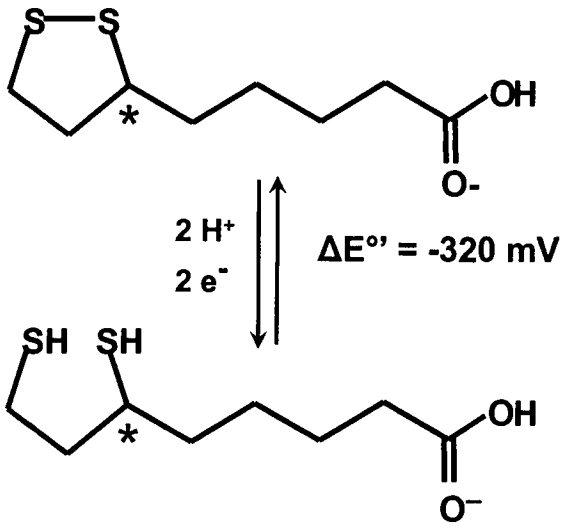
Antioxidants and endothelial function.

Ascorbic acid (vitamin C, AA) and GSH comprise the two most important low molecular weight water-soluble antioxidants in cells [149]. AA effectively scavenges superoxide, hydroxyl radicals, peroxyl radicals, HOCl and some reactive nitrogen species [150, 151]. Studies show that it exerts a protective effect against oxidative stress, atherosclerosis, and vascular dysfunction [152, 153]. Epidemiological studies demonstrate an inverse correlation between the levels of plasma ascorbate and incidence of coronary artery disease [154]. Moreover, AA supplementation enhances endothelium-dependent vasodilation by stabilizing tetrahydrobiopterin availability [90]. Other possibilities have been proposed as mechanisms by which AA may ameliorate endothelial dysfunction. AA lowers oxidized-LDL levels in plasma, which would spare endothelial NO. Furthermore, vitamin C can theoretically reduce nitrite to bioactive NO, or it may directly stimulate eNOS as a cofactor. It has also been proposed that it may favorably enhance smooth muscle guanylyl cyclase activity, leading to improved vessel tone [155].

The therapeutic role of lipoic acid

Lipoic acid (1,2-dithiolane-3-pentanoic acid; LA) and its reduced form, dihydrolipoic acid (DHLA) are naturally occurring compounds, with one chiral center (Fig. 1.3). The *R*-form of LA is the only enantiomer synthesized and used in biological systems as a prosthetic group for mitochondrial α-ketoacid dehydrogenases [156]. In mammals LA is synthesized *de novo* in mitochondria by lipoic acid synthase [157, 158], but it can also be absorbed from the diet [159-164]. It is currently not known whether *de novo* synthesis is adequate to supply all bodily requirements for LA or whether dietary intake is also at least conditionally necessary. Regarding its synthesis, LA is derived from octanoic acid but the immediate precursor substrate is now thought to be the octanoate-acyl carrier protein complex [165, 166]. The source(s) for the two sulfhydryl moieties is presently unknown but as the reaction proceeds without an exogenous thiol source, the donated sulfurs may actually come from the iron-sulfur moiety of the enzyme itself [167, 168]. Further studies will be necessary to elucidate the regulation and enzymology of LA synthesis in mammals.

The chemical reactivity of LA and DHLA is mainly centered in its dithiolane ring (Fig. 1.3). This structure makes LA unique among other common sulfhydryl-containing biomolecules (e.g. glutathione and cysteine). Torsional strain distorts this ring structure and contributes to the reactivity of both thiol groups [169, 170]. Moreover, the position of the two sulfur atoms in the ring creates an exceptionally high electron density, which confers special properties to LA that are not shared by other disulfides [171]. These structural features make LA a highly reactive molecule under physiological conditions, which is evident in a DHLA/LA reduction potential of –0.32V (Fig. 1.3) [172, 173]. In biological systems only the NAD(P)H/NAD(P)$^+$ redox couple has a higher reduction potential. Consequently, DHLA is capable of reducing a host of other compounds including disulfides, the oxidized forms of antioxidants (e.g. glutathione, vitamins C and E), and transition metal ions [174, 175]. DHLA is considered a potent antioxidant in its own right. Thus, the chemical nature



Properties	LA	DHLA
pKa (-COOH)	4.76-5.3	4.85
pKa (-SH)	--	10.7
λ max (nM)	333	--

Figure 1.3. Lipoic acid structure and chemical properties. Lipoic acid (LA; 1,2, octanoylpentacetic acid), is an eight-carbon dithiol compound with a high reduction potential. It has one chiral center that is denoted by an asterisk.

of LA and DHLA make it capable of participating in a variety of biochemical reactions where redox state is important.

Considering that cellular concentrations of nonprotein-bound LA are practically undetectable under post-fed conditions, it is not surprising that little effort, until recently, has been undertaken to discern biological roles for LA outside of acyl group transfer reactions. Despite its limited availability on a sustained basis, numerous reports show that intraperitoneally (i.p.) administered LA results in a transient increase in free cellular LA levels [176]. IP bioavailability studies show that an injected LA dose is rapidly absorbed into the gastrointestinal tract and appreciably increases plasma LA levels.

LA has been used as a potent antioxidant, as a detoxication agent for heavy metal poisoning, and has been implicated as a means to improve age-associated cognitive decline [177, 178]. Most importantly, LA has been used extensively as a therapy for complications associated with diabetes mellitus, especially to improve glucose handling and reduce diabetes-associated polyneuropathies. A quick perusal of this partial list shows the diversity of this compound to mitigate at least certain effects of both chronic and acute conditions [for a comprehensive review see reference 179].

The problem of aging and vascular function

With the progression of modern sanitation and medical technology, we have drastically reduced the number of deaths due to pathogenic diseases; thus the human lifespan has dramatically increased. The outcome of these advances has narrowed the focus of biomedical research and directed it towards understanding the pathologies of aging and age-related diseases. While the incidence of neurological disorders (Alzheimer's and Parkinson's diseases) and cancer increase with age, cardiovascular-related diseases remain the single largest cause of death in western society irrespective of age or gender [180]. In fact, statistics show that age is the number one risk factor for cardiovascular diseases (CVD) of all types [181, 182]. Clinical hypertension is

observed in over 30% in the aged populus (those over the age of 65) and over 80% of reported cases of coronary disease occur in this population [183]. Since proper circulation and the distribution of oxygen and nutrients is crucial to maintenance and repair of all tissues, age-associated vascular dysfunction is ontologically involved in the progression of nearly all age-associated diseases. Therefore, underlying the epidemiological observations of age-related diseases and CVD, as a causative force, is a specific decline in vascular function which is nearly universal in the elderly [182, 184-191]. Unfortunately, the social responsibility for this problem will only escalate; Statistical projections forecast that the population of persons over the age of 65 in the U.S.A. will reach approximately 70 million by the year 2030 [192]. Thus, the physiological importance of the effect of age on the vascular system cannot be overstated with respect to age-related diseases of all types.

The decline in vascular function brought about by age is evident by gross changes in the anatomy and physiology of the large arteries of the circulatory system. The arterial wall, is composed of an outer adventitial layer, a medial vascular smooth muscle layer and an intimal layer composed of an elastic matrix and endothelial cells, all of which are affected by the process of aging. Analysis of the morphological changes to the intimal layer show that it becomes more fibrous by deposition of excess collagen and fibronectin [193]. Observed changes in the medial layer include enlargement of vascular smooth muscle cells accompanied by decline in actual cell number, again leading to loss of arterial elasticity and dilation of the luminal area of the vessels [193]. Thus, the conduit arteries become enlarged and less plastic with the advance of age [189, 193].

Though age leads to morphological alterations to large vessels which undoubtedly contributes to gross alterations in vessel function, a more specific and profound change is specifically observed in endothelial cell function. Why might this occur? Though the vascular endothelial cell is capable of mitotic proliferation (to meet the needs of angiogenesis and wound healing), the cells are characterized as long-living [194, 195]. In the conduit arteries, the half-life of a typical endothelial cell is estimated to be 30 years [194, 195]. Therefore, excluding the contribution of new

endothelium from wound healing and angiogenesis, the typical aortic endothelial cell may undergo only a few divisions in a person's lifetime. Considering this, endothelial cells are cumulatively exposed to a relatively long term in which damage may accumulate. Thus, considering this potential to accumulate damage, perhaps it is not surprising that endothelial function is specifically impaired during the aging process.

In particular, aging leads to specific losses in endothelial NO-dependent vasodilatory function of the conduit arteries and this occurs independent of any particular disease state [196-202]. Since the endothelium is the principle mediator of vasomotor tone in conduit arteries via synthesis of NO, its loss during aging has profound implications toward overall health and wellbeing. The physiological outcome of age-specific endothelial NO loss is first noted as an inability of the vasculature to adapt to physical states such as stress and exertion. But, more importantly, the loss of NO-dependent relaxation is important because it has a compounding effect, complicating and exacerbating other age-related cardiovascular pathologies and ultimately leading to advanced complications such as chronic hypertension, stroke, thrombosis, pulmonary edema and death [181, 182, 190, 199, 203].

It must be noted that all arteries are not affected to the same degree. The major conduit arteries such as the aorta, carotid and coronary arteries suffer from age-related NO loss and resultant vasoimpairment, where peripheral arteries and the microvasculature show little or no decline in vessel tone [204, 205]. Evidence of age-related impairment of NO-mediated vessel tone comes from both human and animal studies showing that conduit arteries of aged subjects have significantly reduced responses to classical vasodilatory agents such as acetylcholine and bradykinin which stimulate eNOS activation [186, 204]. There is no age-related decline in vessel relaxation response to NO analogs such as nitroglycerine, nitroprusside or other NO-donating drugs [185]. Also, there is no decline in VSM guanylyl cyclase activity associated with aging [8]. Thus, evidence illustrates that the age-associated impairment of NO-dependent vasodilation is particularly due to endothelial dysfunction and not to a VSM pathology. These age-related changes in the conduit

arteries occur *independently of any particular disease or pathology.* Yet, since aging is characteristically associated with a host of other pathologies (including atherosclerosis, diabetes and chronic hypertension) which directly contribute to further loss of conduit vessel function, it is difficult to resolve the specific effects of aging from age-associated diseases.

Aging and the specific loss of endothelial NO.

What is specifically known with regard to age-associated loss of endothelial NO? In order to answer this question, we must consider what scenarios could possibly lead to loss of NO-dependent vasodilation. A strong body of work has focused on alterations to the endothelial redox environment which could cause inactivation of the NO signal. NO is an uncharged radical molecule and is thus highly susceptible to chemical inactivation by oxidants. A general phenomenon of aging which has been characterized in many different tissue types is increased production of reactive oxygen species (ROS) including the radical anion superoxide [206-211]. These ROS are thought to emanate from decaying mitochondria, NAD(P)H oxidases and in the endothelium, potentially from eNOS itself [206, 208-210]. Additionally, there is typically a loss of antioxidants in many tissues, including the endothelium, with age [47, 155, 212, 213]. While little definitive work has been done as to the specific site(s) of reactive oxygen species formation in the endothelium, it does appear that increased oxidative stress occurs in vascular endothelial cells with age [44, 206, 207, 214]. Superoxide or other reactive oxygen species may react rapidly with endothelial NO forming nitrate, nitrite, peroxynitrite or other reactive nitrogen species, which are not viable vasodilatory signal molecules. Though this field of research appears quite promising with respect to our quest for an understanding of the age-associated loss of NO, it presents numerous technical difficulties. First, the endothelium, being a monolayer of cells, is difficult to isolate and analyze without significant alteration to cellular function. Furthermore, while the detection of 'oxidizing species' may be

reasonably straightforward in cells, the accurate identification and quantitation of specific ROS species is inherently plagued by inaccuracies and artifacts.

Another potential explanation for the age-associated loss of NO is focused on the potential for cellular alterations which affect the eNOS enzyme. Altered expression, aberrant regulation or limitation of cofactors and substrates could possibly lead to loss of NO. Several laboratories have attempted to answer the question: does aging alter endothelial expression of eNOS? Chou *et al* reported age-associated loss of eNOS activity in Wistar rats with *no alteration* of eNOS protein expression [215]. An *increase* of eNOS protein expression concomitant with a loss of NO synthetic capacity was reported in aged F344 x Brown Norway hybrid and Wistar rats by van der Loo *et al* and Cernadas *et al*, respectively [196, 207]. Alternatively, two other laboratories reported that age leads to significant *decreases* in eNOS protein expression in F344 rats [216, 217]. Therefore, the subject of eNOS protein expression during age does not seem to be governed by any general phenomenon and may be dramatically different depending on the animal model used. Though the age-associated loss of NO and NO-dependent vasodilation is well documented in humans, the potential contribution of age on eNOS protein expression remains entirely unknown.

Another potential mechanism which may explain the observed loss of endothelial NO could be limitation of eNOS substrates or cofactors in the endothelium. As discussed previously, tetrahydrobiopterin deficiency can cause eNOS to function aberrantly, producing superoxide rather than NO. Though this endpoint seems promising, only one laboratory has ever reported quantitative analysis of tetrahydrobiopterin in vascular tissues of young and old animals. Blackwell *et al* recently showed that though endothelial NO was lost, tetrahydrobiopterin levels in the carotid artery were unaltered with age in mice. Again, though several laboratories have reported that tetrahydrobiopterin supplementation improves vasodilatory function in various models of hypertension and hypercholesterolemia, none demonstrated that tetrahydrobiopterin levels were initially altered or limiting in any way in vascular tissues [48, 218, 219].

Loss of arginine availability in the endothelium could potentially lead to limitation of NO synthesis. This is well documented *in vitro* [39, 71]. However, to date, no research has demonstrated that age leads to any particular loss of arginine transport or availability during aging. In fact, Ahlers *et al* showed that endothelial arginine transport is *unaltered* with age in humans [220]. Additionally, age-specific hypertension is not responsive to arginine supplementation [91, 221].

In the present work, a *novel* theory is explored with respect to the nearly universal observation that age leads to loss of endothelial NO synthetic capacity. It is based on a newer understanding of the cell-signaling cascades in the vascular endothelium, which regulate eNOS activity. During the last several years, our understanding of port-translational regulation of eNOS has advanced greatly. It is now well known that eNOS activity in the endothelial cell is tightly controlled by phosphorylation, by complexing with regulatory proteins, and by subcellular translocation. The goal of this work was to characterize whether any of these parameters might be altered during the aging process, whether any potential alterations had relevance to endothelium-dependent vasodilation and to search for potential mechanisms which might lead to the observed loss of endothelial NO.

1.2 Dissertation hypotheses and aims

It has been demonstrated that the cellular regulation of eNOS localization and activity contributes immensely to proper circulatory function and homeostasis. Accordingly, it is surprising that little or no research has focused on investigating the potential alterations of signal transduction pathways which govern eNOS activation in the aging setting. Advances in our understanding of the complex signal transduction networks in the endothelium have made a critical analysis of the aging endothelial cell overdue. In this work, we will investigate several of the regulatory elements which control the function of eNOS *in vivo* in addition to searching for mechanistic links which may cause this aging pathology.

This work was guided by two distinct hypotheses. The first hypothesis is that aging adversely affects eNOS regulatory components, in part by alteration of its cellular localization, protein-protein interactions and phosphorylation state. This hypothesis has been explored by addressing the following specific aims:

1. Assess endothelial regulation of eNOS by determining whether eNOS expression, localization, accessory protein binding and phosphorylation status are altered with age. This work was accomplished by the isolation and characterization of eNOS from freshly isolated, unstimulated rat aortic endothelium from young and aged animals.

2. Investigate whether ceramides, glutathione status or sphingomyelinase activity is altered in the endothelium with age and whether any potential alterations correlate with the age-related loss of endothelial NO-dependent vasodilation.

Secondly, this work was guided by the hypothesis that acute lipoic acid administration may partially restore endothelium-dependent vasodilation by manipulation of endpoints which are characteristic of eNOS regulatory control. This hypothesis has been explored by addressing the following specific aims:

1. Measure any effect that acute LA administration may have on endothelium-dependent vasodilation by pharmacologic (i.p.) administration to old animals over 24 h.

2. Determine whether acute LA administration alters eNOS phosphorylation state.

3. In searching for a mechanistic link for potential LA-effects on endothelial function, determine whether LA treatment might affect endothelium-dependent vasodilation-NO by alteration of cellular ceramides, glutathione status or sphingomyelinase activity.

Chapter 2

Cellular organization of the endothelial nitric oxide synthase enzyme is altered with age.

Anthony Smith

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2.1 Abstract

A consistent body of evidence effectively demonstrates that age progressively leads to a profound loss of endothelial nitric oxide (NO) and hence, NO-dependent vasodilation of the conduit arteries such as the aorta, carotic and femoral arteries. The mechanism of this loss remains unclear. To determine why this loss might occur we investigated the phenomenon in aged Fisher 344 x Brown Norway (F344 x BN) hybrid rats (32-34 months; corresponding to a 65 –75 year-old human) and compared results to those found in young animals (2-4 months; corresponding to an adolescent human adult). Freshly isolated endothelium from young and old rats were first analysed to determine if endothelial nitric oxide synthase (eNOS) protein levels changed or whether aging lead to alterations in the subcellular compartmentalization of eNOS. We also compared the pattern of eNOS association with its regulatory accessory proteins caveolin, Hsp90 and Akt in young and old aortic endothelium. Results show that maximal eNOS activity significantly declines with age ($n=4$; $p\leq0.05$) though there was no change in eNOS protein levels in the aortic endothelium. Endothelial NOS exists in two distinct subcellular fractions. No alterations were detected in the soluble, inactive fraction while significantly less eNOS protein is detected in the active, plasma membrane fraction of the endothelium ($n=4$; $p\leq0.02$). In the aged endothelial membrane fraction, significantly less eNOS protein is found associated with the stimulatory kinase Akt and the chaperone protein Hsp90 both of which , when bound to eNOS form a trimeric complex which activates eNOS. Since these declines correlate well with the observed loss of endothelium-dependent NO, the data suggests that the loss, and resulting physiological complications characteristic of age, may be caused by an alteration of the cellular regulation of eNOS in the endothelial cell.

2.2 Introduction

Cardiovascular diseases (CVD) and related complications are the leading cause of hospitalization and death in western societies and CVD of all types affect nearly 80% of persons over the age of 65 [180]. The cardiovascular pathologies that contribute to these statistics are numerous, including hypertension, stroke, heart attack, atherosclerosis, thrombosis, inflammation, diabetes, and restenosis [193]. However, a hallmark of aging is an overall decline in vascular endothelial function, leading to dramatic losses of vasomotor activity in the major conduit arteries [182, 193]. Loss of vasomotor function leads to systemic hypertension and ultimately to the progression of nearly all types of CVD. Interestingly, this human phenomenon has been successfully approximated by animal models that show an equal loss of vascular tone [188, 190, 193, 204, 222]

The vascular endothelium affords the vessel with an ability to sense blood flow and chemistry and react by directing dilation and contraction of the vessels in a controlled manner by synthesis and secretion of second messenger molecules which are targeted to the vascular smooth muscle layer. The principal effector of endothelium-dependent vasodilation in the large conduit arteries of the cardiovascular system is nitric oxide (NO). In endothelial cells, NO synthesized by activation of the homodimeric enzyme endothelial nitric oxide synthase (eNOS) via a five electron oxidation of the terminal guanidino-group of L-arginine [71, 80]. NO is an uncharged, lipophilic radical which diffuses rapidly to the smooth muscle layer where it activates soluble guanylyl cyclase, causing a cGMP-dependent sequestration of cellular Ca^{+2} and subsequent relaxation of the muscle [223]. Aging impairs the ability of the endothelium to synthesize vasodilatory concentrations of NO in humans and in rats[12, 89, 190, 207, 215]. However, in both of these animal models, the aged vessels maintain proper vasodilatory response when exposed to exogenous sources of NO such as sodium nitroprusside or DEA-NONOate[8, 187, 205, 207]. This indicates that the vascular smooth muscle functions properly despite age and that aging results in a *specific* loss of endothelium-derived NO (EDNO).

Since aging results in a specific impairment of EDNO production, research has focused on elucidating the mechanisms leading to this loss. One hypothesis is that aging may alter expression of the eNOS protein. Several laboratories have attempted to answer the question of whether aging alters endothelial expression of eNOS Chou *et al* reported age-associated loss of eNOS activity in Wistar rats with *no alteration* of eNOS protein expression [215]. An *increase* of eNOS protein expression concomitant with a loss of NO synthetic capacity was reported in aged F344 x Brown Norway hybrid and in Wistar rats by van der Loo *et al* and Cernadas *et al* (respectively) [196, 207]. Alternatively, two other laboratories reported that age actually leads to significant *decreases* in eNOS protein expression in F344 rats {Woodman, 2002 #1302;Tanabe, 2003 #1294.

A second hypothesis is that age may decrease concentrations of specific substrates and cofactors required for proper eNOS function {Bredt #194}. For example, Blackwell *et al* recently showed that though endothelial NO was lost, the eNOS cofactor tetrahydrobiopterin was unaltered in the carotid artery of aged mice [202]. Though several laboratories have reported that tetrahydrobiopterin supplementation improves vasodilatory function in various models of hypertension and hypercholesterolemia, none have demonstrated that tetrahydrobiopterin levels are actually altered or limiting in any way in aging vascular tissues [48, 218, 219].

Alternatively, loss of the eNOS substrate, arginine, could also lead to limitations in NO synthesis. This is well documented *in vitro* [39, 71]. However, to date, no research has demonstrated that age leads to any particular loss of arginine transport or availability. In fact, Ahlers *et al* specifically showed that endothelial arginine transport is *unaltered* with age in humans [220]. Additionally, age-specific hypertension is not responsive to arginine supplementation [91, 221].

The present work, investigates a novel theory to explain the age-associated loss of endothelial NO-dependent vasodilation which is derived from more recent understanding of the cellular regulation of eNOS. eNOS bioactivity is regulated in part by its subcellular compartmentalization and by its complexing with accessory proteins (see reference [61] for review). Once translated, nascent eNOS protein is

transported from the Golgi apparatus (where it is inactive and insensitive to intracellular Ca^{+2}) to the endothelial cell plasma membrane. There, it is complexed with the scaffold protein caveolin in an inactive state which is sensitive to intracellular Ca^{+2} [58]. It is regulated in a biphasic manner by Ca^{+2} mobilization and also by phosphorylation. In particular, rises in intracellular Ca^{+2} initiate eNOS activity by promoting disassociation from the scaffold protein caveolin (which maintains eNOS in an inactive conformational state) and subsequent association with Hsp90 and concomittent insertion of Ca^{+2}/calmodulin into its reductase domain. Subsequently, eNOS activity is down regulated by cellular sequestration of Ca^{+2} to the mitochondria and the endoplasmic reticulum. In a second mode of regulation, the eNOS/Hsp90 complex may be phosphorylated by complexing with the protein kinase B (known as Akt) in response to growth factors, shear stress, estrogen, bradykinin or corticosteroid hormones [97, 98, 111, 113]. Phosphorylation by Akt preserves eNOS association with Hsp90 and allows sustained EDNO synthesis independent of intracellular free Ca^{+2} concentrations [95]. Thus, Golgi associated eNOS may be considered to be 'quiet' or unavailable for cellular activation, particularly by intracellular Ca^{+2}. However, plasma membrane-associated eNOS may be considered to be 'primed' for activation because, though inactive at rest, it is acutely sensitive to initiation of NO synthesis by endothelial mechanisms which release Ca^{+2} into the cytosol from intracellular stores.

