Original article

Involvement of NADPH oxidase in age-associated cardiac remodeling

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A B S T R A C T

Increased activation of the renin–angiotensin–aldosterone system (RAAS) and an increase in oxidative stress are both implicated in age-related cardiac remodeling but their precise interrelationship and linkage to underlying molecular and cellular abnormalities remain to be defined. Recent studies indicate that NADPH oxidases are major sources of oxidative stress and are activated by the RAAS. This study investigated the relationship between the NADPH oxidase system, age-related cardiac remodeling and its underlying mechanisms. We studied male Fisher 344 cross Brown Norway rats aged 2 months (young rats), 8 months (young adult rats) or 30 months (old rats). Aging-dependent increases in blood pressure, cardiomyocyte area, coronary artery remodeling and cardiac fibrosis were associated with increased myocardial NADPH oxidase activity attributable to the Nox2 isoform. These changes were accompanied by evidence of local RAAS activation, increased expression of connective tissue growth factor (CTGF) and TGF-β1, and a significant activation of MMP-2 and MT1-MMP. The changes in old rats were replicated in 8 month old rats that were chronically treated with angiotensin II for 28 days. Increased RAAS activation may drive age-related cardiac remodeling through the activation of Nox2 NADPH oxidase and subsequent increases in MMP activation, fibrosis and cardiomyocyte hypertrophy.

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1. Introduction

Aging substantially increases the risk of cardiovascular diseases such as hypertension, atherosclerosis, left ventricular hypertrophy (LVH), heart failure, atrial fibrillation and stroke. The steep rise in incidence of LVH and chronic heart failure with advancing age is thought to be related at least in part to a continuum of structural and functional alterations in the heart, which increase the propensity to develop clinical cardiac disease [1,2]. Age-related cardiac remodeling includes significant changes in cardiac chamber stiffness and volumes and in diastolic and systolic contractile function. These changes are the result of major alterations in cellular and extracellular phenotype, including cardiac myocyte hypertrophy, an increase in perivascular and interstitial fibrosis, remodeling of the extracellular matrix, and a reduced capillary to myocyte ratio. In addition, increases in ventricular afterload secondary to alterations in the peripheral vasculature and in ventricular-vascular coupling also contribute to the cardiac remodeling process.

The underlying mechanisms that drive age-related cardiac remodeling remain to be fully defined. Many experimental studies have shown that increased activation of the renin-angiotensin-aldosterone system (RAAS) is a prominent feature of age-related

cardiac remodeling which may account for many of the phenotypic changes that are observed [1,2]. An increase in oxidative stress is also implicated in age-related cardiac remodeling [3,4], and aging is indeed well recognized to be associated with increased production of reactive oxygen species (ROS) in many different tissues [5]. Moreover, it is increasingly appreciated that RAAS activation may itself be a significant driver of increased oxidative stress [6]. However, the precise interrelationship between RAAS activation and increased oxidative stress with respect to age-related cardiac remodeling and its underlying cellular and extracellular abnormalities remains to be clarified.

In the last few years, studies in other cardiovascular settings have established an important role for ROS-generating NADPH oxidases in redox signaling pathways that underlie cardiovascular remodeling, both experimentally and in humans [7]. The NADPH oxidases are multi-protein enzyme complexes that generate superoxide through the catalysis of electron transfer from NADPH to molecular oxygen [8]. Five oxidase isoforms have been identified each of which is based on a distinct catalytic subunit, i.e., Nox1 to Nox5. Among these isoforms, Nox1, Nox2 and Nox4 are known to be expressed in cardiovascular cells. Nox5 has also been identified in human vascular cells but is not expressed in rodents. In contrast to other sources of ROS, such as mitochondria, xanthine oxidase and uncoupled nitric oxide synthases, the generation of ROS by most NADPH oxidases is a regulated process that is initiated by specific agonist stimulation. Notably, NADPH oxidase family members are activated by angiotensin II, aldosterone, endothelin I, cytokines (e.g., tumor necrosis factor α [TNFα]), growth

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factors (e.g., transforming growth factor β [TGFβ], platelet derived growth factor [PDGF]), and mechanical forces [7,8]. The regulated production of small amounts of ROS in response to such stimuli appears to be ideally designed for involvement in redox signaling pathways, and an involvement of NADPH oxidases in such signaling has indeed been demonstrated in many studies [8,9]. Experimental studies have confirmed that NADPH oxidase-derived ROS play important roles in the pathogenesis of cardiovascular pathologies such as endothelial dysfunction, atherosclerosis, diabetic vasculopathy, RAAS-related hypertension, and ischemic vascular remodeling [6,7]. Recently, it has also been shown that NADPH oxidase activation is involved in several types of cardiac remodeling, including angiotensin II-induced cardiac hypertrophy, aldosterone-induced cardiac fibrosis and cardiac remodeling after myocardial infarction (MI) [10–15]. In addition, increased myocardial NADPH oxidase activity is found in human heart failure [16–18]. However, the possible involvement of the NADPH oxidase system in age-related cardiac remodeling remains unclear. In the current study, we have investigated the relationship between the NADPH oxidase system, age-related cardiac remodeling and some of its underlying mechanisms in a rodent aging model.

2. Material and methods

2.1. Animals

All animal experimentation was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and using protocols approved by the institutional Animal Use and Care Committee. Male Fisher 344 cross Brown Norway rats aged 2 months (young rats), 8 months (young adult rats) or 30 months (old rats) were housed and maintained under identical conditions. A cohort of young adult rats received angiotensin II (200 mg/kg/day) via subcutaneous osmotic minipump (Alzet Corp., Cupertino, CA) infusion for 28 days. Systolic blood pressure (SBP) was measured by tail-cuff plethysmography in trained animals. At the end of the experiments, animals were sacrificed by an overdose of sodium pentobarbital and the hearts were immediately removed. Tissues for histology were fixed in 4% neutralized formalin, while those for immunoblotting or other assays were frozen in liquid nitrogen and stored at −70 °C.

