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Silent Partner in Blood Vessel Homeostasis? Pervasive Role of Nitric Oxide in Vascular Disease

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Abstract

The endothelium generates powerful mediators that regulate blood flow, temper inflammation and maintain a homeostatic environment to prevent both the initiation and progression of vascular disease. Nitric oxide (NO) is arguably the single most influential molecule in terms of dictating blood vessel homeostasis. In addition to direct effects associated with altered NO production (*e.g.* vasoconstriction, excessive inflammation, endothelial dysfunction), NO is a critical modulator of vaso-relevant pathways including cyclooxygenase (COX)-derived prostaglandin production and angiotensin II generation by the renin-angiotensin system. Furthermore, NO may influence the selectivity of COX-2 inhibitors and ultimately contribute to controversies associated with the use of these drugs. Consistent with a central role for NO in vascular disease, disruptions in the production and bioavailability of NO have been linked to hypertension, diabetes, hypercholesterolemia, obesity, aging, and smoking. The ability of the vessel wall to control disease-associated oxidative stress may be the most critical determinant in maintaining homeostatic levels of NO and subsequently the prospect of stroke, myocardial infarction and other CV abnormalities. To this end, investigation of mechanisms that alter the balance of protective mediators, including pathways that are indirectly modified by NO, is critical to the development of effective therapy in the treatment of CV disease.

Keywords

Vascular Disease; Nitric Oxide; Nitrotyrosine; Cyclooxygenase; Prostaglandins; Angiotensin

Introduction

Cardiovascular disease (CV) is the leading cause of death in most Western countries and many of its associated risk factors have been linked to pathophysiological changes acting individually or in concert to promote vascular injury. Nitric Oxide (NO) is well established as a vasodilator [1] and an inhibitor of platelet aggregation [2]. There is little doubt that defective vascular NO synthesis and/or bioactivity, often termed endothelial dysfunction, is a principal contributor to pathological alterations to the CV system [3–6]. Thus, the endothelium is critical in the regulation of vascular function by maintaining homeostatic levels of NO and, as discussed herein, a variety of other vasoactive substances [7–9].

It has become evident that interpretation of data from studies investigating the role of defective endogenous NO production in CV disease is both arduous and potentially ambiguous due to a variety of reasons: 1) NO synthesis can derive from three nitric oxide synthase (NOS) isoforms whereby most cell types may express more than one NOS. Therefore, compensatory NOS

actions and additive amounts of NO must be considered during basal and inflammatory conditions. For example, endothelial nitric oxide (eNOS) is present under basal conditions, inducible nitric oxide synthase (iNOS) is strongly induced and activated by inflammatory stimuli [10]. This occurs in numerous cell types including vascular smooth muscle cells, macrophages and the endothelium itself [11,12]. To this end, it is exceedingly challenging to differentiate between excessive, balanced and deficient NO production in the local tissue environment, 2) NO and NO-derived species mediate diverse biological actions that are greatly influenced by the surrounding milieu. For example, NO can kill invading pathogens as well as participate in oxidative reactions that modify biomolecules and contribute to perpetuation of damage, and 3) NO can indirectly modify blood vessel homeostasis by targeting other physiological and pathophysiological pathways. In the present review, we investigate the diverse impact of endogenous NO on the vasoregulatory systems that ultimately define endothelial function and blood vessel homeostasis (Fig. 1).

Endothelial Dysfunction

Maintaining a delicate balance between vasoregulatory factors is vital to the maintenance of a healthy blood vessel. The inner layer of the vasculature, called the endothelium, regulates complex functions including vascular tone, hemostasis, blood coagulation and fibrinolysis, and platelet and leukocyte interactions with the vessel wall. The inability of blood vessels to perform these endothelium-dependent functions is broadly defined *endothelial dysfunction*, a hallmark of chronic blood vessel disorders, such as diabetes, hypertension and atherosclerosis [13–16].