It is now clear that cellular, post-transcriptional regulation of eNOS plays a critical role in normal endothelial NO synthesis. Alterations in the cellular regulatory elements which control eNOS would dramatically alter the endothelial synthesis of NO in response to classic vasodilatory stimuli. Therefore, this project was directed by the hypothesis that age alters eNOS post translational regulation in a manner that changes its subcellular compartmentalization and its resting association with accessory proteins giving way to age-associated loss of NO-dependent vasodilatory ability.

2.3 Materials and Methods

The animal model. Throughout this study, male Fischer 344 × Brown Norway hybrid (F344×BN) rats were used as the experimental model. This is a well-characterized rat strain that is an approved rodent model for aging studies by the National Institutes of Health and the National Institutes of Aging (NIH/NIA). Young (2-4 months; corresponding to an adolescent human adult) and old (32-34 months; corresponding to a 65 –75 year-old human) rats were used for all studies. Although F344×BN rats do not develop atherosclerosis, they do develop the same age-related decline in vascular function as do humans and other mammalian species [224].

Isolated aortic ring myography. Segments of thoracic aorta were cleaned of adherent connective tissue, cut into 3-5 mm long rings and suspended in an organ-bath chamber containing Krebs-Henselheit solution, gassed with 95% O2 and 5% CO_2, and maintained at 37°C. Tissues were mounted on an isometric force-displacement transducer (Kent Scientific, Torrington, CT) and changes in isometric forces were continuously recorded. Rings were gradually stretched to 1-1.5 g and allowed to equilibrate for 90 min. Maximal contractility was evaluated by the addition of KCl 60 mM. After washing and further equilibration, the rings were contracted with $3x10^{-7}$ M norepinephrine. After stabilization (10-15 min), relaxation was assessed by the cumulative addition of acetylcholine (10^{-10} to 10^{-4} M). Sodium nitroprusside (10^{-10} to 10^{-5} M) was used to evaluate endothelium-independent vasorelaxation. As a negative control to demonstrate the specificity of ACh stimulation of endothelial NO, myography was performed on rings in which the endothelium was mechanically damaged by rubbing.

Preparation of vascular endothelium samples. Freshly isolated aortae from male F344×BN rats were perfused with Hank's Buffered Saline Solution (HBSS) pH 7.4 containing protease and phosphatase inhibitors, and then removed to a petri-dish. The aortae were opened longitudinally and adhered to poly-L-lysine coated glass, then

frozen over liquid nitrogen. After freezing, the endothelial surface was carefully scraped from the vessel segments with a surgical scalpel and collected into homogenization buffer containing protease and phosphatase inhibitors.

Primary culture of rat aortic endothelium. The aortae of young and old rats were perfused in situ with sterile endothelial culture basal medium (MCDB131), then removed to a fibronectin-coated petri-plates. (BD Biosciences, San Jose, CA). The fibronectin-matrix was overlaid with complete MCDB131 medium containing 2% FCS. On the fourth day after isolation the aortic segments were removed from the fibronectin-matrix and the media was changed. After an additional four days, the cells were harvested by trypsinization, resuspended in complete MCDB131 medium and seeded into 24-well plates or 75 cm^2 flasks and grown to confluency for experiments without further passage. 70% to 80% of the cells stained positive for von Willebrand's factor as assessed by fluorescence-microscopy. Importantly, the expression patterns of ENOS, Akt, β-actin and von Willebrand's factor in the old and young primary cultured endothelium remained consistent with those seen in freshly isolated endothelium (data not shown).

Immunochemical analysis of endothelial proteins. Vascular endothelium was scraped from freshly isolated aortae from young and old rats as described above. Protein homogenates were quantified and prepared for either Western blotting (after SDS-PAGE under reducing conditions) or co-immunoprecipitation. The former were quantified by horse radish peroxidase-linked secondary antibodies and subsequent chemiluminescent detection. The latter were performed using primary antibodies coupled to Sieze-AminoLink® IP beads (Pierce, Rockford, IL) in non-reducing, non-denaturing conditions. Then, proteins were eluted from the IP beads and analyzed by Western blot. In some experiments, plasma membrane proteins were fractioned from the soluble cellular proteins by ultracentrifugation at 100,000g for 90 m at 4° C.

2.4 Results

The age-associated decline in endothelium-dependent vasorelaxation has been observed in mouse, rabbit, rat, pig and human conduit vessels [89, 196, 221, 225-227]. We sought to recapitulate this phenomenon in our rat model and to determine if physical alterations to eNOS might be responsible for this aging-phenotype. We first performed vessel myography with freshly isolated aortic rings from young and old animals. After a period of equilibration and precontraction with phenylepherine (norepinepherine), dose-dependent relaxation response to acetylcholine (ACh) was measured. The rings from young animals showed dose-dependent relaxation beginning at 10^{-7} M ACh with maximal relaxation response (~80% relaxation) at 10^{-4} M ACh (Fig. 2.1A). The old vessel rings began relaxing at similar low-end concentrations, however, maximal relaxation was only ~40% at 10^{-4} M ACh (Fig 2.1A). Relaxation of old vessel rings were significantly lower than relaxation of young rings at concentrations above the range 10^{-5} - 10^{-4} M ACh ($P \leq 0.05$). There were no differences in the relaxation profiles of young and old aortic rings in response to the NO-donor compound sodium nitroprusside (Fig. 2.1B). This data demonstrates that endothelium-dependent relaxation is affected with age while vascular smooth muscle response to NO is unaffected.

In searching for an underlying cause or mechanism to explain this loss of endothelial NO, eNOS protein expression was estimated by Western blot of freshly isolated endothelium form young and old animals. Endothelial NOS protein expression in the aged endothelial layer of the aorta does not differ from eNOS expression in the young (Fig 2.2A). Therefore, the age-associated loss of NO and NO-dependent vasodilation is not likely due to altered expression of eNOS protein in the cell.

To determine whether the observed loss of endothelium-dependent vasorelaxation was due to a loss of maximal NO synthetic capacity, eNOS enzymatic activity was measured in young and old aortic endothelial cells by analyzing cellular

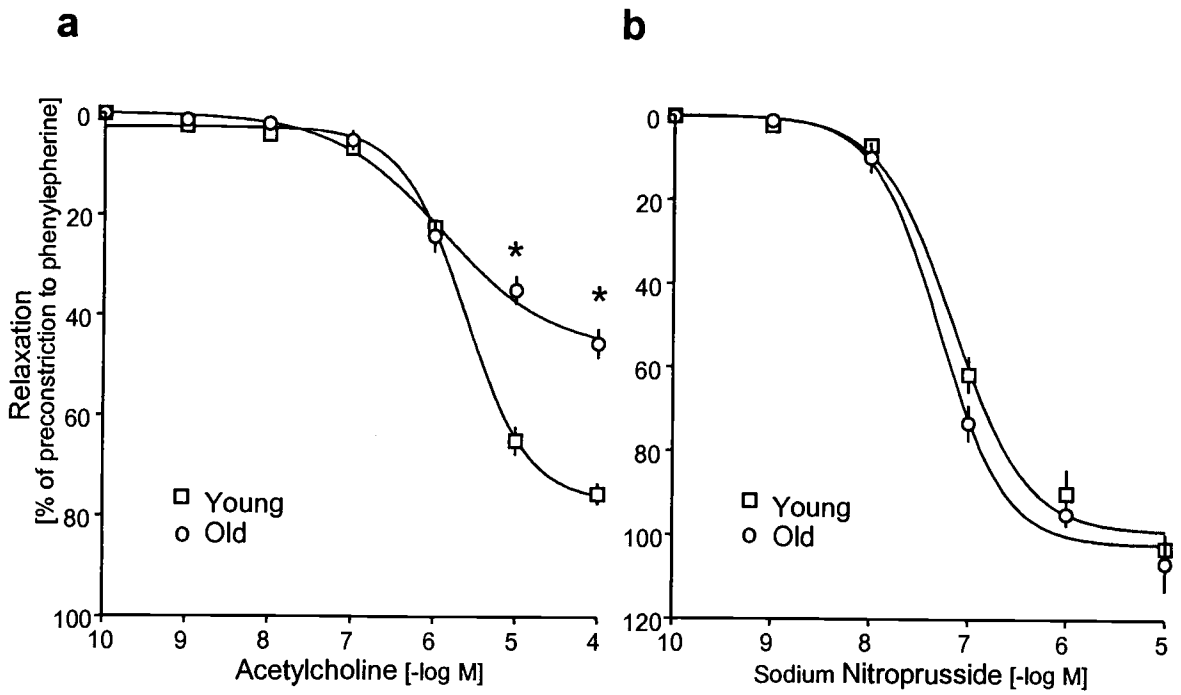

Figure 2.1. Aging is associated with significant loss of endothelium-dependent vasodilation. (a) Aortic rings from old rats exhibited a significantly lower response to acetylcholine-induced vasorelaxation, as compared with young animals. (b) Addition of the NO analog, sodium nitroprusside, to the medium induced complete vasorelaxation indicating maintenance of proper smooth muscle cell function through age. *$p \leq 0.05$ according to unpaired Student's two-tailed *t* test; n= 3

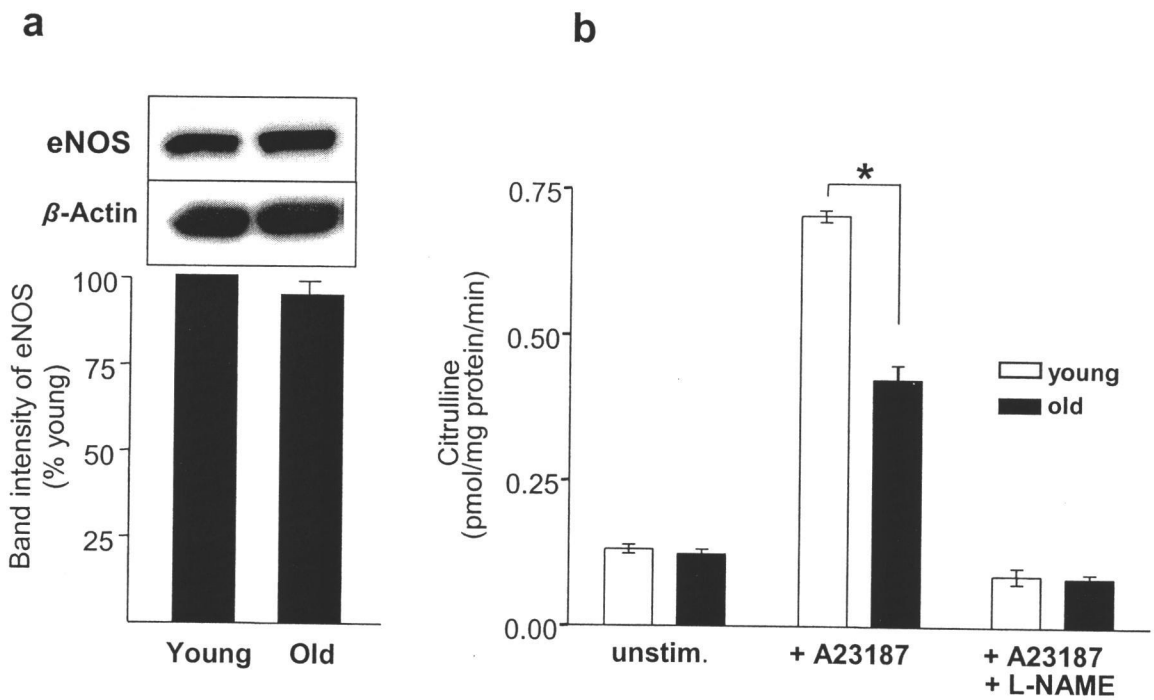

Figure 2.2. Age leads to significant loss of NO production with no change in eNOS protein expression. (a) eNOS protein levels are unchanged with age in the aortic endothelium. (b) Maximal NO synthetic capacity of endothelial cells was determined by stimulation with the calcium ionophore A23187. The NOS inhibitor L-NAME was used to show specificity of eNOS activity. Significantly decreased eNOS activity is a characteristic endothelium from old animals. n=4; *$p \leq 0.04$ according to unpaired Student's two-tailed *t* test; Western blots representative of n=4.

conversion of ^{3}[H]-arginine to ^{3}[H]-citrulline. Maximal NO production by young endothelium was estimated at 0.70±0.01 pmol/mg protein and was only 0.43±0.02 pmol/mg protein in old endothelium, representing a 40% loss of maximal NO synthetic ability with age (Fig. 2.2B; $P \leq 0.04$).

It is now widely understood that eNOS activity is governed by complex intracellular signaling mechanisms which include subcellular localization of eNOS, and by association of eNOS with inhibitory and stimulatory accessory proteins. To determine whether the subcellular localization of eNOS is altered in the aged endothelial cell, freshly isolated endothelium was isolated from the aortae of young and old rats and fractionated into soluble and particulate fractions by ultracentrifugation. Endothelial NOS protein levels in the particulate (plasma membrane vesicles) and soluble (cytosol and Golgi apparatus) fractions were analyzed by Western blot. Results demonstrate that here is significantly less eNOS protein (50%) in the plasma membrane fraction of old endothelium when compared to young (Fig. 2.3). This observation is important since eNOS in this fraction is considered to be the 'active pool' while Golgi-associated eNOS (found in the soluble fraction) does not contribute to vascular tone. No age-associated change in eNOS in the soluble fraction was observed (Fig 2.3). Therefore, significantly less eNOS is localized at the plasma membrane of the aged endothelial cell during the resting state. This result suggests that the age-associated loss of ACh-dependent vasodilation (Fig 2.1A) is due to a diminished presence of 'primed' (Ca^{+2} sensitive) eNOS.

The observed loss of eNOS protein in the active membrane pool correlates well with the age-associated loss of endothelial NO. Furthermore, since structural association with accessory proteins may govern the retention and activation of membrane associated eNOS we sought to assess whether aging affects eNOS association with the accessory proteins caveolin, Hsp90 and Akt. For this, caveolin, Hsp90 and Akt were immunoprecipitated from separate, freshly isolated endothelial membrane preparations. The immune complexes were then immunoblotted for eNOS protein. When caveolin-associated eNOS was compared between young and old endothelium, a significant doubling of eNOS bound to this inhibitory scaffold protein

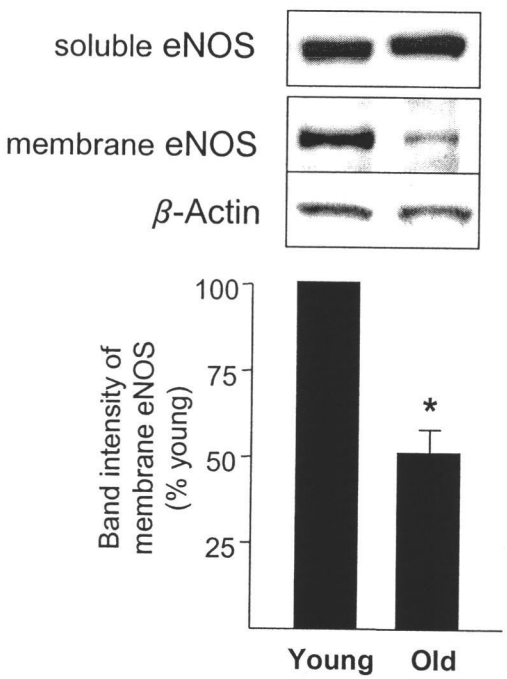

Figure 2.3. Endothelial NOS from old rats show a decreased presence at the endothelial cell membrane while the soluble fraction of eNOS remains unchanged. Translocation of eNOS from the Golgi apparatus to the cell membrane is a requisite for its proper function. Western blot analyses of baseline, unchallenged endothelial membrane fractions from young and old rats reveals that the latter have a significantly lower degree of association with the cell membrane. *$p \leq 0.02$ according to unpaired Student's two-tailed *t* test; Western blots representative of n= 4.

in the aged endothelium was noted (Fig 2.4A). This further reinforces the suggestion that though total cellular eNOS levels may not change with age, physical regulatory elements which control the enzyme may contribute to the aging phenotype.

Hsp90 facilitates the simultaneous dissociation from caveolin and insertion of Ca^{+2}/CaM into eNOS during the promotion of eNOS into the active complex. Therefore, the relative amount of eNOS associated with Hsp90 represents the active fraction of eNOS. Endothelial NOS association with Hsp90 was analyzed by immunoprecipitation of Hsp90 followed by Western blotting of the immune complex for eNOS. Results demonstrate the opposite of the previous findings with respect to eNOS-caveolin association. Hsp90-associated eNOS was approximately 50% lower in the endothelium of old animals when compared to young (Fig 2.4B). This significant decline ($P \leq 0.02$) suggests that at rest, the aged endothelium has less eNOS available for activation of NO synthesis.

Akt is the predominant kinase which phosphorylates eNOS at serine-1176 (rat sequence; serine 1177 human sequence). Upon stimulation of endothelial cells by growth factors, shear stress, estrogens and insulin, Akt binds to eNOS in an Hsp90-dependent manner. The Akt association with eNOS is enhanced by concomitant binding with Hsp90, forming a trimeric active complex of eNOS, Hsp90 and Akt. Therefore, like Hsp90 association with eNOS, Akt association with eNOS represents the active fraction which consumes arginine and produces NO. We analyzed Akt-associated eNOS by immunoprecipitation of Akt from young and old endothelial preparations and probed the immune complex for eNOS by Western blotting. Results show that old endothelial cells possess less than 50% of the Akt-bound eNOS found in young endothelium (Fig. 2.4C). This significant decline ($P \leq 0.02$) correlates well the previous data and further reinforces the suggestion that aging significantly alters the dynamics of subcellular localization and accessory-protein binding of eNOS in the endothelium and hence adversely affects the formation of the eNOS active-complex.

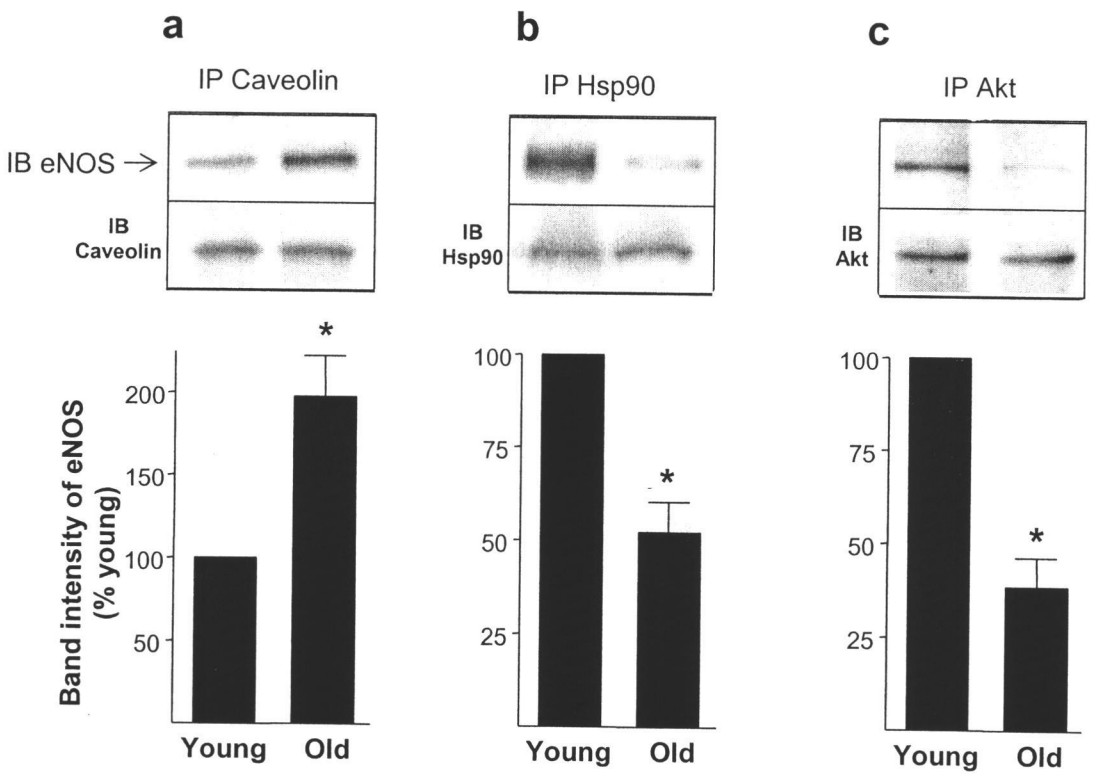

Figure 2.4. Endothelial NOS is less associated with positive-regulatory accessory proteins during aging. Endothelial NOS was co-immunoprecipitated (IP) from endothelial membrane fractions with antibodies to the accessory proteins caveolin, Hsp90 and Akt) and subsequently immunoblotted (IB) for eNOS. (a) Results show that the proportion of eNOS associated with the inhibitory scaffold protein caveolin is significantly elevated in the old endothelial membrane fraction when compared to the young animals. (b,c) eNOS in old endothelial membrane fractions bind less to Hsp90 and Akt under basal conditions when compared with the young. *$p \leq 0.02$ according to unpaired Student's two-tailed *t* test, n= 4.

2.5 Discussion

The age-related loss of endothelium-dependent vasorelaxation is an important observation which relates directly to personal and public health by contributing significantly to age-associated pathologies. This study suggests a potential mechanism which might mediate this loss on a cellular level. As shown by others [196, 207, 215], we also measure specific loss of endothelial-dependent vasodilatory ability in conjunction with significantly diminished maximal NO producing ability. This was performed in a physiological model using aortic ring myography as well as biochemically by measuring maximal NO synthesis in intact endothelium from old and young rats. These findings reinforce the near universality of the age associated decline in endothelial function [89, 187, 190, 207, 215] A unique aspect of the present work is that post-translational regulatory elements which are known to control eNOS activation are altered in the endothelium during the aging process. This work suggests for the first time that the cell biology of the aged endothelial cell is fundamentally altered in a way which leads to inactivation of the eNOS enzyme.

