NADPH oxidase mediates angiotensin II-induced endothelin-1 expression in vascular adventitial fibroblasts

Sheng Jun An\textsuperscript{a}, Ryan Boyd\textsuperscript{a,1}, Min Zhu\textsuperscript{b,1}, Alexander Chapman\textsuperscript{a}, David R. Pimentel\textsuperscript{c}, Hui Di Wang\textsuperscript{a,*}

\textsuperscript{a} Department of Community Health Sciences, Brock University, St. Catharines, ON, Canada L2S 3Y6
\textsuperscript{b} Department of Pharmacology, University of Saskatchewan, Saskatoon, SK, Canada
\textsuperscript{c} Myocardial Biology Unit, Boston University Medical Center, Boston, Massachusetts, USA

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Abstract

Objective: We have recently reported that adventitial fibroblasts are able to express endothelin-1 (ET-1) in response to angiotensin II (Ang II) stimulation. However, the mechanism by which this occurs in the adventitia remains unclear. As Ang II has been reported to increase oxidant production by NADPH oxidase, we examined the role of this complex in Ang II stimulated ET-1 expression in vascular adventitial fibroblasts.

Methods and results: Adventitial fibroblasts were isolated and cultured from mouse aorta. Cells were treated with Ang II (100 nmol/L) in the presence or absence of NADPH oxidase inhibitors, apocynin (100 \mu mol/L) and diphenyleneiodonium (10 \mu mol/L), superoxide scavengers, SOD (350 units/mL), tempol (100 \mu mol/L), tiron (100 \mu mol/L), and ET-receptor antagonists (10 \mu M), BQ123 (for ETA-) and BQ788 (for ETB-). PreproET-1 mRNA and ET-1 level were determined by relative RT-PCR and ELISA, respectively. Type I procollagen-\alpha-I (collagen) level was detected by Western blot. Superoxide anion (superoxide) production was determined by coelenterazine or lucigenin chemiluminescence. Ang II-induced collagen expression was inhibited by BQ123, suggesting that adventitial ET-1 plays a significant role in regulating the extracellular matrix. NADPH oxidase inhibitors and superoxide scavengers significantly decreased Ang II-induced ET-1 mRNA and peptide expression, superoxide production as well as collagen expression. Furthermore, deletion of gp91phox (a key subunit of NADPH oxidase) and overexpression of SOD1 attenuated Ang II-induced responses.

Conclusion: Ang II-evoked expression of ET-1 in adventitial fibroblasts appears to be mediated, at least in part, by NADPH oxidase. Functionally, this mechanism stimulates collagen expression thereby implicating the adventitia as a potential contributor to the vascular pathophysiology associated with oxidative stress and vascular remodeling.

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1. Introduction

Angiotensin II (Ang II) plays a major role in the development of hypertension and atherosclerosis. Ang II may elicit its vascular effects through the generation of reactive oxygen species (ROS) [1], or via its interaction with the endothelin (ET) system [2]. Past studies indicate that ET-1 contributes to the vascular actions of Ang II [3,4]. ET-1 is the most potent endogenous vasoconstrictor as well as a potent mitogenic agent [5]. The most abundant source of ET-1 in vivo is the vascular endothelium [6]. ET-1 synthesis however, has also been reported in non-endothelial cells, including vascular smooth muscle cells [7], cardiomyocytes [8], and cardiac fibroblasts [9]. Recently, we found that Ang II stimulates ET-1 synthesis and release, which lead to collagen synthesis in vascular adventitial fibroblasts and lead to collagen synthesis [10].
An increase in type I procollagen-α-1 (I) transcripts and protein levels in adventitial fibroblasts and neointimal cells have been reported following coronary injury and dissecting medial injury [11,12]. Activated adventitial fibroblasts induce the formation of myofibroblasts through the expression of α-smooth muscle actin, and myofibroblasts appear to contribute to the deposition of collagen and tissue repair in the form of vascular remodeling [13]. Both Ang II and ET-1 have been reported to stimulate vascular collagen synthesis via transforming growth factor-β (TGF-β) [14,15]. While vascular collagen production is regulated by many factors, recent findings suggested that ET-1 could also contribute to Ang II-evoked collagen expression in adventitial fibroblasts [10,16].

