[Frontiers in Bioscience 14, 3168-3187, January 1, 2009]

Redox sensitive signaling pathways in cardiac remodeling, hypertrophy and failure

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1. ABSTRACT

Adaptation of the heart to intrinsic and external stress involves complex modifications at the molecular and cellular level that lead to tissue remodeling and functional compensation or failure depending upon the nature, intensity and chronicity of the stress. Signaling mechanisms mediated by reactive oxygen species (ROS) are now known to play important roles in many aspects of this complex process. In particular, the tightly regulated generation of ROS by NADPH oxidases appears especially important in key signaling events that drive the development of cardiomyocyte hypertrophy, fibrosis, extracellular matrix remodelling and cell apoptosis. This review discusses the signaling pathways modulated by ROS during the development of cardiac remodelling and failure with a particular emphasis on the role of NADPH oxidases.

2. INTRODUCTION

"Cardiac remodeling" describes a complex pathophysiological process which has major effects on organ function and subsequently on clinical condition. Remodeling occurs in response to a variety of 'insults' or 'stresses'; those that are encountered most commonly in clinical practice are (i) an increased resistance to ventricular output, known as pressure overload, e.g. systemic arterial hypertension or aortic stenosis; (ii) an abnormally high volume of blood pumped during each cardiac cycle, known as volume overload, eg, as a result of aortic or mitral valve regurgitation; and (iii) myocardial infarction (MI), where there is an increased stress on the non-infarcted myocardium.

At a cellular level, the processes that contribute to cardiac remodeling include cardiomyocyte hypertrophy,

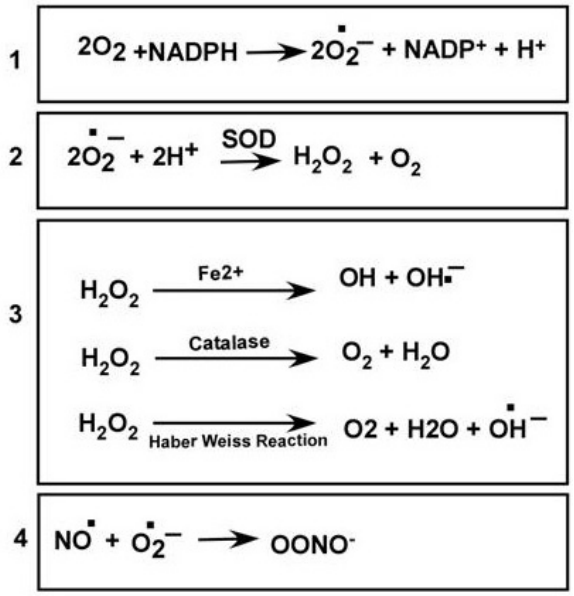

Figure 1. Reactions involved in the production and clearance of main reactive oxygen species. NADPH oxidases generate superoxide ($O_2^{\bullet-}$) by electron transfer from NADPH to molecular O_2 (reaction 1). $O_2^{\bullet-}$ is dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutases (SOD) – reaction 2. In the presence of Fe, H_2O_2 may form the highly reactive hydroxyl ($OH^{\bullet}$) species through the Fenton and Haber-Weiss reactions (reactions 3). $O_2^{\bullet-}$ also reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$) (reaction 4), while H_2O_2 may be degraded to H_2O by catalase or various peroxidases (reaction 5).

apoptosis, altered excitation-contraction coupling, abnormal energetics, interstitial fibrosis, remodeling of the extracellular matrix, reduced capillarisation, vascular dysfunction and inflammation, while at a molecular level these processes are driven by significant alterations in gene and protein expression (1). At a macroscopic level, cardiac remodeling is associated with significant changes in cardiac chamber size, shape, wall thickness and contractile function. Different morphological patterns of size and shape change are recognized clinically and specific patterns are associated with particular types of stress. For example, chronic pressure overload initially induces "concentric hypertrophy", where wall thickness is increased without a change in cavity size (the cellular correlate being the deposition of new sarcomeres in parallel). This yields the potential benefit of reduced wall stress through Laplace's law. In contrast, volume overload induces "eccentric hypertrophy", where an increase in ventricular cavity size dominates over accompanying increases in wall thickness (the cellular correlate here being the deposition of new sarcomeres in series).

At least some components of the above changes may have a beneficial adaptive function, analogous to the cardiac remodeling that occurs in the healthy athlete's heart (known as "physiological hypertrophy or remodeling"), but in the context of disease cardiac remodeling ultimately leads to the development of overt contractile dysfunction, arrhythmia and heart failure. The clinical heart failure syndrome is characterized by symptoms of exertional fatigue, breathless and dependent oedema and signs of fluid overload which are the ultimate consequence of impaired cardiac contractile function and/or its inability to meet its requirements without an abnormal elevation in ventricular filling pressures. In addition to major morbidity, heart failure is associated with a markedly elevated mortality rate and represents an important and increasingly prevalent clinical problem, especially in the elderly. Accordingly, elucidation of the signaling events that drive adverse cardiac remodeling, and those potentially distinct pathways that may drive adaptive remodeling programs, is a major research goal that holds the promise of defining novel therapeutic targets for patients with heart failure. While many different signaling pathways have been implicated in the development of cardiac remodeling and failure, in the current review we focus on the role of redox-sensitive pathways.

3. REACTIVE OXYGEN SPECIES (ROS) AS SIGNALING MOLECULES

ROS, including both radical species such as superoxide ($O_2^{\bullet-}$) and non-radical species such as hydrogen peroxide (H_2O_2), have increasingly been recognized to play important physiological and pathophysiological roles (Figure 1). The concept of oxidative stress – namely, an imbalance between ROS and opposing antioxidant defences in favour of the former – and its resultant increased propensity for detrimental oxidative modification of proteins, lipids, nucleic acids and other macromolecules is very well established. More recently, however, it has become appreciated that ROS can also act as targeted signaling molecules that reversibly modulate the activities of a diverse range of proteins, enzymes, ion channels and components of signal transduction cascades (2). ROS generated by at least some sources fulfill several criteria for suitability as physiological signaling molecules, namely (i) local production upon specific stimulation; (ii) demonstrable biological effects either within the cell where they are generated or on neighboring or more distant cells; (iii) reversibility of action. In particular, the small size, relative stability and diffusibility of H_2O_2 makes it especially suitable as a signaling molecule. In addition, the presence of complex cellular systems (eg, peroxiredoxins, catalase, peroxidases) that are capable of degrading ROS and thereby terminating the ROS-dependent signal provides another important component for a robust signaling system.

4. EVIDENCE FOR INVOLVEMENT OF ROS IN CARDIAC REMODELING AND FAILURE

Three broad types of clinical and experimental studies support an involvement of ROS and ROS-modulated processes in the pathophysiology of cardiac remodeling and failure.

Firstly, observational studies have consistently demonstrated an association between increased oxidative