To confirm that aging leads to loss of endothelial NO-dependent dilation, a classical Ca^{+2} mobilizing agent, ACh was used to stimulate NO release from the endothelial layer of aortic rings. It is important to note that no age-associated differences were observed in the threshold concentration of ACh required to initiate vasorelaxation but that significant differences in vasorelaxation were observed at concentrations above $\sim 10^{-6}$ M ACh. Alterations in muscarinic receptor number or function could possibly explain this loss. However, van der Loo *et al* and Cernadas *et al* showed that this did not happen in old rats. They showed that the same age-associated vasodilatory loss is measurable in rings treated with either A23187 or Bradykinin—Ca^{+2} agonists which operate independently of the muscarinic receptor system [196, 207]. Furthermore, disturbance of the gyanylyl cyclase system in the aged vessel was also ruled out because no alteration in vasorelaxation was observed in nitroprusside-treated rings of young and old animals. Finally, the ACh concentration which gave 50% maximal relaxation of each respective ring (young and old) was

approximately the same (Fig 2.1). This subtlety suggests that Ca^{+2} mobilization in the aortic endothelium is unchanged with age. Taken together with the lack of change in total eNOS protein expression suggests that the levels of 'primed' eNOS (eNOS which is resting at the plasma membrane and thus is sensitive to Ca^{+2}) diminishes with age. This is supported by our results examining association of eNOS with regulatory proteins that, together for an active eNOS complex associated with the caveolae (Figs 2.3 and 2.4).

The fact that no age-related changes in eNOS total protein expression was observed is in line with that of Cernasad *et al* [196]. However, our results are on contrast to van der Loo *et al* who observed an age-related *increase* in eNOS expression using the same rat model as this study [207]. Still, other laboratories have reported losses in eNOS expression with age [216, 217]. Though the animal model used was the same in these two studies, the methodology of sample preparation was quite different. Our method of rapid freezing and scraping the endothelium from the luminal surface is theoretically superior at preserving protein-protein interactions and preventing degradation when compared to traditional methods of endothelial isolation which utilize prolonged periods at warm temperatures using collagenase preparations to loosen the endothelium from the vessel wall.

The observation that there is a nearly 50% decline in plasma membrane associated eNOS suggests that the loss should be observable when whole or unfractioned endothelium is probed for eNOS, though this is not the case. Thus it is important to note that approximately one-third of the total amount of protein analyzed in the membrane fraction was needed to observe eNOS in the soluble fraction. This suggests that a relatively large amount of presumably inactive eNOS is present in the Golgi apparatus of both young and old endothelium and the 50% loss of eNOS at the membrane fraction appears insignificant when total cellular eNOS is measured. Though a loss of membrane eNOS is observed, the sensitivity of Western blot techniques do not afford the resolution necessary to detect an age-associated difference in the overall amount of eNOS in the cell. This suggests that the major portion of eNOS in both young and old endothelium may reside in non-plasma

membrane cellular compartments. It would be interesting in future experiments to utilize fluorescence microscopy and immunohistochemical analysis of eNOS localization in the aortic endothelium to determine whether a similar age-associated alteration in eNOS subcellular localization could be visualized.

To initiate NO synthesis, the endothelial cell must execute two basic cellular processes: 1) Transport nascent eNOS protein from the Golgi apparatus to the plasma membrane. i.e. place it in the active or 'primed' pool of eNOS; and 2) Prime it for calcium sensitivity by presenting it with regulatory accessory proteins which facilitate its activation and deactivation. This work shows for the first time the resting levels of membrane eNOS which actively complex with Hsp90 and Akt is significantly lower in aged endothelium while the remainder of membrane eNOS remains with the inhibitory scaffold protein caveolin (Fig 2.4). This finding is made even more profound when viewed in context of the data presented in Figure 2.3 showing a 50% loss of plasma membrane associated eNOS. Thus the relatively robust eNOS band seen in Figure 2.3A generated by immunoprecipitation of Hsp90 from plasma membrane preparations of endothelial cells may represent *nearly all* of the eNOS present there. This notion is supported by the observation that the eNOS bands in Figure 2.3B and 2.3C of old endothelial membrane preparations is very low, in fact, near detection limits of Western blot visualization. Taken together, these findings, in part, begin to explain why the aged vasculature system is unable to adapt to physical exertion as well as the young since the pool of eNOS available for activation is low.

Why is there less eNOS in the membrane fraction of the aged animal? Relatively little is known of the regulatory aspects of the control of eNOS movement from the Golgi to the membrane. It is believed to be transported rapidly to and from there via vesicular transport in a similar manner as caveolin, and other caveolar constituents [108]. However, partial disruption of the Golgi apparatus with the compound brevifeldin-A *does not* inhibit agonist-induced NO-mediated vasodilation in porcine arterial rings, thus reinforcing the suggestion that the Golgi-associated eNOS pool is not active in making NO[100]. It has been suggested that though vesicular transport of eNOS to the plasma membrane my be governed by 'cellular

need' [85, 102, 111], *retention* of eNOS at the plasma membrane may be what is altered with age. Research suggests that Akt-associated eNOS remains at the plasma membrane even after deactivation and is thus re-associated with caveolin and remains in the 'primed' Ca^{+2}-sensitive pool of eNOS [95, 102, 228]. Thus diminished contribution of Akt to eNOS retention may be one mechanism which could ultimately lead to decreased association of eNOS with the plasma membrane over time.

Taken together, this work presents a good argument for further investigation of potential alterations in the cellular regulation of eNOS during age. In light of this research, the lack of clarity generated by previous research investigating this phenomenon is not surprising as its focus did not take into account the role of cellular regulation of eNOS. Future work which seeks to ascertain the specific mechanisms which cause the age-associated loss of endothelial NO must look deeper into this still-developing understanding of eNOS regulation if a thorough answer is to be gained. Ultimately, though the dysregulation of eNOS accessory protein association and subsequent alteration of subcellular localization in the aged endothelial cell may lead to the observed phenotype (e.g. loss of NO producing ability), the cause of this dysregulation remains unknown. Thus, a systematic search for a targeted therapy or candidates for pharmacologic intervention remain elusive. Future work should be directed at determining whether eNOS retention at the plasma membrane interface might be due to altered Akt status and concomittent alteration in eNOS phosphorylation. Further work is also warranted to investigate whether the loss of endothelial NO is fundamentally lost, suggesting something physiologically irrepairable, or whether a particular physiological stimulus is lost with age.

Chapter 3

Alterations in signal transduction lead to eNOS inactivation during aging.

Anthony Smith

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3.1 Abstract

Aging is the single largest risk factor for cardiovascular diseases, which in turn are the leading cause of death of individuals over the age of 65 years. In part, this risk is due to a profound loss of vasomotor function of the major conduit arteries, primarily because of lower levels of nitric oxide (NO), a potent vasorelaxing agent synthesized and released by the endothelium. The source of NO is the endothelial nitric oxide synthase (eNOS) protein. Alterations to eNOS which limit its synthetic activity are thought to be involved in the age-associated loss. An important component of the cellular regulation of NO synthesis is by phosphorylation of eNOS. The enzyme is regulated both positively and negatively by phosphorylation, affecting its sensitivity to intracellular Ca^{+2}. This work hypothesized that altered eNOS phosphorylation could contribute to the age-associated loss of endothelial NO. Results show that age alters eNOS phosphorylation in a pattern consistent with cellular inactivation (38% increase in inhibitory phosphorylation at eNOS threonine-494; 50% decline in stimulatory phosphorylation at serine-1176; n=4; $p \leq 0.02$). A significant increase in activity of the ceramide activated protein phosphatase 2A (PP2A) was also detected in the aged rat endothelium (n=4; $p \leq 0.01$), taken as evidence that protein kinase B (Akt)-mediated activation of eNOS might be undone by PP2A. Indeed, Akt activity levels decline significantly with age in the endothelium and these changes are concomitant with a decline in endothelial glutathione (GSH), increased sphingomyelinase activity (n=4; $p \leq 0.01$) and free ceramide lervels. The results suggest that age-related changes in eNOS phosphorylation, and perhaps more global alterations in endothelial signal transduction, may contribute to the physiological effects of aging and resultant loss of vasomotor function.

3.2 Introduction

Aging is one of the leading risk factors for cardiac diseases of all types. In particular, the aging process results in progressive loss of vascular endothelial function and resultant loss of vasomotor control (i.e. the inability of the vascular endothelium to relax the smooth muscle layer). The endothelium is a single cell layer which lines the entire vascular lumen. Endothelial cells synthesize and release the second messenger, nitric oxide (NO), which is the principal vasodilator of the vascular smooth muscle in large, conduit arteries. Age-related endothelial dysfunction is most predominantly observed in the conduit arteries such as the aorta, carotid, femoral, and brachial arteries, while little or no decline in endothelium-dependent vasodilation is seen in the peripheral and resistance arteries [204, 205]. While the mechanisms which lead to this loss are undoubtedly multifactorial, it is clear that there is loss of endothelial NO production and concomitant loss of NO-dependent vasorelaxation of the vascular smooth muscle layers [89, 187, 190, 202, 205, 215]. This age-dependent decline in endothelial function is not limited to humans and appears to be a part of the basic biology of aging; loss of vascular tone occurs in the large arteries of nearly all mammals with age [182, 186, 204, 205, 215].

Endothelial nitric oxide synthase (eNOS) is the enzyme which produces NO by oxidation of the terminal guanidine group of arginine. It has been hypothesized that the age-related loss of endothelial NO might be due to alterations in its bioavailability rather than the outright loss of NO synthesis. A strong body of work has focused on alterations to the endothelial redox environment, leading to oxidative inactivation of the NO signal. Most results reinforce the so-called, *oxidative stress theory* of aging in which cells of the body accumulate damage due to increased production of reactive oxygen species (ROS) which react with proteins, lipids, DNA and sugars. It is well known that superoxide reacts at diffusion-limited rates with NO. Several independent laboratories have reported age-associated increases in the appearance of ROS in many different tissue types, including the aortic endothelium, though specific identification of superoxide remains questionable [206-211]. Also supporting the *oxidative stress*

theory of aging is the observation that there is typically a loss of antioxidants in many tissues, including the endothelium, with age [47, 155, 212, 213]. While little definitive work has been done as to the specific site(s) of reactive oxygen species formation in the endothelium, it does appear that increased oxidative stress occurs in vascular endothelial cells with age [44, 206, 207, 214]. Elevated levels of ROS could play a significant role by contributing to the degradation of endothelial NO into nitrate, nitrite, peroxynitrite or other reactive nitrogen species, none of which are viable vasodilatory signal molecules. While this is a promising area of vascular aging research, the accurate and reliable measurement of specific ROS or their cellular sources, *in situ,* is beyond current technology. On a more concrete level, there is specific evidence that, independent of the loss of endothelial *NO bioactivity*, the enzymatic activity of eNOS significantly declines with age. Therefore, while ROS-mediated destruction of NO may contribute to the loss of endothelium-dependent vasodilation, alterations in cellular and biochemical regulation of the enzyme are likely to contribute independently to the age-dependent loss of NO.

It has also been hypothesized that age may alter the availability of critical substrates and cofactors required for NO synthesis. Specifically, limitation of the primary substrate, arginine, severely limits eNOS activity. The redox active cofactor tetrahydrobiopterin is also required for proper eNOS activity. However, recent research shows that arginine and tetrahydrobiopterin are *not limited* during aging in the endothelium [48, 218, 219]. Furthermore, arginine supplementation of elderly human patients suffering from essential hypertension has no clinical effect on blood pressure or tissue perfusion rates [89, 91]. To date, no work has been published which has analyzed endothelium for age specific changes in other eNOS cofactors such as NAD(P)H, FAD, FMN, iron or zinc.

Ruling out to possible loss of eNOS activity due to cofactor or substrate loss, the importance of the independent involvement of the *cellular* regulation of eNOS in the aging phenomenon is reinforced. On a cellular level the location of eNOS within the endothelial cell governs its ability to produce NO. The work described in chapter 2 of this dissertation characterized how a specific age-related loss of NO-dependent

vasomotor control is highly correlated with a decline in resting levels of eNOS protein localized to the endothelial cell plasma membrane. Furthermore, eNOS binding to its regulatory accessory proteins was altered with age, while no changes in overall expression of eNOS protein were seen in the endothelium of the aged animals. Therefore is follows that the signal transduction elements which control eNOS location and function may also e altered with age.

In addition to eNOS regulation by intracellular location and complexing with regulatory accessory proteins, the role of phosphorylation in eNOS control is now widely known [58, 61, 71, 97, 98, 112, 113]. Endothelial NOS, in its resting state, is constitutively phosphorylated at threonine 494 (T494 rat sequence; human T495). Endothelial NOS is a Ca^{+2}-dependent enzyme and T494 phosphorylation prevents the association of Ca^{+2}/CaM with the eNOS reductase domain, inhibiting NOS activity by blocking electron flow within the enzyme. Removal of T494 phosphorylation is a requisite for eNOS activation and is mediated by the phosphatase PP1 in direct response to intracellular Ca^{+2} ($[Ca^{+2}]_i$) [93, 114]. Endothelial NOS is also phosphorylated at serine 1176 (S1176 rat sequence; human S1177) predominantly by Protein kinase B (PKB, known as Akt), but also by PKA, AMP-activated kinase (AMPK), and calmodulin-dependent kinase (CaMK) [93, 117]. Phosphorylation at S1176 upregulates eNOS activity by stabilizing its association with Ca^{+2}/CaM after $[Ca^{+2}]_i$ declines. Thus S1176 phosphorylation is not a requisite for eNOS activation, but it amplifies the rate of NO synthesis as well as the duration of NO synthesis post $[Ca^{+2}]_i$ transient. Removal of S1176 phosphorylation is specifically mediated by protein phosphatase 2A (PP2A) which returns eNOS to its $[Ca^{+2}]_i$ –sensitive state. Thus, coordinated control of sustained endothelial NO production is balanced by the rates of Akt and PP2A activities.

Recently, there has been renewed interest in the role of cellular ceramides and sphingolipids in signaling. This is partly due to a renewed appreciation of the ceramide activated phosphatases (CAPs) in cellular signal transduction, including PP2A and additionally because of our rapidly evolving understanding of the role of oxidative stress and cellular inflammation in this complex system of lipid second-

messengers. Due in part to its abundance, glutathione (GSH) is one of the most important low molecular weight antioxidants in the cell. It functions not only as a scavenger of reactive oxygen species, but also as a detoxicant and as a redox buffer system which maintains the redox balance of intracellular thiols. Intracellular GSH levels are usually in the range of 1-5 mM. Our laboratory and others have showed that a hallmark of aging in many cell types is a loss of intracellular GSH levels compared to young cells [229-234]. Cellular GSH status is linked to control of the ceramide-activated phosphatases (i.e. PP2A) via its direct effect on the enzyme sphingomyelinase, which liberates ceramide from membrane sphingolipids.

In the plasma membrane of endothelial cells, sphingomyelinases with neutral pH optima (nSMase) are predominantly expressed; they are found concentrated in the caveolae in close proximity to enzymes such as eNOS, Akt, PP2A, PKC and others [134, 135]. There, it functions as a rapid source of ceramides which may act as activators and/or stimulators of various membrane component enzymes. Ceramidases, also localized in the plasma membrane, degrade ceramides to its components, serine and fatty acyl-CoAs, thus maintaining a homeostatic balance of ceramide levels. Neutral SMase is directly regulated by cellular redox status and by oxidative stress. Glutathione plays a major role in maintaining homeostatic cellular redox status and simultaneously nSMase activity *in vitro* and *in vivo* [140, 142, 143] The mechanism is thought to be due to GSH-mediated maintenance of critical thiol moieties on nSMase rather than allosteric binding [141-143]. In fact, depletion of GSH pharmacologically, or by TNF-α stimulation initiates rapid sphingomyelin hydrolysis by nSMase, leading to apoptotic cell death [138, 142]. Furthermore, Hannun's group has demonstrated that inhibition of nSMase or application of exogenous GSH (by GSH-ethyl ester repletion) prevents this TNF-α dependent apoptosis [142]. The observation that GSH inhibits nSMase is of utmost importance to our understanding of ceramide-mediated cellular dysfunction, since we and others have observed significant losses of intracellular GSH in the aged tissues of several animal models [234, 235].

To date little research has focused on whether eNOS control by the cellular signal transduction system might be altered with age. The work presented in chapter 2

of this dissertation serves to reinforce the concept. Therefore it was hypothesized that age leads to alterations in the phosphorylation state of eNOS which correlate with the observed loss of NO. Furthermore, it was hypothesized that alterations to eNOS phosphorylation might be brought about by low-GSH mediated activation of ceramides through sphingomyelinase activation.

3.3 Materials and Methods

The animal model. Throughout this study, Fischer 344 × Brown Norway hybrid (F344×BN) rats were used as the experimental model. This is a well-characterized rat strain that is an approved rodent model for aging studies by the National Institutes of Health and Aging (NIH/NIA). Young (2-4 months; corresponding to an adolescent human adult) and old (32-34 months; corresponding to a 65 –75 year-old human) rats were used for all studies. Although F344×BN rats do not develop atherosclerosis, they do develop the same age-related decline in vascular function as do humans and other mammalian species [224].

Preparation of vascular endothelium samples. Freshly isolated aortae from male F344×BN rats were perfused with Hank's Buffered Saline Solution (HBSS) pH 7.4 containing protease and phosphatase inhibitors, and then removed to a petri-dish. The aortae were opened longitudinally and adhered to poly-L-lysine coated glass, then frozen over liquid nitrogen. After freezing, the endothelial surface was carefully scraped from the vessel segments with a surgical scalpel and collected into homogenization buffer containing protease and phosphatase inhibitors.

Immunochemical analysis of endothelial proteins. Vascular endothelium was scraped from freshly isolated aortae from young and old rats as described above. Protein homogenates were quantified and prepared for Western blotting (after SDS-PAGE under reducing conditions). The immunodetected proteins were quantified by HRP-linked secondary antibodies and subsequent chemiluminescent detection. In some

experiments, plasma membrane proteins were first prepared by fractionation from the soluble cellular proteins by ultracentrifugation at 100,000g for 90 min at 4° C.

Vitamin C measurement. Vitamin C levels were measured as described by Frei *et al* [151]. Briefly, endothelial preparations were lysed in 10% (w/v) metaphosphoric acid containing 1 mM diethylamine triamine pentacetic acid (DTPA) to chelate transition metals. Insoluble debris was pelleted by centrifugation. The resulting supernatant was pH corrected and subjected to HPLC separation using a Hitachi L-6000 solvent delivery system and a Supelco LC-8 column (4.75 mm × 100 mm; 5 μm pore size). Ascorbate was monitored using electrochemical detection (applied voltage: +0.6 V). Vitamin C concentrations were quantified relative to standards.

Glutathione measurement. Endothelial preparations were lysed in 10% (w/v) perchloric acid. The acid soluble fractions containing GSH and glutathione disulfide (GSSG) were derivatized with iodoacetic acid (40 mM). The resulting carboxymethyl derivatives were dansylated by conjugation with dansyl chloride (75 mM) and separated by the method of Jones *et al* [236]. GSH and GSSG were separated by HPLC using fluorescent detection (Hitachi L7000; Ex/Em: 330/515nm). Quantification was achieved relative to standards using γ-glutamyl-glutamate as an internal control to assess completeness of derivatization.

Phosphatase activity. Total serine/threonine phosphatase activity in endothelial extracts was estimated using the IQ assay platform (Pierce, Rockford, IL). Briefly, cellular protein extracts were incubated with a proprietary synthetic phosphopeptide (LRRApSLG) which functions as a substrate for endogenous serine/threonine phosphatases. A developing agent confers fluorescent activity upon non-phosphorylated peptide. Thus, fluorescence intensity of the sample, post-development, is directly correlated with phosphatase activity over the time course of the assay. To insure specificity and accuracy, control experiments were run in parallel with the experimental samples including: omission of endothelial protein, omission of

the peptide substrate, addition of a phosphatase inhibitor cocktail (Sigma), 100% phosphorylated peptide substrate and 0% phosphorylated peptide substrate (Pierce, Rockford, IL).

In order to estimate the relative contribution of the phosphatase PP2A to overall phosphatase activity, phosphatase activity was measured in young and old endothelial samples in the presence of increasing concentrations of the phosphatase inhibitor okadaic acid (Sigma). The concentration of okadaic acid required to inhibit PP1 and other phosphatases is over 100 times that required to inhibit PP2A.

Ceramide measurement. Free ceramides in the endothelium were estimated using a modification of the diacylglycerol (DG) kinase assay [237]. Briefly, lipids were extracted from a known amount of endothelial protein and dried under N_2. The dried lipids were solublized by bath sonication in a detergent solution (7.5% (w/v) n-octyl-β-D-glucopyranoside and 5 mM 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]) and incubated with 5 U recombinant bacterial DG kinase and 4 μCi [γ-^{32}P]ATP for 2 h at 25°C. The reaction was quenched by addition of ice-cold methanol and the lipids were extracted and dried under N_2. The resultant [γ-^{32}P]-labelled phospholipids were seperated by thin layer chromatography. [γ-^{32}P]-labelled 1-phospho-ceramide bands were visualized by autoradiography and scraped from the TLC plates and quantified by scintillation counting. Since the DG kinase reaction is an entirely *in vitro* method, synthetic C_6-ceramide was included in all reactions as an internal standard to control for completeness of the reaction. Endogenous ceramide levels were quantified according to external standards which consisted of the use of synthetic C_{16}-C_{18}-ceramide in DG kinase reactions in place of sample lipids.

Sphingomyelinase assay. Endogenous neutral sphingomyelinase and ceramidase activities were estimated by incubation of endothelial membrane fractions with fluorescently-derivatized substrates (NBD-sphingomyelin, NBD-ceramide; Molecular Probes, Eugene, OR) *in vitro*. Briefly, endothelial membrane fractions are incubated with either NBD-sphingomyelin or NBD-ceramide in PBS pH 7.4 containing 5mM

MgCl for 1 h at 37°C. The reaction is terminated by addition of ethanol and the resulting solution is separated by HPLC with fluorescence detection (455/530nm; Ex/Em). Liberated NBD-ceramide (from sphingomyelinase activity) and NBD-fatty acid (from ceramidase activity) were quantified according to NBD-ceramide NBD-fatty acid standards.

3.4 Results

In order to determine whether age affected signal transduction controlled phosphorylation-dependent eNOS activation in the aorta, we analyzed the phosphorylation status of eNOS (S1176 and T494 residues) in the membrane and cytosolic fractions of endothelial cells. Results showed a 50% decline of eNOS S1176 phosphorylation in the membrane fraction of aortic endothelial cells (Fig 3.1A). No S1176 phosphorylation was detected in cytosolic fractions (data not shown), reinforcing the concept that eNOS in that fraction is not active in NO synthesis. When phosphorylation of T494 was analyzed, the opposite trend occurred; a 38% increase in phosphorylation was observed in the endothelial membranes from old rats compared to the young (Fig 3.1B). The ratio of S1176 to T494 phosphorylation, a proxy of the enzyme ability to properly catalyze NO formation, was 71% lower in old as compared with young rats (Fig. 3.1C).

