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Angiotensin II upregulates Kv1.5 expression through ROS-dependent transforming growth factor-beta1 and extracellular signal-regulated kinase 1/2 signalings in neonatal rat atrial myocytes

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ABSTRACT

Kv1.5 potassium channel represents a promising target for atrial fibrillation (AF) therapy. During AF, the renin–angiotensin system is markedly activated. Recent evidence indicates that angiotensin II (Ang II) can upregulate Kv1.5 channel, but the mechanism remains unknown. In this study, we report that Ang II-mediated transforming growth factor-beta1 (TGF-β_1)/Smad2/3 and extracellular signal-regulated kinase (ERK) 1/2 signalings are involved in atrial Kv1.5 expression. In neonatal rat atrial myocytes, quantitative PCR and Western blotting revealed that Ang II upregulated TGF-β_1, synapse-associated protein 97 (SAP97) and Kv1.5 expression in a time- and concentration-dependent manner. The Ang II-induced upregulation of Kv1.5, SAP97 and phosphorylated Smad2/3 (P-Smad2/3) were reversed by the Ang II type 1 (AT_1) receptor antagonist losartan, an anti-TGF-β_1 antibody and the ERK 1/2 inhibitor PD98059 but not by the AT_2 receptor antagonist PD123319. mRNA knockdown of either Smad2 or Smad3 blocked Ang II-induced expression of Kv1.5 and SAP97. These data suggest that AT_1 receptor/TGF-β_1/P-Smad2/3 and ERK 1/2 signalings are involved in Ang II-induced Kv1.5 and SAP97 expression. Flow cytometry and Western blotting revealed that losartan and the anti-TGF-β_1 antibody diminished Ang II-induced reactive oxygen species (ROS) generation and that the antioxidants diphenyleneiodonium and N-acetyl cysteine inhibited Ang II-induced expression of P-Smad2/3, phosphorylated ERK (P-ERK) 1/2, Kv1.5, SAP97, suggesting that ROS participate in Kv1.5 and SAP97 regulation by modulating Ang II-induced P-Smad2/3 and P-ERK 1/2 expression. In conclusion, we demonstrate that ROS-dependent Ang II/AT_1 receptor/TGF-β_1/P-Smad2/3 and Ang II/ERK 1/2 signalings are involved in atrial Kv1.5 and SAP97 expression. Antioxidants would be beneficial for AF treatment through inhibiting atrial Kv1.5 expression.

1. Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia observed in clinical practice [1,2]. A major goal for developing new AF therapies is the identification of the repolarization mechanisms that are present in the atrium and not in the ventricle [3]. The potassium current I_{Kur} has been shown to be selectively involved in atrial repolarization in human atria [3]. Hence Kv1.5, the corresponding channel for I_{Kur}, is considered to be a promising target for an atrial-specific AF therapy. Many potent Kv1.5 inhibitors, such as diphenylphosphine oxide, isoquinolinone, S9947 and AVE0118 have been developed and have been shown to be beneficial for AF treatment [3,4]. Despite the importance of Kv1.5 blockers in AF therapies, the regulatory mechanisms underlying Kv1.5 expression in atrial myocytes remain elusive.

In the heart, Kv1.5 colocalizes with its anchoring partner synapse-associated protein 97 (SAP97) [5,6]. SAP97 modulates the expression of Kv1.5 by stabilizing its localization in the plasma membrane [6]. Kv1.5 is reported to be redox-sensitive. Hypoxia and decreased reactive oxygen species (ROS) generation mediated by mitochondrial hyperpolarization have been shown to

Abbreviations: AF, atrial fibrillation; Ang II, angiotensin II; AT_1, angiotensin II type 1; AT_2, angiotensin II type 2; SAP97, synapse-associated protein 97; TGF, transforming growth factor; ERK, extracellular signal-regulated kinase; P-ERK, phosphorylated ERK; P-Smad, phosphorylated Smad; DPI, diphenyleneiodonium; NAC, N-acetyl cysteine; RAS, renin–angiotensin system; ROS, reactive oxygen species; siRNA, small interfering RNA; HUVECs, human umbilical vein endothelial cells; PASMCs, pulmonary artery smooth muscle cells.

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downregulate Kv1.5 expression in pulmonary artery smooth muscle cells (PASMCs) [7,8]. AF is associated with oxidative stress. During AF, the activated renin–angiotensin system (RAS), especially angiotensin II (Ang II), triggers a great amount of cardiac ROS [9]. Recent evidence has shown that Ang II increases Kv1.5 expression in a time- and concentration-dependent manner in parallel with intracellular ROS generation in human umbilical vein endothelial cells (HUVECs) [10]. However, whether Ang II is involved in cardiac Kv1.5 expression remains unknown.