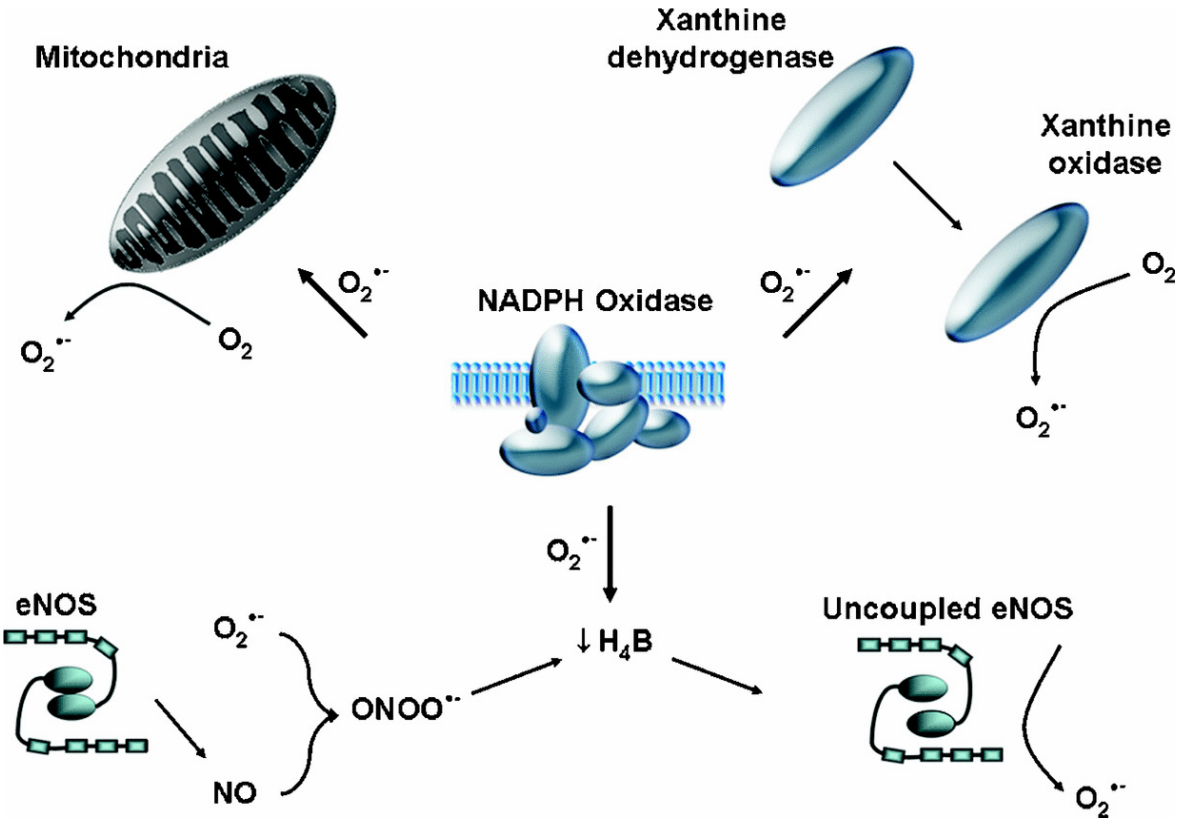

Figure 2. Interplay between NADPH oxidase and other ROS sources. $O_2^{\cdot-}$ generated from NADPH oxidase can potentially enhance ROS production by other enzymes. For example, xanthine dehydrogenase is converted to $O_2^{\cdot-}$generating xanthine oxidase through oxidation. Similarly, mitochondrial ROS generation can be increased by ROS derived from other sources. Finally, $O_2^{\cdot-}$ or $ONOO^-$ can degrade the essential NO synthase co-factor H_4B, thereby promoting NOS uncoupling and further $O_2^{\cdot-}$ generation. Reproduced with permission from (145).

stress and cardiac remodeling or failure. For instance, increased indices of oxidative stress are found in hypertension (3), post-MI (4) and heart failure (4-6). In addition, the expression and activity of enzyme systems that generate ROS, is also found to be increased – eg, xanthine oxidases (7-9), mitochondria (10-12) and NADPH oxidases (13-16).

Secondly, interventional studies using a variety of "anti-oxidant" approaches support a role of ROS. For example, the antioxidants agents probucol and dimethylthiourea have both been shown to ameliorate left ventricular remodeling after experimental MI (17,18). In contrast, the use of an agent to block Cu-Zn-superoxide dismutase resulted in increased hypertrophy and apoptosis of cultured neonatal cardiomyocytes (19).

Thirdly, studies using various genetically-modified animal models have provided more direct molecular evidence for an etiological role of ROS in cardiac remodeling and failure. For example, overexpression of the endogenous antioxidant glutathione peroxidase is able to reduce left ventricular remodeling after experimental MI (20).

5. SOURCES OF CELLULAR ROS IN HEART FAILURE

ROS are generated within cells by a wide variety of enzymes and processes, including the mitochondrial electron transport chain, the xanthine oxidase/dehydrogenase system, 'uncoupled' nitric oxide synthases (NOSs), cytochrome P450 and NADPH oxidases – all of which generate $O_2^{\cdot-}$ in the first instance, which may then be dismutated to H_2O_2. While ROS generation is generally a by-product of the actions of most of these enzymes, the NADPH oxidase family are an exception in that ROS production appears to be the primary function of these enzymes (21-23). Furthermore, these enzymes have several attributes that make them especially suitable for involvement in signal transduction (as discussed below). Another interesting point about NADPH oxidases is the increasing evidence that they are capable of initiating or stimulating further ROS production by other enzymes, eg, by (i) 'uncoupling' NOS, (ii) favoring conversion of xanthine degydrogenase to xanthine oxidase and (iii) increasing mitochondrial ROS generation (Figure 2). Amplification of ROS production may thus occur and may be important in exerting effects on signaling pathways.

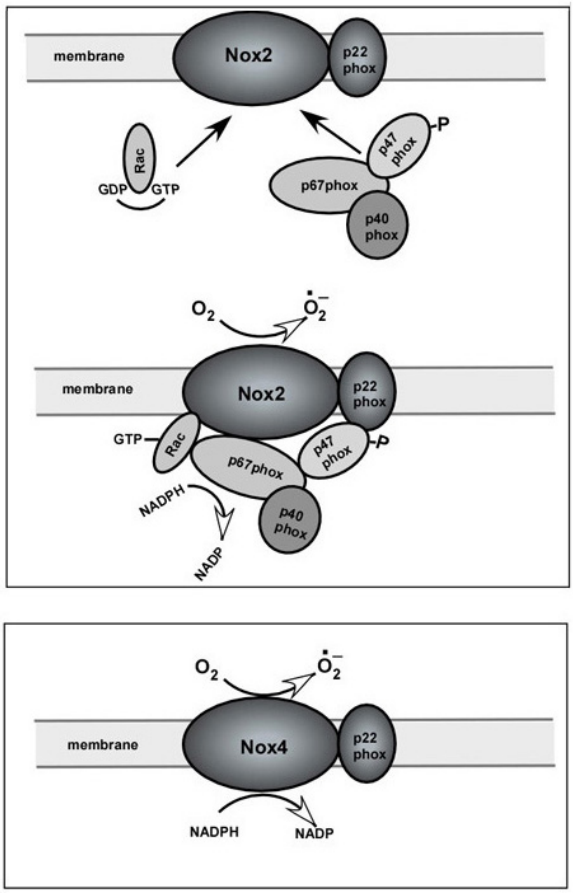

Figure 3. Schematic diagram of the structure of Nox2 and Nox4. Top panel shows the classical Nox2 oxidase under basal and activated conditions. Activation of the oxidase involves the stimulus-induced translocation of the cytosolic subunits p47phox, p67phox, and p40phox and GTP-bound Rac to the cytochrome b558 which is composed of Nox2 and p22phox. Bottom panel shows the structure of the Nox4-based oxidase.

In the current article, we focus primarily on the NADPH oxidases but do provide a brief description of the other important ROS sources. It should be noted at this point that multiple cell types within the heart are involved in ROS production, including cardiomyocytes, vascular endothelium, vascular smooth muscle, fibroblasts and infiltrating inflammatory cells of different types.

5.1 Mitochondrial ROS

Cardiac myocytes have a very high volume density of mitochondria compared to most other cells and there is a significantly increased mitochondrial generation of ROS in the diseased myocardium, which may damage mitochondrial macromolecules either at or near the site of their formation (24,25). The mtDNA could be a major target for such ROS-mediated damage. For example, overexpression of the mitochondrial antioxidant, peroxiredoxin-3, is reported to ameliorate mitochondrial DNA damage and inhibit adverse left ventricular remodeling after MI (26). Another recent study demonstrated that the development of right heart failure is associated with an increased capacity for ROS production by NADPH oxidase as well as mitochondria. A selective increase in expression and activity of mitochondrial Complex II was suggested to be particularly important (27).

5.2 Xanthine oxidase

Xanthine oxidoreductase (XOR) exists in two interconvertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). They differ in that XO only reduces oxygen (thereby generating $O_2^{\cdot-}$) whereas XDH can reduce either oxygen or NAD^+ but has greater affinity for the latter (7,28). Both forms catalyse the conversion of hypoxanthine to xanthine and xanthine to uric acid (UA), the terminal two reactions of the purine degradation pathway. Several studies have demonstrated upregulation of XOR in animal models and in human dilated cardiomyopathy (reviewed in (28). Functionally, XOR inhibition acutely enhances myocardial mechanical efficiency in both animals and humans. XOR inhibition has been demonstrated to improve cardiac performance and induce reverse remodeling in a spontaneously hypertensive rat model (7). A more detailed discussion of the role of XOR in cardiac pathology can be found elsewhere in this issue.

5.3 NADPH oxidases

The major source of signaling ROS in most cell systems appears to be NADPH oxidases (or "Noxs"), which catalyse electron transfer from NADPH to molecular oxygen thereby generating $O_2^{\cdot-}$. NADPH oxidase was first discovered in neutrophils which, during activation and phagocytosis, undergo the so-called respiratory burst with the release of $O_2^{\cdot-}$ into the phagosome. This high local concentration of ROS appears to be necessary for the process of killing of ingested pathogens within phagosomes. Several distinct but functionally related members of the NADPH oxidase family have been identified in recent years (21), each of which contains an isoform of the core oxidase catalytic unit termed Nox. The classical neutrophil NADPH oxidase is designated 'Nox2' while other oxidases include Nox1, Nox3, Nox4 and Nox5 (23). The Nox2 oxidase is a multimeric protein complex, the major subunit of which is Nox2 (or gp91phox). Nox2 is a lipid membrane-associated molecule with 6 membrane-spanning domains, which associates with a lower molecular weight subunit known as p22phox (Figure 3). To be functionally active, the gp91phox-p22phox complex must associate with other subunits that are recruited to the plasma membrane upon activation of the enzyme system. These comprise an activator subunit (known as p67phox), a regulatory subunit (known as p47phox), a small GTPase (Rac) and a p40phox subunit. Post-translational modification of these subunits, notably phosphorylation of p47phox and geranyl-geranylation of Rac, is essential for their recruitment to and association with the gp91phox-p22phox heterodimer.