As Akt is one of the predominant kinases responsible for S1176 phosphorylation and eNOS activation, we sought to determine whether the observed losses of eNOS activity were due to concomitant alterations of Akt status. Interestingly, no difference in T308 phosphorylation were observed, though Akt S473 phosphorylation declined by 37%, (Fig. 3.2A). Further, Akt kinase activity was measured by using a synthetic GSK-3β substrate and was found to decline significantly with age (Fig. 3.2B). T308 is the site of initial Akt activation by PI3-kinase dependent phosphorylation, though S473 phosphorylation is a requisite for Akt kinase activity. These results suggest an age-specific loss in endothelial Akt activation, while the differential decline in S473 phosphorylation further indicates the

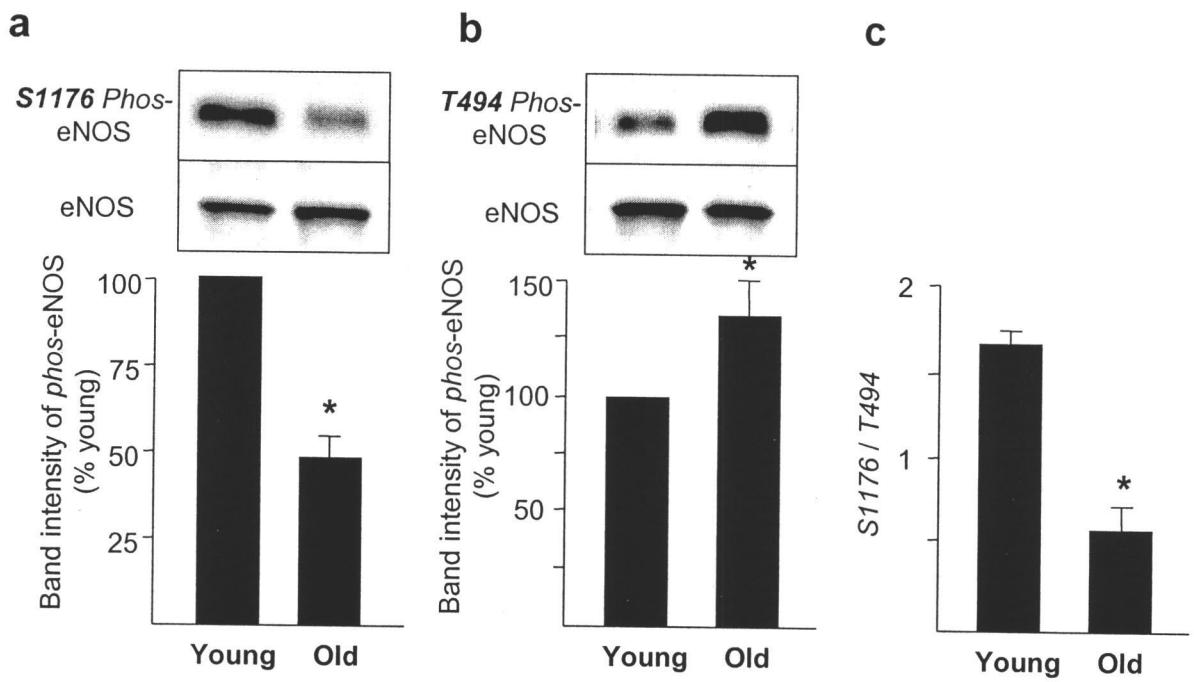

Figure 3.1. Endothelial NOS phosphorylation is adversely affected by age. The phosphorylation status of eNOS in membrane fractions of young and old rat aortic endothelium was analyzed. (a) Phosphorylation of eNOS at S1176 (which upregulates eNOS activity) is significantly ($p \leq 0.01$) decreased by age. (b) Conversely, phosphorylation of T494 (which prevents NO synthesis) is significantly ($p \leq 0.02$) increased. (c) As a result, the ratio of phosphorylation (S1176/T494) significantly ($p \leq 0.02$) declines by 71% in the aged endothelium. n= 4 for each assay.

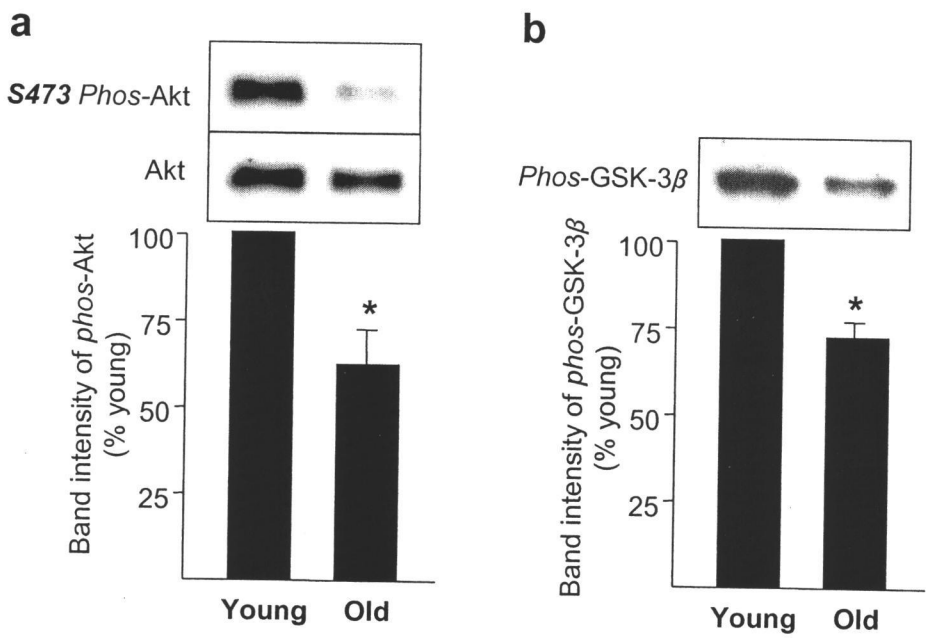

Figure 3.2. Akt is chronically hypoactivated in the aged endothelium. Phosphorylation of Akt at S308 remains unchanged during age. (a) However, in old endothelium, we observed a 37% loss of S473 phosphorylation of Akt ($p \leq 0.02$; n=6) which is required for Akt activity. (b) Akt-kinase activity was similarly impaired with a significant decline of 27% as assessed by the ability of Akt to phosphorylate a synthetic GSK-3β substrate ($p \leq 0.05$; n=4).

potential involvement of phosphatase activity, as loss of upstream activation of Akt would have resulted in loss of *both* S437 and T308 phosphorylation

Of significance, it is known that PP2A, a ceramide-activated phosphatase, is responsible for dephosphorylation of both S473 on Akt and S1176 of eNOS.

Together, this data suggests that the status of Akt in the old aortic endothelium would possess significantly less ability to properly phosphorylate eNOS.

To further establish this differential loss of phosphorylation and the potential role of PP2A in this process, experiments were performed to determine PPA activity in both young and old endothelium. First, because PP2A protein levels have not been previously determined in aging rat vascular endothelium, PP2A levels were determined by Western blot. PP2A levels in membrane fractions of young and old rat endothelium were unaltered indicating no alterations in either expression or turnover of the protein with age (data not shown).

To estimate the relative contribution of PP2A to overall serine/threonine (S/T) phosphatase activity, we capitalized on the heightened sensitivity of PP2A to okadaic acid compared to other S/T phosphatases (the IC_{50} of okadaic acid for PP2A is reported as ~ 0.5×10^{-9} M, while the IC_{50} for PP1 is ~1×10^{-8} M [238]. Overall S/T phosphatase activity was measured by incubating endothelial cell extracts from young and old animals with a fluorescently derivatized synthetic phosphorylated peptide whose fluorescence intensity was directly related to phosphorylation state. In unstimulated endothelial extracts, S/T phosphatase activity in aortic endothelial cells from old animals was more than double that seen in the young endothelium (Fig. 3.3A). When the assay was performed in the presence of increasing concentrations of okadaic acid, the observed age-associated increase in S/T phosphatase activity was abolished at very low concentrations (Fig. 3.3B). At higher concentrations of okadaic acid, there were no differences in phosphatase activity between young and old cells. This measurement strongly suggests that with age, there is higher PP2A phosphatase activity in the endothelium and that this alteration may be the cause for the observed loss of eNOS and Akt phosphorylation.

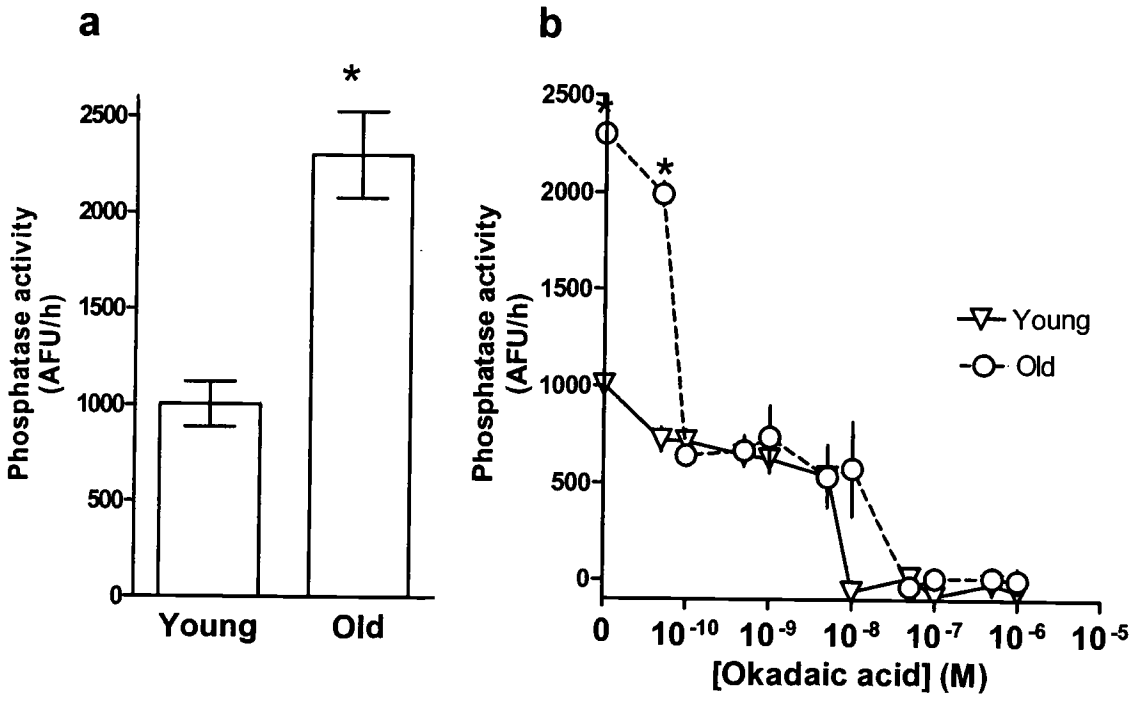

Figure 3.3. PP2A phosphatase activity is significantly elevated in the aged endothelium. Total serine/threonine (S/T) phosphatase activity was estimated in freshly isolated, unstimulated endothelium. (a) In the aged endothelium, S/T phosphatase activity is significantly elevated by a factor of 2.25 compared to the young ($p \leq 0.01$; n= 4). (b) S/T activity was inhibited by increasing concentrations of the S/T phosphatase inhibitor okadaic acid (OA). PP1 and other S/T phosphatases require ~100 times higher concentration of OA than PP2A for inhibition. The age-associated difference in activity is abolished at 10^{-10} M suggesting that the age-associated difference in phosphatase activity is due to PP2A.

Since PP2A is known as a ceramide-activated phosphatase, we hypothesized that the observed increase in PP2A activity seen with age might be due to an increase in the levels of free ceramides in the endothelium. When ceramides were measured in lipid extracts from young and old rat aortic endothelium, it was found that the levels significantly increased from 0.48±0.05 to 1.22±0.09 pmol/mg protein (Fig. 3.4; mean ± S.E.M.; $P \leq 0.01$).

nSMase and ceramidase activity in membrane fractions of endothelial cells from young and old rats was also analyzed since it is a major source of ceramide in cells. Results show that with age, nSMase activity increased significantly from 0.067±0.005 to 0.203±0.004 pmol ceramide/mg protein/h (Fig. 3.5A; mean ± S.E.M.; $P \leq 0.01$), though no change was detected in ceramidase levels (Fig 3.5B).

Due in part to its abundance, glutathione (GSH) is one of the most important low molecular weight antioxidants in the cell. Intracellular GSH levels are usually in the range of 1-5 mM. Age-associated declines in GSH status have been reported for many tissues and cells, but never in rat aortic endothelium. Therefore GSH was estimated in aortic endothelial preparations from young and old rats. (reduced GSH and GSH/GSSG ratio) in order to determine whether this might be one mechanism responsible for the observed increases in nSMase activity and the resultant increase in free ceramides. In young aortic endothelium, reduced GSH was 20.36±0.26 nmol/mg protein while old endothelial GSH was only 13.98±0.37 pmol/mg protein, a significant decline of 31% (Fig. 3.6A; mean ± S.E.M.; $P \leq 0.01$). When GSH/GSSG was plotted, statistically significant age-associated loss of 36% was noted (Fig. 3.6B; 41.11±6.38 vs. 26.11±2.6, young vs old respectively; mean ± S.E.M.; $P \leq 0.05$). This result reinforces the notion that aging leads to progressive impairment mediated in part by oxidative stress.

In addition to analyzing endothelial GSH, ascorbate levels were also measured in endothelial preparations from young and old animals. In the old cells, ascorbate was 6.69 ± 0.86 nmol/mg protein which was significantly lower than the young level of 14.60 ± 0.74 nmol/mg protein (Fig 3.7; n=4; $p \leq 0.01$).

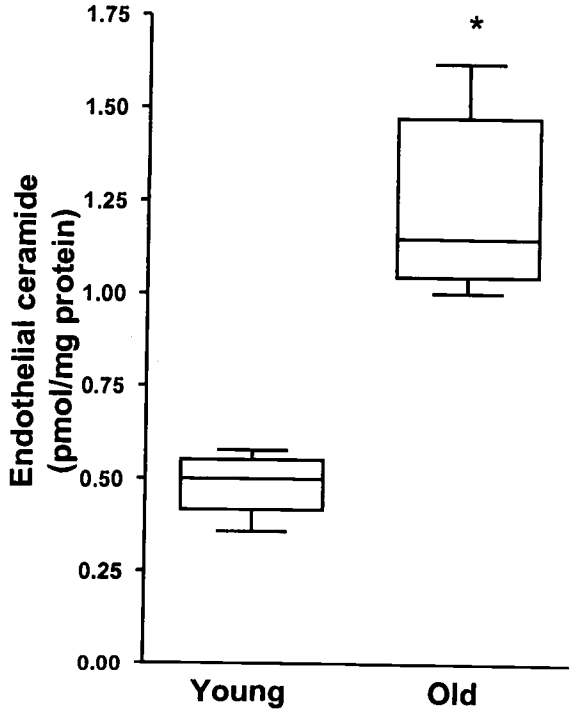

Figure 3.4. Free ceramide levels are elevated with age in the endothelium. Total ceramide levels were measured in freshly isolated endothelium from young and old animals. In the aged endothelium, ceramides were more than double the level in young animals. *$p \leq 0.01$ according to unpaired Student's two-tailed *t* test, n= 4.

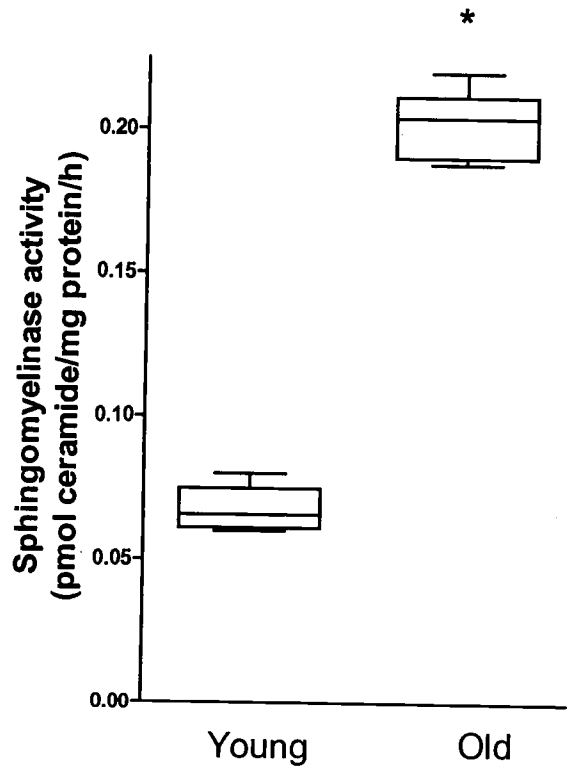

Figure 3.5. Neutral-isoform sphingomyelinase (nSMase) activity is significantly elevated in the aged endothelium. Cellular ceramides are generated in part by the action of sphingomyelinases on membrane sphingolipids. Enzymatic activity of nSMase was measured in endothelium isolated from young and old animals. Enzymatic activity in the old endothelium was nearly triple the activity seen in the young (*$p \leq 0.01$ according to unpaired Student's two-tailed *t* test, n= 4). In order to confirm specificity of the assay, the nSMase inhibitor desipramine was used as a negative control and inhibited the assay by over 85% (data not shown).

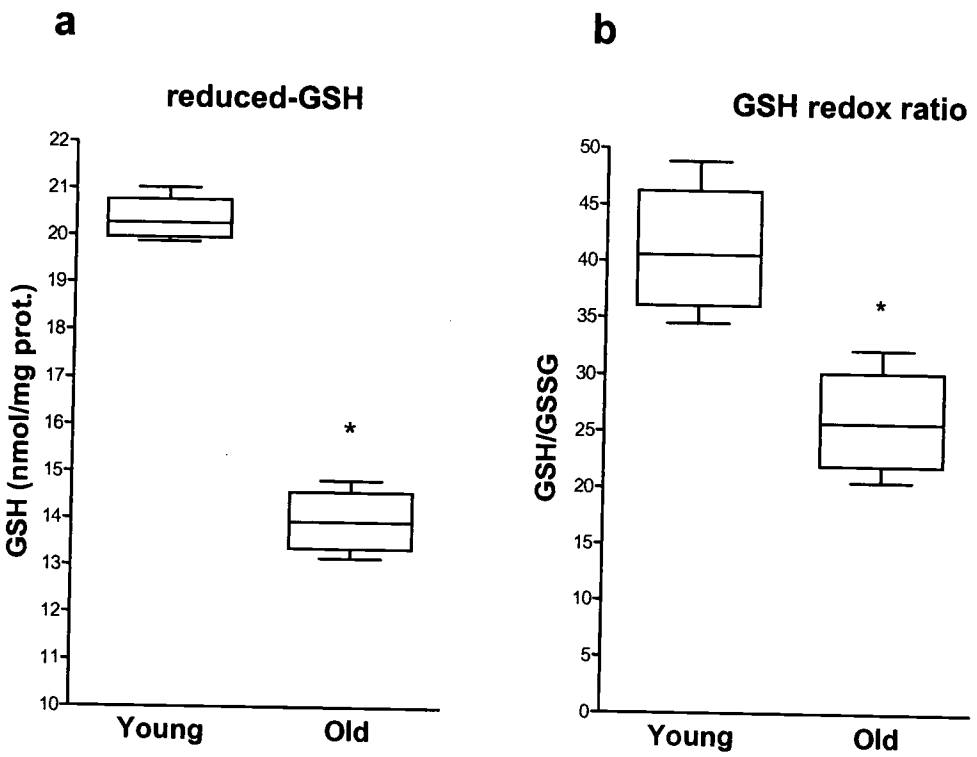

Figure 3.6. Glutathione levels in the aortic endothelial cells is significantly compromised with age. (a) Reduced GSH levels were measured by HPLC in acid extracts from young and old endothelium. Age results in a 31% decline in endothelial GSH. (b) The thiol redox ratio is an indication of cellular health and was estimated by calculation of the reduced:oxidized glutathione ratio (GSH:GSSG). In the aged endothelium, the GSH redox ratio was significantly lower (36%) in the old compared to young animals. *$p \leq 0.01$ according to unpaired Student's two-tailed *t* test, n= 4 for all groups.

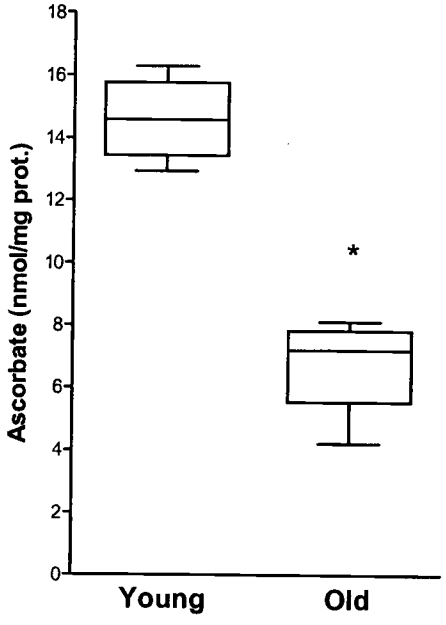

Figure 3.7. Endothelial ascorbate levels significantly decline with age. Ascorbate (vitamin C) was estimated in endothelial preparations from young and old animals. In the old cells, ascorbate was 6.69 ± 0.86 nmol/mg protein which was significantly lower than the young level of 14.60 ± 0.74 nmol/mg protein. *$p \leq 0.01$ according to unpaired Student's two-tailed *t* test, n= 4.

We determined that heightened phosphatase activity may be responsible for losses in eNOS and Akt phosphorylation and activation. In part, the phosphatase stimulation may be due to excess nSMase activity. Therefore, to test the concept, we determined whether inhibition of the nSMase in vascular rings could restore eNOS-dependent vasodilation. Rings pre-incubated with the nSMase inhibitor GW4869 were pre-contracted with phenylepherine and dose dependently relaxed with acetylcholine. We found that GW4869 pre-incubation partially restored endothelium-dependent vasodilation response of the old vessel rings, while it did not affect the relaxation profile of the young vessels (Fig. 3.8).

3.5 Discussion

Based in part on the findings of our previous work which assessed eNOS binding with accessory proteins and in particular on the observation that eNOS is significantly less-associated with Akt in old endothelium, we hypothesized that the phosphorylation state of eNOS might also be altered with age as well. Indeed this work demonstrates that basal phosphorylation status of plasma membrane eNOS suggests a state of inactivity. This observation regarding eNOS signal transduction is mirrored by a similar decline in Akt phosphorylation and activation. Though Akt is the predominant kinase which acts on eNOS, phosphorylating it at S1176, the potential role or contribution of other kinases such as PKA, AMPK and CaMK cannot be ruled out. Ultimately, any further study in this field might benefit from an analysis of expression and activity of these kinases during the aging process in order to clarify whether the mechanisms explored in this work have relevance to those pathways as well. Regardless, the data suggests that either the Akt-dependent pathways are fundamentally understimulated in the aged endothelium, or that another mechanism is responsible for overactivation of phosphatases.