2.2. Histology

Hearts sections were stained with hematoxylin and eosin (H&E), toluidine blue, Picrosirius Red or Elastic van Gieson (EVG) and quantitation of myocyte cross-sectional area and coronary vessel histomorphometry were performed as described previously [19]. Immunohistochemistry was performed according to the biotin–streptavidin kit instructions (Zymed Laboratories, Inc). Antigen was recovered in citrate buffer at pH 6.0 (Zymed Laboratories, Inc) for 1–3 min in a microwave oven. The slides were incubated overnight at 4 °C with primary antibodies. Smooth muscle cells in vessels were identified by staining with an antibody against α-smooth muscle actin and endothelial cells by staining with an anti-CD31 antibody.

2.3. In situ hybridization

Digoxigenin (DIG)-labeled MMP-2 (330 bp), TIMP-2 (361 bp), and MT1-MMP(2338 bp) riboprobes were obtained from plasmid cDNA (kindly provided by Michael Crow, Gerontology Research Center, Baltimore, MD) by standard RNA synthesis, using SP6, T7, or T3 RNA polymerase. Incorporation of DIG-conjugated uridine-5′-triphosphate (UTP) was determined using a commercial kit (Boehringer Mannheim Corp) following the manufacturer's instruction. In situ hybridization was performed by modification of the methods previously described [20].

Corresponding sense probes, hybridization buffer without probes, and RNase digestion of tissue sections for 30 min before hybridization were used as controls to determine specific hybridization.

2.4. Western blotting

Left ventricle lysates were resolved by SDS-PAGE and transferred onto PVDF membrane (Immobilon). The transferred membranes were incubated in PBS containing primary antibodies to MMP-2, TGF-β, angiotensin converting enzyme (ACE), angiotensinogen, angiotensin II, aldosterone, AT$_1$ receptor, Nox2 or Rac1 at 4 °C for 24 h. HRP-conjugated IgG (Amersham Pharmacia Biotech, Buckinghamshire, GB) was used as secondary antibody and detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Immunoblotting for α-sarcomeric actin was used as a protein loading control. Band intensities were quantified by densitometric analysis. We found in pilot studies that the protein levels of several potential "housekeeping" proteins changed with aging but α-sarcomeric actin showed less alteration and had the additional advantage of being a cardiomyocyte protein. Furthermore, in most cases, changes in expression by immunoblotting were substantiated by another method (e.g., immunohistochemistry).

2.5. In situ gelatin zymography

The presence of MMP-2 activity within the epicardial coronary arteries *in situ* was detected as described [20]. Zymography was repeated in the presence of an inhibitor antibody against MMP-2 to determine the enzymatic specifity of gel digestion.

2.6. NADPH oxidase activity

NADPH oxidase activity was assayed in heart homogenates as NADPH-dependent superoxide generation measured using lucigenin-enhanced chemiluminescence, as described previously [11]. Tissue was homogenized in liquid nitrogen and placed into a 96-well microplate luminometer together with dark-adapted lucigenin (5 μmol/L), and NADPH (300 μmol/L) was added before recording chemiluminescence. Superoxide production was expressed as arbitrary light units over 20 min. The effects of the following agents, pre-incubated for 15 min, were used to assess potential sources of superoxide production: diphenyleneiodonium (DPI, 10 μmol/L), a flavoprotein inhibitor; N^G-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L), a nitric oxide synthase inhibitor; oxypurinol (100 μmol/L), a xanthine oxidase inhibitor; rotenone (2 μmol/L), a complex I mitochondrial electron chain inhibitor; or apocynin (1 mmol/L). The superoxide scavengers, Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid, 20 mmol/L) or superoxide dismutase (SOD, 200 U/mL), were used to confirm that the detected signal resulted from superoxide.

2.7. Real-time polymerase chain reaction (RT-PCR)

RNA was isolated from snap-frozen hearts using the RNeasy Fibrous Tissue Kit (Qiagen). cDNA was synthesized using the Omniscript kit (Qiagen) and random decamers. Real-time PCR was performed using the ABI PRISM 7700 and SYBR Green master-mix (Applied Biosystems). All quantitative assays used the universal thermal cycling parameters (initial steps: 50 °C for 2 min, 95 °C for 10 min; and 95 °C for 15 s, 60 °C for 1 min, for 40 cycles). The level of expression of all transcripts was determined using standard curves from known amounts of template and normalized to β-actin. The primer sequences were as follows:

Nox1 F: CCAAACGTGACAGTGATGTATGC, R: AGCTGAAGTTACCAT-GAGAACCAA; Nox2 F: CGTATTGTGGGAGACTGGACTGA, R:

AGGGCCCATCAACTGCTATCT; Nox4 F: GCCTAGGATTGTGTTTGAG-CAGA, R: GCGAAGGTAAGCCAGGACTGT; p47phox F: GCGTACGCTGCTGTTGAAGA, R: CGGTGATGTCCCCTTTCCT; p22phox F: CCGTCTGCCTTGGCCATTG, R: GGTAGGTGGCTGCTTGATGGT; connective tissue growth factor (CTGF) F: GCTGCCTACCGACTGGAAGAC, R: GAACAGGCGCTCCACTCTG; β-actin F: CGTGAAAAGATGACCCA-GATCA, R: TGGTACGACCAGAGGCATACAG.