In addition to neutrophils, the Nox2 oxidase complex is now known to be also expressed in endothelial cells (29-31), cardiomyocytes (32,33), fibroblasts (34) and some vascular smooth muscle cells (VSMC) (35). Other Noxs expressed in cardiovascular cells include Nox1, Nox4

Table 1. Expression of Nox isoform mRNAs in cardiovascular cells and tissue

	Cardiomyocytes	Endothelial cells (EC)	Fibroblasts	Vascular smooth muscle cells (VSMC)
Nox1		Isolated human coronary artery EC Human umbilical vein EC (HUVEC) Rat aortic EC Rat basilar artery EC	Isolated human cardiac fibroblasts	Isolated human coronary artery SMC Human aortic SMC Rat VSMC from mesenteric arteries Rat aortic VSMC Rabbit pulmonary arterial SMC Rabbit SMC from resistance arteries Mouse aortic VSMC
Nox2	Mouse left ventricle Isolated mouse cardiomyocytes Isolated rat cardiomyocytes	HUVEC Isolated human coronary artery Porcine pulmonary artery EC Rat cardiac microvascular EC Rat aortic EC Rat basilar artery EC	Isolated human cardiac fibroblasts Adventitia of human coronary arteries Adventitia of mouse aorta	Isolated human coronary artery SMC HVSMC from resistance arteries Human aortic intimal SMC Intimal cells from human coronary arteries Rat aortic VSMC
Nox4	Mouse left ventricle Isolated mouse cardiomyocytes	Isolated human coronary artery EC HUVEC Rat aortic EC Rat basilar artery	Isolated human cardiac fibroblasts Adventitia of human coronary arteries Isolated adult rat fibroblasts	Intimal cells of human coronary arteries Human aortic media Media of human coronary arteries Isolated human coronary artery SMC Human VSMC from resistance arteries Human aortic SMC Rat VSMC from mesenteric arteries Medial smooth muscle within rat carotid arteies
Nox5		HUVEC	Human cardiac fibroblasts	Human VSMC Human aortic SMC

and in human cells Nox5 (Nox5 is not expressed in rodents) (21,36). The cardiac tissue expression pattern of different Noxs is shown in Table 1. Like Nox2, the Nox1 oxidase has an analogous activation process which requires association with regulatory subunits. However, Nox4 appears to be quite distinct in that the only subunit that appears to be necessary for its activity is p22phox (37,38, Figure 3). It is notable that ROS production by Noxs, in particular Nox2 and Nox1, is activation by a range of stimuli that are implicated in cardiovascular pathophyisology (Figure 4). More detailed biochemical and pathophysiological aspects of NADPH oxidases have been extensively reviewed elsewhere (21,22).

A further area of variation amongst Nox members is their intracellular localization, which is likely to be of significant relevance for a role in signal modulation. The classical Nox2-containing oxidase is found to be a membrane-associated protein in both phagocytic (39) and non-phagocytic cells (37,40-42) but a proportion of the enzyme may also be found in an intracellular location in proximity to the perinuclear cytoskeleton – eg, in endothelial cells (29,37). In contrast to Nox2, the subcellular localization of Nox4 remains ambiguous. Published studies have variously reported Nox4 localization to the focal adhesions in VSMC (43), the endoplasmic reticulum (37,44), stress fibres (45,46) and the nucleus (45,47) in different cell types. Nox4 has also been reported to be at the plasma membrane (48) where it colocalized with TASK-2 and acted as an oxygen sensor in transfected HEK293 cells (49). The intracellular localization of Nox4 may clearly determine its potential downstream targets (43,45,47). Co-localisation of Nox1 and p22phox to the plasma membrane and sub-membrane regions has been reported in VSMC (50) whereas another study found intracellular co-localisation with protein disulfide isomerase (an ER marker) in VSMC (51).

6. REDOX SIGNALING IN CARDIAC HYPERTROPHY

6.1 Pro-hypertrophic effects OF ROS and anti-hypertrophic effects of antioxidants

H_2O_2 induces the activation of key pro-hypertrophic signaling molecules in a concentration-dependent fashion. Exposure of adult ventricular cardiomyocytes to low concentrations of H_2O_2 resulted in the activation of ERK1/2, increased protein synthesis and cellular hypertrophy (52). Higher concentrations of H_2O_2 (100 μM) led to the activation of p38MAPK, JNK and AKT and subsequent cellular apoptosis (53). Sustained increases in intracellular ROS have been shown to cause hypertrophy by increased intracellular Ca^{2+} and activating ERK1/2 (54).

Several antioxidant agents have been shown to modulate hypertrophic signaling. Resveratrol, which is proposed to account in part for the protective effect of red wine on the cardiovascular system, has been demonstrated to inhibit angiotensin II-induced cardiomyocyte hypertrophy via attenuation of ROS generation (55). Isorhapontigenin, a resveratrol analog, also attenuated cardiac hypertrophy by blocking angiotensin II-induced activation of PKC, ERK1/2, JNK and p38MAPK (56). Green tea extract blocks the development of cardiac hypertrophy by inhibiting cardiac myocyte ROS production (57). Epigallocatechin-3-gallate (EGCG) is a major bioactive polyphenol present in green tea and attenuated angiotensin II- and pressure overload-mediated cardiac hypertrophy (58). The antioxidant edaravone significantly attenuated pressure overload-induced cardiac hypertrophy mediated through its antioxidative function and subsequent inhibition of ASK1 signaling pathway (59). HMG CoA-reductase inhibitors (“statins”) such as simvastatin inhibit ROS-mediated hypertrophy in cultured neonatal rat cardiac myocytes (60-62).

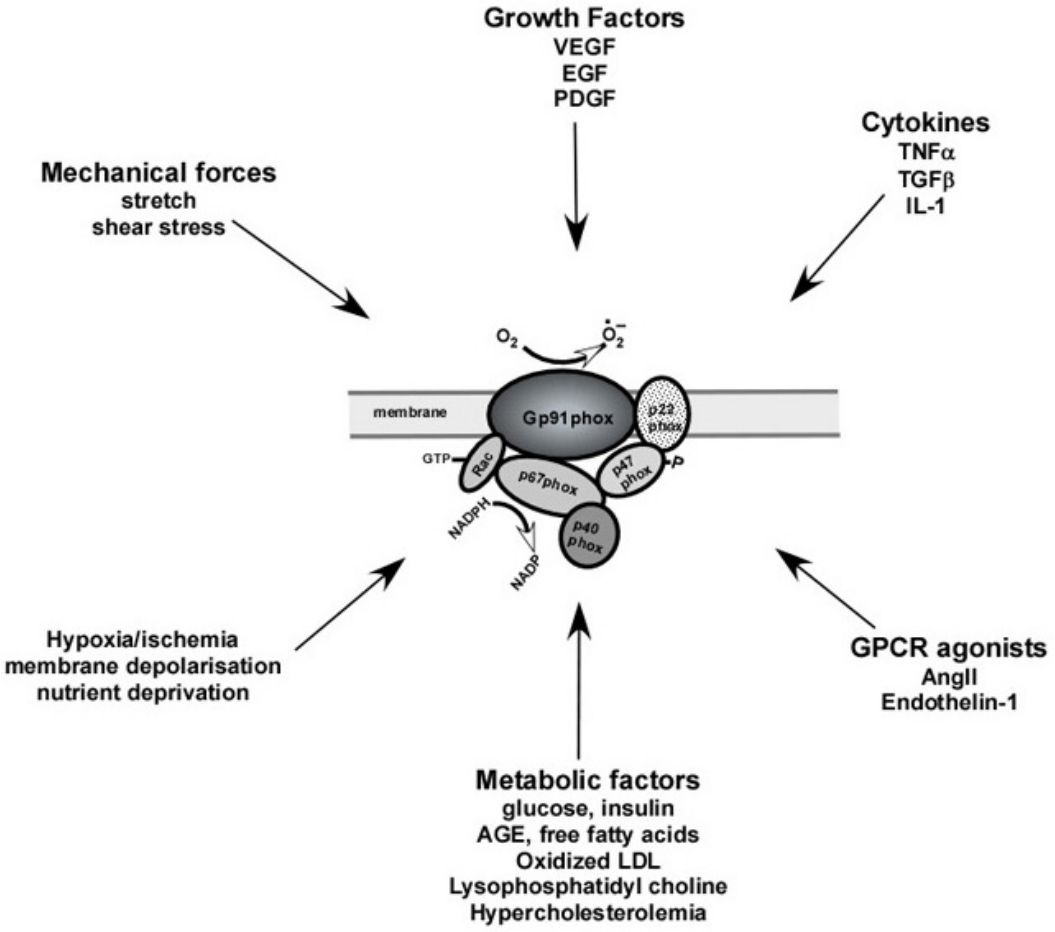

Figure 4. Main activators of the Nox2 oxidase. A diverse range of signals activate the oxidase, including G-protein coupled receptor (GPCR) agonists such as angiotensin II (Ang II) and endothelin-1, mechanical forces, ischemia-associated factors, metabolic factors, and growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), advanced glycation end-products (AGE) and low density lipoprotein (LDL).