Though he decline in Akt phosphorylation and concomitant loss of Akt kinase activity in the aged endothelial cell may suggest understimulation by the kinase, we hypothesized that the phenotype was due to aberrant phosphatase activity. This

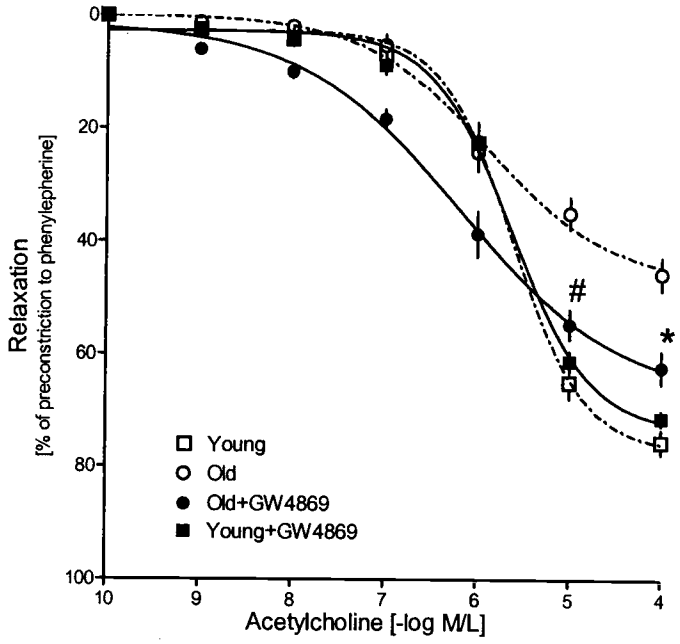

Figure 3.8. Inhibition of neutral sphingomyelinase partially restores endothium-dependent vasorelaxation. The specific neutral sphingomyelinase (nSMase) inhibitor GW4869 was preincubated with vessel rings from young and old animals for 1h prior to myography. Inhibition of nSMase partly restored the age-associated loss of acetylcholine (ACh)-dependent vasorelaxation in the old vessel rings while it had no effect on vasomotor action of the young vessels (solid lines). ACh-dependent dilation of untreated young and old vessels is shown for comparison (dotted lines). *$p \leq 0.05$ compared to old untreated using Student's unpaired *t* test; # $p \leq 0.05$ compared to young or to old untreated; n= 3 for GW4869-treated vessels.

hypothesis was drawn for the following reasons. First, while multiple kinases stimulate eNOS by phosphorylating it at S1176, only one enzyme is known to remove that phosphorylation, PP2A [127]. Interestingly, PP2A is responsible for the removal of phosphorylation from numerous substrates of Akt including Bad, FKHR, GSH-3β, CREB and IRS-1 [115, 238-240]. Secondly, global hypoactivation of Akt and/or other pro-survival kinase such as CaMK, AMPK and PKA would presumably result in cell death or gross endothelial dysfunction with complications greater than the observed alteration in eNOS activity. Finally, despite the observed loss of Akt activity and S473 phosphorylation, the subtle observation that there was no difference in Akt T308 phosphorylation (the target site for PI_3/PDK-dependent activation of Akt) suggests that the signal transduction elements upstream of Akt are functional. This observation is reinforced by the work of Schubert *et al* who demonstrated that exposure of human TF-1 cell lines to ceramide results in PP2A-dependent removal of S473 from Akt and concomitant loss of Akt activity, with no alteration in S308 phosphorylation [145]. Together evidence suggested that serine/threonine phosphatase activity might be the cause of eNOS and Akt attenuation rather than a kinase effect.

When endothelial serine/threonine (S/T) phosphatase activity was measured, endothelial preparations from young animals displayed approximately 40% of the baseline phosphatase activity as old animals. Seeking a measure of specificity in this assay, phosphatase activity was titrated with the selective PP2A inhibitor okadaic acid (OA). In vitro, the reported IC_{50} of PP2A for OA is approximately 0.5 nM, while the IC_{50} for PP1 and other S/T phosphatases is greater than 50 nM [145, 238]. In this assay, the age-related increase in S/T phosphatase activity was abolished at approximately 0.5 nM OA, suggesting that the age-associated difference in phosphatase activity in the absence of OA is attributable to PP2A. PP2A is classified as a ceramide-activated phosphatase. Furthermore a large body of research supports the assertion that nearly all metabolically active tissues suffer from various oxidative and inflammatory stresses which are brought about by the aging process [193, 207,

213, 221, 241, 242]. Increased ceramide is a classical cell response to both oxidative and inflammatory stressors [123, 235].

Ceramides are derived biologically either from *de novo* synthesis of ceramide from fatty acids and serine substrates, or by liberation of free ceramide from sphingolipids by sphingomyelinases. The potential contribution of *de novo* ceramide synthesis towards the observed age-related increase in endothelial ceramides was not addressed and therefore it cannot be ruled out. However, a significantly large increase in nSMase activity is seen in the aging endothelium suggesting that sphingomyelinase is in part responsible for the observed increase in ceramides. This assertion is reinforced by the concomitant loss of endothelial GSH, which is a known stimulus for nSMase induction [141, 143]. Future work in this field should address the potential for the *de novo* pathway to contribute to this phenomenon. The rationale for this is that aging may affect mitochondrial metabolism, leading to alterations in free fatty-acid levels. Surplus fatty acids are a stimulus for *de novo* ceramide synthesis. Furthermore, inhibition of ceramide *de novo* synthesis has been shown to lower cellular ceramides and stimulate eNOS/Akt activity in pig and rat aortae [24, 243].

The observed age-related losses in endothelial GSH and the thiol redox ratio are not surprising since depletion of intracellular GSH seems to be a common characteristic in many aging tissues [229-234]. The same result has been observed in many different cell types by this laboratory and in others [229-234]. However, since compromised GSH status is a potent inducer of nSMase activity this work suggest that in the endothelium, this may account for a major portion of the net losses in the signal transduction lesions which lead to loss of eNOS activation.

In an attempt to test the concept that sphingomyelinase hyperactivation is responsible for the age-related losses in NO-dependent vasodilation, the enzyme was inhibited with the nSMase inhibitor GW4869. The working theory was that if nSMase activity was the causative mechanism which leads to elevation of cellular ceramides and resultant PP2A-mediated blunting of eNOS/Akt, its inhibition should at least in part, should restore endothelium-dependent vasodilation in response to acetylcholine (ACh). While significant improvement was seen in the vasorelaxation profile of

GW4869 treated rings from old animals compared to untreated rings from old animals, the improvement in vasodilation was not complete. Interestingly, preincubation of rings from young animals with the GW4869 compound had no effect on vasodilation, Several possibilities might explain the lack of complete restoration of endothelium-dependent vasodilation in the old vessel rings. One probability is that pharmacological alteration of cellular ceramide levels is likely to affect numerous signal transduction pathways. This is because ceramide-dependent signaling exerts its effects on cell signaling by alteration of membrane lipid microdomains near the signal transduction machinery [135, 138, 244]. Thus the use of GW4869 may involve unrelated signaling elements which alter endothelial function. Alternatively, the lack of complete restoration of endothelium-dependent vasodilation may be due simply to insufficient time or GW4869 concentrations.

Taken together, this work demonstrates for the first time that alteration of cellular signal transduction can occur in the vasculature during aging and that these alterations can lead to profound effects on the function of cells. To date, most aging research has focused on assessment of cellular damage, depletion of cellular substrates or altered gene expression. Therefore the findings presented here may expand the scope of what we know to occur during aging. The findings presented here are also promising because they show a connection between a physiological outcome (loss of endothelial NO) and a common phenotype seen in aging cells and tissues (loss of antioxidants and oxidative stress). Future work should focus on strengthening two main concepts. First evidence for the *direct* involvement of PP2A is necessary. Secondly it must be determined whether repletion of endothelial GSH is possible; If so, does it reverse the age associated increases in nSMase activity, ceramide and ultimately endothelial NO?

Chapter 4

Acute administration of lipoic acid partially restores eNOS function.

Anthony R. Smith

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4.1 Abstract

Aging results in a dramatic loss of endothelium-dependent vasorelaxation of conduit arteries due to in part to age-specific loss of NO synthetic capacity. Our previous work with the thiol agent (R)-α-lipoic acid (LA) showed that the compound improved eNOS activity in cultured human endothelial cells that it increased stimulatory phosphorylation of eNOS and Akt in the endothelium of aged rats. However, no research focused on determining mechanism by which LA effects the endothelium We tested the role of pharmacologic administration of LA (40 mg/kg, i.p. over 24 h) on old endothelium in F 344 x BN hybrid rats (32-34 months; corresponding to a 65 – 75 year-old human). Results show that LA significantly improved the age-associated loss of endothelial glutathione (GSH; n=3; $p \leq 0.05$ compared to saline controls). LA treatment of the old animals significantly lowered membrane sphingomyelinase activity (n=4; $p \leq 0.01$) resulting in complete reversal of the age-associated increase in ceramide in old animals (n=4; $p \leq 0.01$). Finally, LA significantly improved endothelium-dependent vasodilation of aortic rings from old animals, suggesting that it might be a good therapeutic agent for age-related vascular endothelial dysfunction.

4.2 Introduction

Aging leads to profound and specific losses in endothelial nitric oxide-dependent vasodilation of conduit arteries. Our previous work characterized specific alterations to endothelial nitric oxide synthase (eNOS) which occur during aging, finding that eNOS subcellular localization to the active plasma membrane pool of the enzyme was significantly diminished with age. Further research demonstrated that this loss was concomitant with altered eNOS phosphorylation status suggesting that it is less sensitive to classical activation pathways in the endothelium. Searching for a biochemical mechanism for these observations, we discovered that the root of this age-associated alteration in eNOS control stemmed from depleted thiol redox status and diminished intracellular glutathione (GSH). Though the finding that GSH was lost with age in the aortic endothelium has not been documented previously, the observation is not surprising since GSH loss is a common feature of aging cells [230-232, 234, 245]. Furthermore, an association between the age-related loss of GSH and activation of endothelial sphingomyelinases was noted. This correlation establishes a rationale that altered redox state affects endothelial ceramide status and in turn leads to increased free cellular ceramides, which are known to activate the protein phosphatase 2A (PP2A). PP2A is a major cellular phosphatase which directly lowers both eNOS and Akt activity by removing phosphorylation from critical serine residues. Thus pharmacological agents that could break the cycle of redox changes and deramide-dependent PP2A activation would provide significant therapeutic potential for maintenance of vasomotor function during aging.

α-Lipoic acid (LA) is a dithiol compound derived from octanoic acid and is a necessary cofactor for mitochondrial α-ketoacid dehydrogenases and thus serves a critical role in energy metabolism. Historically, research related to LA has focused on its role as a protein-bound cofactor for active acyl-group transfer reactions. However, there is a growing awareness that LA, which transiently accumulates in a variety of tissues following supplementation, may be an effective pharmacotherapeutic agent against a host of pathophysiologic insults. LA has been described as a potent

antioxidant, as a detoxication agent for heavy metal poisoning, and has been implicated as a means to improve age-associated cognitive decline [177, 178]. Most importantly, LA has been used extensively as a therapy for complications associated with diabetes mellitus, especially to improve glucose handling and reduce diabetes-associated polyneuropathies [246-253]. A quick perusal of this partial list shows the diversity of this compound to mitigate at least certain effects of both chronic and acute conditions.

While the structural/chemical properties of LA that may be partly responsible for some of its therapeutic roles are understood, other biochemical and cellular mechanisms where LA may act are only now becoming known. One of the most commonly reported pharmacologic activities of LA is an improvement in tissue and cellular GSH levels [234, 254-260]. Considering this, we hypothesized that if pharmacologic treatment with LA (40 mg/kg, i.p. over 24 h) could be used to improve endothelial GSH status in the aged rat, it might also improve endothelial-NO-dependent vasomotor function by lowering ceramides and preventing PP2A phosphatase hyperactivation, thus preserving eNOS phospho-activation. This hypothesis is already strengthened by previously published work from our laboratory which showed that improvement in the age-related decline of eNOS *and* Akt phosphorylation is seen 12 h after intraperitoneal (i.p.) administration of LA (40 mg/kg) [261]. With this in mind old animals were treated with either saline-vehicle or pharmacologic doses of *R*-α-lipoic acid (40 mg/kg, i.p.) over 24 h and the effects of LA on endothelial GSH, sphingomyelinase activity, free ceramides and endothelium-dependent vasodilation via aortic ring myography were observed. Results demonstrate that LA restored a number of critical factors related to control of eNOS activity and a potential for lipoic acid as a therapeutic agent for age-associated endothelial dysfunction.

4.3 Materials and Methods

The animal model. Throughout this study, Fischer 344 × Brown Norway hybrid (F344×BN) rats were used as the experimental model. This is a well-characterized rat strain that is an approved rodent model for aging studies by the National Institutes of Health and Aging (NIH/NIA). Young (2-4 months; corresponding to an adolescent human adult) and old (32-34 months; corresponding to a 65 –75 year-old human) rats were used for all studies. Although F344×BN rats do not develop atherosclerosis, they do develop the same age-related decline in vascular function as do humans and other mammalian species [224].

Preparation of vascular endothelium samples. Freshly isolated aortae from male F344×BN rats were perfused with Hank's Buffered Saline Solution (HBSS) pH 7.4 containing protease and phosphatase inhibitors, and then removed to a petri-dish. The aortae were opened longitudinally and adhered to poly-L-lysine coated glass, then frozen over liquid nitrogen. After freezing, the endothelial surface was carefully scraped from the vessel segments with a surgical scalpel and collected into homogenization buffer containing protease and phosphatase inhibitors.

Isolated aortic ring myography. Segments of thoracic aorta were cleaned of adherent connective tissue, cut into 3-5 mm long rings and suspended in an organ-bath chamber containing Krebs-Henselheit solution, gassed with 95% O2 and 5% CO_2, and maintained at 37°C. Tissues were mounted on an isometric force-displacement transducer (Kent Scientific, Torrington, CT) and changes in isometric forces were continuously recorded. Rings were gradually stretched to 1-1.5 g and allowed to equilibrate for 90 min. Maximal contractility was evaluated by the addition of KCl 60 mM. After washing and further equilibration, the rings were contracted with $3x10^{-7}$ M norepinephrine. After stabilization (10-15 min), relaxation was assessed by the cumulative addition of acetylcholine (10^{-10} to 10^{-4} M). Sodium nitroprusside (10^{-10} to 10^{-5} M) was used to evaluate endothelium-independent vasorelaxation. As a negative

control to demonstrate the specificity of ACh stimulation of endothelial NO, myography was performed on rings in which the endothelium was mechanically damaged by rubbing.

Glutathione measurement. Endothelial preparations were lysed in 10% (w/v) perchloric acid. The acid soluble fractions containing glutathione (GSH) and glutathione disulfide (GSSG) were derivatized with iodoacetic acid (40 mM). The resulting carboxymethyl derivatives were dansylated by conjugation with dansyl chloride (75 mM) and separated by the method of Jones *et al* [236]. GSH and GSSG were separated by HPLC using fluorescent detection (Hitachi L7000; Ex/Em: 330/515nm). Quantification was achieved relative to standards using γ-glutamyl-glutamate as an internal control to assess completeness of derivatization.

Ceramide measurement. Free ceramides in the endothelium were estimated using a modification of the diacylglycerol (DG) kinase assay [237]. Briefly, lipids were extracted from a known amount of endothelial protein and dried under N_2. The dried lipids were solublized by bath sonication in a detergent solution (7.5% (w/v) n-octyl-β-D-glucopyranoside and 5 mM 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]) and incubated with 5 U recombinant bacterial DG kinase and 4 μCi [γ-^{32}P]ATP for 2 h at 25°C. The reaction was quenched by addition of ice-cold methanol and the lipids were extracted and dried under N_2. The resultant [γ-^{32}P]-labelled phospholipids were seperated by thin layer chromatography. [γ-^{32}P]-labelled 1-phospho-ceramide bands were visualized by autoradiography and scraped from the TLC plates and quantified by scintillation counting. Since the DG kinase reaction is an entirely *in vitro* method, synthetic C_6-ceramide was included in all reactions as an internal standard to control for completeness of the reaction. Endogenous ceramide levels were quantified according to external standards which consisted of the use of synthetic C_{16}-C_{18}-ceramide in DG kinase reactions in place of sample lipids.

Sphingomyelinase assay. Endogenous neutral sphingomyelinase and ceramidase activities were estimated by incubation of endothelial membrane fractions with fluorescently-derivatized substrates (NBD-sphingomyelin, NBD-ceramide; Molecular Probes, Eugene, OR) *in vitro*. Briefly, endothelial membrane fractions are incubated with either NBD-sphingomyelin or NBD-ceramide in PBS pH 7.4 containing 5mM MgCl for 1 h at 37°C. The reaction is terminated by addition of ethanol and the resulting solution is separated by HPLC with fluorescence detection (455/530nm; Ex/Em). Liberated NBD-ceramide (from sphingomyelinase activity) and NBD-fatty acid (from ceramidase activity) were quantified according to NBD-ceramide NBD-fatty acid standards.

4.4 Results

LA has proven to be an excellent compound for improving intracellular GSH [256, 258, 262-266]. In the previous chapters, we characterized certain age-related changes to the signal transduction network which governs eNOS activation. Since the mechanistic explanation suggested a correlation with the age-associated loss of antioxidants, namely GSH, we sought to determine whether endothelial GSH status in the aged animal might be sensitive to pharmacologic manipulation by LA. In the previous chapter, we measured a 31% decrease in endothelial GSH levels and a 36% decline in the GSH/GSSG thiol redox ratio (Fig 3.6). Because of this, LA (i.p.; 40 mg/kg body weight) or sterile saline solution was administered to old rats, twice over a 24 hr period (at 0 h and 12 h, followed by sacrifice at 24 h) and isolated aortic endothelium. In order to determine whether any effect LA might have on the endothelium might be specifically attributable to increases in intracellular GSH, GSH-ethyl ester (i.p.; 40 mg/kg body weight) was also administered to a group of old animals according to the same schedule. Results showed that saline injections had no effect on GSH status (data not shown). However, the endothelial cells of LA treated rats showed a statistically significant improvement in reduced-GSH levels compared to either untreated (or saline treated) old animals (Fig. 4.1; 18±0.7 vs. 14±0.4 pmol/mg

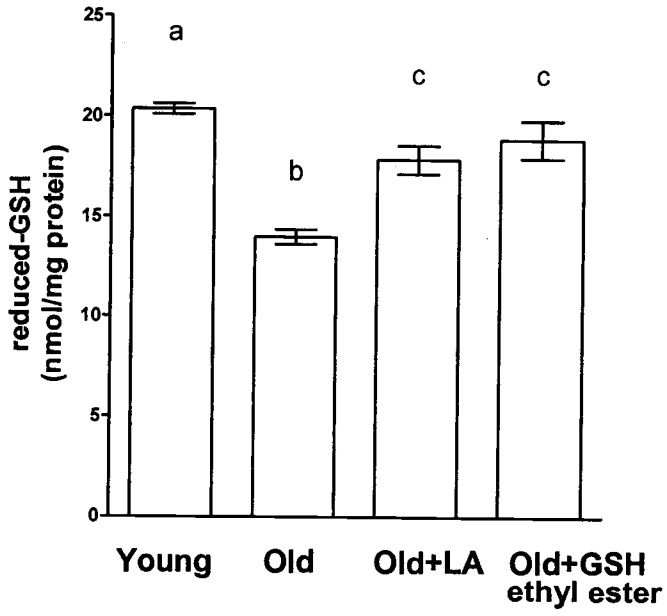

Figure 4.1: Lipoic acid (LA) improves the age-associated loss of endothelial glutathione (GSH). Pharmacologic administration of LA to old animals over 24 h significantly improved reduced-GSH levels. This was comparable with similar administration of GSH-ethyl ester which also significantly improves endothelial GSH in the old animals. Unique letters designate statistically significant differences in results ($p \leq 0.05$) using ANOVA with Tukey's post hoc analysis; n= 3 for LA and GSH-ethyl ester treated groups; n= 4 for others.

protein respectively; n=3; $p \leq 0.05$ compared to old controls). A similar effect on endothelial GSH levels was observed when GSH-ethyl ester was administered to old

animals. After this treatment, endothelial GSH levels increased to 19±0.9 pmol/mg protein (compared to 14±0.4 pmol/mg for untreated old animals), a significant improvement of nearly 33% over untreated old rats (Fig 4.1; n=3; $p \leq 0.05$ compared to old controls). An important observation stemming from this experiment comparing the effects of LA and GSH-ethyl ester is the suggestion that one specific effect of pharmacologic administration of LA is improvement of cellular GSH status.

Assessment of cellular thiol redox ratio (GSH/GSSG) was also determined by measuring both reduced and oxidized GSH by HPLC. Though LA treatment tended to increase the endothelial GSH/GSSG (35±2.9 vs. 26±2.6 pmol/mg in old controls), the difference did not reach statistical significance. However, GSH-ethyl ester administration significantly increased the GSH/GSSG by 42% over old control animals (Fig. 4.2; 36±1.5 vs 26±2.6 pmol/mg protein; $p \leq 0.05$).

In the previous chapter, we described an aging mechanism in the aortic endothelium whereby neutral sphingomyelinase (nSMase) activity is elevated in old endothelium when compared to young. Low GSH is a potent trigger for activation of nSMase activity. Since pharmacologic administration of LA partially reversed the age-associated loss of intracellular GSH in the endothelium, we hypothesized that LA treatment might be able to reverse the activation of nSMase in the aged endothelium. LA treatment significantly lowered the activity of nSMase in freshly isolated endothelium from old animals (Fig. 4.3; 0.14±0.01 vs 0.20±0.01 pmol ceramide/mg protein/h in old controls; $p \leq 0.01$) while ceramidase activity was unaffected (data not shown).

We previously hypothesized that an age-related increase in free ceramides in the endothelium was responsible for PP2A hyperactivation and resultant loss of eNOS activity. Since LA treatment improved GSH in the aged endothelium and significantly lowered nSMase activity, we measured free ceramide levels in the endothelium of

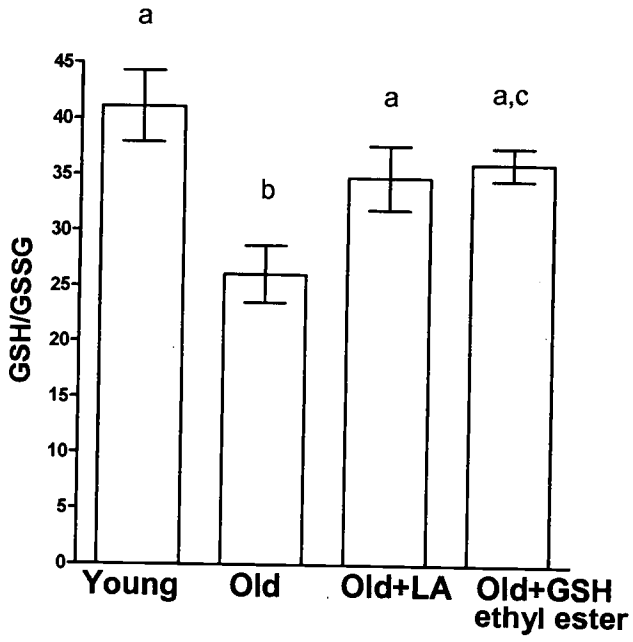

Figure 4.2. LA improves the thiol redox ratio in old animals. The thiol redox ratio tended to increase with 24h LA administration, though did not reach statistical significance over saline controls. Similar administration of GSH-ethyl ester did increase the GSH redox ratio significantly over saline and untreated controls. Unique letters designate statistically significant differences in results ($p \leq 0.05$) using ANOVA with Tukey's post hoc analysis; n= 3 for LA and GSH-ethyl ester treated groups; n= 4 for others.

