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REVIEW ARTICLE

The molecular sources of reactive oxygen species in hypertension

PAOLO PUDDU, GIOVANNI MARIA PUDDU, ELEONORA CRAVERO, MARZIA ROSATI & ANTONIO MUSCARI

Department of Internal Medicine, Aging and Nephrological Diseases, University of Bologna and S.Orsola-Malpighi Hospital, Bologna, Italy

Abstract
In both animal models and humans, increased blood pressure has been associated with oxidative stress in the vasculature, i.e. an excessive endothelial production of reactive oxygen species (ROS), which may be both a cause and an effect of hypertension. In addition to NADPH oxidase, the best characterized source of ROS, several other enzymes may contribute to ROS generation, including nitric oxide synthase, lipoxygenases, cyclo-oxygenases, xanthine oxidase and cytochrome P450 enzymes. It has been suggested that also mitochondria could be considered a major source of ROS: in situations of metabolic perturbation, increased mitochondrial ROS generation might trigger endothelial dysfunction, possibly contributing to the development of hypertension. However, the use of antioxidants in the clinical setting induced only limited effects on human hypertension or cardiovascular endpoints. More clinical studies are needed to fully elucidate this so called ''oxidative paradox'' of hypertension.

Key Words: *Endothelial dysfunction, hypertension, mitochondrial dysfunction, oxidative stress, reactive oxygen species*

Introduction

A large body of evidence supports the involvement of endothelial dysfunction in the pathogenesis of both arterial hypertension and atherothrombosis (1,2). Endothelial dysfunction is characterized by a decreased bioavailability of nitric oxide (NO), which, under normal conditions, mediates both endothelium-dependent vasodilation and the regulation of other protective properties of endothelial cells. In human hypertension, endothelial dysfunction would be mainly associated with an increased NO degradation, due to NO interaction with superoxide anions, while in experimental models of hypertension, a decreased NO production has also been observed (3). A defect of NO activity causes an imbalance in favor of endothelial contracting factors, such as endothelin-1 (ET-1), angiotensin II (Ang II), vasoconstrictor prostanoids and superoxide anions (4).

In both animal models and humans, increased blood pressure (BP) and endothelial dysfunction have been associated with increased oxidative stress, i.e. an excessive endothelial production of reactive oxygen (ROS) and nitrogen (RNS) species (5,6). Oxidative stress contributes to vascular pathophysiology by promoting increased vascular tone, inflammation, cell growth, as well as the activation of matrix metalloproteinases and the deposition of extracellular matrix proteins, which are processes associated with the vascular phenotype of hypertension (1). However, it still remains unclear whether elevated levels of ROS (including superoxide, hydrogen peroxide and hydroxil anion) and RNS (such as peroxynitrite) initiate the development of hypertension, are a consequence of hypertension, or both (7).

The present paper will mainly deal with the molecular mechanisms underlying the generation

Correspondence: Antonio Muscari, Department of Internal Medicine, Aging and Nephrological Diseases, University of Bologna - S.Orsola-Malpighi Hospital, Via Albertoni, 15, 40138 Bologna, Italy. Tel: +39-051-6362280. Fax: +39-051-6362210. E-mail: antonio.muscari@unibo.it

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of ROS in excess in endothelial cells, with particular consideration for their implications in hypertension.

Oxidative stress and hypertension

Oxidative stress, an excessive production of ROS outstripping antioxidant defense mechanisms, has been associated with several pathophysiological processes affecting the cardiovascular system including hypertension, hypercholesterolemia, diabetes, hyperhomocysteinemia and cigarette smoking (8–11).

In animal models, oxidative stress has been observed in spontaneous hypertensive rats (12), renovascular hypertension (13), salt-sensitive hypertension (14), and in a rat model of obesity-induced hypertension (15). Although its pathogenesis is complex and multifactorial, human hypertension may be also considered a state of increased oxidative stress (16,17), associated with a decreased antioxidant activity and a reduced ability to scavenge oxygen-derived free radicals (6,18).