The mechanisms involved in the induction of ET-1 by Ang II are still unknown. Free radical generated F2-isoprostane released during oxidative injury can cause ET-1 release in endothelial cells [17], while H2O2 (hydrogen peroxide) increases ET-1 expression in vascular smooth muscle cells [18]. Moreover, the ROS and extracellular signal-regulated kinase (ERK) pathways are also involved in ET-1 gene expression in rat cardiac fibroblasts [9]. Ang II is well known to cause the induction of reactive oxygen species via NADPH oxidase [19–21]. Although gp91phox is substituted by its homologue mitogenic oxidase-1 in rat vascular smooth muscle cells, rat adventitial fibroblasts, endothelial cells and human vascular smooth muscle cells only express gp91phox, not mitogenic oxidase1 [22]. Using knockout mice we have also shown that it is gp91phox that is chiefly responsible for Ang II-stimulated vascular oxidative stress and smooth muscle growth in vivo [23]. Thus, we examined the hypothesis that Ang II mediates ET-1 release from fibroblasts via regulation of NADPH oxidase. We measured collagen production as a functional correlate of this pathway.

2. Methods

2.1. Cell culture

The method used to culture aortic adventitial fibroblasts has previously been established [10]. Male C57BL and gp91phox (a major subunit of NADPH oxidase) knock out (gp91phox–/–) mice, 16 to 18 weeks of age, were obtained from Jackson Laboratory (Bar Harbor, ME). The mice were anesthetized with inhaled isoflurane and then killed by vertebral dislocation. Thoracic aortas were removed and cleaned under sterile conditions. The adventitia was separated from the media under a surgical microscope, cut into 1–2 mm² flat segments, and left to adhere and proliferate in dishes coated with poly-d-lysine. Subcultures for up to 3 passages were used in our experiments. All animal protocols were approved and conducted according to the recommendations from Research Sub-Committee of Brock University on Animal Care and Use and the Canadian Council on Animal Care, which conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

To demonstrate that cultured cells used in our studies were not contaminated by endothelial cells or vascular smooth muscle cells, cultured cells were characterized for specific cell markers by means of RT-PCR. The following cell markers were used to differentiate among different cell types: von Willebrand Factor (vWF) for endothelial cells; myosin heavy chain (MHC) and desmin for differentiated vascular smooth muscle cells (VSMC). Unlike the aorta which displayed heavy staining for vWF, MHC and desmin, the cultured cells from the adventitia did not express these markers, while both cultured fibroblasts and aorta tissue expressed β-actin. These findings exclude the possibility of contamination due to endothelial and smooth muscle cells.

2.1.1. Pharmacological interventions

Cells were grown to sub-confluence and incubated in serum-free medium for 24 h. Cells were treated with Ang II at 100 mmol/L for 1.5 h, the optimal incubation for mRNA of preproET-1 expression, or superoxide anion, and 24 h for type I procollagen-α-1 expression and ET-1 peptide measurements. The cells and medium were then collected for cell culture medium via ELISA. Western blot analysis was used for type I procollagen-α-1 protein from whole cell lysates. Superoxide anion generation was measured by coelenterazine or lucigenin chemiluminescence from cells after treatments.

The role of NADPH oxidase and its derived oxidative stress was tested by comparing the effect induced by Ang II release and type I procollagen-α-1 production with or without superoxide anion scavengers tempol (100 μmol/L), tiron (100 μmol/L) or superoxide dismutase (SOD, 350 units/mL), and specific NADPH oxidase inhibitors, diphenyleneiodonium sulphate (DPI) (10 μmol/L) or apocynin (100 μmol/L). The contribution of ET-1 in collagen synthesis was assessed by comparing the Ang II-induced type I procollagen-α-1 expression in the presence or absence of BQ123 (1 μM), an ET₄-receptor inhibitor, and BQ788 (1 μM), an ET₃-receptor inhibitor.

2.2. Qualitative and relative quantification of mRNA of prepro ET-1 by RT-PCR

Total RNA was extracted from confluent cultured adventitial using Trizol (Molecular Research Center, Cincinnati, OH). For amplification of preproET-1, the sequence of the primers and RT-PCR procedures were previously published [10].