2.8. Statistics

Analyses were performed using the SPSS 13 statistical package (SPSS, Inc, Chicago, Il). Data were expressed as mean ± SEM. The comparisons between experimental groups were performed using one-way ANOVA followed by a Scheffe post hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Age-related cardiac remodeling in the Fisher 344 cross Brown Norway rat

SBP progressively increased with advancing age such that it was approximately 30 mm Hg higher in 30 month compared to 8 month rats (Supplementary Fig. 1A). There was no significant change in the heart weight over body weight ratio in old versus young adult rats (Supplementary Fig. 1A) but the cardiomyocyte cross-sectional area was significantly higher in old animals (Supplementary Fig. 2), indicating the development of hypertrophy. Aging was associated with significant remodeling of the epicardial coronary arteries. Coronary artery thickness increased progressively from age 2 months to 8 months to 30 months (Supplementary Fig. 1A) and this was accompanied by a significant decrease in both smooth muscle and endothelial cell density (Supplementary Fig. 1B). In addition, there was a substantial increase in perivascular fibrosis and interstitial cardiac fibrosis as assessed by Picrosirius red or EVG staining (Supplementary Fig. 3). Furthermore, the increased birefringence pattern under polarized light in Picrosirius Red stained sections (Supplementary Fig. 3, middle panels) suggested that much of the increased collagen was abnormally cross-linked. Taken together, the above data show typical changes of cardiac and coronary vascular remodeling with advancing age.

Since RAAS activation is implicated in age-associated cardiac remodeling and is a potent stimulus for NADPH oxidase activation, we also studied a cohort of young adult rats that were chronically treated with angiotensin II infusion. Angiotensin II-treated young adult rats demonstrated similar cardiomyocyte hypertrophy (Supplementary Fig. 2) and vascular remodeling (data not shown) to that found in old rats.

3.2. Myocardial NADPH oxidase activity

Myocardial NADPH-dependent ROS generation was significantly increased in 30 month old rats compared to 8 month old rats (Fig. 1A). Angiotensin II-treated young adult rats also had a similar increase in ROS generation. The lucigenin signal was inhibited by Tiron or SOD in all groups, confirming superoxide as the measured ROS (Fig. 1B). NADPH-dependent superoxide generation was abolished by the flavoprotein inhibitor DPI but was unaffected by L-NAME, oxypurinol or rotenone (Fig. 1B), consistent with NADPH oxidase being the major source of detected ROS.

3.3. NADPH oxidase subunit expression

In order to assess which NADPH oxidase isoforms may be responsible for the increased oxidase activity, we measured the expression of Nox1, Nox2 and Nox4 mRNA by real-time PCR. Nox1
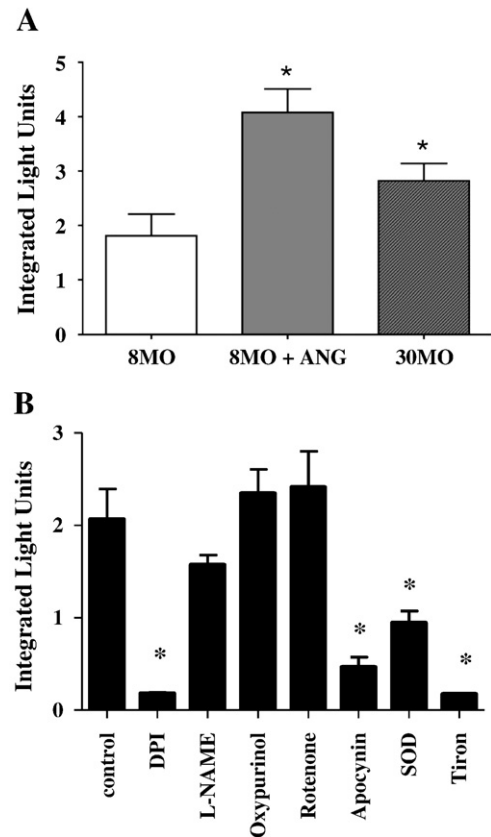

Fig. 1. NADPH-dependant ROS generation in heart homogenates, assessed by lucigenin-enhanced chemiluminescence. (A) ROS generation in the absence of inhibitors. (B) ROS generation in the presence of inhibitors in 8 month old rats treated with angiotensin II, as described in the text. *$P < 0.05$ cf. 8 month group; $n = 6$/group.

mRNA levels were at the limit of detection, consistent with previous data in the rodent LV [7]. There was a significant nearly 3-fold increase in Nox2 mRNA expression in 30 month old rats compared to 8 month old rats, while Nox2 mRNA levels were also significantly elevated in the angiotensin II-treated young adult group (Fig. 2A). Nox4 mRNA expression levels were not significantly different among the groups (Fig. 2B). Since Nox2 oxidase activity requires several other protein subunits, we also measured the expression of the p22phox and p47phox subunits. No significant differences in mRNA expression level were found (Figs. 2C, D). Nox2 protein levels were significantly increased in the 30 month old rats and in the angiotensin II-treated young adult group compared to untreated young adult rats by immunoblotting (Supplementary Fig. 4). Immunohistochemistry also confirmed a robust increase in cardiomyocyte Nox2 expression in the aging group (Fig. 3A). Since Rac1 is an important activator of Nox2 oxidase [6–8], we assessed Rac1 levels and localization. Rac1 protein levels were substantially increased in 30 month old rats and in the angiotensin II-treated 8 month old group compared to young adult rats (Fig. 4A). Immunostaining of cardiac sections indicated that Rac1 levels were increased in cardiomyocytes with clear evidence of enhanced staining at the sarcolemma (Fig. 4B).