The Transformation of Endothelial Nitric Oxide Synthase (eNOS) during Pathogenesis

The diverse actions of NO as a regulator of the cardiovascular, immune and nervous systems, are mediated by three isoforms of NOS. Inducible NOS, neuronal NOS (nNOS) and eNOS function as dimers, share great sequence homology, and share binding sites for substrate L-arginine, heme and tetrahydrobiopterin (BH4) in their oxygenase domains. Catalysis by NOS isoforms is tightly regulated whereby the c-terminal reductase domain of one monomer supplies electrons to heme in the n-terminal oxygenase domain of the companion monomer. BH4 is an essential redox-active cofactor in NOS catalysis and provides structural support to NOSs by facilitating dimerization [17] and substrate binding [18,19].

The ability of NOS-bound BH4 to undergo redox cycling *in situ* is now appreciated as an essential mechanism for driving NOS catalysis. Notably, BH4 donates an electron to the heme during hydroxylation of bound arginine and the resulting oxidized pterin (BH3[•]) is reduced back to BH4 using NOS flavin-domain electrons. In the final step, hydroxylated arginine is converted to citrulline and NO [20,21]. Recent findings demonstrate that BH3[•] reduction in nNOS is a calmodulin-dependent process and dependent on the heme moiety, which serves as a conduit for electron transfer [22]. Differences in BH4 redox cycling may exist between the three NOS isoforms and provides an explanation for the selective vulnerability of eNOS during oxidative stress. Notably, two of these isoforms (eNOS and nNOS) are constitutively expressed and synthesize NO in response to intracellular Ca⁺² levels. On the other hand, iNOS protein expression is induced by inflammatory stimuli and plays an important role in host-defense, producing sustained levels of NO independent of intracellular Ca⁺² levels.

eNOS is the predominant NOS isoform in endothelial cells and plays a vital role in blood vessel homeostasis. eNOS-derived NO serves to stimulate soluble guanylate cyclase in adjacent smooth muscle cells. This causes accumulation of the second messenger cyclic guanosine monophosphate and a subsequent reduction in actin-myosin interactions [23–25]. In addition to its vasodilatory properties, physiological levels of eNOS-derived NO protects against atherogenesis by preventing platelet aggregation and adhesion to the blood vessel lumen and

by inhibiting smooth muscle cell proliferation [26]. Paradoxically, a number of CV and metabolic disorders can transform eNOS into an oxidative stress-amplifying enzyme. Importantly, disruption of NO production (NOS uncoupling) is accompanied by a switching of the NOS product to superoxide (O_2^-), thereby favoring the formation of reactive nitrogen species (RNS; *i.e.* NO_x ; Fig. 1). Excess generation of ROS and NO_x are potentially harmful, owing to their ability to overwhelm antioxidant defense mechanisms and promote oxidative modifications that impair vascular enzymatic functions [27,28]. Importantly, while iNOS-derived NO plays an important protective role in innate host-defense, its overproduction may also damage local host tissues when formed in conjunction with excessive ROS [29,30]. Since eNOS has the lowest catalytic activity of all NOS isoforms [31], blood vessels may be particularly prone to NO insufficiency arising from ROS overproduction and altered NO chemistry in the setting of chronic inflammation (*e.g.*, with atherosclerosis, diabetes and hypertension). Thus, the preservation of balanced NO levels *via* regulation of its synthesis and the prevention of its oxidation is critical in the management of chronic blood vessel disorders.

Endothelial NO insufficiency may lead to the collapse of a delicate equilibrium of mediators that protect our vascular system. Thus, we will consider the following mechanistic bases for decreased NO bioavailability and endothelial dysfunction: 1) tetrahydrobiopterin (BH4) oxidation, NOS uncoupling, and accelerated NO destruction, and 2) diminished L-arginine availability.

Tetrahydrobiopterin (BH4) oxidation, NOS uncoupling and NO destruction—

Proinflammatory conditions associated with an increase in oxidative stress cause insult to the cellular environment by compromising NO bioavailability. Simultaneous overproduction of iNOS-derived NO and O_2^- and the resultant formation of their product $ONOO^-$ promotes significant damage to components in the vessel wall [32,33]. $ONOO^-$ is linked to a multitude of deleterious cellular events including uncoupling of NO synthase, depletion of antioxidants, enzymatic inactivation, increased lipid peroxidation, BH4 oxidation and increased protein oxidation and 3-nitrotyrosine (3-NT) formation [27]. To this end, we and others have recently shown that progressive NOS uncoupling, concomitant with BH4 oxidation and an increase in 3-NT formation, can drive vascular dysfunction in animal models of atherosclerosis [34] and type 2 diabetes [35]. In fact, iNOS deletion in atherosclerotic mice (ApoE-null mice on a high cholesterol diet) reduced 3-NT accumulation and offered significant protection against BH4 oxidation in a tissue-selective manner, indicating that iNOS-derived species contribute to the pathogenesis of atherosclerosis [34].