2.3. Measurement of endothelin-1 release by ELISA

The cell culture medium was collected for ET-1 ELISA measurement. The concentration of ET-1 in culture medium...
was determined by using a commercial ELISA kit (ALPCO, NH) according to the manufacturer’s instructions. The cellular protein concentration was determined with Bradford Reagent (Bio-Rad). The ET-1 release was expressed as fmol of ET-1 per $10^{-5}$ cells.

### 2.4. Measurement of type I procollagen-α-I production by Western blot

The cells were cultured with or without different treatments for 24 h and lysed with a lysis buffer containing 50 mmol/L HEPES (pH 7.5), 100 mmol/L NaCl, 1% Triton, 2.5 mmol/L β-glycerophosphate, 10% glycerol, 5 mmol/L MgCl₂, 1 mmol/L EGTA, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride. Equal amounts of total proteins (20 μg) were separated by electrophoresis in 7% SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane (Hybond-C, Amersham Pharmacia Biotechnology). Type I procollagen-α-I was detected by incubating the membrane with 1:7000 diluted polyclonal antibody against type I procollagen-α-I (Santa Cruz Biotechnology Inc) overnight at 4 °C. The membranes were then incubated with a 1:2000 diluted secondary peroxidase-
conjugated antibody and detected by the ECL detection system (Amersham Pharmacia Biotech) according to manufacturer’s instructions.

2.5. Superoxide anion measurement by chemiluminescence

The superoxide detection method has been published previously [24]. Five μM (final concentration) of coelenterazine or lucigenin was added into cells. Tiron (10 mmol/L, final concentration) was then added to quench all superoxide anion-dependent chemiluminescence. The superoxide anion generation was expressed as milli units of light per minute and cell protein.

2.5.1. Construction of pAdCMV-SOD1

Mouse SOD1 cDNA was amplified by attB PCR with primers: 5′-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC AAGGAGATAAGCATTGCCATGA AAGCGGTTGTCGTCGCT-3′ (forward) and 5′-GGGG ACC ACT TTG TAC AAG AAA GCT GGG TC TTACTGCCAATCCCCAATCACTCA-3′ (reverse) and cloned into entry pDONR221 according to the manufactures instruction. To generate pAdCMV-SOD1 expressing constructs, LR recombination reaction was performed by using pAd/CMV/V5-DEST™ vector and pDONR221-SOD1 entry vector according to manufacturer’s instruction. The sequence of SOD1 was confirmed by sequence analysis. To generate SOD1 expressing virus, pAdCMV-SOD1 viral plasmids were linearized by Pac I and transfected into 293A cells with Lipofectamine 2000. Virus was harvested, tittered and stocked at 1×10⁹ plaque forming units/mL.

2.5.2. Cell transduction and treatment

Cells were transduced at sub-confluence with 100 multiplicity of infection (MOI) of adenovirus for 24 h and then incubated in fresh medium for 48–72 h to allow for recovery. The success of overexpression was confirmed by comparing SOD1 expression levels in cells with or without adenovirus carrying SOD1 genes. The adenovirus-transduced cells were stimulated with Ang II (100 nmol/L) and then collected for preproET-1, ET-1 peptide, superoxide and type I procollagen-α-I measurements.

3. Data analysis

Relative RT-PCR gels images were digitized with an automated digitizing system UN-SCAN-IT (Ver.5; Silk Scientific Corporation). Data were expressed as mean±SEM. Statistical comparisons were made by ANOVA. Significance was accepted when p value was less than 0.05.

Fig. 3. Superoxide anion in cultured aortic adventitial fibroblasts. The cells were incubated with Ang II (100 nmol/L) for 1.5 h. The cellular superoxide generation was determined by coelenterazine chemiluminescence. Apocynin (100 μmol/L) inhibited the Ang II-induced superoxide anion generation. Results are means±SE of 4 experiments. *P<0.01 compared with control cells.