6.2. GPCR-mediated cardiac hypertrophy

Several studies have revealed that angiotensin II, acting through AT_1 receptors, induces the activation of NADPH oxidase and the subsequent activation of key signaling molecules that drive cardiomyocyte hypertrophy. Data from our lab demonstrated that chronic angiotensin II infusion significantly increased heart/body weight ratio, myocyte area, atrial natriuretic factor and beta-myosin heavy chain mRNA expression (these being molecular markers of ventricular hypertrophy), and cardiac fibrosis in wild-type but not in Nox 2 null mice. Angiotensin II treatment concomitantly increased myocardial NADPH oxidase activity in wild-type but not Nox2 knockout mice (32). AT_1-induced Nox2-dependent hypertrophic responses have also been described in isolated cardiomyocytes (63). In mice with cardiomyocyte-specific deletion of Rac1, chronic *in vivo* angiotensin II stimulation induced less hypertrophy, decreased gp91phox-p67phox interaction, NADPH oxidase activity and myocardial oxidative stress (64) – indicating a critical role of Rac1 (Figure 5). Ang II has been shown to induce cardiac hypertrophy with increases in cardiomyocyte size, beta-myosin heavy chain expression, c-fos expression, increased gp91phox expression and increased superoxide generation in myocytes and all these effects were significantly inhibited by atrial natriuretic peptide (65). The antioxidant actions of atrial natriuretic peptide may thus contribute to their antihypertrophic effects.

Members of the MAPK family are known to be important regulators of hypertrophy of the heart and NADPH oxidase-dependent activation of ERK1/2 by angiotensin II is reported (66). Tanaka *et al* observed that phenylephrine or endothelin-1 treatment of adult cardiac myocytes led to NADPH oxidase-derived ROS production and hypertrophy through ERK activation (67). In this study, neither p38 MAPK nor JNK were activated during endothelin-1 or phenylephrine-induced hypertrophy. In a similar setting, angiotensin II caused an induction of TGF-β1 expression in adult ventricular cardiomyocytes and induced AP-1 binding activity; this was found to be redox sensitive and p38 MAPK dependent (68). Using neonatal cardiomyocytes, Cheng *et al* showed that ET-1 induced myocyte hypertrophy is redox sensitive and is mediated by

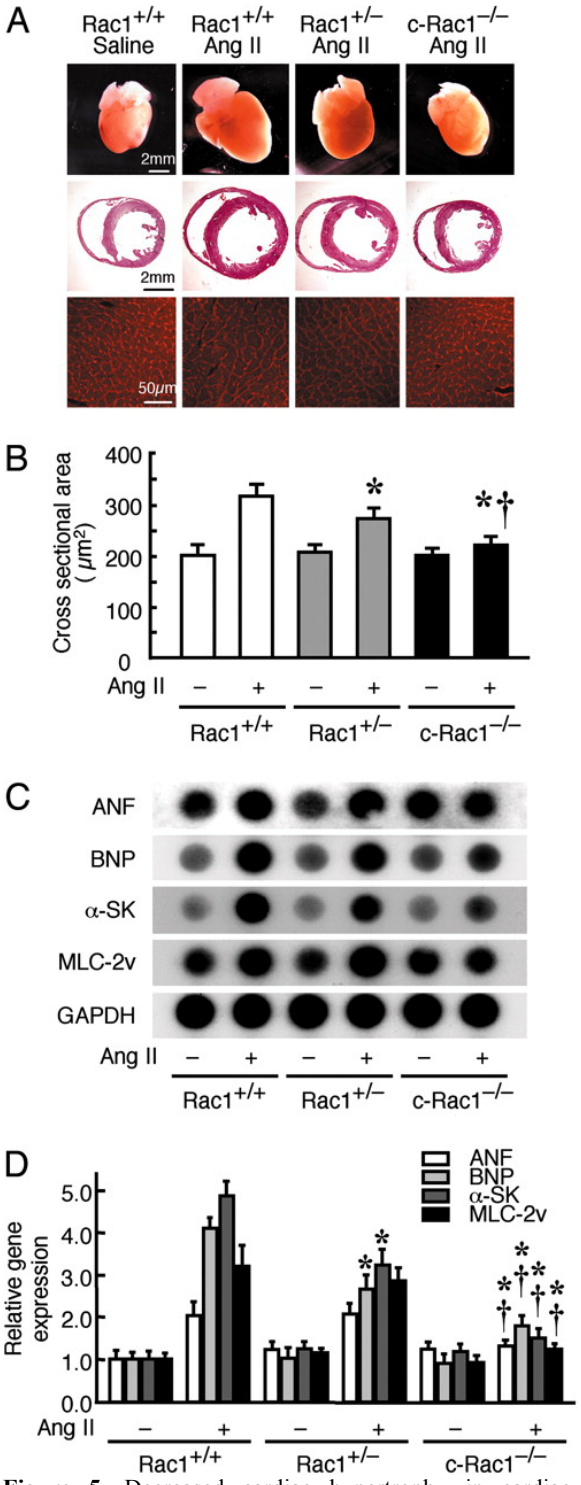

Figure 5. Decreased cardiac hypertrophy in cardiac-specific *Rac1* deletion mice (A) Hearts from Rac1$^{+/+}$, Rac1$^{+/-}$, and c-Rac1$^{-/-}$ mice at 2 weeks after saline or Ang II infusion. Below: Hematoxylin and eosin and wheat-germ agglutinin stains of heart sections. (B) Myocardial cross-sectional area. (C) RNA dot-blot analysis of cardiac gene expression from Rac1$^{+/+}$, Rac1$^{+/-}$, and c-Rac1$^{-/-}$ mice. (D) Quantitative analysis of mRNA expression. Reproduced with permission from (64).

ERK, p38MAPK and JNK. A possible involvement of the ras-raf-MAPK pathway has been suggested (69). In this context, it is interesting to note that ET-1 treatment of cardiomyocytes also increased constitutive NO synthase activity and NO production and that NO inhibited endothelin-1-induced cardiomyocyte hypertrophy through cGMP-mediated suppression of ERK phosphorylation (70).

A potentially important role of Rho kinase in angiotensin II-dependent cardiac hypertrophy is suggested by a study of long-term infusion of angiotensin II for 4 weeks in rats. These investigators found hypertrophic changes of vascular smooth muscle and cardiomyocytes which were significantly suppressed by concomitant oral treatment with a specific Rho-kinase inhibitor (71). Involvement of other signaling molecules such as a Ca^{2+}-sensitive non-receptor tyrosine kinases, proline-rich tyrosine kinase 2 (Pyk2) and c-Src was observed in Rac-mediated hypertrophy induced by ET-1 or PE, by the same group (72).

A key role of Akt activation in angiotensin II-mediated cardiac hypertrophic response was evidenced in a study by Hingtgen *et al* (73). Using dominant negative forms of Rac1 and Akt in neonatal cardiomyocytes, they demonstrated that increased formation of $O_2^{\cdot-}$ from a Rac1-regulated Nox2-containing NADPH oxidase is a key upstream mediator of angiotensin II-induced Akt activation, and that this signaling cascade plays a crucial role in angiotensin II-induced cardiomyocyte hypertrophy (73). However, other investigators using a similar cellular system reported that GPCR agonist-induced hypertrophic response was mediated through the activation of the apoptosis signal regulated kinase (ASK-1), followed by NFκB activation (74). An involvement of ASK1 in angiotensin II-induced cardiac hypertrophy and remodeling was further supported by studies in ASK$^{-/-}$ mice, where hypertrophy was inhibited in these animals (75). Interestingly, both p38MAPK and JNK were activated downstream of ASK-1 in this study.