3.4. Hormonal and cytokine mediators of remodeling

The expression of CTGF, which is strongly implicated in NADPH oxidase-dependent cardiac remodeling [11,15], was significantly increased in the angiotensin II-infused 8 month old group and 30 month compared to 8 month rats (Fig. 3B). We also found a significant increase in CTGF mRNA expression in 30 month old rats (data not shown).
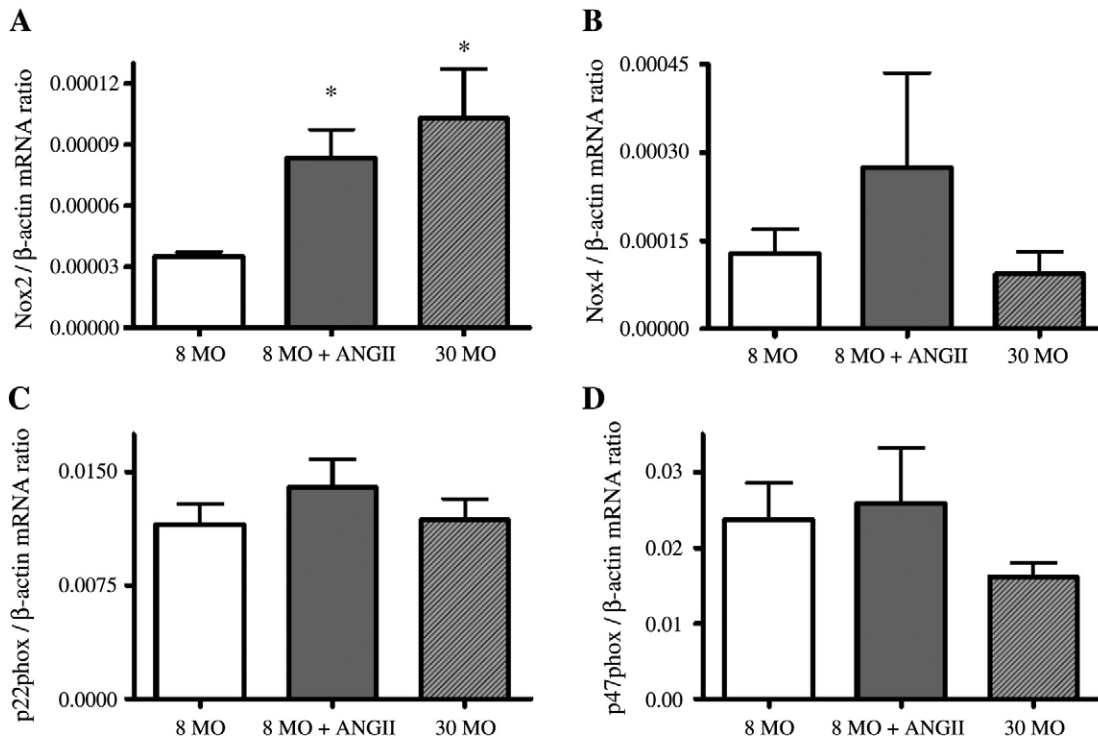
Fig. 2. mRNA expression of NADPH oxidase subunits Nox2, Nox4, p22phox and p47 phox. *$P<0.05$ cf. 8 month group; $n=6$/group.

Immunoblot analyses showed that protein levels of aldosterone and activated TGF-β1 were significantly elevated in the myocardium of 30 month old rats and in the angiotensin II-treated 8 month old rats compared to 8 month old untreated rats (Figs. 5A, B). Both these agents not only promote remodeling but are known to activate NADPH oxidase [7].

To establish evidence of local RAAS activation, we measured protein levels of components of the RAAS in the myocardium. Immunoblotting of LV samples showed that 30 month old rats and angiotensin II-treated 8 month old rats had significantly increased levels of angiotensinogen, ACE and AT$_1$ receptor compared to 8 month

old untreated rats (Figs. 5C, D). This was accompanied by evidence of increased local angiotensin II levels by immunostaining (Supplementary Fig 5).

3.5. MMP expression and activation

Alterations in MMP activity are centrally involved in cardiac remodeling [21] while NADPH oxidase activation has been shown to be upstream of the MMP activation that occurs in response to angiotensin II stimulation [11,22] or during post-MI remodeling [15].
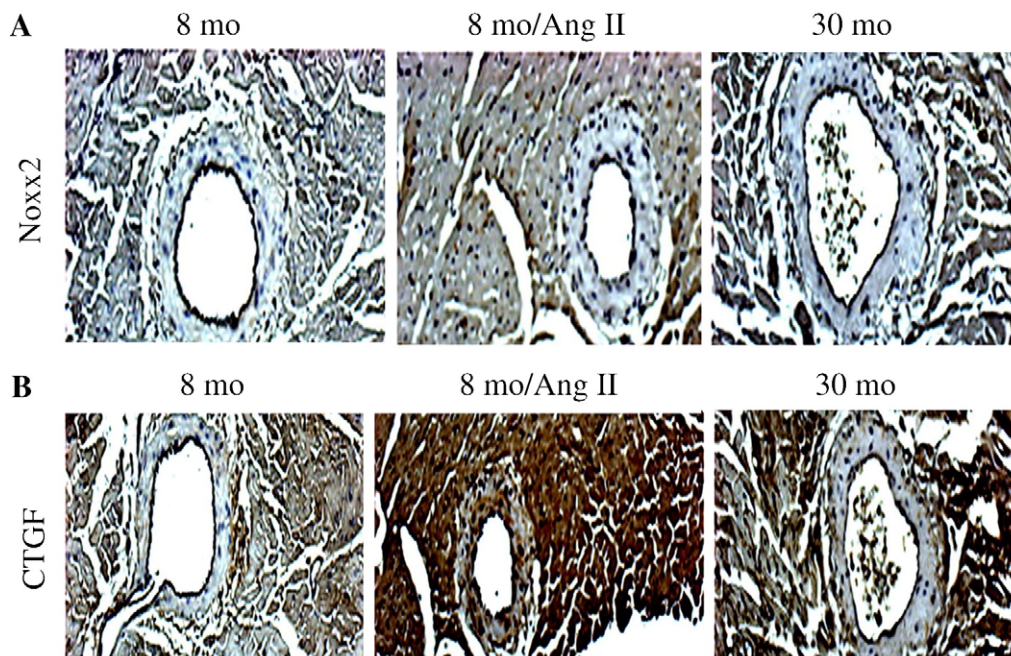