Guzik et al. (19) have shown an association involving cardiovascular risk factors, vascular NADPH-dependent superoxide production and reduced Ach-induced vasorelaxation. However, other authors have shown a dissociation between altered superoxide production and BP (20–22). Moreover, results can vary depending on the marker of oxidative stress being investigated (6). For example, Kumar and Das (23) have observed a higher superoxide and hydrogen peroxide production in hypertensive patients. Similar results have also been obtained by Lacy et al. (24), who found a significant correlation between hydrogen peroxide production and systolic BP. Furthermore, in untreated hypertensives, reductions in superoxide dysmutase (SOD) and glutathione peroxidase activity have been observed compared with controls (25), while the treatment of hypertensive patients with candesartan or amlodipine caused a significant reduction in oxidized glutathione (26). On the other hand, studies with more specific markers of oxidative stress, such as F2-isoprostanes measured in plasma and/or urine, have demonstrated no differences between untreated hypertensives and controls (27,28). Thus, human studies have not been as conclusive as animal studies.

Redon et al. (29) have recently studied the oxidative status, antioxidant activities and ROS byproducts in whole blood and mononuclear peripheral cells in untreated hypertensive subjects and in normotensive volunteers. The blood and mononuclear cells from hypertensive patients exhibited significant deficiencies of physiological antioxidants and an accumulation of large quantities of peroxidation and DNA oxidation byproducts. However, no significant relationship between oxidative stress and BP values was observed. These data may indicate that factors other than BP alone, inherent to the hypertensive status (such as hyperadrenergic reactivity, enhanced activity of the renin–angiotensin system or hyperinsulinemia), may be responsible for the altered oxidative status, which, in this case, would be a consequence, rather than a causative factor, of hypertension (29).

Molecular sources of ROS in hypertension

Although NADPH oxidase is considered the best characterized cellular source of ROS, there are a number of further possible sources of free radical production within the vasculature. These include NO synthase (NOS), lipoxygenases, cyclo-oxygenases, xanthine oxidase (XO) and cytochrome P450 enzymes (CYP450) (30,31). Mitochondria may also be a significant source of superoxide in hypertension.

Nicotamide adenine dinucleotide phosphate reduced form (NADPH)-oxidases (Noxs)

Since increased Nox activity has been reported as a major source of superoxide, it may be relevant to consider the potential genetic influences on this enzymatic system, which is specifically designed to modulate cellular redox signaling (22). Moreno et al. (32) have recently described a variant of the human p22phox (an essential Nox subunit) gene promoter, the 930A/G polymorphism, which is associated with essential hypertension. Further studies have confirmed that among the polymorphisms of the gene encoding p22phox, the 930A/G polymorphism may significantly contribute to the susceptibility of hypertensive patients to oxidative stress (33,34).

The role of Noxs in cardiovascular health and disease has recently and extensively been reviewed by Cave et al. (22). Nox isoforms represent an enzyme family containing at least five members, which are known to differ in relation to cell- specific expression and intracellular sites of localization (35). It has recently been suggested that the overexpression of the Nox1 isoform in SMCs may produce a basal increase in vascular ROS production, an Ang II-induced hypertensive response and medial artery hypertrophy (36). Conversely, in Nox1-null mice Ang II-induced hypertension is mild and without effect on medial hypertrophy (37). These findings are consistent with the data of Wang et al. (38)

showing that a deficit in another NADPH oxidase isoform (Nox2), although without effect on Ang II-induced hypertension, causes a significant attenuation of medial hypertrophy in mice. Thus, these data could provide important insights on the possibility that abnormalities of vascular structure may precede the development of hypertension (35). In addition, these studies support the idea that Noxs may be not only an important source of ROS within the vessel wall, but also play a more specific role in the pathogenesis of hypertension.