Fig. 4. Effect of overexpressing SOD1 on Ang II-induced prepro-ET-1 mRNA and type I procollagen-α-I protein expression. A is the representative western blot of SOD1 in total cell lysates from 3 experiments. B and C show the effect of overexpressing SOD1 on Ang II-induced prepro-ET-1 mRNA and type I procollagen-α-I protein expression in cultured adventitial fibroblasts, respectively. Results are means±SE of 3 to 5 experiments. *P<0.05 compared with control and Adv-B-galactosidase infected (Adv-gal) cells.
4. Results

4.1. Effect of superoxide anion scavengers and NADPH oxidase inhibitors

4.1.1. Ang II-induced prepro-ET-1 mRNA and ET-1 expression

The effect of various superoxide scavengers, and NADPH oxidase inhibitors on Ang II-induced preproET-1 mRNA and ET-1 peptide release are shown in Fig. 1A and B, respectively. Cells treated with 100 nmol/L of Ang II for 1.5 h significantly increased preproET-1 mRNA by 1.82 fold compared to control ($P < 0.01$). In addition, pre-treatment with superoxide anion scavengers, tempol and tiron, inhibited Ang II-induced preproET-1 mRNA to basal levels. Pre-treatment with two specific NADPH oxidase inhibitors, DPI and apocynin, also reduced prepro-ET-1 mRNA expression to near basal levels ($P < 0.01$ compared to Ang II treated cells), indicating that NADPH oxidase contributes to Ang II-induced ET-1 expression (Fig. 1A). Minimal quantities of ET-1 are secreted under basal conditions; however, addition of Ang II evokes an increase in ET-1 by 3 fold ($P < 0.01$). Moreover, tiron and apocynin decrease the Ang II-induced ET-1 peptide expression ($P < 0.05$) to near basal levels (Fig. 1B).

4.1.2. Ang II-induced collagen I protein expression

The expression of type I procollagen-α-1 protein in aortic adventitial fibroblasts is presented in Fig. 2. Ang II (100 nmol/L) causes a significant increase in type I procollagen-α-1 protein expression by 1.40 fold ($P < 0.01$), which was inhibited by the ETA-receptor antagonist, BQ123, but not by the ETB-receptor antagonist, BQ788 (Fig. 2A). Strikingly, the increase in type I procollagen-α-1 expression evoked by Ang II was inhibited by the superoxide scavengers and NADPH oxidase inhibitor (Fig. 2B).

4.2. Effect of overexpressing SOD1

4.2.1. Ang II-induced prepro-ET-1 mRNA, collagen I protein expression and superoxide anion generation

To confirm the role of superoxide anion in Ang II-induced ET-1 expression, we over expressed SOD1 in aortic adventitial fibroblasts using recombinant adenovirus. Adenovirus expressing $\beta$-galactosidase was used as the control. There was a 2.5 fold overexpression of SOD1 as seen in Fig. 4A. Overexpression of SOD1 in cells significantly inhibited Ang II-induced responses to preproET-1 mRNA (Fig. 4B), type I procollagen-α-1 protein expression (Fig. 4C) and superoxide anion generation (Fig. 5) to near control levels ($P < 0.05$ compared to Ang II treated cells); however, no effects of viral overexpression were noted under basal conditions. In addition, $\beta$-galactosidase had no effect in the presence or absence of Ang II.

4.3. Effect of gp91phox knockout on ET-1 release

The effects of Ang II on ET-1 release in cells derived from gp91phox$^{-/-}$ mice are shown in Fig. 6. Although the basal release of ET-1 peptide was similar, Ang II caused a much smaller release of ET-1 from fibroblasts of gp91phox$^{-/-}$ mice when compared from cells of wild-type mice ($P < 0.01$).

5. Discussion

In this study, we demonstrate for the first time that a functional local ET system in vascular adventitial fibroblasts...
is activated by oxidative stress, specifically the generation of superoxide anion by activation of NADPH oxidase by Ang II. These events contribute to the increased collagen synthesis observed with Ang II. The importance of superoxide anion is demonstrated by the inhibition of mRNA of proET-1 and ET-1 peptide expression by chemical and genetically induced superoxide scavengers and NADPH oxidase inhibitors.