Numerous studies now explicitly associate diminished NO bioavailability with a BH4 deficiency-mediated compromise in NOS catalysis although the mechanisms by which attenuated BH4 levels directly induce eNOS uncoupling remain unknown. Since BH4 is readily oxidized, it follows that the extent of BH4 oxidation and NOS uncoupling in endothelial cells will be increased by oxidative stress. Considerable effort has been invested into understanding mechanisms of BH4 redox chemistry and how BH4 levels may be preserved for maintenance of eNOS bioactivity and the correction of vascular dysfunction. BH4 supplementation can acutely improve vascular conditions in patients with CV and metabolic disorders such as type 2 diabetes, hypercholesterolemia, and hypertension [13,15,36]. However, oral BH4 supplementation has been challenging due to the compound's instability [37]. Additionally, it is not yet clear how BH4 dosing and bioavailability determine eNOS coupling in the vasculature. To this end, a recent clinical trial of patients with poorly controlled hypertension determined that a daily oral dose of 400 mg BH4 is necessary for a significant reduction in blood pressure and improvement of endothelial function [38]. Other studies in patients with coronary artery disease determined that vascular, but not systemic BH4 levels, inform on the extent of eNOS coupling [39]. These studies highlight the importance of site-specific delivery of BH4 therapy in determining the effectiveness of these agents.

An alternative approach aimed at preserving BH4 by reducing ROS is the administration of oxidant scavengers such as folates, vitamin E and vitamin C. These methods proved effective in augmenting eNOS activity and BH4 levels in cell culture [40,41], in animal models of atherosclerosis [42] and diabetes [43] and in improving endothelial function in humans [44]. However, current lines of evidence indicate that endothelial dysfunction involves mechanisms that extend beyond ROS scavenging and the protection of absolute BH4 levels. Notably, Crabtree et al. have demonstrated that in an animal model of type 2 diabetes, the oxidation product of BH4, (7,8-dihydrobiopterin, BH2) is not inert and can paradoxically promote eNOS uncoupling by competing with BH4 for binding site occupancy [35]. More recently, Crabtree et al. also confirmed in BH4 deficient cells that the intracellular stoichiometric ratio between eNOS and BH4 are essential and direct determinants of eNOS uncoupling and superoxide production [45]. These findings ascertain BH4-eNOS stoichiometry as well as BH2 binding to eNOS in oxidative stress and eNOS uncoupling and provide a novel view that BH4 depletion is not the only relevant factor [35,45]. Collectively, these findings provide important leads toward a better understanding of mechanisms that can reverse endothelial dysfunction associated with CV disease.

Diminished L-Arginine Availability—As L-arginine is the substrate for NO synthesis; this molecule has been proposed to be a critical determinant of NO bioavailability. However, arginase II, the enzyme that metabolizes L-arginine to L-ornithine, can compete with eNOS for L-arginine. Altered arginase II activity has been implicated in vascular dysfunction in numerous animal models of vascular disease including atherosclerosis and hypertension[6, 46]. Therefore, modulation of arginase II activity is considered a potential avenue for therapy. However, many studies that involve arginine supplementation or the use of arginase inhibitors in animal models of hypercholesterolemia and atherosclerosis, as well as in atherosclerotic patients, have been challenged due to contradictory outcomes, particularly with respect to endothelial function[4,47–50]. These inconsistencies can derive from ineffective targeting of arginase in the blood vessel and/or the involvement of arginine in other metabolic pathways, most notably the urea cycle. Nevertheless, recent studies in ApoE-null mice showed that inhibition or deletion of the arginase II gene significantly increased NO production and improved vascular dysfunction while reducing atherosclerotic plaque formation[51]. The process of evaluating new approaches for the treatment of endothelial dysfunction may not be as simple as oral administration of L-arginine or arginase inhibitors. Nevertheless, insight into mechanisms that lead to endothelial dysfunction can bring us closer to finding safe and effective therapeutic strategies for the treatment of chronic CV disease.