Investigations into the contribution of PI3-kinase (PI3K) isoforms in angiotensin II and α-adrenoceptor-mediated hypertrophic signaling in cardiomyocytes reported that PI3K inhibition decreased ROS formation, phosphorylation of p38 MAPK, and TGFβ expression (76). While down-regulation of the p110β isoform inhibited the angiotensin II-induced signaling pathway, down-regulation of the p110α isoform decreased α-adrenoceptor-mediated hypertrophic growth of cardiomyocytes. α1-adrenoceptor mediated ERK1/2 activation was also blocked by inhibitors of NADPH oxidase (52).

Ras is a signaling protein which is known to have redox-sensitive cysteine residues and it has been shown that α adrenoceptor-stimulated hypertrophic signaling is mediated via oxidative modification of Ras thiols. Activation of Ras leads to the activation of signaling molecules such as MEK1/2, ERK1/2 and p90RSK, leading to cellular hypertrophy, sarcomere reorganization, and protein synthesis. Thus α adrenoceptor-stimulated

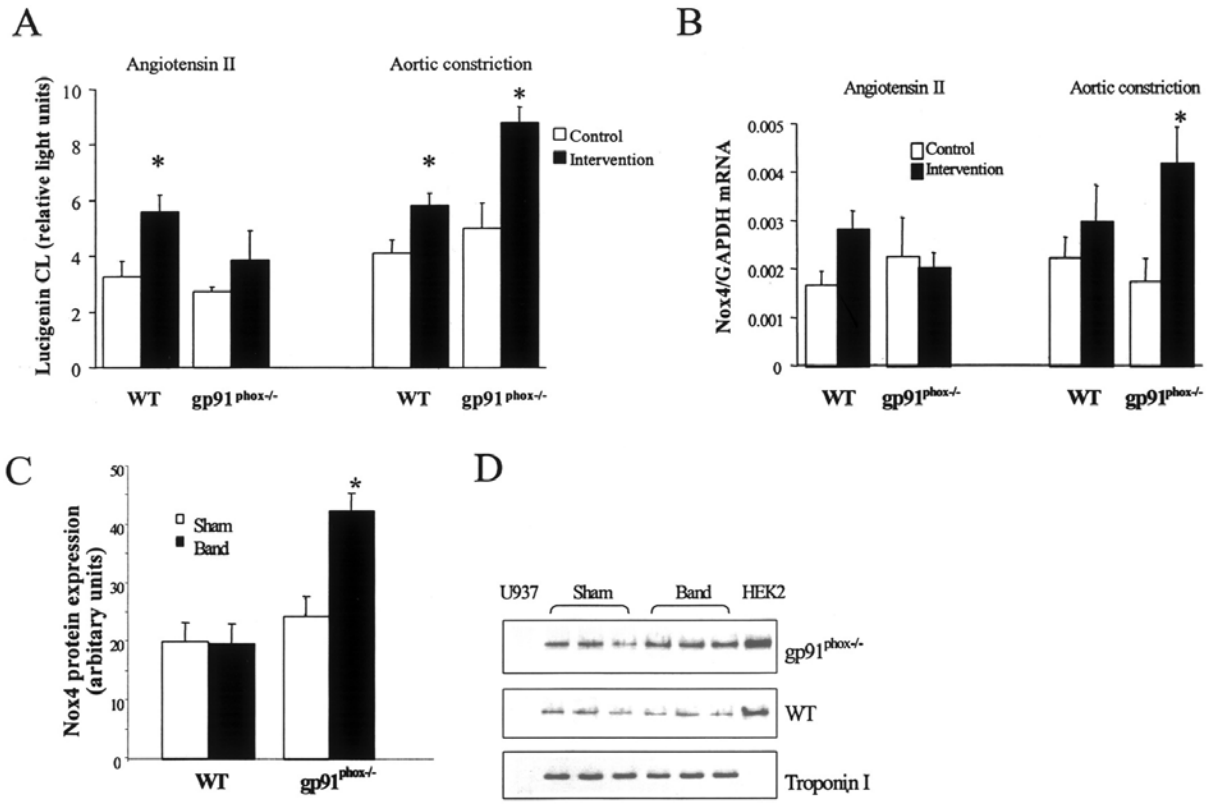

Figure 6. Expression of Nox4 after aortic constriction. (A) NADPH oxidase activity measured by lucigenin chemiluminescence increases after aortic constriction in Nox2 knockout mice, unlike the situation with chronic angiotensin II infusiob. (B) Increased Nox4 mRNA expression after aortic constriction in Nox2 knockout mice. (C,D) Increased Nox4 protein expression after aortic constriction in Nox2 knockout mice. Reproduced with permission from (78).

hypertrophic signaling in adult rat ventricular myocytes is suggested to be mediated via a thioredoxin 1-sensitive posttranslational oxidative modification of thiols on Ras (77).

6.3. Pressure-overload and mechanical strain-induced hypertrophy

Analysis of the pro-hypertrophic signaling mechanisms in a guinea pig model of chronic pressure-overload LV hypertrophy in our laboratory suggested a key role of NADPH oxidase. A progressive increase in expression of the NADPH oxidase subunits p22phox, gp91phox, p67phox, and p47phox were observed in parallel with a significant increase in activation of ERK1/2, ERK5, JNK1/2, and p38 MAPK (33). However, studies in Nox2 knockout mice showed that there was no difference in hypertrophy or upregulation of molecular markers such as ANF in response to chronic pressure overload compared to wild type animals (78,79), suggesting that Nox2 may be dispensable for pressure-overload hypertrophy. Interestingly, the expression of an alternative Nox isoform, Nox4, was found to be increased by pressure overload (Figure 6), raising the possibility that this isoform might be involved in the hypertrophic response (78). On the other hand, mice with cardiomyocyte-specific Rac deletion were protected against pressure overload-induced hypertrophy (64) while other studies also suggest an involvement of Nox2 (80). Therefore, further studies are required to clarify this issue.

At a cellular level, *in vitro* stretch of cultured cardiomyocytes may provide a useful system for exploring potential pro-hypertrophic signaling pathways. Such *in vitro* studies suggest that ROS are a key factor in inducing hypertrophy (81). Aikawa *et al* found that mechanical stress-induced cardiac hypertrophy is mediated by a Rac1-ROS-p38MAPK pathway. Similarly, using cyclically stretched neonatal rat ventricular myocytes, Pimental *et al* (82) showed that stretch caused a graded increase in O_2^- production. Stretch-induced increases in protein synthesis and cellular protein content were completely inhibited by SOD/catalase mimetics. While low amplitude stretch induced hypertrophy, high amplitude stretch led to apoptosis and this effect was inhibited by scavenging ROS. Both low- and high-amplitude stretch caused rapid phosphorylation of ERK1/2, while high but not low amplitude stretch caused JNK phosphorylation in a ROS-dependent fashion (82). Other work has recently demonstrated that mechanical strain causes cellular hypertrophy via ROS-dependent post-translational modification of Ras, leading to activation of the Raf/MEK/ERK growth pathway (83). ROS-dependent S-glutathiolation of Ras at Cys118 was found to be a key event in this process.

6.4. Cardiac hypertrophy in response to other stimuli

TNF-α induces ROS generation and hypertrophy in a dose-dependent manner, with the hypertrophy inhibited by antioxidants (84). TNF-α is a known activator of Nox2 oxidase and the cardiomyocyte hypertrophy is reported to be mediated through NFκB activation, as with GPCR agonists (85). TNFα also induces the expression of AP-1.

7. Redox signaling in cardiac fibrosis and extracellular matrix remodeling

Cardiac fibrosis occurs as part of the natural response to myocardial injury. Following MI, the deposition of collagen in the region of the infarct provides mechanical strength in this area, and hence can be seen as appropriate. However, fibrous tissue can also affect other regions in the heart in the absence of overt injury and produce significant problems in this setting. Significant fibrosis occurs in myocardium subjected to chronic pressure overload and in the non-infarcted regions of the ventricle following MI. Indeed, the occurrence of fibrosis is a key distinguishing feature of pathological compared to physiological hypertrophy (1). Additionally, perivascular fibrosis (around intracardiac coronary arteries) is also seen in these conditions. The potential physiological consequences of fibrosis include (i) increased myocardial 'stiffness', which results in a requirement for greater filling pressures in diastole (so-called diastolic dysfunction); (ii) disruption of myofibre organization, which contributes to impaired systolic/contractile function; (iii) altered electrical properties within the ventricle (and atria), which produces a substrate for arrhythmias; and (iv) an impairment of gas exchange between blood vessels and cardiac cells due to perivascular fibrosis, which contributes to relative ischemia even in the absence of overt coronary artery disease.