Fig. 3. Representative heart sections immunostained for (A) Nox2 and (B) CTGF. Similar results were obtained in 4 experiments.
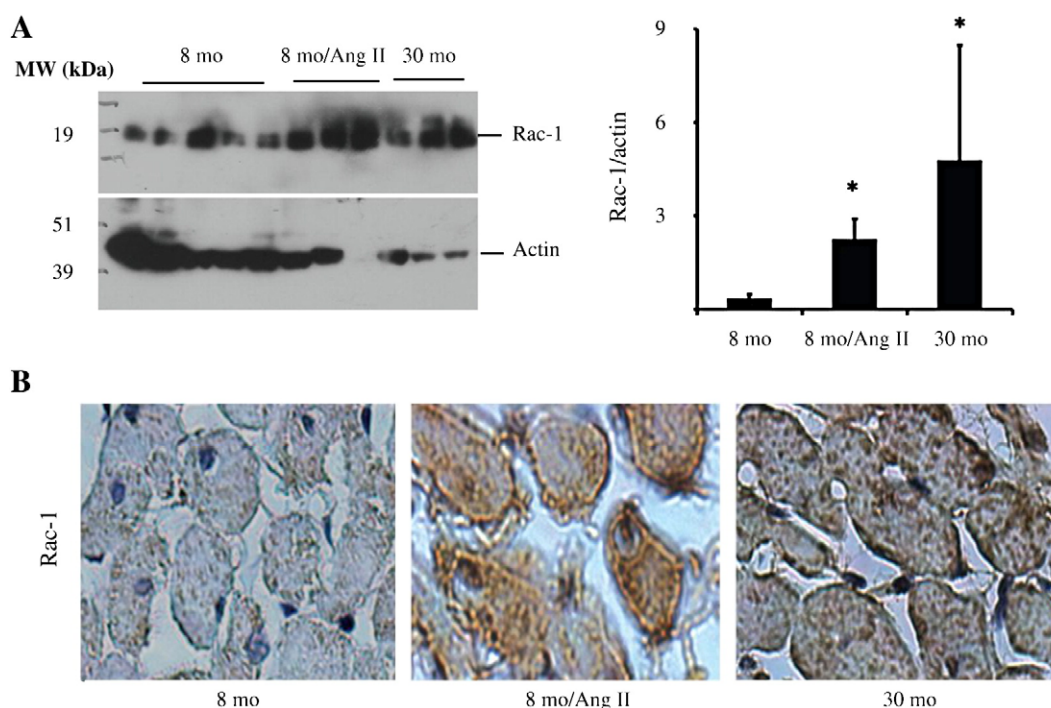
Fig. 4. Changes in Rac1 expression in myocardium. (A) Immunoblots showing Rac1 expression and densitometric quantification. *$P < 0.05$ cf. 8 month group; $n = 4$/group. (B) Representative heart sections (×400 magnification). Similar results were obtained in 4 experiments.

We therefore undertook a detailed analysis of MMP expression and activation.

In situ hybridization studies demonstrated that the expression of both MMP-2 and MT1-MMP (which is involved in TGFβ activation) was increased in the intima and media of epicardial coronary arteries of 30 month old rats, whereas TIMP-2 expression (which inhibits MMP activity) was reduced (Fig. 6). Similar results were obtained when immunostaining for these proteins was undertaken (Fig. 7). *In situ* zymographic studies confirmed that these changes in MMP/TIMP expression were accompanied by an increase in MMP gelatinolytic activity in the intima and media of epicardial coronary arteries of 30 month old rats (Supplementary Fig. 6). The *in situ* gelatinolytic activity was almost completely inhibited by an antibody against MMP-2, indicating that it mainly resulted from MMP-2 activity. MMP2 activity assessed in total myocardial tissue homogenates by zymography was also significantly increased in the 30 month old rat group and the 8 month old rat group treated with angiotensin II as compared to the 8 month old untreated group (Fig. 8).

4. Discussion

This study examined the possible involvement of NADPH oxidase in aging-associated cardiac remodeling in a rodent model of aging. We found that aged Fisher cross Brown Norway rats developed characteristic phenotypic changes of cardiac remodeling [23,24] comprising an increase in cardiomyocyte hypertrophy, interstitial and perivascular fibrosis, and a reduction in cellularity and a thickening of coronary vessels. These changes were associated with an increase in myocardial NADPH oxidase activity which was attributable mainly to the Nox2 oxidase isoform and increased Rac1. The increases in NADPH oxidase activity were accompanied by increased levels of aldosterone and active TGFβ. Since both these agents (as well as angiotensin II) are known to be activators of NADPH oxidases [6,7], it is likely that the increased oxidase activity may have been attributable to increased RAAS activation and increased TGFβ. Indeed, we found evidence of local RAAS activation in myocardium of old rats in that there were increased protein levels of ACE, angiotensinogen, angiotensin II and

the AT_1 receptor. Consistent with the possibility that angiotensin II was an important activator of NADPH oxidase, we found that young adult rats chronically infused with angiotensin II also developed increases in Nox2 expression, Rac1, NADPH oxidase activity and cardiovascular remodeling. The increases in NADPH oxidase activity were paralleled by a significant increase in MMP expression and activity, in line with previous findings that NADPH oxidase can drive MMP activation [11,22]. Taken together, these results suggest that an increase in Nox2 NADPH oxidase activity plays an important role in aging-associated cardiac remodeling.