Interactions between Nitric Oxide and Vasoactive Pathways in Blood Vessels

Nitric Oxide Insufficiency and the Cyclooxygenase Controversy

It is important to recognize that blood vessel homeostasis is additionally maintained by mediators other than NO, and that processes are indirectly influenced by NO and its derivatives. For example, prostacyclin (PGI₂), a cyclooxygenase-derived prostaglandin produced by the blood vessel endothelium, is a vasoprotective molecule that mediates vasodilation, circulating inflammatory cell adherence and lipid accumulation in the blood vessel wall. Substantial evidence has demonstrated that an imbalance between vasoactive prostaglandins in the blood vessel wall can trigger CV disease similarly to attenuated NO bioactivity[52–54]. Of note, while NO and PGI₂ are both released from the endothelium, NO release is continual while PGI₂ release is intermittent[55]. Therefore, it is anticipated that temporal differences in the synthesis of blood vessel mediators would present therapeutic dilemmas.

Under normal physiological conditions, cyclooxygenase enzymes (COX-1 and COX-2) catalyze the conversion of the fatty acid arachidonic acid into prostaglandin precursors, which are subsequently converted into thromboxane A₂ (TxA₂), prostaglandin E₂ (PGE₂),

prostacyclin (PGI₂) and other prostanoids. These products are biologically active and play a wide range of regulatory roles in physiological systems including the cardiovascular, renal, nervous and gastrointestinal. While structurally similar, COX-1 and COX-2 have marked functional differences and their metabolites often produce opposing effects on the body. For example, COX-1 is found in almost all tissues, particularly in circulating blood platelets; COX-2 is greatly upregulated at sites of inflammation and enriched in the endothelium and some other organs (*e.g.* kidneys). While TxA₂ and PGE₂ are physiological antagonists of NO and prostacyclin and thus traditionally viewed as detrimental to blood vessels, TxA₂ is essential in preventing excessive bleeding and PGE₂ provides critical protection to the lining of the stomach. The complexity of defining the precise action of prostaglandins and their corresponding receptors is highlighted by studies in COX-2-deficient and PGI₂ synthase-deficient mice[56–58]. Results from these studies collectively report that diminished levels of PGI₂ contribute to renal disorders. Surprisingly, PGI₂ receptor-deficient mice displayed no associated renal abnormalities[59].

The dichotomous properties of prostaglandins indicate that a critical balance of these products must be maintained to avoid vascular dysfunction. The seemingly straightforward differences between beneficial and deleterious prostaglandins led to the development and use of selective COX-2 inhibitors as anti-inflammatory drugs that effectively suppressed pro-inflammatory prostaglandins derived from the enhanced expression of COX-2 at sites of inflammation. However, adverse CV events were observed in patients taking COX-2 inhibitors, likely due to a blockade of a crucial source of COX-2-derived PGI₂ in the endothelium while leaving the unopposed production of COX-1-derived TxA₂. Accordingly, low-dose aspirin is beneficial in reducing CV disease due to the effective inhibition of platelet COX-1 (the primary source of TxA₂ formation). However, emerging evidence indicates that high-risk vascular patients receiving a regular aspirin regimen continue to generate residual TxA₂ levels that increase their risk of subsequent CV events[60]. Understandably, the clinical outcome in these aspirin-treated patients would improve from total suppression of serum TxA₂ levels. Thus, designing and selecting COX inhibitors is a paradox; the assumption that an improvement in the therapeutic index can be achieved through selective targeting of action sites may only partially apply to COX inhibition in vascular disease.