The extracellular matrix (ECM) has several important functions in the heart. In addition to its obvious role in providing structural support to the cardiomyocytes that are embedded in it, the ECM is critical in signaling processes. There are complex interactions between the ECM and cardiac cells, for example through integrins and a variety of matricellular proteins (86), which facilitate the transmission of messages in response to mechanical and chemical triggers. Integrins, for example, play important roles in mechanotransduction. In addition, the ECM and matricellular proteins have a key role in regulating inflammation within the myocardium. The majority of settings in which adverse cardiac remodeling occurs involve substantial changes in the ECM, for example those that are required to allow ventricular dilatation. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes with differing substrate specificities for various components of the ECM, which are key regulators of ECM structure as well as mediators of ECM signaling (87). Alterations in MMP activity, and in that of tissue inhibitors of MMPs (TIMPs), are crucial in ECM remodeling.

7.1. Clues from noncardiac settings to the importance of redox regulation in fibrosis

Evidence from extracardiac systems implicates intracellular redox balance as a key regulator of fibrosis and remodeling. ROS modulate fibroblast proliferation and their transformation into matrix-producing myofibroblasts (88-91), and oxidative stress is profibrotic in the liver, lungs, kidney and vasculature (90). Profibrotic stimuli such as angiotensin II, aldosterone and cyclic load all stimulate intracellular ROS production (92,93). In addition, many signaling pathways and transcription factors implicated in fibrogenesis are redox-sensitive (92-94). Notably, MMP expression and activation are exquisitely redox-sensitive (88,95-100). In the liver, NADPH oxidase plays a central role in fibrogenesis. The proliferation of hepatic stellate cells is a critical step in hepatic fibrogenesis and NADPH oxidase has been shown to play a crucial role in PDGF-induced proliferation of hepatic stellate cells (101). Both pharmacological inhibition with DPI and genetic studies using p47phox knockout mice provided evidence for a central role of NADPH oxidase in the regulation of stellate cell activity and liver fibrosis (102). An important role of the renin-angiotensin system in liver fibrogenesis has been demonstrated (103,104).

7.2. The role of nadph oxidase-generated ros in cardiac fibrosis

Several recent studies have clearly demonstrated the important role of NADPH oxidase-derived ROS in the development of cardiac fibrosis. Studies from our laboratory found that subcutaneous administration of subpressor or pressor angiotensin II significantly increased NADPH oxidase activity and interstitial fibrosis in wild-type mice but not in Nox2 knockout animals (32,105). The activation of NF-kB, expression of connective tissue growth factor (CTGF), fibronectin, procollagen I and procollagen III mRNAs, and the activation of MMP-2 were all found to be Nox2-dependent (105), (Figure 7,8). Furthermore, we and others observed that aldosterone induces similar Nox2-dependent effects and also that the pro-fibrotic effects of angiotensin II were mediated, at least in part, through mineralocorticoid receptor activation (105,106). Similarly, in another study, aldosterone infusion was found to increase procollagen I and III expression in LV compared to placebo-infused controls, while an NADPH oxidase inhibitor apocynin decreased procollagen expression (106).

Similar pro-fibrotic effects of Nox2 were also found in mice subjected to chronic pressure overload (107). Interestingly, Nox2 knockout mice in this study had reduced interstitial fibrosis and better preserved contractile function than wild-type animals even though the degree of hypertrophy was similar. Perivascular inflammation and cardiac fibrosis occurring during pressure overload have also been demonstrated to be mediated by oxidative stress (108).

While the above studies suggest an important role for Nox2 in fibrosis, experiments in cultured cardiac fibroblasts have suggested an important role for the Nox4 isoform in mediating TGFβ-induced differentiation of cardiac fibroblasts into myofibroblasts (109). TGFβ1 irreversibly converts fibroblasts into myofibroblasts, which express smooth muscle α-actin *de novo* and produce extracellular matrix. Nox 4 was reported to mediate TGFβ1-induced conversion of fibroblasts to myofibroblasts

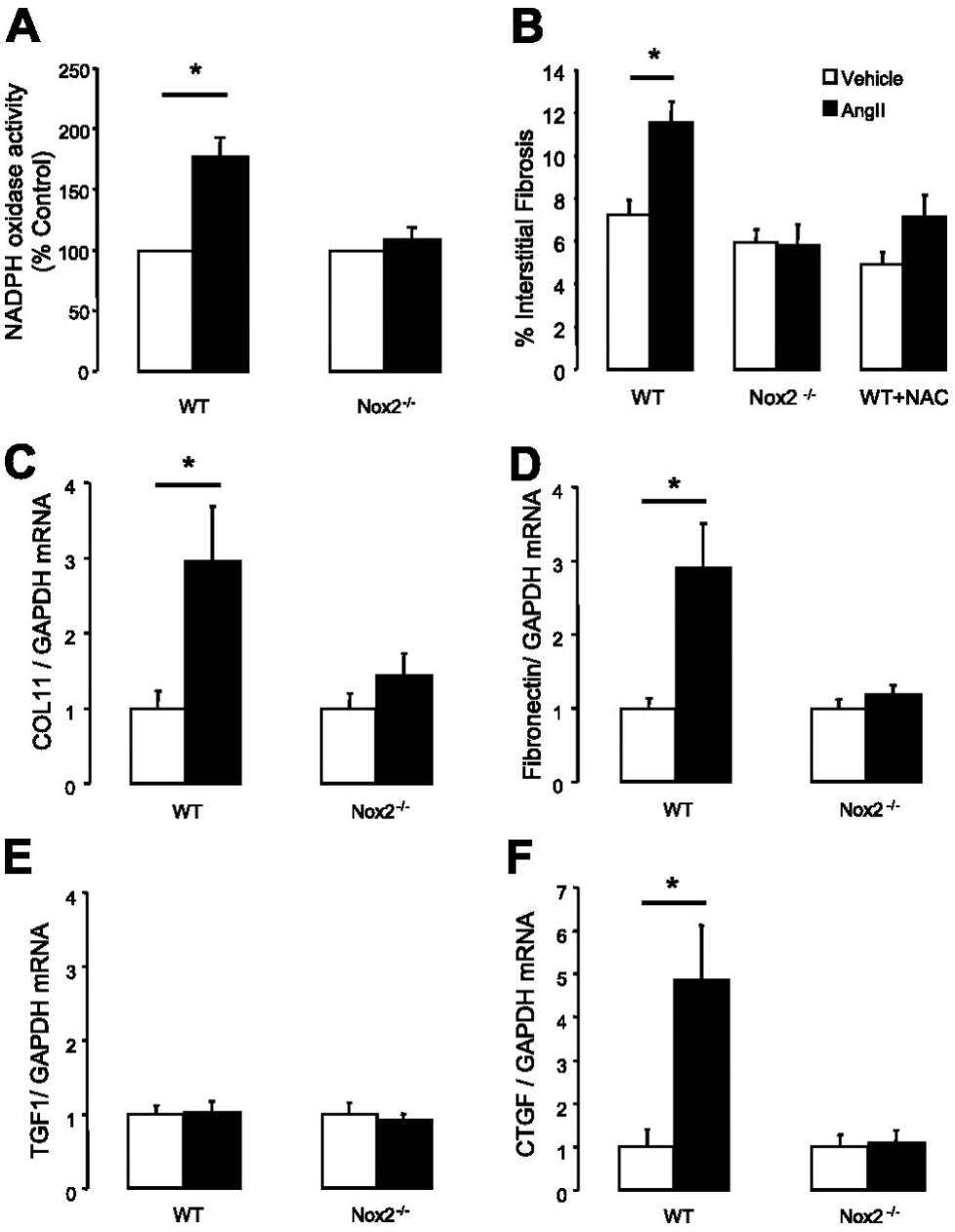

Figure 7. Role of Nox2 in pro-fibrotic gene expression in response to *in vivo* angiotensin II infusion. Nox2 knockout and wild-type (WT) mice were treated with subcutaneous angiotensin II (AngII) infusion for 2 weeks. (A) Myocardial NADPH oxidase activity measured by 5 μM lucigenin-enhanced chemiluminescence. (B*)* Interstitial fibrosis assessed by image analysis of Masson's Trichrome stained sections. The third pair of columns show the effects of N-acetylcysteine treatment on the response to AngII. (C–F) Changes in mRNA expression of pro-fibrotic genes and growth factors. CTGF = connective tissue growth factor. Reproduced with permission from (156).

by regulating Smad 2/3 activation. (109). Although the *in vivo* role of Nox4 in the development of cardiac fibrosis remains unknown, one possibility that could reconcile these studies implicating either Nox2 or Nox4 in the development of interstitial fibrosis would be that both isoforms are required.

