

## Experimental Physiology – Symposium Report

# Oxidative stress and vascular remodelling

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Oxidative stress plays an important role in the pathophysiology of vascular diseases. Reactive oxygen species, especially superoxide anion and hydrogen peroxide, are important signalling molecules in cardiovascular cells. Enhanced superoxide production increases nitric oxide inactivation and leads to an accumulation of peroxynitrites and hydrogen peroxide. Reactive oxygen species participate in growth, apoptosis and migration of vascular smooth muscle cells, in the modulation of endothelial function, including endothelium-dependent relaxation and expression of proinflammatory phenotype, and in the modification of the extracellular matrix. All these events play important roles in vascular diseases such as hypertension, suggesting that the sources of reactive oxygen species and the signalling pathways that they modify may represent important therapeutic targets. Potential sources of vascular superoxide production include NADPH-dependent oxidases, xanthine oxidases, lipoxygenases, mitochondrial oxidases and nitric oxide synthases. Studies performed during the last decade have shown that NADPH oxidase is the most important source of superoxide anion in phagocytic and vascular cells. Evidence from experimental animal and human studies suggests a significant role of NADPH oxidase activation in the vascular remodelling and endothelial dysfunction found in cardiovascular diseases.

(Received 9 March 2005; accepted after revision 5 May 2005; first published online 12 May 2005)

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Oxidative stress is defined as the imbalanced redox state in which pro-oxidants overwhelm antioxidant capacity, resulting in increased production of reactive oxygen species (ROS). ROS have been implicated in the pathogenesis of virtually every stage of vascular lesion formation in atherosclerosis (Madamanchi *et al.* 2005; Mueller *et al.* 2005). Increasing evidence supports a role for oxidative stress in the pathogenesis of experimental and essential hypertension (Touyz, 2004). It has been extensively described that ROS contribute to blood pressure elevation by influencing vascular function and structure (Zalba *et al.* 2001b; Dröge, 2002).

### Production and metabolism of reactive oxygen species

Traditionally, macrophages have been assumed to be the source of the most of the ROS in the vessel wall, and there is no doubt that these cells play an important role in vessel pathology (Cathcart, 2004). However, it has become clear that virtually all cells in the vessel wall (endothelial, smooth muscle and adventitial cells) produce ROS, in varying

amounts and in response to diverse stimuli, which can then act in an autocrine or paracrine fashion to modulate cellular function (Griendling *et al.* 2000).

ROS play a physiological role in the vessel wall and participate as second messengers in endothelium-dependent function, in smooth muscle cell and endothelial cell growth and survival, and in remodelling of the vessel wall. Each of these responses, when uncontrolled, contributes to vascular diseases (Griendling & Harrison, 1999; Irani, 2000; Taniyama & Griendling, 2003). The major vascular ROS is superoxide anion ( $\cdot\text{O}_2^-$ ), which inactivates nitric oxide (NO), the main vascular relaxing factor, thus impairing relaxation (Kojda & Harrison, 1999; Cai & Harrison, 2000). Dismutation of  $\cdot\text{O}_2^-$  by superoxide dismutase (SOD) produces hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), a more stable ROS, which, in turn, is converted to water by catalase and glutathione peroxidase.  $\text{H}_2\text{O}_2$  and other peroxides appear to be important in the regulation of growth-related signalling in vascular smooth muscle cells (VSMCs) and inflammatory responses in vascular lesions (Li *et al.* 1997; Irani, 2000). High levels of  $\cdot\text{O}_2^-$ , the consequent accumulation of  $\text{H}_2\text{O}_2$  and diminished