Xanthine oxidoreductase

Xanthine oxidoreductase (XOR) is a metalloflavoprotein enzyme that exists in two interconvertible although functionally distinct forms, namely xanthine dehydrogenase (XD) and XO. Of them, only the latter generates superoxide by oxidation of hypoxanthine to xanthine, and xanthine to urate (which requires the reduction of molecular O_2) (39–42).

XO is activated by proinflammatory mediators and is upregulated by NADPH oxidase activation. *In vitro* experiments with endothelial cells have shown that incubation with apocynin or disruption of the p47phox gene markedly decrease XO expression and activity (43). However, the precise *in vivo* regulation of endothelial XO is still unclear (40).

The putative contribution of XO to ROS elevation in hypertension has been assessed using specific inhibitors, such as allopurinol and oxypurinol. For example, oxypurinol increases endothelial-dependent arterial relaxation in rats fed a high-salt diet (44), in spontaneously hypertensive rats (SHR) (45), and in rats overexpressing renin and angiotensinogen genes (46). These data suggest that XO may be another source of ROS elevation in several disease settings associated with impaired vascular function (39,42). XO activation may also contribute to the genesis of redox signaling dysfunction leading to altered gene–protein expression (47). Finally, polymorphisms of the XO gene have recently been found to be associated with BP levels (48).

Endothelial NOS (eNOS)

eNOS, like all NOSs, catalyzes the oxidation of L-arginine to L-citrulline and NO (49). Under abnormal circumstances, eNOS can become ''uncoupled'' and reduce molecular oxygen (rather than transferring electrons to L-arginine), thereby generating superoxide (50–54). The essential NOS cofactor tetrahydrobiopterin (BH_4) plays a key role in regulating NOS function by coupling the reduction of O_2 to L-arginine oxidation (55,56). Thus, a relative deficiency of BH_4 or arginine may lead to eNOS uncoupling. Further, an increased ROS production by NADPH oxidases can cause the oxidative loss of BH_4, with following amplification of oxidative stress through decreased NO production and increased NOS-dependent superoxide generation (57). Peroxinitrite itself (a product of the interaction between excess superoxide and NO) may also oxidize BH_4, causing eNOS uncoupling (58). Increased production of superoxide rather than NO, and increased generation of peroxynitrite, have been implicated in the pathophysiology of several cardiovascular diseases, including hypertension (53,40).

Table I. Targets and actions of NO.

Target	Action
Endothelium	Modulates permeability
	Inhibits leukocyte adhesion
	Promotes migration
	Increases proliferation
Vascular smooth muscle	Initiates relaxation
	Inhibits proliferation
	Diminishes migration
Platelets	Reduces activation
	Decreases aggregation
	Limits adhesion
Leukocytes	Inhibits adhesion and diapedesis

Cytochrome P450 (CYP) enzymes

In a recent editorial (59), Fleming has briefly discussed the available data concerning the link between CYP enzymes and the regulation of vascular tone. CYP enzymes fall into two classes: the epoxygenases, which generate vasodilator epoxyeicosa-trienoic acids (EETs), and the ω/ω-1 hydroxylases, which generate the vasoconstrictor 20-hydroxy-eicosa-tetraenoic acid (20-HETE).

Since EETs act as NO, a decrease in their formation and tissue levels can contribute to the development of some forms of experimental hypertension (60), including salt-induced hypertension in salt-sensitive Dahl rats (61). Moreover, during the CYP reaction cycle, when the electrons of the central heme iron are transferred on the activated bound oxygen molecule in a NADPH-dependent reaction, the CYP epoxygenases generate superoxide anions, hydrogen radicals and hydroxyl radicals (60). Thus, the CYP epoxygenases expressed in endothelial cells could contribute significantly to the production of ROS within the vascular wall. This production of ROS would be sufficient to impair NO-mediated

vasorelaxation, and to elevate the activity of nuclear factor kB (Nf-kB) and the expression of vascular cell adhesion molecules (VCAM) (30).