Studies from our laboratory (110) and others (111) have also examined the impact of ROS on fibrosis and matrix remodeling in the setting of post-MI ventricular remodeling. After experimental MI induced by coronary artery ligation, Nox2 knockout mice (110) or p47phox knockout mice (111) developed significantly less cardiac chamber dilatation and had better preserved contractile function than wild type animals. Microscopically, both mutant strains had significantly less interstitial cardiac fibrosis in the remote non-infarcted myocardium (Figure 9). Looi *et al* (110) also found that MMP-2 activity was

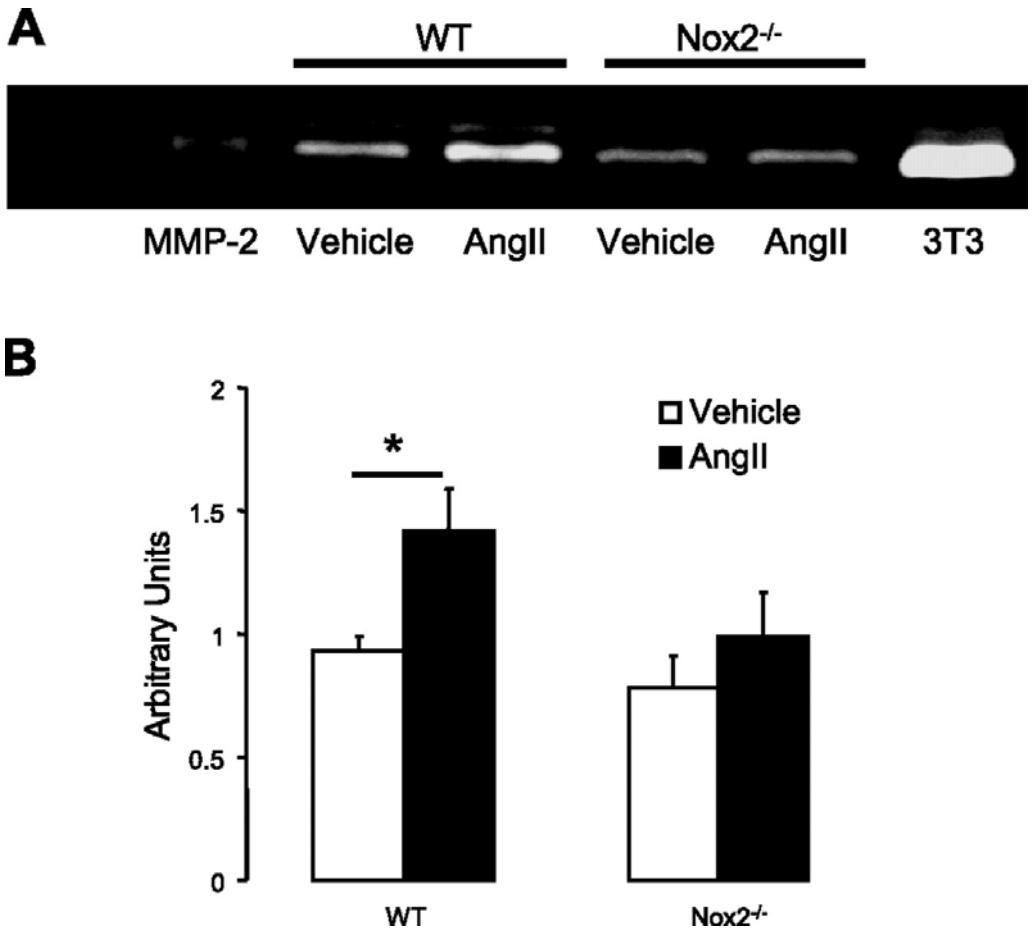

Figure 8. Inhibition of angiotensin II-induced MMP2 activation in Nox2 knockout mice. Gelatin zymography of LV homogenates from mice treated with AngII infusion or vehicle. The top panel shows a representative gel from mice infused with AngII and their respective controls. The bottom panel shows corresponding quantification results. Reproduced with permission from (156).

significantly attenuated in Nox2 knockouts compared to wild-type mice post MI, which may underlie the protection against cardiac chamber enlargement. At a transcriptional level, Nox2$^{-/-}$ mice showed significantly less upregulation of mRNAs for ANF, connective tissue growth factor (CTGF), procollagen I and fibronectin (Figure 10). Collectively, these data indicate that NADPH oxidase-derived ROS (particularly those from Nox2) play major roles in the development of interstitial fibrosis and adverse ventricular remodeling in a wide variety of pathological settings.

8. ROS IN CARDIAC CELL APOPTOSIS

Apoptosis (a form of programmed cell death) is characterized by nuclear chromatin condensation, DNA fragmentation and cellular shrinkage, followed by breaking up of the nucleus and formation of apoptotic bodies. It may occur via 2 convergent pathways, one involving "death receptors" and exemplified by Fas-mediated caspase-8 activation and the other a stress or mitochondria-mediated caspase-9 activation pathway. Both pathways converge onto caspase-3 activation, ultimately resulting in nuclear degradation. The mitochondrial pathway involves the release of cytochrome C into the cytosol, which is dependent upon the opening of the mitochondrial permeability transition pore (MPTP). Once released, cytochrome C forms an activation complex with apoptotic caspase-9 and triggers the apoptotic pathway. Significant evidence indicates that apoptosis makes an important contribution to the cardiac remodeling process and the progression of heart failure. Numerous studies have documented low levels of cardiomyocyte apoptosis in experimental and human heart failure while, recently, transgenic overexpression of caspase within the murine myocardium which produced very low levels of apoptosis has been shown to lead to dilated cardiomyopathy (112). Such approaches provide strong support for a possible causative role for apoptosis in the progression of heart failure.

The general evidence for an involvement of redox state in apoptosis has been reviewed in detail elsewhere (113). ROS are involved in various steps in apoptosis. Firstly, ROS may act as second messengers following the binding of specific ligands (eg, TNFα) to

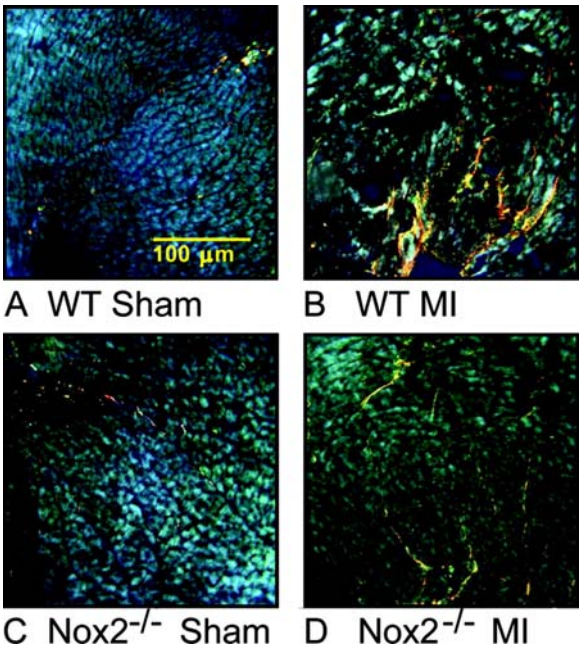

Figure 9. Inhibition of interstitial fibrosis in Nox2 knockout mice after MI. Picrosirius red stained LV sections viewed under circular polarized light. Reproduced with permission from (110).

death receptors. Depending on the specific system in question, ROS can induce either survival or death pathways within the cell. The amount, duration of production and species of ROS are all likely to be important in determining this. An example of redox modulation of downstream proteins involved in apoptosis is seen with ASK-1, which is normally inhibited through binding to thioredoxin (Trx). The oxidation of Trx leads to its dissociation from ASK-1, thus relieving the latter of its basal inhibition; ASK-1 can then bind to adaptor molecules such as TRAF-2 and TRAF-6 and subsequently activate p38MAPK (114). A number of studies have indicated activation of ASK-1 by ROS (115-117). Evidence linking plasma membrane-generated ROS and the activation of a lethal cascade initiated by Ask-1 has been presented by Van Laethem *et al* (116). This study showed that in human keratinocytes exposed to UVB the generation of reactive oxygen species (ROS) acts as a mediator of ASK-1. An NADPH oxidase inhibitor diphenylene iodonium chloride and the EGFR inhibitor AG1487 prevented UVB-mediated ROS generation, the activation of the ASK1-p38MAPK stress response pathway, and apoptosis. Furthermore, H_2O_2 has been demonstrated to predispose neonatal rat ventricular cardiomyocytes to Fas mediated apoptosis by activating the ASK-1 signalling pathway (115).