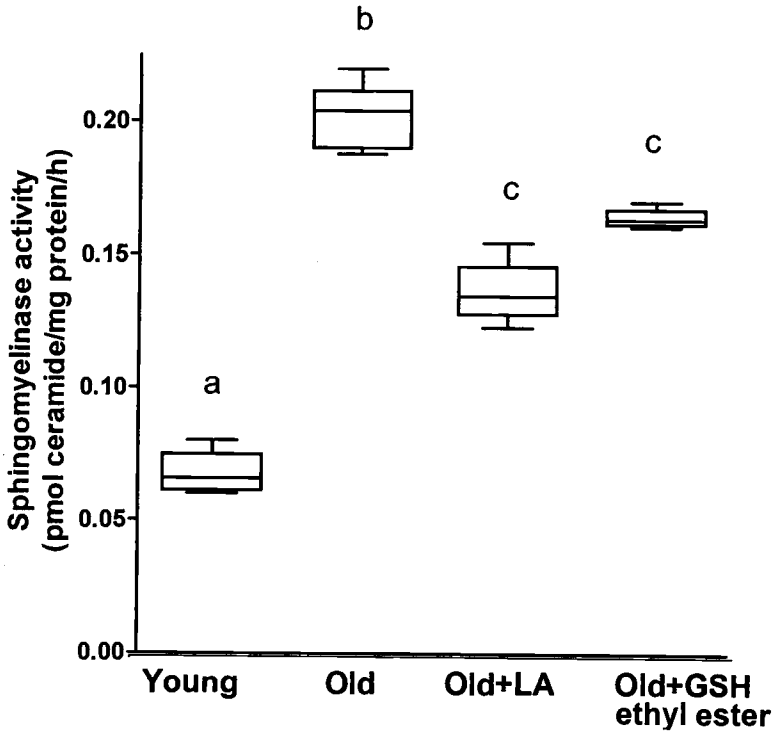

Figure 4.3. LA lowers endothelial neutral SMase activity. After pharmacologic treatment of old animals with LA or GSH-ethyl ester, nSMase activity was estimated in plasma membrane preparations of aortic endothelium. Both LA and GSH-ethyl ester significantly lowered nSMase activity. The observation that GSH-ethyl ester worked in a similar manner to LA suggests that the effect by LA is due to improvement in endothelial GSH. Unique letters designate statistically significant differences in results ($p \leq 0.05$) using ANOVA with Tukey's post hoc analysis; n= 4.

aged rats after administration of LA. Compared to the old controls, LA treatment significantly lowered free ceramide levels by 46% (Fig. 4.4; 0.66±0.04 vs. 1.2±0.09 pmol/mg protein in old controls: $p \leq 0.01$). Thus, LA nearly restored ceramide to the levels typically found in young endothelium ($p \geq 0.05$ compared to young).

The elevated levels of free ceramides noted in the aortic endothelium may be responsible for our previous observation that PP2A-dependent de-phosphorylation of eNOS and Akt lead to significantly lower eNOS activity in response to calcium and other classical endothelial-NO agonists. Therefore since LA treatment lowered both nSMase and free ceramide levels in the aged endothelium, we theorized that LA administration might partially restore endothelium-dependent vasorelaxation of old aortic rings After a period of equilibration and precontraction with phenylepherine, dose-dependent relaxation response to acetylcholine (ACh) was measured. The saline control old vessel rings began relaxing at 10^{-7} M ACh and almost exactly paralleled the relaxation curve of the untreated old animals (Fig 4.5). However, maximal relaxation of both was only ~40% and was reached at 10^{-4} M ACh. Rings from LA treated animals relaxed significantly more than saline-injected and old controls at concentrations above 10^{-6} M ACh (Fig 4.5; $p \leq 0.02$). However, the LA did not completely restore vasodilation of old vessel rings in spite of its near reversal of cellular ceramide levels. This suggests that other factors contribute to the age-associated loss of endothelium-dependent vasodilation besides elevated ceramide.

4.5 Discussion

In the previous chapter, the age-related decline in endothelial GSH was suggested to be mechanistically responsible for the observed losses in eNOS synthetic capacity and the resulting loss of endothelium-dependent vasorelaxation seen with age. One of the major therapeutic effects reported from LA administration is improvement of intracellular GSH. This research evidence is reported both during conditions of toxicity which lower GSH as well as the restoration of age-associated declines in GSH [256-258, 260, 264]. Additionally, previous data showed that

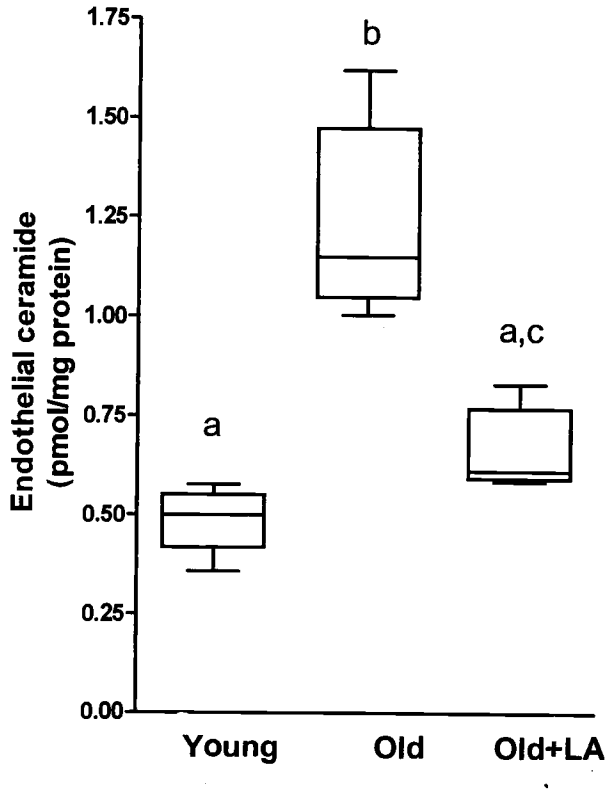

Figure 4.4. LA lowered the age-associated increase in ceramide compared to saline controls. Ceramides were measured after administration of pharmacologic administration of LA or saline controls to old animals. After treatment with LA, the improvement in ceramide levels was vigorous enough to eliminate statistical differences between the LA-treated old animals and untreated young animals ($p \geq 0.05$). Unique letters designate statistically significant differences in results ($p \leq 0.05$) using ANOVA with Tukey's post hoc analysis; n= 4.

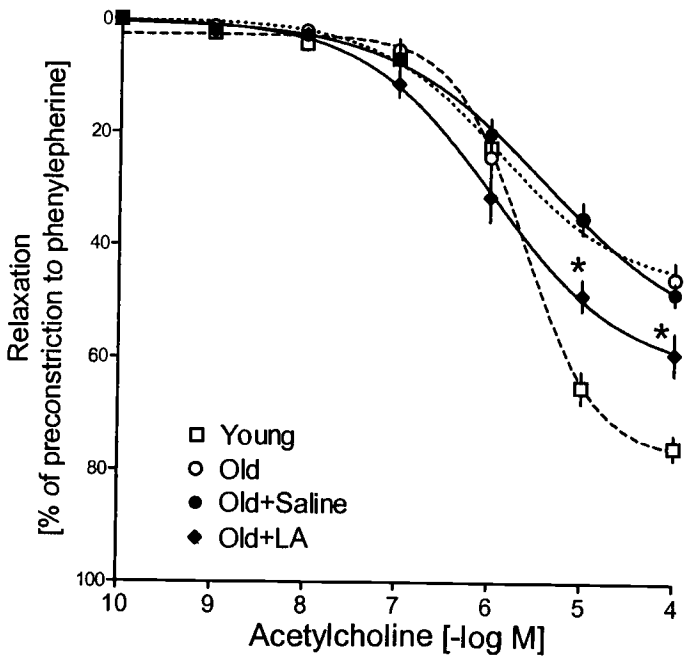

Figure 4.5. LA administration partially restores endothelium-dependent vasodilation of old rat aortic rings. Treatment of animals with pharmacologic administration of either LA or saline solution was followed by myography to determine endothelium-dependent relaxation in response to acetylcholine. Maximal relaxation of vessels from LA treated old animals was significantly improved compared to untreated or saline treated old animals. *$p \leq 0.05$ compared to young, old or old-saline according to ANOVA with Tukey's post hoc analysis; n= 4.

membrane neutral sphingomyelinase activity and concomitantly, free ceramides were elevated in the endothelium with age, leading to losses in eNOS phosphorylation and activation. Taken together, these observations suggest a good rationale for testing the effects of LA on these endpoints in the endothelium in the aging setting.

We discovered that LA can partially restore the age-related loss of endothelial GSH in the aged animal. Again, this effect of LA matches previous research from this lab and others which effectively improve age-associated declines in cellular GSH The fact that GSH-ethyl ester also significantly improved endothelial GSH does not yield information regarding the mechanism of *how* LA improves GSH. However it provided us with a control with which to benchmark the possibility that further LA effects might be due specifically to its effect on GSH. Though LA treatment in this study significantly improves endothelial GSH in the old animals, the improvement was not complete (e.g restoration to the levels seen in young endothelium). The most likely explanation for this incomplete reversal is the mode of LA administration utilized in this study. Much of the research previously published on this topic utilized dietary administration of LA, usually food mixed with 0.2% LA (w/w) fed for two weeks or more. Considering a typical 500 g rat consuming 18-20 g food/day, a 0.2% LA diet exposes the animal to approximately 80 mg LA/kg/day. These numbers are similar to the protocol used in this study where animals were treated with 40 mg LA/kg *twice* over 24 h. Though dietary absorption is expected to be lower than i.p. injection, the dose ammounts remain similar with the difference being the *duration* of the LA exposure. Thus, one might expect to see a more significant improvement or even a reversal of the age-related loss of endothelial GSH if a longer regimen of LA administration is used.

LA administration significantly lowered membrane-associated nSMase activity after 24 h. This finding is important because it presents a direction for investigating and understanding how LA works to improve endothelial NO. Interestingly, administration of GSH-ethyl ester also lowered membrane nSMase activity, suggesting that the improvement of intracellular GSH is responsible for the apparent inhibition of nSMase in the aged endothelium. This is a reasonable assumption since

it is well known that low GSH (low thiol-redox status to be more accurate) is a potent inducer of the neutral isoform of SMase [141, 143]. LA increased GSH in the aged endothelium by approximately 20% while the effect of LA on nSMase activity was nearly a 33% reduction. These results suggest that the age-associated decline in GSH *does* have a significant effect on cellular functions, perhaps one of which is the induction of nSMase activity in the endothelium. This assertion is strengthened by the observation that even a relatively small improvement in GSH made a significant improvement in nSMase activation. Treatment of aged animals with GSH-ethyl ester also significantly lowered nSMase activity in the endothelium (Fig. 4.3; 0.16±0.002 vs 0.20±0.004 pmol ceramide/mg protein/h in old controls; $p \leq 0.01$), suggesting that the mechanism of action for LA's effect on nSMase was due to improvement of intracellular GSH status.

The reduction of nSMase seen in the aged endothelium correlates well with a significant decline in the levels of free ceramides. The LA-mediated reduction of ceramide levels was nearly complete, lowering ceramide levels on the aged endothelium to levels which are not statistically different from those measured in the young. The age-associated increase in free ceramide which was reported in the previous chapter suggested that phosphatase hyperactivation might be responsible for the deactivation of Akt and correspondingly, eNOS. Indeed, in a previously published report, we documented how similar treatment of old animals with LA partially restored the phosphorylation of both eNOS and Akt [261]. Why did LA have such a profound effect on ceramide levels considering that we only realized a 20-30% improvement in GSH and nSMase activity using LA? One possibility is a potential effect of LA on free fatty acid levels in the cell. Aging and several age-related pathologies are characterized by diminished mitochondrial efficiency and accumulation of free fatty acids due to altered β-oxidation. LA has been shown to improve mitochondrial efficiency and metabolic function by improving β-oxidation and lowering free fatty acid levels [179, 265, 267-270]. An important source of cellular ceramide is *de novo* synthesis. *De novo* synthesis of ceramide may can be

upregulated in response to excess free fatty acids. Thus, LA may have an effect on cellular ceramide levels independently of nSMase modulation.

Vessel myography was performed in order to assess the effects of acute LA treatment on endothelium-dependent vasorelaxation. Compared to saline control-treated old animals and to untreated old animals, LA significantly improved the relaxation response to the NO agonist, ACh. The effect of LA was partial, suggesting the possibility that longer exposure to LA might need to be assessed or that the age-associated loss of eNOS function in only partly dependent on intracellular thiol status. The near restoration of ceramides to young levels is not accompanied by complete restoration of vasorelaxation which suggests that *other* mechanisms may also play a role in the age-associated loss of endothelial function. These may include factors such as premature degradation of NO, loss of eNOS cofactors, loss of growth factors and cell-cell and call-matrix associations which regulate Akt-dependent activation of eNOS. Though these concepts were beyond the scope of this project, their importance should not be underestimated.

This research is important not only to aging-researchers but to those interested in the biochemical role of LA *in vivo*. Here, evidence is presented reinforcing the suggestion that the therapeutic aspects of LA stem, in part, from its restoration of intracellular GSH. However, future research should be aimed at a more definitive mechanistic understanding. For example, the co-administration of buthionine sulfoxide (BSO) with LA to prevent GSH synthesis would be helpful in understanding whether LA induced GSH synthesis or recycling by chemical reduction of GSSG might be responsible for the observed effects. This is important because a rationale exists for a mechanism by which LA may activate Akt in a more direct manner by thiol-mediated stimulation of receptor-tyrosine kinases and hence PI3-kinase pathways. Klip *et al* and preliminary work by the author showing that LA may potentiate eNOS and Akt phosphorylation in a PI3-kinase dependent manner in cultured human aortic endothelium (data not shown). Indeed, other thiol agents such as N-acetylcysteine, sulphoraphane and anetholedithione could be important tools tested in parallel with LA do determine whether to observed LA effects are related to

its dithiol nature, its antioxidant status or other points such as uptake, metabolism or conjugation.

Chapter 5

Vitamin C matters. Increased Oxidative Stress in Cultured Human Aortic Endothelial Cells Without Supplemental Ascorbic Acid

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5.1 Abstract

Because standard culture media for human aortic endothelial cells (HAEC) do not contain vitamin C, we hypothesized that HAEC may be under significant oxidative insult compared to the situation *in vivo*. To assess parameters of oxidative stress, intracellular vitamin C, glutathione (GSH), GSH/GSSG and NAD(P)H/NAD(P)$^+$ ratios as well as oxidant appearance and oxidative damage were measured in HAEC with or without vitamin C addition. The effect of vitamin C on eNOS activity was also determined.

Results showed that HAEC without vitamin C treatment were essentially scorbutic. Upon addition of 100 μM vitamin C to the culture media, intracellular vitamin C levels increased and peaked at 6 hr. A concomitant increase in the total GSH and the GSH/GSSG ratio was also observed; the NAD(P)H/NAD(P)$^+$ ratio increased more slowly over the 24 hr time course.

Significantly lower ($P < 0.05$) oxidant appearance and steady-state oxidative damage were also observed following vitamin C repletion. Vitamin C treatment increased eNOS activity by 600%. Thus, HAEC are scorbutic under normal culture conditions and exhibit higher oxidative stress than vitamin C repleted cells. Vitamin C supplementation should be considered when using cultured cells, especially when experimental endpoints are related to cellular redox status and eNOS activity.

5.2 Introduction

Vascular endothelial cells (EC) mediate vasomotor relaxation by synthesis and release of nitric oxide (NO) [4, 271] and other bioactive compounds [212, 272]. NO synthesis and availability are sensitive to cellular antioxidant status and redox balance [88, 273-275]. In general, vitamin C is a major water-soluble intracellular antioxidant that significantly affects endothelial-dependent vasorelaxation [155]. Specifically, tetrahydrobiopterin, a critical cofactor for endothelial nitric oxide synthase (eNOS), is kept in the reduced state by vitamin C. In addition, vitamin C may spare endothelial-derived nitric oxide (EDNO) from oxidation and conversion to non-vasoactive reactive nitrogen species (RNS) such as nitrate, nitrite and peroxynitrite [276]. Thus, conditions under which vitamin C becomes limiting could adversely affect EDNO levels and ultimately vasomotor response.

Elucidation of endothelial function and the role that vitamin C plays in vasomotor activity has been greatly advanced by the use of EC in culture. Cultured human aortic endothelial cells (HAEC) have been characterized with respect to adhesion molecule expression, harvest conditions, cell cycle control and signaling [277, 278]. However, little work has focused on characterizing the extent of culture-induced alterations to cellular vitamin C status. This is surprising given the overall importance that vitamin C plays in mitigating oxidative stress and maintaining EDNO availability.

Striking differences in vitamin C status exist when comparing standard HAEC in culture with the blood vessel environment. Human plasma vitamin C levels are typically maintained at 50-120 μM with resulting intracellular EC concentrations in the millimolar range [151]. In contrast, culture media does not typically contain vitamin C since it is unstable in aqueous solutions.

We hypothesized that HAEC grown using standard culture conditions (where vitamin C is not routinely supplied) are likely to contain very little vitamin C. This compromised antioxidant status could lead to altered redox balance and heightened oxidative stress, which may not accurately mimic the characteristics of HAEC *in vivo*.

To examine this hypothesis, we analyzed parameters in cultured HAEC that are important in maintaining a pro-versus anti-oxidant balance. We measured vitamin C levels, reduced/oxidized glutathione ratios, pyridine nucleotide redox ratio, oxidant appearance, steady-state oxidative damage and EDNO synthesis in HAEC under typical culture conditions and following addition of vitamin C to the culture media. Herein, we show that cultured HAEC are scorbutic and do not reflect typical physiological conditions with respect to basal eNOS activity and EDNO bioavailability.

5.3 Materials and Methods

Materials. HAEC and culture media were obtained from Clonetics (Rutherford, NJ). The secondary antibody, goat anti-mouse IgG-Oregon Green 488, and the fluorophores 2',7'-dichlorodihydrofluorescein diacetate (DCF), dihydrorhodamine 123 (DHR) and Mitotracker Red™ were obtained from Molecular Probes (Eugene, OR). The eNOS inhibitor, L-Nitro-arginine methylester (L-NAME), calcium ionophore A23187, Hanks buffered saline solution (HBSS), Dowex anion exchange resin, glutathione reductase, glucose-6-phosphatase and vitamin C were from Sigma (St. Louis, MO). L-[^{14}C]-arginine was purchased from Amersham (Piscataway, NJ). The primary antibody, mouse anti-8-hydroxyguanine was purchased from Trevigen (Gaithersburg, MD).

Cell Culture. HAEC were cultured to confluence with endothelial growth medium (EGM™, Clonetics), which includes 2% fetal bovine serum, 10 ng/ml human recombinant epidermal growth factor, 1 mg/ml hydrocortisone, 3 mg/ml bovine brain extract and antibiotics. Cells were used between passages 5-7.

Vitamin C measurement. Confluent cells (1.5-2 $\times 10^6$) were treated with EGM™ medium supplemented with 100 μM vitamin C. Vitamin C levels were measured as described by Frei *et al* [151]. Briefly, cells were washed three times with HBSS,

trypsinized and lysed in 10% (w/v) perchloric acid (PCA). Insoluble debris was pelleted by centrifugation. The resulting supernatant was subjected to HPLC separation using a Hitachi L-6000 solvent delivery system and a Supelco LC-8 column (4.75 mm × 100 mm; 5 μm pore size). Ascorbate was monitored using electrochemical detection (applied voltage: +0.6 V). Vitamin C concentrations were quantified relative to standards. EGM™, fetal bovine serum and M199, another common culture medium, were also analyzed for vitamin C content.

Glutathione measurement. Confluent cells (1.5-2 × 10^6) were treated with EGM™ with or without 100 μM vitamin C added to the media. Changes in GSH levels were followed over 24 hr following vitamin C addition. Cells were washed, trypsinized and lysed in 10% (w/v) PCA. The acid soluble fractions containing glutathione (GSH) and glutathione disulfide (GSSG) were derivatized with iodoacetic acid (40 mM). The resulting carboxymethyl derivatives were dansylated by conjugation with dansyl chloride (75 mM) and separated by the method of Jones *et al* [236]. GSH and GSSG were separated by HPLC using fluorescent detection (Hitachi L7000; Ex/Em: 330/515nm). Quantification was achieved relative to standards using γ-glutamyl-glutamate as an internal control to assess completeness of derivatization.

NAD(P)H/NAD(P)$^+$ ratio measurement. The pyridine nucleotide status was determined as previously described [279]. Briefly, HAEC (2 ×10^6) growing in EGM™ or EGM™ supplemented with vitamin C (100 μM) were collected by trypsinization. A time course of NAD(P)H/NAD(P)$^+$ was followed over 24 hr following vitamin C addition. Cells were washed twice with HBSS and sonicated on ice in an extraction buffer (0.1 M Tris-HCl, pH 8.0, 0.01 M EDTA and 0.05% (v/v) Triton X-100). Reduced pyridine nucleotides (NAD(P)H, NADH$^+$) were quantified by spectrophotometry at 340 nm using the extinction coefficient of 6220 M^{-1} cm^{-1}. For all samples, spectral analysis was performed in duplicate and averaged. Three readings were determined: Absorbance of the untreated sonicate was measured (A_1) representing the reduced pyridine nucleotide pool in the sample. A second aliquot of

the sonicate (A_2) was converted to NAD(P)H by treatment with glucose-6-phosphate dehydrogenase (5 IU), glucose-6-phosphate (5mM) and MgCl (10mM). Finally, the total NAD(P)H pool was measured by treating an aliquot of the sonicate (A_3) with glutathione reductase (5 IU), GSSG (5 mM) and EDTA (10mm). Subtraction of A_3 from A_1 represents the total NAD(P)H of the sample, while $A_2 - A_1$ yields the $NAD(P)^+$ content. In all assays, reaction buffers without sample or NAD(P)H were measured to correct for background absorbance.

Oxidant appearance. HAEC (2.5×10^4 cells/well) were allowed to adhere for 48 h to gelatin-coated 96-well Costar™ microtiter plate wells. Cells were rinsed with HBSS and incubated for 30 min with or without vitamin C (100 μM) or L-NAME (300 μM). Cells were washed and loaded with DCF (100 μM) or DHR (30 μM). Cellular (DCF; Ex/Em: 494/520nm) and mitochondrial (DHR; Ex/Em: 507/529nm) oxidant production were continually monitored by evaluating the rate of fluorescence appearance over time using a Cytofluor fluorescence plate reader (Applied Biosystems, Foster City, CA).

In experiments where indices of oxidative stress were examined histologically, HAEC were grown for 48 hr on gelatin-coated microscope cover slips. These coverslips were incubated for 30 min with HBSS containing vitamin C (100 μM). Cells were washed then incubated with HBSS containing DHR (30 μM). The slips were mounted on microscope slides and scanning laser confocal microscope images (Leica, Bannockburn, NJ) were taken after 25 minutes.