Changes in the production of 20-HETE, which is currently characterized as a vasoconstrictor metabolite of arachidonic acid, may also be linked to the development of experimental (31,62) and human hypertension (63,64). In particular, Ward et al. (63) found that urinary 20-HETE is associated with endothelial dysfunction in humans with blunted flow-induced vasodilatation of the brachial artery. More recently, Gainer et al. (64) reported that a functional variant of the human renal 20-HETE synthase, CYP 4A11, was associated with essential hypertension in a white population from Tennessee. These studies support the hypothesis that abnormalities in the 20-HETE pathway could play a role in various forms of human hypertension.

Mitochondria as a source of ROS in hypertension

During aerobic metabolism in the mitochondrial respiratory chain, a small quantity of O_2 may be incompletely reduced, and superoxide be produced by complexes I (NADH coenzyme Q reductase) and III (ubiquinol Cyt C reductase). The rate of ROS generation can be modulated by the mitochondrial inner membrane potential (65,66). Indeed, mitochondria can be considered a major source of ROS. On the other hand, the increase in oxidative stress associated with atherosclerosis risk factors might cause a mitochondrial oxidative damage, so that mitochondria can also be important targets for ROS (67,68).

An excessive production of ROS in mitochondria will damage lipids, proteins and mitochondrial DNA (mtDNA), which is located close to the inner membrane and is not protected by histone proteins, as is the case for nuclear DNA (69,70). The accumulation of mtDNA mutations can cause cell dysfunction by inducing the synthesis of defective protein subunits of the respiratory chain (71), with further oxidative stress and ROS production. A vicious cycle may therefore be generated, which leads to progressive accumulation of mutations and oxidative damage to mtDNA (72) Interestingly, NO itself at high concentrations can damage mtDNA by stimulating the mitochondrial production of superoxide, hydrogen peroxide and peroxinitrite (73).

mtDNA mutations may also be inherited. For example, variants of the mitochondrial genome have been reported by Schwartz et al. in both African American and white hypertensive families (74), and an excess of maternal transmission of predisposition to hypertension has been found in hypertensive families from three ethnic groups (75).

The participation of mithocondria in the pathogenesis of hypertension is also suggested by the involvement of mitochondrial uncoupling proteins (UCPs) in experimental and human hypertensive states. In particular, mice with doxycycline-inducible expression of UCP1 in arterial walls develop hypertension and dietary atheroscerosis. UCP1 expression also increases superoxide production and decreases the availability of NO (76). These results are in agreement with the study by Douette et al. (77), which showed that UCP1 has a dual influence on free radical generation. On the one side, FFA-activated UCP1 is able to decrease superoxide anion production, demonstrating that a decrease in ROS generation is an obligatory outcome of UCP1 activity. On the other side, an increase in UCP1 content was concomitant with an increase in the basal release of superoxide by mitochodria as a side consequence of the overall increase in oxidative metabolism.

As far as UCP2 is concerned, its content in kidney mitochondria of SHR was lower than in normotensive controls (78), suggesting that a major function of UCP2 would be to increase the proton conductance across the inner mitochondrial membrane, thus reducing superoxide production (79). Accordingly, a common polymorphism of the UCP2 gene was associated with hypertension in a Japanese population, and with hypertension and obesity in Caucasians (80).

The possible mitochondrial involvement in hypertension is supported by several studies in both animals and humans. Hypertension is associated with the deterioration of mitochondrial energy production in SHRs (78,81,82) and mice (76). The occurrence of mitochondrial dysfunction has been associated with decreases in cytochrome oxidase activity (83), ATP production (76) and inorganic phosphate translocator activity (84).

Similarly to eNOS, mitochondrial NOS (mtNOS) might be involved in hypertension. This hypothesis was not confirmed after measurement of brain mtNOS in SHR (85). However, further studies will be required to assess the physiological significance of vascular mtNOS and to elucidate its regulatory effects on cellular energy metabolism in mitochondria (86).

Other possible sources of ROS in hypertension

Myeloperoxidase (MPO) may be an important source of ROS in the presence of vascular damage, contributing to exacerbate vascular diseases (87).