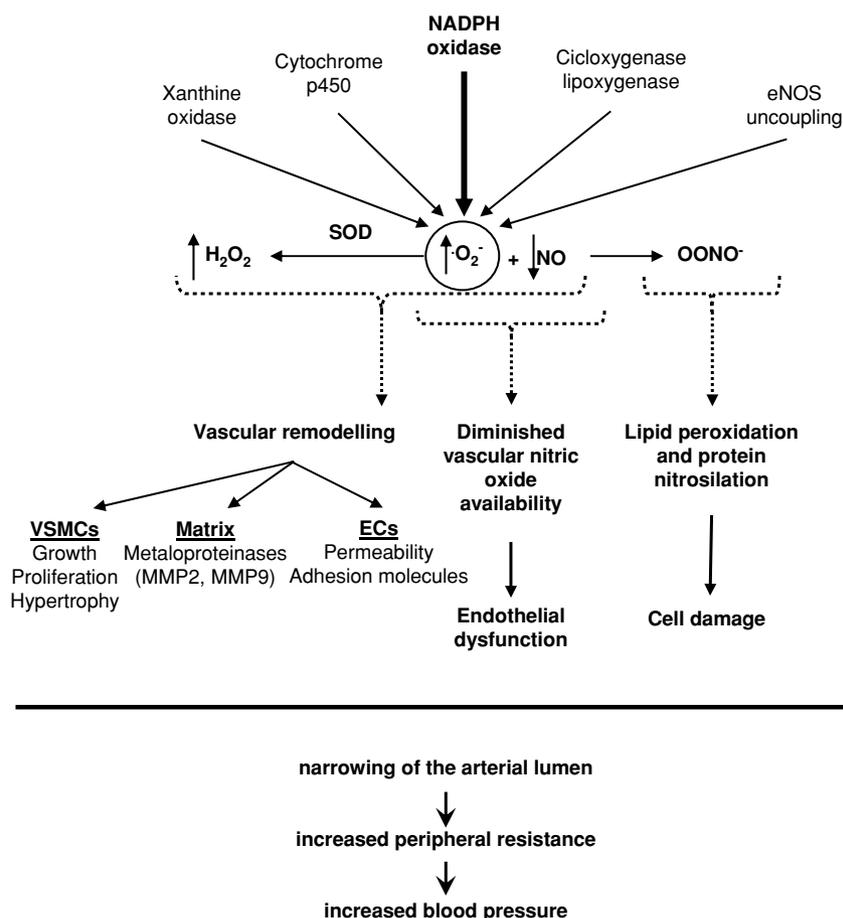
NO bioavailability play a critical role in the modulation of vascular remodelling. Finally, the reaction product between  $\cdot\text{O}_2^-$  and NO, peroxynitrite, constitutes a strong oxidant molecule, which is able to oxidize proteins, lipids and nucleic acids, causing cell damage (Beckman & Koppenol, 1996). These pathological processes are associated with hypertension because they contribute to the narrowing of the arterial lumen and consequently to increased peripheral resistance and blood pressure (Fig. 1).

### NADPH oxidase systems

Not only are there different cellular sources of ROS, but also cells use different enzymes to produce ROS in different circumstances. In addition to mitochondrial sources of ROS,  $\cdot\text{O}_2^-$  can be derived from xanthine oxidase, NADPH

oxidases, cyclooxygenases, lipoxygenases and uncoupled nitric oxide synthase. On the basis of experimental and clinical studies it has been proposed that NADPH oxidase is the predominant  $\cdot\text{O}_2^-$ -producing enzyme in the context of oxidative stress in cardiovascular diseases (Mueller *et al.* 2005).

The best-characterized NADPH oxidase is that found in phagocytes, where this enzyme is also the major inducible source of  $\cdot\text{O}_2^-$  and where it plays a role in immune protection owing to its bactericidal activity (Cathcart, 2004). The phagocytic oxidase consists of a membrane-associated cytochrome that comprises a large subunit, gp91phox ('phox' being derived from *phagocytic oxidase*), and a small one, p22phox. Besides these, there are at least three cytosolic subunits (p47phox, p67phox and p40phox) and a low-molecular-weight G protein (rac2; Cross & Segal, 2004). All the subunits present on phagocytic



**Figure 1. Generation of reactive oxygen species**

Many enzymatic systems have the potential to generate reactive oxygen species. This flow diagram illustrates the functional and structural consequences of reactive oxygen species in arterial hypertension. An enhanced superoxide production, mainly due to NADPH oxidase activation, is involved in endothelial dysfunction and vascular remodelling by decreasing NO bioavailability and increasing production of  $\text{H}_2\text{O}_2$  and  $\text{OONO}\cdot$ . eNOS, endothelial nitric oxide synthase; SOD, superoxide dismutase; VSMCs, vascular smooth muscle cells; ECs, endothelial cells.