Immunocytochemical determination of oxidative damage. Vitamin C supplemented EGM™ (100 μM) was added daily to each culture well for seven consecutive days prior to confluency. Cells were washed, fixed with 4% (w/v) *p*-formaldehyde and permeablized with ice-cold acetone. Blocking buffer containing 1% (w/v) bovine serum albumen, 1% (w/v) goat serum and 0.1% (v/v) Tween-20 was added to prevent non-specific binding. Cells were incubated overnight at 4°C with mouse anti-8-hydroxyguanine (anti-8-oxo-dG) antibodies. This monoclonal antibody recognizes

several compounds containing guanine bases hydroxylated at the C-8 position and as such, can be used to determine oxidative damage to both DNA and RNA. Following three washes with blocking buffer, a secondary antibody (goat anti-mouse IgG-Oregon Green 488; Ex/Em: 494/520nm) was added and incubated for one hour at room temperature. Images were taken using a scanning laser confocal microscope (Leica, Bannockburn, NJ). To monitor whether immunoassaying co-localized with mitochondria, HAEC were co-stained with Mitotracker Red™ following the manufacturer's instructions. In some experiments, RNAse A was incubated with fixed cells to assess the relative levels of RNA versus DNA damage.

Endothelial nitric oxide synthase activity. Confluent HAEC grown in six-well culture plates were incubated for 30 minutes with HBSS with or without 100 μM vitamin C. The cells were washed and allowed to equilibrate for 30 min at 37 °C in HBSS containing 10 μM indomethacin (16). L-[^{14}C] arginine (230 pmol; specific activity: 320 mCi/mmol) was added to each well. Five minutes later, cells were stimulated with 2 μM (final conc.) A23187. After 5 min of incubation, the reaction was stopped with ice-cold phosphate-buffered saline containing 5 mM L-arginine and 4 mM EDTA. The cells were denatured with absolute ethanol and, after evaporation, the cellular residue was dissolved in 500 μl water and added to test tubes containing 1 ml of an aqueous suspension of Dowex AG 50WX8-400 anion exchange resin, Na^+ form [280]. After vortex mixing and brief centrifugation, 750 μl of the supernatant was mixed with an appropriate amount of Beta Blend® scintillation cocktail (ICN, Costa Mesa, CA) and the radioactivity was determined in a scintillation counter (Beckman Instruments, Fullerton, CA).

Protein determination. Protein was quantified by the method of Lowry *et al* [281].

Statistical methods. Experimental data is presented as means ± standard deviation of the mean. Experimental means were either compared to their respective controls using the Student's t-test, or subjected to ANOVA with Scheffe non-parametric, post hoc

analysis using a confidence interval of 0.05. Differences were considered significant if P-values were less than 0.05.

5.4 Results

Antioxidant status under defined culture conditions. Vitamin C and GSH, the most important low molecular weight, intracellular, water-soluble antioxidants, are usually found in millimolar concentrations in mammalian cells [282]. To determine the relative levels of low molecular weight antioxidants in cultured HAEC, vitamin C and GSH were determined in confluent cells under typical culture conditions. Analysis of cellular vitamin C content showed that cultured HAEC had no detectable vitamin C ($1.5\text{-}2 \times 10^6$ cells; detection limit ≥ 5 pmol) under the assay conditions employed (Fig. 5.1A). To determine whether EGM™ or fetal bovine serum contained appreciable levels of vitamin C these compounds were analyzed for ascorbate content. Additionaly, M199, another medium commonly used for HAEC culture, was analyzed. None of these solutions contained ascorbate (data not shown). These results thus suggest that under standard culture conditions, HAEC are essentially scorbutic.

To determine whether supplementing the culture medium could restore vitamin C status, cells were treated with 100 μM vitamin C in EGM™. This regimen led to a rapid cellular appearance of vitamin C, such that after 1 hr, ascorbate content was 2.01 ± 0.21 nmol/mg protein (Fig. 5.1A; N= 4). Maximum vitamin C levels were seen approximately 6 hr (4.80 ± 0.95 nmol/mg protein) after addition and fell to 0.47 ± 0.22 nmol/mg protein (N= 4) at 24 hr. These results show that HAEC are scorbutic when grown under standard culture conditions but that exogenously added vitamin C is rapidly taken up.

To determine whether the scorbutic nature of HAEC in culture also affected glutathione redox state, GSH and GSSG levels were determined in cells treated with or without vitamin C. GSH and GSSG levels in scorbutic cells were 18.67 ± 3.42 and 0.334 ± 0.046 nmol/mg protein (N = 4), respectively, which gives a GSH/GSSG ratio of 54.29 ± 6.26 (Fig 5.1B). Considering that EC have an approximate intracellular

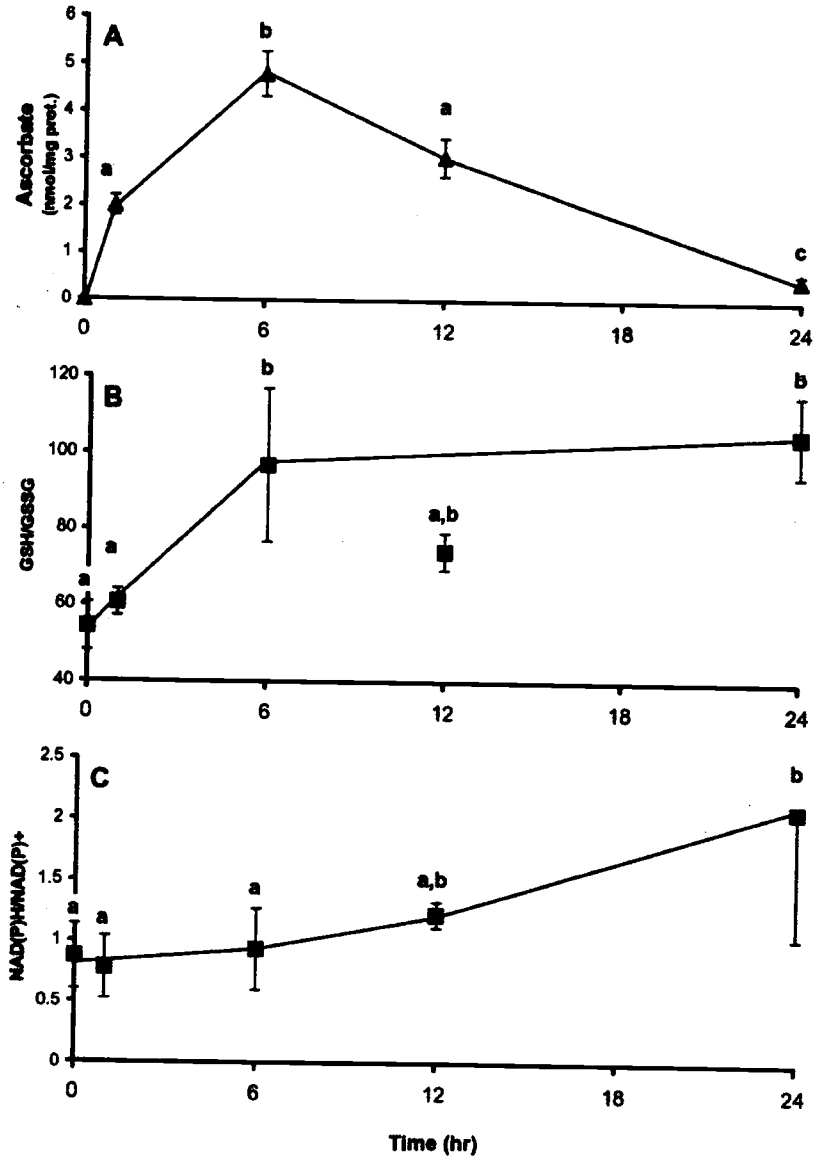

Figure 5.1. ***A.*** **HAEC grown under standard conditions contain no detectable vitamin C.** However, they are rapidly repleted when given 100 μM vitamin C in growth medium. Maximal vitamin C content was seen 6 hr after addition of the vitamin (n=4). *B.* Vitamin C also increased the GSH/GSSG ratio, which remained elevated over the 24 hr time course (n = 4). *C.* NAD(P)H/NAD(P)$^+$ ratio improved significantly only after 24 hr following vitamin C repletion (n = 3), suggesting that in contrast to the thiol redox status, pyridine redox state does not directly correlate with vitamin C repletion. Unique lettering denotes statistical significance ($p \leq 0.05$).

volume of 1 μl/10^6 cells, this value translates to a GSH concentration of 5 mM, which is similar to reported GSH levels for other mammalian cell types [283]. Monitoring GSH status following addition of vitamin C (100 μM) to the culture media revealed that vitamin C repletion resulted in a significant doubling of total GSH levels after 24 hr (38.41 ± 3.58 pmol/mg protein; $P< 0.05$; $N = 4$) and also an increase in the GSH/GSSG ratio, which also almost doubled to 104.78 ± 10.66 ($P< 0.05$; $N = 4$). Most of the changes in GSH levels and the GSH/GSSG ratio occurred during the first 6 hr following vitamin C addition, which was also the time of greatest vitamin C accumulation (Fig. 5.1A and B). GSH and the GSH/GSSG ratio remained elevated over the 24 hr time course though ascorbate levels began declining after only 6 hr. Thus, reversing the scorbutic state in HAEC by vitamin C addition also correlates with improved GSH status and further indicates that lack of vitamin C leads to a compromised intracellular thiol redox ratio.

Redox of HAEC. To further explore how vitamin C affected metabolic redox ratio, the pyridine nucleotide redox status of the cell was measured following vitamin C addition to the culture media. NAD(P)H/$NAD(P)^+$ ratio for cells grown without vitamin C was 0.88 ± 0.27 (Fig. 5.1C, N =3). Again, this suggests that these cells exhibit an altered cellular redox balance considering that this ratio is usually greater than 1.0 in most cell types measured [63, 284, 285]. The pyridine nucleotide redox ratio improved significantly ($P >0.05$) to 2.08 ± 1.04 only after 24 hr following vitamin C repletion (Fig. 5.1C). Thus, changes in pyridine redox state do not directly correlate with vitamin C repletion or GSH/GSSG ratio following vitamin C addition. However, indirect effects of vitamin C on NAD(P)H/$NAD(P)^+$ cannot be ruled out. In summary, vitamin C status influences both without vitamin C have distinctly lower overall redox balance compared to vitamin C repleted cells.

Indices of oxidative stress and oxidative damage. To determine whether the lack of vitamin C resulted in increased oxidant appearance, cells were incubated with DCF, a dye that fluoresces upon oxidation by reactive oxygen and reactive nitrogen species

(ROS/RNS) [286-292]. The rate of oxidant-induced fluorescence in scorbutic cells was 7.95 ± 1.04 AFU/min (Fig. 5.2A, N= 5) whereas vitamin C supplementation decreased oxidant appearance to 5.13 ± 0.75 AFU/min (N= 5), a significant decline of 35.5% ($P<0.05$). These results suggest that vitamin C markedly lowers general oxidant levels in cultured HAEC.

Endothelial NOS becomes a superoxide-generating enzyme when tetrahydrobiopterin becomes limiting or oxidized; the latter situation may occur in HAEC when cultured without vitamin C. We sought to determine whether the enhanced oxidant appearance in vitamin C-deprived cells was partly due to eNOS-generated oxidants. Cells were incubated with the eNOS inhibitor L-NAME and oxidant appearance was determined as before. Results showed that oxidant levels in L-NAME treated cells were significantly lower than scorbutic HAEC (3.69 ± 0.38 AFU/min vs. 7.95 ± 1.04 AFU/min; $P<0.05$). Oxidant levels were not completely inhibited by L-NAME, suggesting that in addition to eNOS, other cellular sources also contribute to ROS and RNS production.

Because mitochondria produce significant amounts of oxidants as a by-product of normal metabolism, we determined whether treatment with vitamin C affects mitochondrial oxidant production. To this end, cells were incubated with DHR, a dye that specifically associates with the mitochondria and, like DCF, fluoresces upon oxidation by ROS/RNS [293-297]. Scorbutic HAEC exhibited a rate of fluorescence of 2.05 ± 0.13 AFU/min (Fig. 5.2B, N= 4). Conversely, the rate of mitochondrial fluorescence in vitamin C-repleted cells was approximately half the rate seen in scorbutic cells (1.03 ± 0.06 AFU/min, N= 4), reflecting a significant decline ($P<0.01$). Similarly, the eNOS inhibitor l-NAME also decreased DHR fluorescence (1.39 ± 0.03 AFU/min vs. 2.05 ± 0.13 AFU/min; $P<0.01$). This result may indicate the presence of eNOS-derived RNS as well as ROS in the mitochondria.

To confirm these results, mitochondrial oxidant production was also monitored using scanning-laser confocal microscopy. Intensely bright, punctate, mitochondrial staining was seen in control cells stained with DHR (Fig. 5.3A). However, a markedly lower intensity of fluorescence was observed after treating HAEC with vitamin C

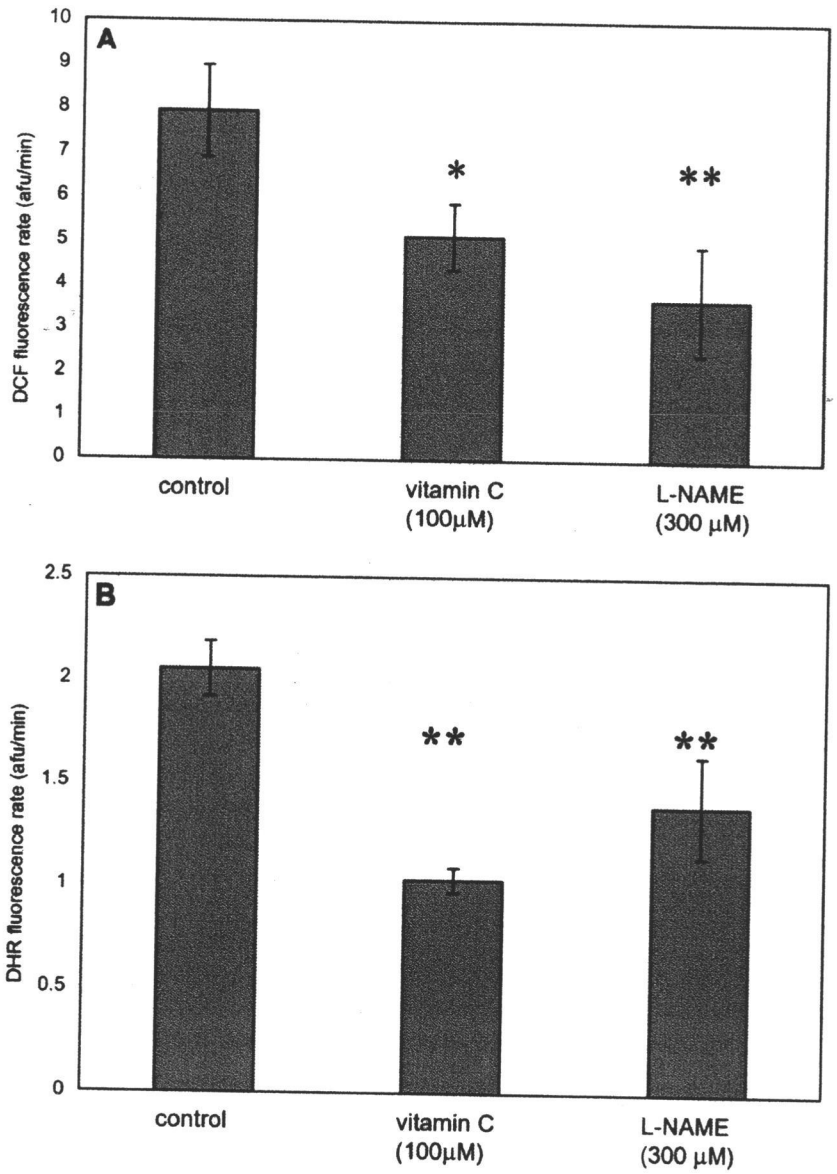

Figure 5.2. ***A.*** **The rate of appearance of ROS/RNS in HAEC was measured by DCF fluorescence.** Treatment with 100 μM vitamin C lowered the rate of appearance of ROS/RNS in HAEC (n=5). The eNOS inhibitor L-NAME also lowered DCF fluorescence, indicating that at least some of the signal is due to eNOS-derived ROS/RNS. *B.* The rate of appearance of ROS/RNS in the mitochondria was measured by DHR fluorescence (n=4). Treatment with vitamin C significantly lowered the rate of appearance of ROS/RNS in the mitochondrial compartment of HAEC. The eNOS inhibitor L-NAME significantly lowered DHR fluorescence, indicating that at least some of the signal is due to appearance of eNOS-derived ROS/RNS in the mitochondrion.
* $p<0.05$; ** $p<0.01$

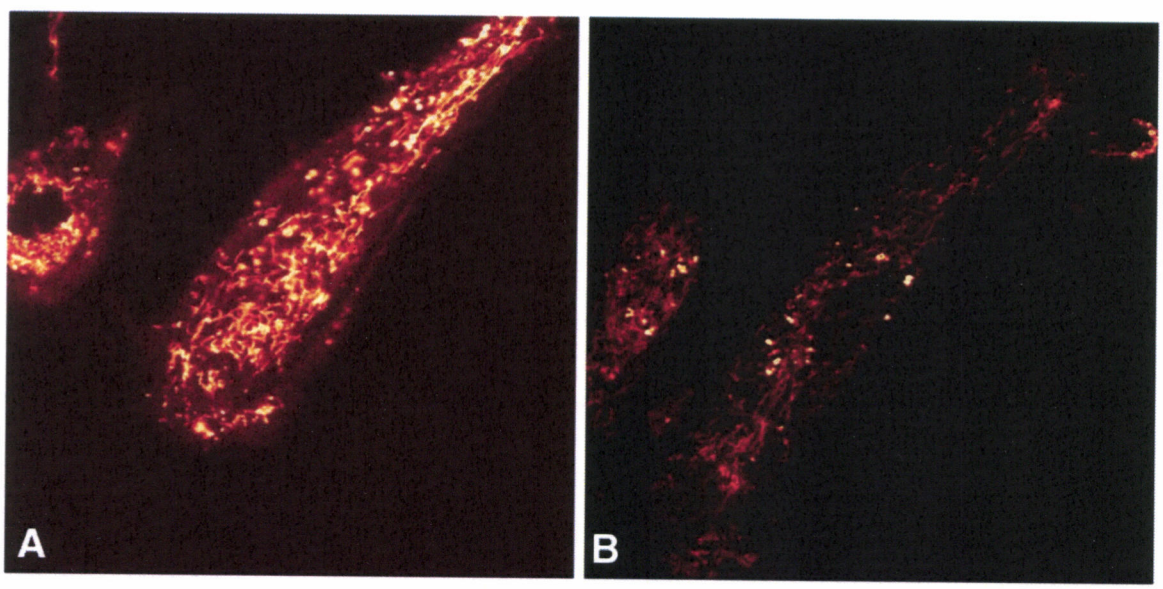

Figure 5.3. ***A.*** **Scanning confocal laser images of DHR-stained mitochondria in scorbutic HAEC**. *B.* Vitamin C treated HAEC; Images were aquired using the same laser settings and z-axis for each treatment. As with Fig. 2B, untreated cells show intense mitochondrial DHR staining (*A*), indicating higher mitochondrial ROS/RNS appearance than in vitamin C repleted cells (*B*).

(Fig. 5.3B). Together, these results suggest that HAEC maintained under standard culture conditions are under increased oxidative stress compared to vitamin C treated cells, which is in part due to heightened oxidant appearance from the mitochondria.

To determine whether altered antioxidant status and heightened oxidant appearance leads to elevated steady-state oxidative damage, oxidized nucleotide levels were monitored immunocytochemically. We used a monoclonal antibody directed against 8-oxo-dG, an oxidative adduct that arises primarily from hydroxyl radical or peroxynitrite attack on guanine nucleobases [298, 299]. Results showed intense staining in control cells (Fig. 5.4A, 5.4C), suggesting oxidative damage to nucleic acids. In contrast, daily addition of vitamin C (100 μM) to the culture media for seven days resulted in markedly lower fluorescence intensity (Fig.5.4B, 5.4D), which indicates that vitamin C repletion lowers steady-state oxidative damage.

Immunostaining in both vitamin C depleted and repleted cells was predominantly perinuclear in nature (Fig. 5.4A-D). Lack of nuclear staining was not due to inability of the antibody to bind nuclear DNA, because experiments where HAEC were incubated with a free radical generating system [300] resulted in nuclear staining (Fig. 5.4E). Thus, vitamin C markedly attenuates oxidative damage in general, but perinuclear oxidative damage predominates in both scorbutic and vitamin C repleted cells.