Transition metals, like iron and copper, are strong catalysts for oxidation reactions in the presence of hydroperoxides. However, the blood concentration of free transition metals is rather low *in vivo*. In addition to free iron and copper, biological forms of iron, such as heme, hemoglobin and myoglobin all have the potential to catalyze oxidative reactions in the vessel wall and to oxidize low-density lipoprotein (88).

Conclusions

Experimental evidence and clinical studies suggest that oxidative stress may be involved in the pathogenesis of hypertension. However, whether elevated levels of ROS initiate the development of hypertension or are a consequence of the disease process is not yet completely understood (6,7). Potential sources of free radicals within the vasculature that could be implicated in hypertension include mitochondria, NADPH oxidase, XOR, uncoupled endothelial NOSs, and CYP enzymes. In addition, enzymes such as lipoxygenases and cycloxygenases may also generate ROS, but the evidence for their participation in redox signaling is still scarce (89).

Activation of the renin–angiotensin system has been proposed as a mediator of NADPH oxidase activation and ROS production. In particular, Ang II stimulation of AT1 receptors in the vascular wall leads to the activation of NADPH oxidase. The resultant oxidative stress is considered a unifying mechanism for hypertension and atherosclerosis (90). Ang II can also directly regulate NADPH activation by phosphorylating and translocating p47phox to the cell membrane (91). Therefore, it is possible that some effects of the antihypertensive and vasculoprotective actions of AT1 receptor blockers and ACE inhibitors could be attributed to NADPH oxidase inhibition and decreased ROS production (92–94).

Finally, also mechanical stretch may induce p47phox membrane translocation and NADPH oxidase activation in vascular smooth muscle cells (95). Stretch-stimulated NADPH oxidase might play an important role in hypertension-induced vessel wall remodeling through the activation of metalloproteinase 2 (MMP-2), which is involved in elastic lamina destruction and in the process of arterial stiffening in humans (96,97).

Oxidative stress may contribute to the pathogenesis and/or maintenance of hypertension according to several possible mechanisms. Ward and Croft (6) have outlined these molecular mechanisms as follows: inactivation of NO by superoxide, generation of vasoconstrictor lipid peroxidation products such as F2-isoprostanes, depletion of BH_4 (the cofactor of NOS), as well as structural and functional alterations within the vascular wall, as recently reported by Kals et al. in patients with peripheral arterial disease (98).

Molecular processes underlying ROS-induced vascular changes involve activation of redox-sensitive signaling pathways. Indeed, superoxide and H_2O_2 stimulate mitogen-activated protein kinases, tyrosine kinases and transcription factors such as nuclear factor kB (NFkB), activator protein-1 (AP-1) and hypoxia-inducible factor-1 (HIF-1) (99), and inactivate protein tyrosine phosphatase (100,101). ROS also increase Ca^{2+}, and upregulate protoncogenes and the expression of proinflammatory genes (100).

Despite experimental evidence of the importance of oxidative stress in hypertension-dependent vascular damage, only a few clinical investigations reported limited effects of antioxidant vitamins on BP (99,102,103). The divergence between experimental models and clinical data has been defined "the oxidative paradox" (104). Patterson et al. (104) have recently examined several possible explanations for the rather disappointing results of antioxidants. For example, the endpoints of most trials are usually ischemic events: these might be too far from the initial development of hypertension and atherosclerosis, which may typically involve oxidative stress. Another important reason may be that in all clinical trials patients were not recruited on the basis of an elevated ROS formation. In other words, the patients without a significant oxidative stress might not benefit from antioxidant therapy, which highlights the need to identify the high-risk population with reliable biomarkers. Thus, more clinical studies on antioxidants or other ROS modulators are needed, possibly involving cohorts with evidence of oxidative stress, in order to fully elucidate whether an excess of ROS does play a significant role in the development of human hypertension and its cardiovascular complications.

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