The concept of selective COX inhibition is crucial in light of additional evidence that oxidative stress can differentially activate endothelial cells and smooth muscle cells and allow for their participation in the inflammatory response[61]. In other words, an inflammatory situation may exist whereby COX-2 induction in smooth muscle cells may compensate for defective PGI₂ synthesis due to a dysfunctional endothelium. In this scenario, it would be presumed that the sole use of a selective COX-2 inhibitor would be deleterious[61]. Although blood vessel tone is influenced by PGI₂, other blood vessel-derived mediators are also critical to maintaining vascular equilibrium including but not limited to products of the coagulation cascade (*e.g.* fibrinogen, plasminogen) and the renin-angiotensin pathways[62]. In fact, ongoing research in the field is challenging the notion that selective COX-2 inhibitors increase CV risk solely *via* a reduction in endothelial-derived PGI₂. Indeed, numerous studies in patients with coronary artery disease and hypertension now ascertain that COX-2 induction contributes to endothelial dysfunction *via* a reduction in NO bioavailability[63,64] and associated ROS generation[65]. Results from these studies indicate that certain selective COX-2 inhibitors may possess off-target effects that preserve physiological levels of NO. Specifically, administration of celecoxib improved endothelial function in animal models of salt-induced hypertension[66] as well as in hypertensive patients[64] and patients with severe coronary artery disease[63]. The use of celecoxib was accompanied with a decrease in plasma levels of stress markers such as C-reactive protein, oxidized LDL[63] and 8-isoprostane[66]. In contrast, rofecoxib and diclofenac were found to not share the apparently COX-2-independent beneficial actions of celecoxib on endothelial function[66]. Therefore, a compensatory pathway for the loss of

PGI₂ via enhanced endothelial NO production may be achieved by fine-tuning selective COX-2 inhibition for the restoration of balanced levels of vasoactive molecules in blood vessels. The dual effects observed with celecoxib resemble the actions of a new class of hybrid NO-donating COX inhibitors that are currently being evaluated in clinical trials[67–69]. In addition to total production and rate of synthesis of these vasoregulatory mediators, the scientific community and pharmaceutical industry have a renewed appreciation for the profound interactions between these systems within the endothelium. As state-of-the-art and unbiased proteomic approaches are implemented, it will soon be possible to identify metabolic features that correlate with molecular identities, and an assessment of the biochemical status of cells and tissues in response to pharmacological intervention.

When NO functions in an oxidative milieu, pathogenic mediators, *i.e.* higher oxides of nitrogen are generated (*e.g.* ONOO[−]) which are highly reactive can profoundly perturb signaling events and metabolic functions within the blood vessel. Physiological consequences of NO_x actions vary greatly with respect to inherent differences in the ability of cell types to survive and/or resist toxicity[61,70], therefore recognition of NO_x-mediated reactions *in vivo* becomes a very complicated task. Nevertheless the ability to determine the nature and extent of a particular NO_x-induced modification, and determine the site of modification in a given protein is instrumental in the molecular characterization of underlying pathologies[71]. Consequently, investigation of mechanisms by which NO interacts with COX and modulates biological levels of prostaglandins in a blood vessels is far more telling than the characterization of COX pathways in isolation (Fig. 1). As mentioned previously, iNOS expression is activated in the setting of vasoinflammatory conditions and generates large and continuous quantities of NO in a manner that is not restricted by intracellular calcium levels. Furthermore, COX-2, iNOS and an overall increase in nitrated proteins were found to co-distribute in blood vessel cells and at sites of inflammation in animal models and humans[72–74]. Further evidence that the iNOS and COX pathways interact comes from transgenic studies in mice lacking the ability to produce NO from iNOS. In these studies, TxA₂ synthesis was elevated; indicating that iNOS-derived NO modulates COX activity[75]. In fact, a direct physical interaction between iNOS and COX-2 was observed in macrophages and reportedly resulted in a specific COX alteration (Cys-nitrosylation) that enhanced prostaglandin production[76].

On the other hand, NO_x-induced alterations have also been described which result in a reduction in prostaglandin synthesis. Tyrosine (Tyr) nitration is a stable post-translational modification that is commonly associated with loss of function and is well established as a marker of oxidative/nitrosative stress. While numerous studies associate Tyr nitration with inflammatory diseases, it is not produced abundantly *in vivo* and its effects on protein function can have a range of outcomes, *i.e.*, no change of function, loss of function or gain of function[77–79]. Thus, it is a challenging process to define the specific role of Tyr nitration in disease. It has become apparent that the site and extent of biological Tyr nitration may be dictated by protein structure. For example, protein transition metals have a unique role in selective targeting of nitration by NO_x to certain Tyr residues that are essential to protein function. This phenomenon occurs by a mechanism that allows for the formation of an efficient nitrating species from NO_x and has been documented for many hemoproteins including COX, ferric hemoglobin, cytochrome P450 and prostacyclin synthase (PGI₂S)[78,80–82]. Therefore, while the extent, fate and biological outcome of Try nitration in disease requires further investigation, it is clear that characterization of interactions between NO and the COX pathway underline a mechanistic linkage that impact the production rate of essential mediators in a pro-inflammatory milieu.