The NADPH oxidase family of enzymes is now well recognized as a major source of ROS involved in cardiovascular pathophysiology [6,7] and recent studies in gene-modified mice and in human heart failure [10–18] support an involvement in cardiac remodeling. The Nox2 isoform (also known as gp91phox) was first described in neutrophils but is also known to be expressed in cardiomyocytes, endothelial cells, fibroblasts and a range of inflammatory cells [6–8]. It is found as a heterodimer with a p22phox subunit, and requires several cytosolic subunits (i.e., p47phox, p67phox, and Rac1) for its activation. The Nox1 isoform is found mainly in vascular smooth muscle cells while Nox4 is widely expressed in all cardiovascular cell types. Whereas Nox1 and Nox2 are activated by agonists such as angiotensin II, cytokines and growth factors, Nox4 oxidase is thought to be constitutively active although its expression level can be increased by TGFβ [8]. Prior studies in mice lacking Nox2 or p47phox (which is necessary for Nox2 activation) suggest that this isoform is especially important for cardiac remodeling [10–15,25], although *in vitro* studies in cultured fibroblasts have also implicated Nox4 in the transformation of fibroblasts to myofibroblasts [26]. The results of the present study suggest that it is the Nox2 isoform that is involved in aging-associated cardiac remodeling. This conclusion is further supported by the finding of increased Rac1 levels and membrane location, known to be a key activator of the Nox2 oxidase [6–8]. The precise Nox2-expressing cell types that are involved remain to be fully established but immunohistochemistry clearly showed an increase in Nox2 expression at least in cardiomyocytes. Interestingly, increased Nox2 oxidase activation has recently also
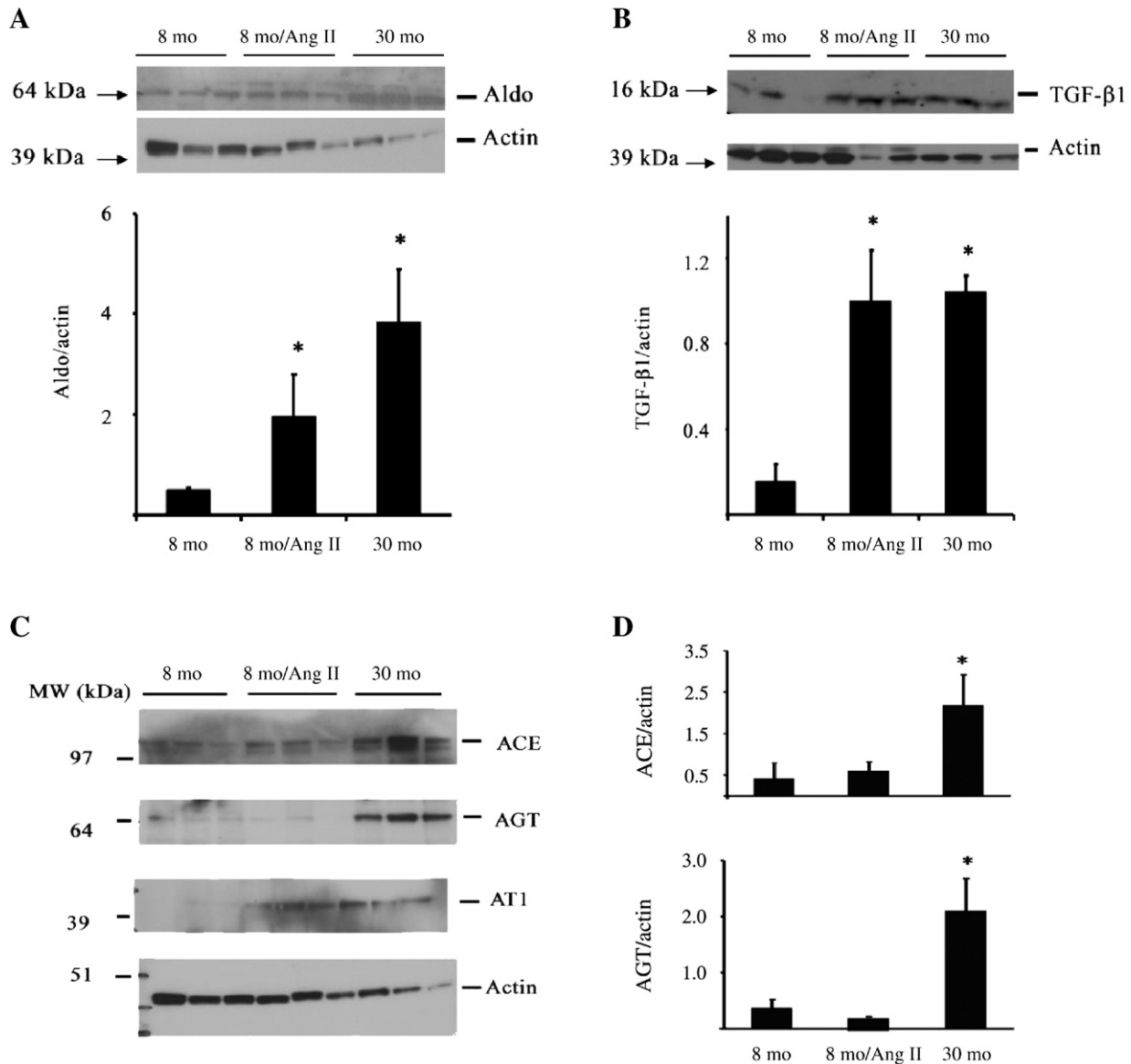
Fig. 5. Changes in expression of local RAAS components and pro-fibrotic factors. (A) Aldosterone expression—immunoblots are shown above with quantitation below. (B) TGFβ expression. (C) Immunoblots showing expression levels of ACE, angiotensinogen (AGT) and AT₁ receptor. (D) Mean densitometric data for ACE and AGT levels (AT₁ data were not quantified since levels were below detection limit in 8 month old rats). *$P < 0.05$ cf. 8 month group ($n = 3$/group).