NADPH oxidase have been identified in vascular cells, as well as p47phox, p67phox and gp91phox (nox2) homologues nox1, nox4 and nox5 (Lassegue & Clempus, 2003). However, several studies have confirmed that p22phox is present in all NADPH oxidase systems and that this subunit is essential for the functionality of the enzyme (Ushio-Fukai *et al.* 1996; Lassegue & Clempus, 2003). Upon cell stimulation, p47phox becomes phosphorylated, and the cytosolic subunits form a complex which migrates to the membrane where it binds to the cytochrome. Then electrons are transferred from the substrate, NADPH, to  $O_2$ , leading to  $\cdot O_2^-$  generation (Brandes & Kreuzer, 2005).

### Role of reactive oxygen species in vascular physiology and pathophysiology

The intracellular and extracellular production of ROS and the consequent activation of specific signalling pathways coordinate several integrated physiological responses in cardiovascular tissues. Numerous studies have shown that ROS influence cellular processes in vascular remodelling by turning on several intracellular signalling cascades. ROS potentially activate extracellular signal regulated kinases (ERKs), mitogen-activated protein kinases (MAPKs) members important in cell growth and differentiation; receptor and non-receptor tyrosin kinases, which have been implicated in cardiovascular remodelling and vascular damage; and protein tyrosin phosphatases and transcription factors, such as NFkB and AP-1, which induce expression of pro-inflammatory genes that play a role in the vascular inflammation associated with hypertension and atherosclerosis (recently reviewed by Touyz & Schiffrin, 2004; Cai, 2005). Nevertheless, in spite of all this evidence, the exact molecular targets of ROS have not been clarified. The physiological processes coordinated by ROS have been analysed in experimental animal models as well as in human disease, showing their important role in the development of hypertensive vascular disease, since they favour structural and functional alterations such as vascular remodelling, augmented deposition of extracellular matrix proteins, inflammatory processes and increased endothelial permeability.

Several studies have established the role of ROS in growth processes that contribute further to vascular injury and remodelling. Angiotensin type 1 (AT1) receptor-mediated production of  $\cdot O_2^-$  generated by NADPH oxidase is followed by an increased intracellular  $H_2O_2$ , which may participate as a second messenger for the long-term responses of angiotensin II, such as hypertrophy or hyperplasia of VSMCs (Griendling *et al.* 1994; Ushio-Fukai *et al.* 1996; Zafari *et al.* 1998). Angiotensin II-induced hypertrophy can be inhibited by the flavoprotein inhibitor DPI (Griendling *et al.* 1994), by antisense p22phox transfection (Ushio-Fukai *et al.* 1996) and by catalase overexpression (Zafari *et al.* 1998), thus

supporting the role of vascular NADPH oxidase. Our group reported an enhanced NADPH oxidase-driven  $\cdot O_2^-$  production in the aorta of the adult spontaneously hypertensive rat (SHR), which develops vascular wall hypertrophy (Zalba *et al.* 2000; Fig. 2). A recent study using transgenic mice that overexpress the p22phox subunit in VSMCs demonstrated that increased  $H_2O_2$  is associated with vascular hypertrophy (Weber *et al.* 2005). The importance of these studies in experimental animal models is confirmed by studies with human VSMCs showing that enhanced NADPH oxidase-dependent oxidative stress is associated with angiotensin II-induced vascular remodelling in essential hypertension (Touyz & Schiffrin, 2001).

ROS also modulate vascular remodelling by increasing deposition of extracellular matrix proteins. Collagen degradation depends on the activity of enzymes known as metalloproteinases (MMPs; Wainwright, 2004). These MMPs are secreted by macrophages and VSMCs in an inactive form (Galis & Khatri, 2002). ROS activate MMP2 and MMP9, which promote degradation of basement membrane and elastin, respectively, in cultured human smooth muscle cells (Rajagopalan *et al.* 1996). In particular, peroxynitrite rapidly increases MMP2 activity in the coronary effluent (Wang *et al.* 2002). Furthermore, increased aldosterone-induced systemic oxidative stress is associated with an increased collagen and fibronectin deposition related to the development of vascular remodelling (Pu *et al.* 2003). This study demonstrates for the first time that the inflammatory response is associated with vascular remodelling and enhanced oxidative stress, which may be induced by endothelin-1 in this experimental model of hypertension.