Cross talk between Nitric Oxide and the Renin-Angiotensin System

The renin-angiotensin system (RAS) is known for its role in the regulation of blood volume, water balance and systemic vascular resistance. This regulation occurs through multiple events

in the RAS cascade. Renin release from the kidney into circulation proteolytically cleaves circulating angiotensinogen to form angiotensin I (Ang I). Endothelial angiotensin converting enzyme (ACE) proteolyzes Ang I and produces circulating angiotensin II (Ang II). Ang II is biologically active and produces a range of effects including the stimulation of aldosterone synthesis and release from the adrenal cortex, sodium and fluid retention in the kidneys, vasoconstriction and an enhancement in sympathetic tone. Consequently, RAS overactivity plays a crucial role in the pathophysiology of chronic vascular diseases and is strongly associated with CV risk conditions, such as hypertension, atherosclerosis, hyperglycemia, hyperlipidemia and hyperinsulinemia[83–86].

As described earlier, impairment of endothelium-dependent vasodilation results from an imbalance of endogenous vasoactive mediators. In addition to the aforementioned NO and prostaglandins, Ang II also plays a role in blood vessel equilibrium, particularly under pathological conditions. The action of Ang II on blood vessels spans beyond vasoconstriction and can include enhanced production of reactive oxygen species (ROS), decreased NO production[87], increased smooth muscle cell proliferation[88], enhanced prostaglandin release and vascular permeability[89] and augmented adhesion molecule expression[90]. These actions collectively mediate increased vascular tone, inflammatory cell infiltration into the blood vessel[91], and often promote blood vessel injury[16].

Endothelial ACE possesses a dual role in its additional capacity to fragment and reduce bradykinin levels produced by the kinin-kallikrein system. Bradykinin is an endogenous peptide that elicits potent vasodilation through endothelial activation and amplification of NO release. Thus, bradykinin antagonizes the vasoconstricting effects of Ang II on blood vessels. In the context of hypertension, decreased NO bioavailability, enhanced ACE activity and elevated Ang II production, ACE inhibition may offer multiple benefits by suppressing harmful levels of Ang II while simultaneously restoring endothelial function through preservation of bradykinin levels and increasing NO production[92]. Accordingly, the therapeutic and CV benefit of using ACE inhibitors stems from their actions on endothelial function. The PROGRESS trial in patients with a history of stroke or transient ischemic attack demonstrated that combination therapy with an ACE inhibitor and a diuretic reduced the risk of recurrent stroke regardless of the patient's blood pressure[93].

Ang II can activate two distinct AT receptor subtypes in cells (AT₁ and AT₂), which elicit opposing cellular responses[94]. AT₁ activation is associated with vasoconstriction and the vascular inflammatory response involving enhanced ROS production and diminished levels of NO[95]. On the other hand, AT₂ activation promotes vasodilation, improves NO bioavailability and reduces bradykinin breakdown[96,97]. Upregulation of AT₂ in a mouse model of atherosclerosis has been reported to oppose the progression of this condition[86]. Alternatively, AT₁ blockade was shown to reverse blood pressure increases in a rat model of diet-induced obesity[84] and prevent aortic aneurysms in a murine model of Marfan's syndrome[98]. Accordingly, the mechanistic actions of ACE regulation and AT receptor blockade or activation appear unambiguous. Nevertheless, ongoing investigations suggest that significant levels of Ang II may be produced by non-ACE dependent pathways[99–101]. Furthermore, local Ang II production can occur in the heart[102], adipose tissue[103] and in mast cells[104], and may contribute to disease independently of the systemic RAS. Understanding mechanisms of tissue-specific Ang II production would predictably reveal additional targets for the therapeutic management of a variety of CV disorders. While numerous studies show that RAS inhibition exerts antihypertensive control and positive clinical outcomes in maintaining endothelial function, CV health and metabolic homeostasis[86,98,105,106], effective therapeutic targeting of RAS continues to require rigorous mechanistic investigations.