been implicated in age-related vascular endothelial dysfunction [27,28].

A key mechanism involved in tissue remodeling downstream of NADPH oxidase-generated ROS is the activation of MMPs [11,15,22]. Altered MMP regulation is well established to be involved in aging-related vascular remodeling [20], which is associated with a significant decrease in the numbers of both vascular smooth muscle cells and endothelial cells, an increase in intimal and medial thickness, and the perivascular accumulation of extracellular matrix and collagen microfibers [1,20]. MMP dysregulation is also implicated in the development of cardiac failure [21]. MMP activity is regulated at multiple levels including transcription, secretion and activation and is under the strict control of specific inhibitors, the tissue inhibitors of MMPs (TIMPs). The change in balance between MMPs and TIMPs in heart tissue and vessels toward an increase in MMP activity results in increased proteolysis causing MMP-dependent degradation of the extracellular matrix and is regarded as an early mechanism in the progression to heart failure [21]. We found that age-related NADPH oxidase activation and heart remodeling were associated with increased mRNA and protein expression of

MMP-2 and a decrease in TIMP-2, leading to a disturbed balance and an overall increase in gelatinolytic activity in old rats. In addition, MT1-MMP expression was also increased in aged rats. Since MT1-MMP is involved in TGFβ-1 activation, which in turn can lead to NADPH oxidase activation and MMP activation, this could be an important positive feedback loop that augments MMP activation and cardiac remodeling. We found changes in overall myocardial MMP activity as well as coronary vessel MMP activation, suggesting that these changes may contribute to both cardiac and vascular remodeling. Although no direct data on coronary vessel NADPH oxidase activation were available in the present study, these results raise the interesting question of crosstalk between coronary remodeling and myocardial remodeling during aging and the possibility that both may be driven by similar stimuli. In addition to TGFβ-1, which is well known to be pro-fibrotic, CTGF is a potent pro-fibrotic cytokine. Furthermore, myocardial Nox2 oxidase activation has previously been found to be upstream of increased CTGF expression [11,15]. It was therefore of interest that the increased age-associated NADPH oxidase activation and fibrosis in the current study were accompanied by increased CTGF expression.
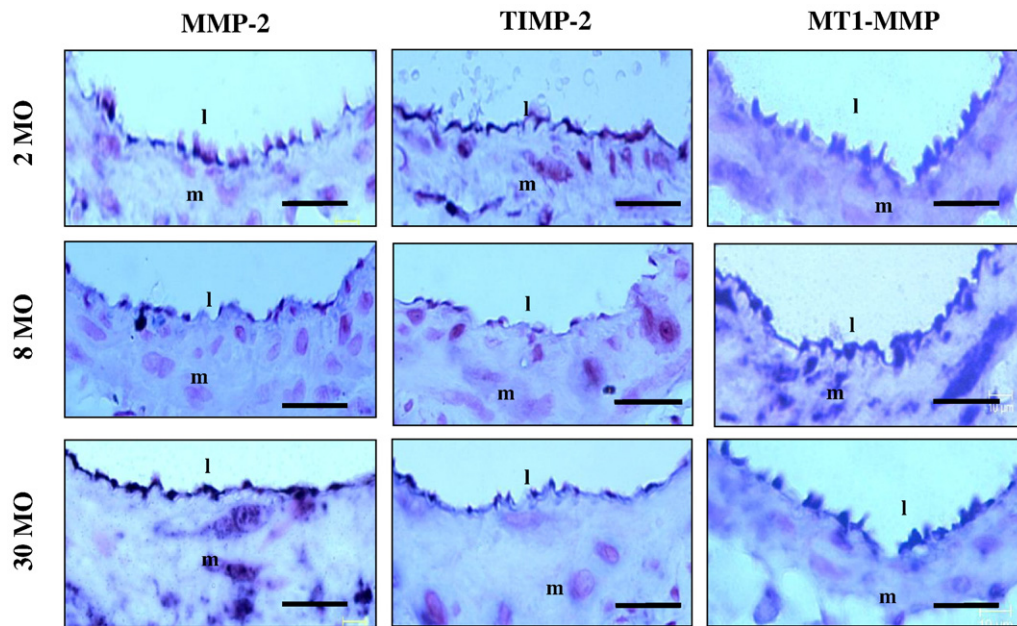
Fig. 6. Representative sections of *in situ* hybridization for MMP-2, TIMP-2 and MT1-MMP. l, lumen; m, media. The blue color indicates mRNA signal and the red color indicates nuclei. Similar results were obtained in 4 experiments.