5.1. Importance of adventitial ET-1

Increasing evidence supports the role for the adventitia in regards to cardiovascular disease. Adventitial fibroblasts have been shown to be involved in arterial repair [25,26] and serve as a major source of vascular oxidative stress [23,27–29]. Inhibition of adventitial oxidative stress has been shown to decrease neointimal proliferation [30], and smooth muscle hypertrophy [32].

We recently reported that adventitial cells express and release ET-1 in response to Ang II, an important hormone involved in the blood pressure regulation and pathogenesis of atherosclerosis. The nucleotide sequence of adventitial preproET-1 cDNA was identical to that of endothelial preproET-1 cDNA. Importantly, we have demonstrated a potential role of vascular adventitial fibroblasts, as released ET-1 can modulate Ang II-stimulated extracellular matrix generation. Collagen is a major component of the extracellular matrix of blood vessels [33,34], produced primarily by fibroblasts, although smooth muscle and endothelial cells may participate. We found that the Ang II-stimulated type I procollagen-α1 mRNA expression and protein synthesis were inhibited by the ETA-receptor inhibitor, suggesting a potentially biological meaningful role for this interaction at the functional level.

5.2. NADPH oxidase derived oxidative stress mediates ET-1 release

While the AT-1 subtype was found responsible for the Ang II-induced ET-1 expression, the mechanism behind the ET-1 signal transduction pathway needs further investigation. Results from the present study support the notion that Ang II increases ET-1 and collagen I expression, and that superoxide anion derived from NADPH oxidase helps to increase the ET-1 and collagen I concomitantly in Ang II-treated adventitial fibroblasts.

We have several lines of evidence to show that superoxide anion is involved in mediating Ang II-induced ET-1. We found that superoxide anion generation in Ang II-treated cells increased concomitantly with that of ET-1 synthesis. Indeed, pharmacological scavengers of superoxide or overexpression of SOD1 has been shown to decrease Ang II-induced ET-1 release, suggesting that superoxide anion plays an important role in mediating ET-1 release from adventitial fibroblasts. Our data are consistent with the previous findings that superoxide anion or H2O2 lead to an increase in ET-1 expression in vascular smooth muscle [18,35,36] and endothelial cells [17,35,37] that is inhibited by superoxide dismutase and/or catalase.

We have demonstrated the role of adventitial gp91phox containing NADPH oxidase in the regulation of ET-1 release in our study by showing that the increase of ET-1 release in response to Ang II was significantly reduced in the presence of NADPH oxidase inhibitors. Furthermore, Ang II-induced ET-1 release was decreased from the cells of gp91phox −/− mice. Therefore, the above data support the role of NADPH oxidase in the generation of ET-1 synthesis in vascular adventitial fibroblasts. NADPH oxidase increases ROS generation in the Ang II-treated cells that in turn increase ET-1 expression.

A role for NADPH oxidase in the vasculature has been widely reported. Gp91phox is one of the major subunits of NADPH oxidase [22,23,38]. It is expressed in native and cultured vascular endothelial and adventitial cells, and in human resistant arterial smooth muscle cells (although gp91phox is substituted by its homologue NADPH oxidase-1 in rat vascular smooth muscle cells [39]). The increase in superoxide anion caused by Ang II was absent in mice that lack gp91phox [23]. Gene transfer of gp91phox inhibitor abolished Ang II-induced vascular hypertrophy [32] and suppressed angioplasty-induced neointimal proliferation [30] and hyperplasia [31] of rat carotid artery. Consistent with these findings, data from the present study indicate that the Ang II-induced ET-1 expression and superoxide anion levels were decreased in cells isolated from the gp91phox −/− mice, indicating the essential functional role of gp91phox in the responses to Ang II.