A second link between ROS and apoptosis is in the modulation of anti-apoptotic systems. An important example here are the heat-shock proteins, which contain cysteine residues susceptible to oxidation by ROS, and which are vital to their activity in protecting against oxidative stress (118). Thirdly, ROS modulate the transcription of (redox-sensitive) genes that may be relevant to apoptosis, eg, NF-κB (119). Fourthly, high ROS levels (for example, as found in inflammatory states) can directly damage mitochondria, leading to the release of cytochrome c into the cytoplasm and triggering apoptosis.

Cardiomyocyte apoptosis has an important role in the transition from compensatory cardiac remodeling to heart failure. Mechanical stretch and pressure overload, which are major initiating factor for cardiac hypertrophy, may lead to apoptotic cell death (120-124).

9. REACTIVE NITROGEN SPECIES IN MYOCARDIAL HYPERTROPHY AND HEART FAILURE

While the main focus of the current article is on ROS, reactive nitrogen species (RNS) may also have important effects in the context of LVH and heart failure. Given their inter-relationship with ROS in settings where there is oxidative stress, we include a brief discussion of RNS effects here although the interested reader is also directed to recent excellent reviews (125,126).

Nitric oxide (NO) has many critical physiological functions in the cardiovascular system including the regulation of cardiac energetics, cardiac contractile function and coronary vasodilatation (125). Its rapid reaction with $O_2^{\cdot-}$ not only results in loss of NO bioactivity but also results in the formation of peroxynitrite ($ONOO^-$), an RNS that may exert important effects on a variety of cellular targets. Notably, $ONOO^-$ may react with cysteine thiol groups through electron transfer reactions which can inactivate mitochondrial enzymes (127), several tyrosine phosphatases (128), and certain MMPs (129,130). $ONOO^-$ may also react with iron-sulfur or zinc-sulfur centers in enzymes, eg, leading to the inactivation of mitochondrial aconitase (131) and eNOS (132), or may sequentially generate other reactive species such as hydroxyl and carbonate. In addition to the above oxidation reactions, $ONOO^-$ nitrates tyrosine residues, resulting in biological effects on enzymes such as creatine kinase (133), SERCA 2a (134), structural proteins (135) and ion channels (136). It is therefore clear that RNS such as $ONOO^-$ could influence many signalling processes, effects being reversible at relatively low intracellular concentrations and irreversible at high concentrations (136,137).

The co-production of ROS and NO (and thus the generation of $ONOO^-$) is likely in settings such as heart failure, ischemia-reperfusion and cytokine activation or when there is increased expression of inducible NOS (iNOS). Indeed, nitrotyrosine formation has been correlated with cardiac dysfunction in heart failure models (138). Furthermore, agents which degrade $ONOO^-$ are reported to improve cardiac function in experimental doxorubicin-induced cardiomyopathy (139). Other novel agents, which inhibit the actions of $ONOO^-$, are reported to reduce adverse cardiac remodelling in a rat coronary ligation model (140). In heart failure, $ONOO^-$-induced changes in polyADP-ribose polymerase (PARP) activity may be especially important. PARP acts as a DNA damage sensor that can trigger DNA repair in response to low levels of

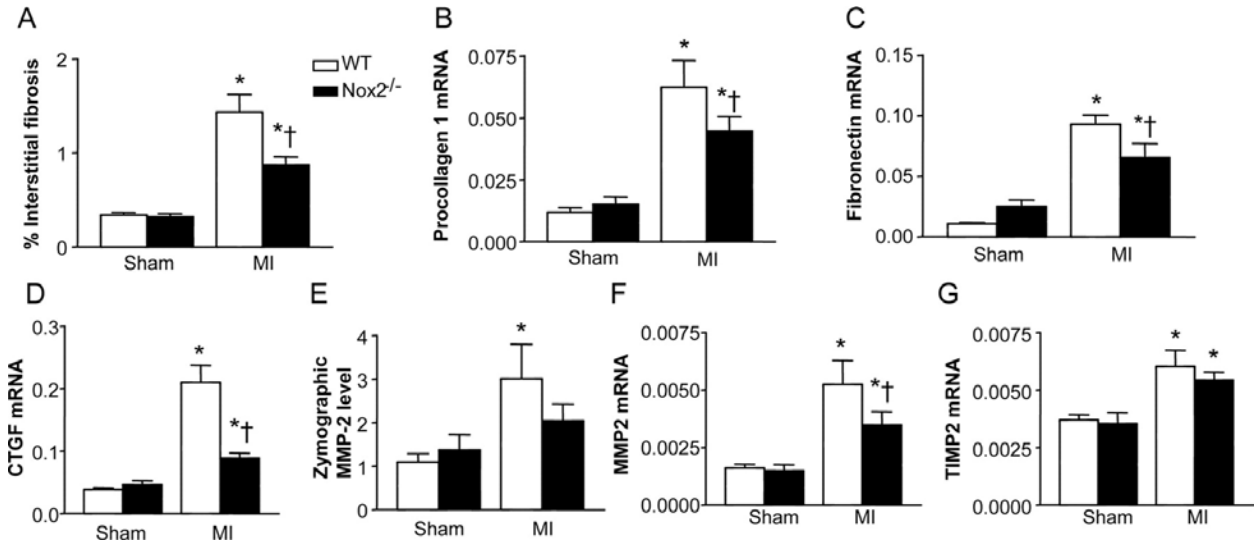

Figure 10. Effect of MI on cardiac fibrosis, MMP-2 activity, and gene expression in WT and Nox2$^{-/-}$ mice. (A) Interstitial fibrosis assessed by Picrosirius red staining. (B-D) mRNA expression of procollagen I, fibronectin, and connective tissue growth factor (CTGF). (E) Mean data from MMP-2 gelatin zymography. (F and G) mRNA expression of MMP-2 and tissue inhibitor of metalloproteinase 2 (TIMP2). Reproduced with permission from (110).

damage (126). After more severe cell damage, death by apoptosis versus necrosis may also hinge on PARP. PARP overactivation leads to depletion of ATP and insufficient energy reserves for apoptosis, hence death by necrosis follows. Alternatively, activation of caspases by pro-apoptotic pathways leads to PARP cleavage, preventing PARP activation and allowing death by apoptosis. In human heart failure, increased PARP-1 expression and PARP-1 activity have been described (141,142). Deletion of the PARP-1 gene is reported to provide protection against angiotensin II-induced cardiac remodelling and contractile dysfunction (143). Similarly, pharmacological inhibition of PARP-1 reduced cardiac hypertrophy and myocardial collagen deposition in murine aortic-banding models (52,144).

RNS-induced changes in pro-MMP cleavage and activation may also be important in cardiac pathology (129). Low $ONOO^-$ levels produce MMP-2 activation whereas higher levels inhibit the enzyme, probably via tyrosine nitration. It has been suggested that activated MMP-2 located within or close to the cardiomyocyte sarcomere can degrade proteins such as troponin I and α-actinin, thereby producing contractile dysfunction (130) – a mechanism that may be important in myocardial stunning after ischemia-reperfusion and possibly also in chronic heart failure.

10. CONCLUSIONS

In this article, we have considered the role of ROS as signaling molecules that modulate processes involved in cardiac remodeling and failure. Whilst considerable evidence supports an important role of ROS and redox-regulated processes, we still need to better understand how these mechanisms are regulated. It is increasingly appreciated that the precise effects of ROS are highly dependent on context – in particular, the species involved, its local concentration, the local antioxidant balance, and perhaps most importantly the intracellular location within the cell. An important advance is the recognition that NADPH oxidases are specific sources of signaling ROS which may play a key role in these processes, and that different Nox isoforms may subserve different effects even in the same cell type. A major challenge to further research remains the development of improved methods for the intracellular detection of specific ROS within cells with high temporal and spatial resolution. While understanding ROS-mediated signal modulation is a highly challenging area for research, the field offers the potential for the development of novel therapeutic interventions for cardiac remodeling and failure.

11. ACKNOWLEDGEMENTS

We are grateful to all members of the Shah laboratory who have contributed to our work in this area. The authors' research is supported by the British Heart Foundation (CVH/99001); EU FP6 grant LSHM-CT-2005-018833, EUGeneHeart; and by the Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's & St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust.

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Abbreviations: ECM:Extracellular matrix, ER: Endoplasmic reticulum, MI: Myocardial Infarction, NOX:NADPH Oxidase, RNS: Reactive Nitrogen Species, ROS : Reactive Oxygen Species, SOD:Superoxide dismutase, XDH : Xanthine dehydrogenase, XOR: Xanthine Oxidoreductase

Key Words: NADPH Oxidase, Nox2, Nox4, Redox signaling, Cardiac remodeling, Hypertrophy, Review

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