Perinuclear staining could be due to RNA damage, mitochondrial DNA (mtDNA) damage or oxidized bases in cytosolic nucleotide and deoxynucleotide pools. Thus, cells treated with or without vitamin C were co-stained with anti-8-oxo-dG antibody along with Mitotracker Red™, a specific mitochondrial stain that is retained after fixation. Histological analysis did not reveal significant co-localization of stains, indicating that mtDNA damage could not account for a significant portion of the noted perinuclear staining (Fig. 5.4C, 5.4D). To determine the extent that RNA damage contributed to perinuclear staining, fixed cells were incubated with RNAse A

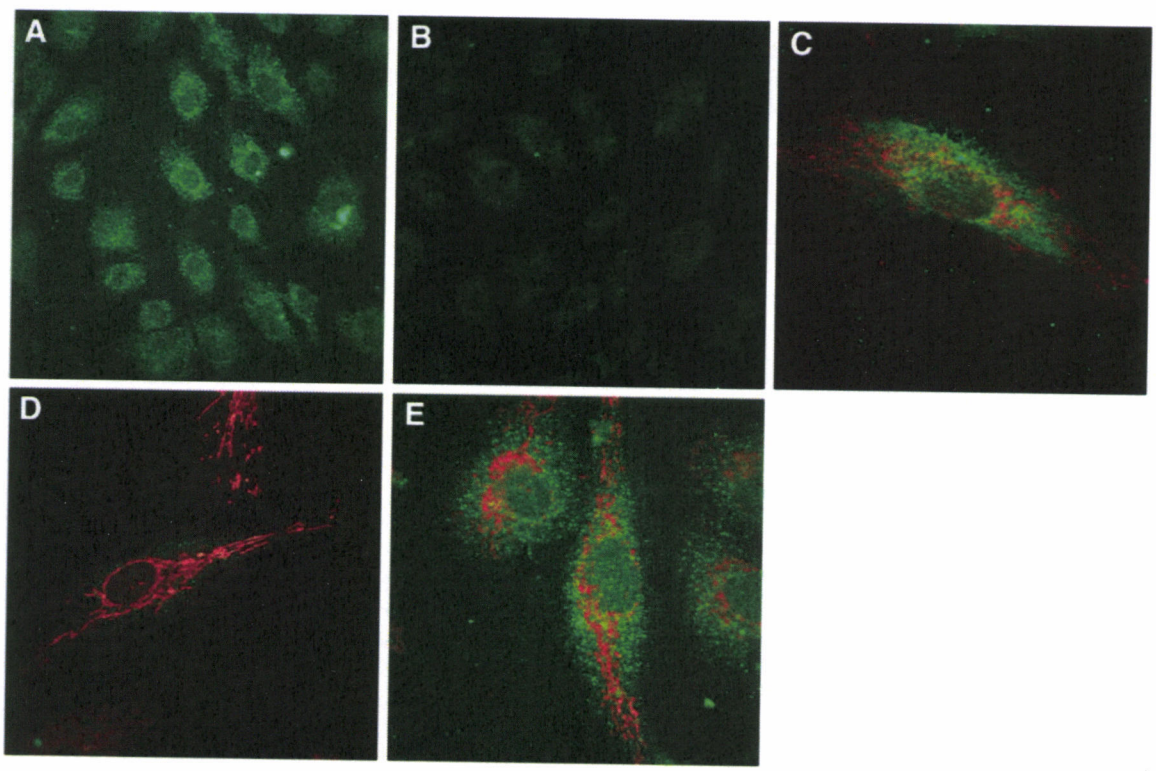

Figure 5.4. ***A.*** **Scorbutic HAEC stained for 8-oxo-dG**. *B.* HAEC treated for 7 days with vitamin C (100μM) and stained for 8-oxo-dG. *C.* Scorbutic HAEC stained for 8-oxo-dG and co-stained with MitoTracker Red™. *D.* Vitamin C (100 μM) treated HAEC stained for 8-oxo-dG and co-stained with MitoTracker Red™. *E.* Positive control HAEC exposed to a free-radical generating system (100 μM Fe^{2+}, 2 mM H_2O_2, 100 μM EDTA, and 100 μM ascorbate) were stained for 8-oxo-dG and co-stained with MitoTracker Red™. Chronic treatment of HAEC with vitamin C (100 μM) markedly lowers immunodetection of 8-oxo-dG (*A, B*). The perinuclear staining is not localized specifically to mitochondria (*C, D*). The positive control shows nuclear staining (*E*).

prior to immunostaining with the 8-oxo-dG antibody. Pretreatment of fixed scorbutic cells with RNAse A did not affect the relative signal intensity. In contrast, pretreatment of the vitamin C supplemented cells with RNAse A abolished the fluorescence signal (data not shown). Thus, repletion of HAEC not only lowers the steady-state levels of oxidative damage, but also alters the nature of perinuclear damage from non-RNA nucleotide pools to RNA.

Endothelial nitric oxide synthase activity. Because eNOS activity can be modified by altered cellular redox status as well as directly influenced by vitamin C levels, we postulated that scorbutic HAEC would have diminished eNOS activity compared to vitamin C repleted cells. This hypothesis was tested by evaluating eNOS activity following stimulation with the calcium ionophore A23187. Vitamin C significantly increased EDNO production by approximately 600%, (Fig. 5.5; N= 3) a highly significant increase over scorbutic cells ($P<0.01$). This effect was blocked by the eNOS inhibitor L-NAME, indicating that vitamin C exerts its tonic effects directly on eNOS.

5.5 Discussion

Standard culture conditions for HAEC do not routinely include vitamin C as a supplement, mainly because vitamin C is not stable in culture media. Other studies showed that cultured EC grown under normal conditions are scorbutic and that vitamin C can be repleted [90, 301]. We show that providing vitamin C (100 μM) results in uptake into cells and intracellular vitamin C levels are maintained for at least 6 hrs before a decline back to scorbutic conditions. Thus, vitamin C supplementation results in a transient, yet marked improvement in steady-state ascorbic acid levels, despite the potential for oxidation or hydrolysis occurring in the media.

After a second addition of vitamin C (24 hr) cellular concentrations rose to a level higher than after the first administration (data not shown). This could be due to

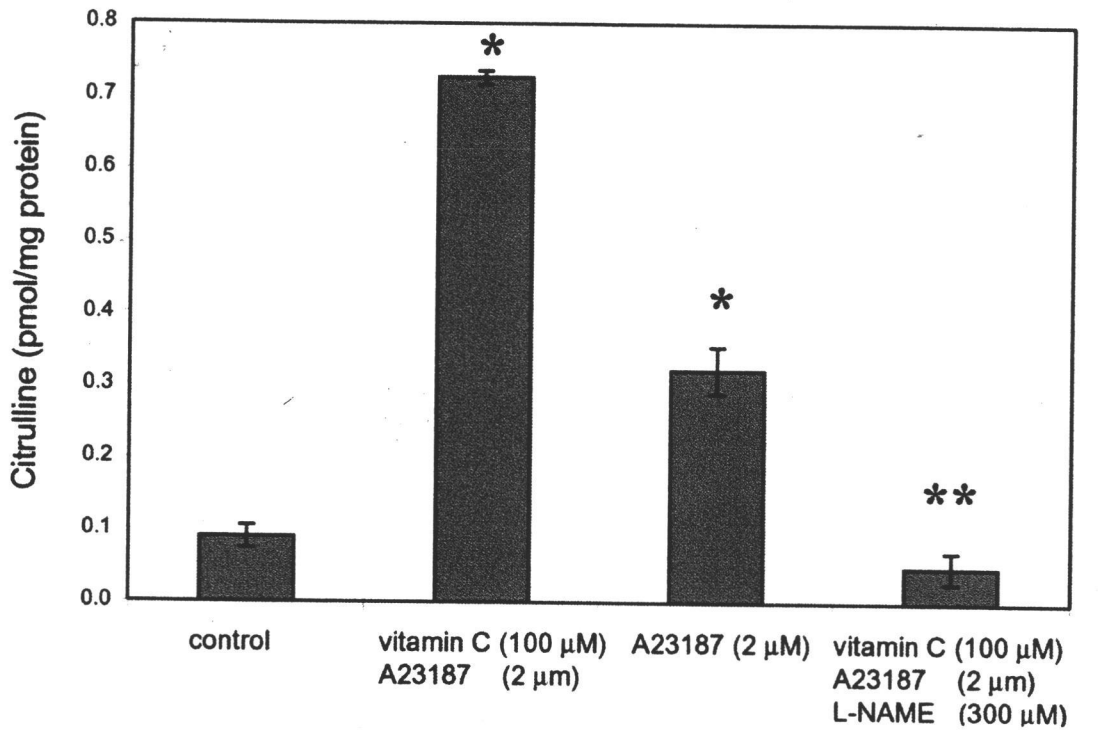

Figure 5.5. Treatment with vitamin C (100 µM) increases agonist-stimulated synthesis of nitric oxide (NO) by eNOS. The calcium ionophore A23187 (2 µM) was used to stimulate maximal production of NO by eNOS. The eNOS inhibitor L-NAME (300 µM) inhibits the reaction, illustrating a direct effect of vitamin C on eNOS activity. Controls using vitamin C alone, L-NAME alone or A23187 + L-NAME were not statistically different from the control treatment (data not shown). NO was measured by conversion of L-[^{14}C]-arginine to L-[^{14}C]-citrulline (n=3). * $p<0.01$; ** $p= 0.05$ compared to control.

increased vitamin C transporters on the cell surface or from uptake of dehydroascorbic acid (DHA) from the media [155, 302]. Our results, coupled with those using phosphorylated vitamin C derivatives showing sustained intracellular levels [303], strongly argue that vitamin C supplementation of standard culture media is feasible and results in a system that more closely resembles physiological conditions.

We show that the scorbutic nature of HAEC in culture leads to major alterations in endothelial function that could directly affect proper interpretation of experimental results. For instance, lack of vitamin C leads to significant increases in ROS/RNS appearance, lower GSH/GSSG and NAD(P)H/NAD(P)$^+$ ratios, and a subsequent increase in oxidative damage. These results suggest that in experiments without the addition of vitamin C, HAEC are in a non-physiological, pro-oxidant environment.

While increased oxidative conditions may actually benefit HAEC growth in culture by causing higher rates of cell proliferation [44], the scorbutic intracellular environment may adversely affect parameters related to endothelial function, especially NO synthesis and availability. NO is a radical that must diffuse to vascular smooth muscle cells to induce vasorelaxation. As a signal molecule, EDNO has a relatively short half-life and can be oxidized to RNS incapable of causing vascular relaxation. Therefore, lack of vitamin C could significantly enhance oxidative inactivation of EDNO and result in a culture model that does not mimic the physiological environment of the intact vessel.

The vitamin C-induced improvement in GSH/GSSG ratio may also enhance EDNO bioavailability in ways other than an improved oxidative environment. While still controversial, it has been proposed that GSH can directly conjugate with EDNO, forming an S-nitrosothiol derivative that stabilizes the NO radical and increases its half-life [304-308]. Thus, vitamin C repletion, by increasing GSH availability, may also improve EDNO levels for vasorelaxation.

Vitamin C treatment also restored the cellular thiol redox ratio (Fig. 1B). Because the one-electron reduction potential of vitamin C is higher than that of GSH, vitamin C likely did not directly reduce GSSG to GSH [309]. Alternatively, vitamin C

may "spare" GSH from oxidation by ROS/RNS by becoming oxidized itself, which would eventually lead higher GSH/GSSG ratios [155].

Improvement of the pyridine nucleotide redox status by vitamin C repletion has broad implications for the functional consequences of inadequate vitamin C levels in cultured cells. NAD(P)H is produced largely by the pentose phosphate pathway and is the primary reducing substrate for a host of anabolic reactions. Thus, lower pyridine nucleotide ratio could broadly affect overall endothelial cell metabolism including analysis of cell proliferation, bioenergetics and apoptosis. Lower redox status may also affect parameters directly related to vasomotor function as NAD(P)H is a necessary cofactor for eNOS, cyclooxygenases, cytochromes P-450, glutathione reductase and NAD(P)H oxidase. Thus, the low GSH/GSSG ratio and pyridine nucleotide redox ratio evident in scorbutic HAEC are more reflective of oxidative stress conditions [310-312] than would be expected normally.

It is interesting that though ascorbate levels peaked 6 hr following addition of vitamin C and began declining afterwards, GSH and the GSH/GSSG ratio remained elevated over the 24 hr time course. The reasons for this are not presently clear but could be due to a variety of factors including a sparing of GSH by vitamin C. In contrast, the NAD(P)H/$NAD(P)^+$ ratio did not change significantly until 24 hr following addition of vitamin C. This may be due to increased dehydroascorbate (DHA); DHA has been shown to stimulate activity of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and transaldolase enzymes of the pentose phosphate pathway [313]. Conversely, the effect may be indirect; if ascorbate spares GSH from oxidation, then less demand is placed on cellular pyridine levels by the glutathione reductase system.

Altered cellular redox ratios translated to an increase in oxidative stress as shown by higher oxidant appearance and oxidative damage in scorbutic versus vitamin C repleted cells. Interestingly, even though cells without vitamin C treatment showed more intense 8-oxo-dG detection than cells given vitamin C, both conditions primarily showed perinuclear oxidative damage. Because the epitope that the antibody was raised against is the hydroxylated C-8 position of the guanine nucleobase, it is

indiscriminant of nucleotide (or nucleoside) type. These results suggest that most steady-state oxidative damage in cultured HAEC is found in RNA or in oxidized cytosolic nucleotide and deoxynucleotide pools. Alternatively, steric hindrance may prevent antibody detection of nuclear DNA damage. Based on control experiments, it appears that most of the damage seen is from RNA. This is suggested because damage patterns did not overlap with mitochondria (Fig. 4C, 4D) and experiments using a free radical-generating system showed that nuclear DNA damage could be detected (Fig. 4E). Perinuclear staining has also been seen by others using this antibody [314, 315], which suggests that it may have greater affinity for non-DNA nucleobases. Surprisingly, RNAse treatment abolished the signal in vitamin C repleted but not scorbutic cells (data not shown). This latter result further suggests that the type(s) of oxidative damage is different in cells lacking vitamin C.

Aside from enhanced oxidative stress and resultant alterations in NO availability, the scorbutic nature of standard culture conditions could also lead to altered EDNO production. EDNO is produced by eNOS, a tetrahydrobiopterin-dependent heme-protein. We show that vitamin C repletion of HAEC in culture leads to significantly higher eNOS activity, which is in agreement with previous data [90, 316, 317]. Furthermore, NAD(P)H is a cofactor for NO synthesis as a reducing substrate and is thus crucial for kinetic activity of eNOS. Though the mechanism(s) by which vitamin C increases eNOS activity were not explored in the present study, previous work suggests that providing vitamin C to HAEC maintains tetrahydrobiopterin levels and/or keeps tetrahydrobiopterin from being oxidized [152, 318]. Vitamin C thereby allows tetrahydrobiopterin to provide reducing power to the enzyme and also allows this critical cofactor to aid in enzyme dimerization. Both the lack of tetrahydrobiopterin reducing power and loss of enzyme dimerization will convert eNOS into a superoxide-generating NAD(P)H oxidase enzyme, which in turn, could exacerbate oxidative stress and lead to lower EDNO availability. Alternatively, it has been proposed that vitamin C can directly reduce critical thiol moieties on the enzyme and thereby upregulate eNOS activity [155].

Finally, use of EC culture models has resulted in significant insights into endothelial cell function. In the year 2000 alone, 1999 papers were published using an EC culture model and, at the time of this manuscript preparation, 1171 additional papers have appeared in 2001. The popularity of cultured EC as a research model is undeniable. However, results as well as interpretation of results are only as good as the model employed. Herein, we argue that the scorbutic nature of HAEC in culture can significantly alter parameters that directly pertain to the known function(s) of vitamin C in endothelial cells. Experimental data related to eNOS activity, oxidative stress, and any mechanism that can be influenced by redox status need to be examined in light of these findings. These other potential mechanisms include altered transcriptional control and gene expression, cell signaling as well as cell proliferation. Furthermore, lack of vitamin C could also alter carnitine synthesis with subsequent adverse effects on cellular bioenergetics [319]. Collagen production and extracellular matrix formation could also be affected [320]. Taken together, these results strongly call for vitamin C supplementation to HAEC to provide a culture model that more closely resembles the in vivo situation.

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Chapter 6

General Conclusion

6.1 General Conclusion

Age related dysfunction and diseases affecting the cardiovascular system present not only a large economic cost to society, but also a moral and philosophical one. In the next 25 years, the population of persons over the age of 65 will reach near 70 million [192]. According to today's statistics, we can count on 30% of these persons suffering from clinical hypertension and that 80% of all heart disease cases will belong to this group. Accordingly, the stated goal of the U.S. National Institute on Aging is "to improve the health and well-being of older Americans through research". Basic research is thus actively searching for the underlying mechanisms of age-associated cardiovascular diseases, to build the grounds for the development of successfull therapies and treatments. From the economical point of view, it should be noted that a multi-billion dollar industry thrives on the current "anti-aging" therapies, profiting from the sale of diet plans, exercise regimens, nutritional supplemements, antioxidants and drugs designed to capitalize on the innate fear of aging that many people feel.

As described in the previous chapters, the vascular system plays a major role in the age-associated cardiovascular diseases. In particular, decline in vascular endothelial function ultimately affects nearly all types of CVD and often may be the precipitating cause for such diseases. Within the last five years, our understanding of vascular function, particularly endothelial cell function has progressed greatly. Our understanding of the molecular, cellular and biochemical parameters which regulate endothelial cell function has advanced so that the hypotheses of full understanding of mechanisms and therapeutical approaches is not unrealistic. For instance, in the last five years, our understanding of eNOS-mediated regulation of NO production had shifted from a simple model of Ca^{+2} and cofactor-dependent regulation to a far more complex model involving intracellular translocation, binding with regulatory accessory proteins and phosphorylation/dephosphorylation by specialized kinases and phosphatases. These regulatory mechanisms were originally mapped and explored in cultured endothelial cell lines and their physiological relevance has been solidified by recapitulation in *in vivo* models. To date, endothelial aging research has sought the

keys to age-associated endothelial dysfunction by investigating simpler, older concepts such as substrate and cofactor availability and eNOS protein expression. Therefore, the field of endothelial aging is primed for application of the newer concepts regarding eNOS regulation. To date little or no research has been published that has looked at age-associated alterations in the molecular, cellular and biochemical regulatory elements which control eNOS.

In this work, we directly addressed these concepts by looking for potential changes in subcellular localization, eNOS accessory protein binding or phosphorylation events which may occur during the aging process. We found that indeed, age results in significant alterations in each of these parameters, all of which suggest that the age-associated loss of endothelial NO may be due to dysregulation of eNOS on a molecular and cellular level.

The consequences of this work are manifold, both in terms of progress in basic biochemical research and as grounds for the development of therapeutical tools. It is well established that age results in a very significant loss of endothelial NO production ability [89, 204, 207, 211, 215]. However conflicting reports have described eNOS expression as either increasing , decreasing or remaining unaltered with age [196, 207, 215]. The work reported in this thesis demonstrates that eNOS protein expression does not change (Fig. 2.2B). Consequently, we refocused our work towards searching for other potential alterations. Though it is well documented that eNOS is regulated by translocation from the inactive Golgi-associate fraction to the plasma membrane caveolar fraction and back, the precise regulatory elements which govern this type of control are almost equivocal. Whether alterations in the regulation of accessory proteins plays a role in this phenotype is also relatively unknown. However, preliminary evidence from this project suggests that there is no change in the total amounts of Hsp90 or caveolin in the aged endothelium. Therefore our finding that aging alters the levels of eNOS normally seen at the endothelial membrane of young animals is novel and must be further analyzed in order to fully understand both its causes and its ramifications toward the progression of age-associated endothelial dysfunction.

When we characterized alterations in the phosphorylation status of eNOS with age, a question arises; Are the subcellular localization of eNOS and its phosphorylation status intimately linked? This is a question which is ultimately larger in scope than the aging question and will likely need to be solved using molecular tools and cultured cell models. However, at this point we may speculate that phosphorylation status seems a likely candidate for the source of signals which regulate eNOS translocation. For other protein types, phosphorylation does function as the regulatory element which mediates translocation [239, 321-323].

Because eNOS is regulated by phosphorylation, it is logical that alterations in phosphatase activity can regulate NO production. Two specific phosphatases have been shown to associate with eNOS; PP1 which is responsible for dephosphorylation of T494 and PP2A dephosphorylates S1176. Our observation that aging leads to heightened phosphorylation of eNOS T494 and decreased phosphorylation of S1176 seemingly presents a conundrum. If S/T phosphatases are globally upregulated in the aged endothelial cell, we might expect to find hypophosphorylation of *both* sites on eNOS. However, the subtleties of phosphatase and kinase balance are complex. It would be equally counterproductive for the cell to co-activate both phosphatases as it would to coactivate both inhibitory and stimulatory kinases. Therefore the endothelium has evolved a set of controlling mechanisms which allow rapid initiation of NO synthesis that can be sustained for a time when the need arises, yet is rapidly turned off at other times. Our current understanding of the temporal regulatory nature of eNOS activation suggests that eNOS T494 is rapidly dephosphorylated by PP1 in response to increases in intracellular Ca^{+2}. Other research has demonstrated that inhibition of PP1 results in PKC-mediated phosphorylation of eNOS T474 and strong inhibition of NO synthesis [93]. Concomitantly, NO synthesis is sustained after the initiating Ca^{+2} transient if Akt-mediated phosphorylation occurs at S1176. However, PKC activation is also implicated in the dephosphorylation of S1176 because it stimulates PP2A [93, 115]. We did not assess the activation state of any of the PKC isoforms in this study. Since PKC upregulation is commonly associated with cellular stress and inflammation, the aging phenotype of endothelium appears to be similar to a

state of chronic inflammation. Further work in this area of aging research should address potential alterations in PKC expression and activity and whether dysregulation of that kinase occurs as a result of, or independently of the mechanisms we have described.

If Akt-mediated signaling was dramatically blunted in the endothelial cell there would be far greater problems than loss of endothelial NO. This is because Akt is known as a principal mediator of cell survival via stimulation of metabolic and anti-apoptotic elements in response to growth factors, shear stress, cell-cell interactions and adhesion to extracellular matrix Therefore we wondered if the perceived alterations in eNOS phosphorylation might be due to alterations in the phosphatases which work in conjunction with kinases to control eNOS phospho-activation. We focused specifically on the phosphatase PP2A, a ceramide-activated phosphatase which specifically removes phosphates from the eNOS S1176 and Akt S473 (and potentially other substrates of Akt). Though it is difficult to ascertain the specific contribution of individual phosphatases towards overall phosphatase activity in cell extracts, this work generated data which strongly suggests a heightened level of PP2A activity in the aged endothelial cell by exploiting the difference in IC_{50} of okadaic acid against PP2A and PP1. Okadaic acid does not seem to be an effective inhibitor of other S/T phosphatases at the concentrations used [24, 238, 324].

Though the alterations which alter eNOS control by signal transduction seem to cause the observed age-associated loss of vasorelaxation, one still must wonder how this occurs or what underlying events cause them. Our work suggests that the observed alterations to eNOS potentially stem from an oxidative-stress related mechanism, namely an alteration of the intracellular thiol redox balance. In fact, GSH loss is a potent inducer of sphingomyelinases which precipitate ceramide-activated downstream events including phosphatase activation. Other research in vascular aging has established the presence of significantly heightened levels of the inflammatory cytokine TNF-α in the serum and vessel walls of the aged [325, 326]. Additionally, the role of the inflammatory marker, C-reactive protein, is now the subject of much research since its presence in the serum of the elderly seems to increase. Further, our

observation that low molecular weight antioxidants (vitamin C, GSH) decrease with age in the endothelium seems to support the 'free radical theory of aging' and that ambient levels of reactive oxygen and nitrogen species (e.g. superoxide, peroxy-radicals and peroxynitrite) may increase significantly with age and specifically in the endothelium [202, 206-208]. Thus the picture of the aged endothelial cell is strongly resemblant of that of a chronically inflamed cell.

As a test of this concept, we utilized cultured human aortic endothelial cells as a model for aging by inducing oxidative stress. Cultured endothelial cells are not routinely supplemented with vitamin C and many vital cellular functions are severely compromised. In vitamin C deficient cells, we observed significant alterations in the pyridine nucleotide redox ratio, GSH, ROS/RNS appearance and importantly, eNOS activity.

Though the molecular clues are there, we are still a long way from specifically addressing the problem of vascular aging with specifically targeted therapeutic interventions. However, our work with LA presents great promise. The partial restoration of endothelial function which is realized even after only 24h of LA treatment is promising and serves two important purposes. The first is the promise that the compound may hold as an actual therapeutic compound for maintaining endothelial health during aging. The second is perhaps more important. That LA may be used as a tool to help us understand, mechanistically, what is occurring in the aging cell. LA restores GSH, but it is capable of much more including metal ion chelation, ROS/RNS scavenging, and stimulation of insulin-dependent signaling pathways (see reference [179]) Certainly much more work must be done in order to determine not only what this compound does to a cell, but also where it acts and over what time course.

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