Redox-sensitive inflammatory processes also contribute to vascular remodelling in hypertension. Expression of intracellular adhesion molecule-1 (ICAM-1), which participates in the inflammatory process, is increased in the aorta from aldosterone-infused rats (Pu *et al.* 2003). Moreover, in an experimental model of angiotensin II-enhanced oxidative stress, where the role of NADPH oxidase is clearly demonstrated, induction of ICAM-1 expression is associated with tissue hypertrophy (Liu *et al.* 2003).

Finally, the endothelial cell redox rheostat is primarily regulated by the dynamic interaction between NO and  $\cdot O_2^-$ . NO inhibits platelet aggregation and adhesion of leucocytes to the endothelium. Increased endothelial permeability results in the extravasation of plasma proteins and the recruitment of inflammatory proteins and cells. Phagocytic cells participate in oxidative stress and inflammation in patients with hypertension (Kristal *et al.* 1998). In the study by Liu *et al.* (2003), angiotensin II-enhanced NADPH oxidase activity plays an important role in vascular hypertrophy, induction of ICAM-1 expression and the consequent leucocyte infiltration.

It is important to note that in cardiovascular diseases not only the vascular oxidase but also the phagocytic NADPH oxidase plays an important role in  $\cdot\text{O}_2^-$  production, since monocytes and lymphocytes can infiltrate cardiovascular tissues (Cathcart, 2004). The relevance of this is underlined by findings showing an enhanced phagocytic NADPH oxidase-dependent  $\cdot\text{O}_2^-$  production in hypertensive patients (Fortuño *et al.* 2004).

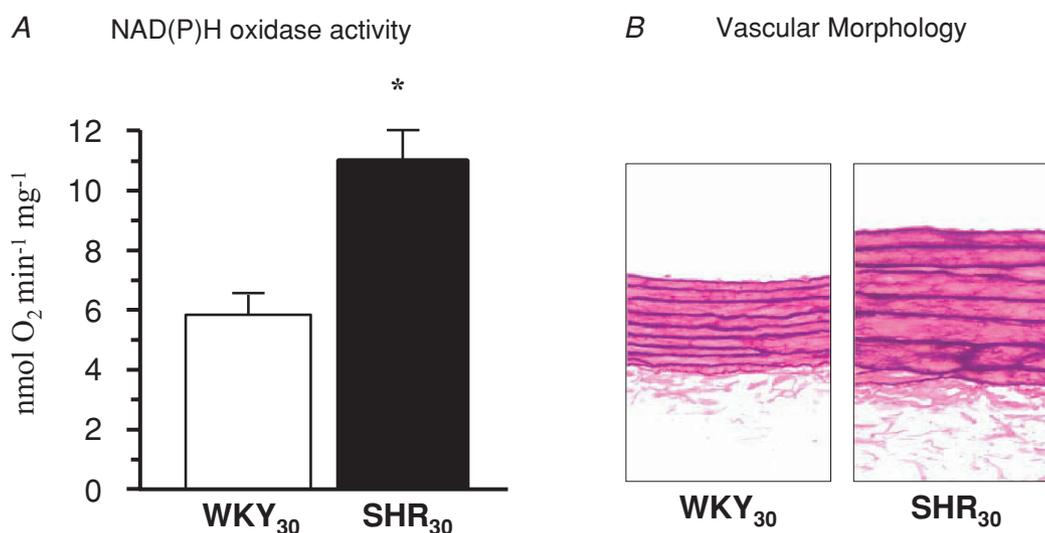
### Activation and regulation of the NADPH oxidase systems

Several potential stimulating factors of NADPH oxidase systems deserve to be considered in the setting of arterial hypertension. Although vascular NADPH oxidases are constitutive enzymes, humoral factors have been shown to regulate their activity. It has been extensively demonstrated that vasoactive agonists, such as angiotensin II, endothelin 1 (ET-1) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), regulate NADPH oxidase in vascular cells (De Keulenaer *et al.* 1998; Liu *et al.* 2003; Pu *et al.* 2003). Furthermore, our group has recently reported that phagocytic cells from hypertensive patients show a higher response to physiological concentrations of angiotensin II and ET-1 than cells obtained from normotensive subjects, supporting a potential role of these factors in the activation of mononuclear cells in the hypertensive state (Fortuño *et al.* 2004).