5.3. Possible mechanisms by which ROS mediates ET-1 synthesis

The mechanisms by which ROS stimulate ET-1 synthesis are unknown. Recent studies have demonstrated that superoxide anion contributes to an activation of the preproET-1 promoter and subsequently increases mRNA concentrations in cultured endothelial cells [37]. Oxidative stress can activate nuclear factor-kappa B [40], which may stimulate the preproET-1 gene expression [41]. Oxidative stress stimulates the generation of TGF-β in glomerular cells [42], which could markedly enhance ET-1 expression in both rat vascular smooth muscle and endothelial cells [43]. Both ROS and Ras–Raf–ERK pathways are required for either Ang II-induced or ET-1-induced ET-1 gene expression in rat cardiac fibroblasts [9]. Antioxidants suppress ET-1-induced ET-1 gene expression [9], which is consistent with the data obtained in the present study. Inhibition of ERK prevents the transcription of the ET-1 gene [9]. Likewise, dominant-negative mutants of Ras, Raf, and MEK1 also decrease ET-1 transcription [44].

Signal transduction pathways that contribute to ET-1 release have been reviewed by Russell and Davenport [45]. Briefly, endothelial cells appear to contain two separate secretory pathways, a constitutive pathway that involves continuous release, and a regulated pathway that involves a
stimulated release. Both pathways have unique methods of regulation, with the constitutive pathway involving mRNA transcription and the regulated pathway involving release from storage granules known as Weibel–Palade bodies. The research defining these pathways has been performed in endothelial cells, vascular smooth muscle cells, and cardiac fibroblasts. It is not known if these pathways also regulate ET-1 release in adventitial fibroblasts in the same manner. Such studies would be a major undertaking and beyond the scope of the current study. Notwithstanding, the data in this article demonstrate that elevated superoxide anion generation in Ang II-treated cells parallels that of ET-1 and type I procollagen expression and that specific blockade or inhibition of the components of this pathway prevent type I procollagen expression. Our findings are consistent with the notion that reactive oxygen species are important signaling molecules mediating ET-1 and type I procollagen expression in adventitial fibroblasts.

Our data showed that there was an increase of ET-1 expression in response to Ang II in the knockout group, although it is less than the ET-1 elevation in wild-type mice. The data suggest that there are mechanisms other than gp91phox containing NADPH oxidase mediating the effect. Mitochondria are also a major source of ROS and we cannot exclude the role of this organelle in remodeling. Therefore, in future we shall study the role of mitochondria-derived oxidative stress in the regulation of adventitial ET-1.

5.4. NADPH oxidase derived oxidative stress mediates collagen production

In agreement with our previous finding, this study showed that Ang II-evoked type I procollagen-α1 expression was inhibited by ET_{α}-receptor inhibitor, indicating the contribution of adventitial ET-1. In addition to examining whether Ang II-induced ET-1 expression is upregulated by Ang II-induced oxidative stress, we determined whether the oxidative stress plays any role on collagen expression in cultured fibroblasts in this study. Consistent with the findings in ET-1 expression, Ang II-induced type I procollagen-α1 expression was inhibited by superoxide scavengers and NADPH oxidase inhibitors. Moreover, Ang II-induced type I procollagen-α1 levels were decreased in cells over expressing SOD1. Our data agree with the findings reported by other investigators that superoxide anion regulates collagen metabolism in cardiac fibroblasts through the activation of matrix metalloproteinase [46]. Hence, Ang II-induced oxidative stress regulates ET-1 release, which in turn mediates collagen synthesis in adventitial fibroblasts. These finding have important implications for disease states associated with remodeling of the vasculature. Rocnik et al. reported that vascular injury may induce a sequence of events in the adventitia that includes the deposition of newly synthesized collagens, which in turn is a key factor for smooth muscle migration [47]. Moreover, fibrillar collagen accumulation could conceivably contribute to vascular stiffness and changes in vascular compliance. Therefore, our findings on mechanisms that might contribute ET-1 release and collagen deposition may suggest an important functional role in the overall remodeling of the arterial wall in various pathological conditions.

5.5. Conclusion

Adventitial fibroblasts synthesize and release ET-1 in response to stimulation by Ang II, which contributes to type I procollagen-α1 expression, suggesting a functional role for Ang II evoked ET-1 release in the regulation of the extracellular matrix. This effect of Ang II is mediated by superoxide anion derived from NADPH oxidase. The increase in adventitial ET-1 may play an important role in the regulation of vascular collagen synthesis and may therefore mediate vascular function through an autocrine or paracrine fashion.

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