The increase in RAAS activation may be a key pathogenic feature that links age-associated NADPH oxidase activation and cardiac remodeling. Angiotensin II mediated oxidative stress has been associated with age-dependent cardiomyopathy [3,4] and inhibition of the RAAS was reported to ameliorate age-related progression of cardiac hypertrophy [29]. As discussed previously, angiotensin II drives cardiac fibrosis and remodeling through NADPH oxidase activation, in part through activation of mineralocorticoid receptors [11]. Angiotensin II can also stimulate TGFβ mRNA expression and promote its conversion to the active form [30] as well as enhancing CTGF expression and thus synergistically increasing the pro-fibrotic effect of both cytokines. Other cytokines that could be involved in the

process include TNF-α, which is implicated in aging-related pathology possibly via an up-regulation of MMP levels [31,32]. TNF-α is also a known activator of NADPH oxidase [33] and its possible involvement in ageing-related cardiac remodeling therefore merits investigation.

4.1. Study limitations

We assessed NADPH oxidase activity in an *ex vivo* assay that does not necessarily translate to similar increases in ROS production *in vivo* nor does it exclude an important contribution by other sources of ROS (e.g., mitochondria). However, the increase in Rac1 that was documented provides supportive evidence of Nox2 activation *in*
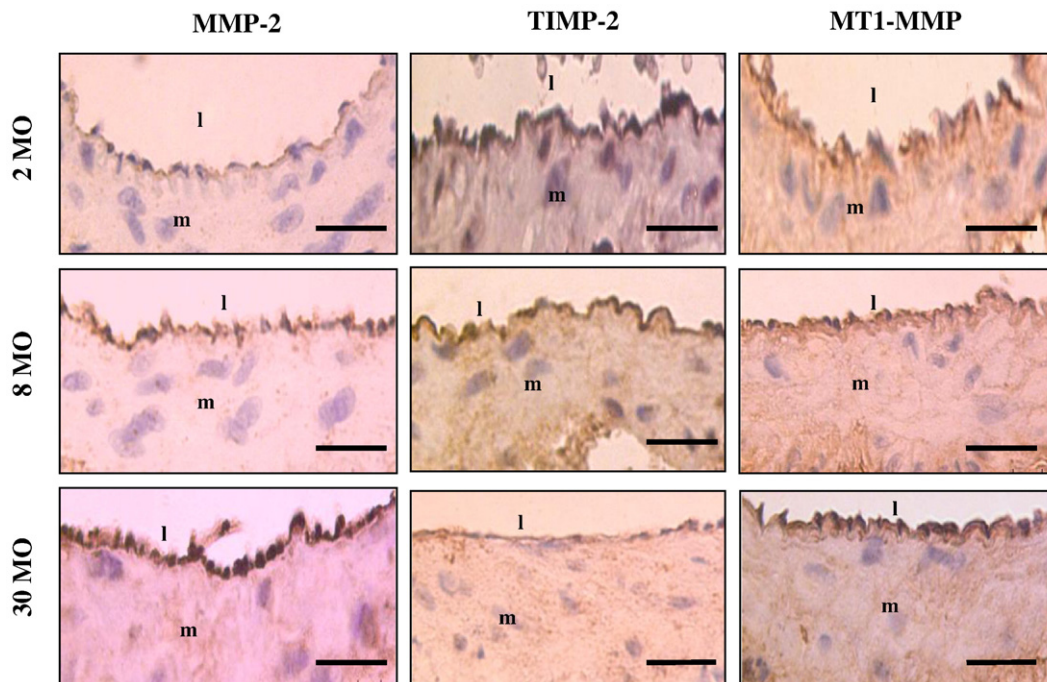

Fig. 7. Representative sections immunostained for MMP-2, TIMP-2 and MT1-MMP. l, lumen; m, media. The brown color indicates protein signal.
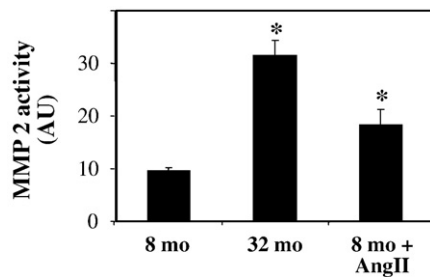
Fig. 8. MMP2 activity in myocardial homogenates assessed by zymography. *$P < 0.05$ cf. 8 month group ($n = 4$–6/group).

vivo. ROS-dependent effects on cardiac remodeling may be significantly influenced by the ambient antioxidant status, which was not assessed in the current study. However, previous studies have reported a significant decrease in heart superoxide dismutase activity in old compared to young Fischer 344 rats [34]. Such a reduction in antioxidant activity, if present in the current study, would have been an important contributing factor to enhanced NADPH oxidase-dependent cardiac remodeling. Changes in protein expression levels assessed by immunoblotting in this study (e.g., aldosterone) could potentially be confounded by alterations in the levels of the "housekeeping" protein used for normalization. However, the results were generally backed up by a second method (e.g., immunohistochemistry, activity assays) and/or the quantification of related molecules. Finally, more definitive evidence for an involvement of NADPH oxidase-derived ROS in aging-associated cardiac remodeling would be provided by studies in appropriate gene-modified models and/or intervention studies.

In conclusion, this study suggests that the Nox2 NADPH oxidase may play an important role in aging-associated cardiac remodeling by enhancing MMP activation, the expression of pro-fibrotic factors such as CTGF and TGFβ, and cardiomyocyte hypertrophy. Since NADPH oxidases are activated by multiple factors, including angiotensin II, aldosterone, endothelin-1 and cytokines, it is arguable that they may be a suitable therapeutic target for the prevention of aging-related cardiac remodeling in addition to targeting the individual agonists (such as the RAAS) that activate the oxidase.

Disclosures

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yjmcc.2010.01.006.

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