However, mechanical forces, including cyclic stretch and laminar and oscillatory shear stress, stimulate NADPH oxidase activity. In angiotensin II-infused rats, a reduction

of blood pressure is associated with normalized aortic  $\cdot\text{O}_2^-$  production (Virdis *et al.* 2002). In fact, high intraluminal pressure itself elicits increased  $\cdot\text{O}_2^-$  production in rat femoral arterial branches, and this effect is mediated by NADPH oxidase activation (Ungvari *et al.* 2003). The question of whether mechanical forces may also play a role in regulation of the phagocytic enzymatic system merits investigation. Nevertheless, our findings that  $\cdot\text{O}_2^-$  production is increased in those treated hypertensive patients in whom blood pressure remains raised suggest that mechanical forces may play an important regulatory role in the activation of the phagocytic NADPH oxidase (Fortuño *et al.* 2004). In this study we showed that there were no differences in the NADPH oxidase activity between treated, although uncontrolled, and not-treated hypertensive patients and, thus, both groups of patients showed elevated  $\cdot\text{O}_2^-$  levels with respect to normotensive subjects and treated hypertensive patients whose blood pressure levels were controlled. Nevertheless, humoral factors, which are also modulated by antihypertensive treatment, may also play a role of in the upregulation of NADPH oxidase activity.

Finally, genetic factors might regulate NADPH oxidase-driven  $\cdot\text{O}_2^-$  production in hypertension. A particular interest has been shown in the gene encoding the p22phox subunit, which possesses a significant number of genetic polymorphisms within the promoter and coding sequences, some of which are able to influence gene expression and NADPH oxidase activity (Zalba *et al.* 2005). Our group reported the existence of five polymorphisms in the promoter region of the p22phox



**Figure 2. NADPH oxidase activity and vascular hypertrophy in rat aorta**

A, NADH-driven  $\cdot\text{O}_2^-$  production in homogenates from aortic tissue from adult Wistar Kyoto (WKY<sub>30</sub>) and spontaneously hypertensive rats (SHR<sub>30</sub>). Bars represent means  $\pm$  s.e.m. of 10 animals in each group.

\* $P < 0.05$  compared with WKY<sub>30</sub>. B, representative cross-sections of aortas from adult WKY<sub>30</sub> and SHR<sub>30</sub> stained with Haematoxylin and showing tissue hypertrophy in the SHR<sub>30</sub>. This figure is adapted from Zalba *et al.* (2000) with permission from Lippincott Williams & Wilkins.

gene, which are associated with higher promoter activity in VSMCs of the SHR (Zalba *et al.* 2001a). More recently, we have characterized the promoter of the human p22phox gene, in which we have discovered a novel variation, the –930A/G polymorphism, which is associated with essential hypertension (Moreno *et al.* 2003). The relevance of this association has been underlined recently by a study demonstrating the functionality of the –930A/G polymorphism associated with diminished NO bioavailability (San José *et al.* 2004).

## Conclusions

Increased oxidative stress, mainly due to an enhanced NADPH oxidase activity, results in an exaggerated  $\cdot\text{O}_2^-$  production that favours the accumulation of  $\text{H}_2\text{O}_2$  and peroxynitrites and that diminishes NO availability. These pathological processes are associated with hypertension, because they contribute to the narrowing of the arterial lumen, and consequently to increased peripheral resistance and blood pressure.

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#### Acknowledgements

This work was supported by the agreement between FIMA and 'UTE project CIMA', Fundación de Aguas de Valencia, FMMA, 55/2002 and 56/2002 from Department of Health of the Government of Navarra, SAF2004-07910 from the Ministry of Science and Technology and RECAVA 2003/ISC from the Ministry of Health of Spain. The authors thank Ana Montoya and Raquel Ros for technical assistance.