An important link between RAS activation and vascular overproduction of ROS may be attenuated NO bioactivity (Fig. 1). Recent studies in multiple animal models of hypertension [107,108] and in patients with renovascular hypertension [109,110] indicate that augmented ROS production is associated with RAS activation [108,111–113], eNOS uncoupling and reduced levels of bioactive NO [15]. Since NO is produced in virtually all tissues and can exert differing actions when expressed at relatively low and high levels by each of the three NOS isoforms, elucidation of its specific role in a given CV or metabolic disease is complex. The generation and use of singly deficient eNOS-null mice demonstrated that endothelial NO deficiency is involved in the genesis of vascular lesions [114] and the manifestation of numerous metabolic disorders *e.g.* hypertension, insulin resistance and hyperlipidemia [115, 116]. Nevertheless, the observation that lesions did not form spontaneously in eNOS-null mice [114] raised questions about compensatory NO production that can arise from other NOS isoforms such as preexisting nNOS and the inducible iNOS [105,117,118]. Thus, Nakata et al. developed mice that are deficient in all three NOS isoforms (n/i/eNOS^{-/-}). In their studies, they sequentially assessed renal and CV abnormalities in the single, double and triple-NOS deficient mice [105]. While survival was markedly reduced, the fact that the triple-NOS deficient mice survived at all is remarkable and suggests that while NO may be important in many systems including the cardiovascular, nervous and immune systems, NO is not essential to development and survival. Their results also suggest that other compensatory mechanisms may support the potentially catastrophic loss of NO. Nevertheless, Nakata et al. provided for the first time, direct and conclusive evidence that defective NO production plays a critical role in metabolic and CV disorders in association with the RAS pathway. Some of their findings (listed below) were derived from RAS activation and inhibition experiments in triple-NOS deficient mice where they compare the use of an AT₁ blocker to an antihypertensive drug, *e.g.* long-term treatment with an AT₁ blocker in NOS deficient mice potently inhibited coronary vascular lesion formation, mast cell infiltration and the incidence of spontaneous myocardial infarctions; AT₁ blockade also reduced blood pressure, levels of plasma LDL and triglycerides while improving glucose tolerance and plasma adiponectin levels [105]. Therefore, the benefit of RAS inhibition via receptor blockade surpasses blood pressure control and provides a mechanistic approach for therapy of numerous vascular disorders that may have originated from endothelial dysfunction and reduced NO bioavailability. To this end, the development of both non-toxic selective NOS inhibitors as well as NO donors that promise selective delivery to vascular sites with compromised NO production are on the horizon.

Concluding Remarks

It is well recognized that endothelial mediators can determine vascular health or vascular damage by processes that are dependent on changes in the intracellular milieu. Often, endothelial pathways are characterized as independent systems; however, emerging evidence underscores the importance of cross interactions from multiple pathways in a local environment. This knowledge adds complexity to the design of potential therapeutic drugs for the management of CV diseases without side complications. While there is still much to learn, ongoing investigations that unveil novel cross interactions between NO and essential pathways in a blood vessel present a promising approach for exerting improved pharmacological treatment of vascular disorders that benefit from improved endothelial function. For example, while the use of selective COX-2 inhibitors will likely remain a controversial issue due to their association with CV risks, it is difficult to dispute the great promise that selective COX-2 inhibition demonstrated in targeting inflammatory prostaglandins. Thus, it is likely that further studies that assess the cross talk between NO and COX as well as levels of balanced essential mediators in a healthy blood vessel may reveal knowledge that enables fine-tuning of selective COX-2 inhibition to offer endothelial protection and bypass adverse CV events. The role of NO in RAS activation represents another interaction of pathways that offers enhanced pharmacological control of CV and metabolic diseases. Indeed, the benefit of AT₁ blockade

in controlling blood pressure extends to improving vascular disorders that derive from endothelial dysfunction. A comprehensive and unbiased analysis of the components of those pathways involved in endothelial dysfunction will lead to a greater therapeutic potential for the treatment of numerous CV diseases.

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Figure 1.

NO at the nexus of vascular disease - blood vessel homeostasis is maintained directly by NO and other endothelial processes that are indirectly influenced by NO and its derivatives, *e.g.* COX pathways and the RAS system.