Ang II is an important peptide that influences the function of cells in the cardiovascular system by activating multiple intracellular signaling pathways. Recent investigations have shown that Ang II stimulates the generation of ROS by activating membrane-bound NAD(P)H oxidase in a variety of cells [11]. ROS may act as a second messenger and regulates various intracellular signal transduction cascades and the activity of various transcription factors [12]. Notably, the transforming growth factor-beta1 (TGF-β_1) and extracellular signal-regulated kinase (ERK) 1/2 signalings are activated by Ang II under pathophysiological conditions and are well-characterized to be influenced by cellular oxidative changes [13–16]. TGF-β_1 is another activator of NAD(P)H oxidase and induces abundant ROS generation in cardiac cells [17]. Studies have shown that Ang II upregulates TGF-β_1 expression via activation of the Ang II type 1 (AT_1) receptor in cardiac myocytes, and induction of TGF-β_1 contributes to pathogenic cardiac remodeling via mechanisms involving oxidative stress [13,14]. Meanwhile, ROS-dependent ERK 1/2 phosphorylation is critical for ERK 1/2 activation and has been reported to be involved in Ang II-mediated regulation of cardiac IL-6 and AdipoR1 [12,16,18]. Although the effects of TGF-β_1 and ERK 1/2 are closely related to ROS, whether TGF-β_1 and ERK 1/2 can regulate Kv1.5 expression through ROS are yet to be determined. In the present study, we hypothesized that Ang II could induce Kv1.5 and SAP97 expression in neonatal rat atrial myocytes through ROS generation, and the mechanism responsible might involve either the Ang II-mediated TGF-β_1 or Ang II-mediated ERK 1/2 signalings.

2. Materials and methods

The experimental protocol was approved by the Animal Ethical and Welfare Committee of Sun Yat-Sen University, Guangzhou, China. All reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified in the text.

2.1. Cell culture

Sprague–Dawley rats (1–3 days old) were supplied by the Experimental Animal Center, Sun Yat-sen University, Guangzhou, China. Neonatal rat atrial myocytes were prepared according to the method used by He et al., with some modifications [19]. Briefly, the hearts were removed from neonatal pups in germfree condition. The atria were separated from the ventricles by cutting through the atrio-ventricular groove, minced into 1-mm^3 cubes and dissociated by 0.125% tryptase (Gibco, USA) and 0.05% collagenase (Gibco). Then atrial myocytes were separated from the non-cardiomyocytes using selective attachment procedures and cultured in DMEM-F12 medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone). The isolated atrial myocytes were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. During the first 24 h, 0.1 mM 5′-bromo-2′-deoxyuridine was used to inhibit the growth of non-cardiomyocytes. Assessments were performed by observing cells pulses and performing immunocytochemistry with an α-actin monoclonal antibody. Using these methods, the purity of atrial myocytes was greater than 90%.

2.2. Small interfering RNA transfection

Silencer small interfering siRNAs targeting Smad2, Smad3, or Smad7 were synthesized by Invitrogen (Carlsbad, CA, USA) based on the sequences of rat Smad2 (GenBank accession No. AB010147), Smad3 (GenBank accession No. BC064437) and Smad7 (GenBank accession No. AF042499) and were used in the knockdown experiments. A control siRNA (Invitrogen) was used to demonstrate that the transfection does not induce any nonspecific effect on gene expression. Neonatal rat atrial myocytes were seeded in a 6-well plate (Corning, USA) at a density of 0.8×10^6/mL and cultured in DMEM-F12 containing 10% FBS for 24 h. These myocytes were later transfected with 10 nM of siRNA using Lipofectamine RNAiMax Reagent (Invitrogen) according to the manufacturer's instructions. Clones of Smad2, Smad3, or Smad7 siRNA that exhibited at least 90% inhibition of the target gene were selected for further analysis.

2.3. Flow cytometry

The molecule 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Beyotime, Shanghai, China) was used as an ROS-sensitive fluorescence probe to determine intracellular ROS production by laser scanning confocal microscopy (FV500, Olympus, Japan, excitation/emission: 488/525 nm). After incubation with Ang II for 24 h, neonatal rat atrial myocytes were washed with serum-free DMEM-F12 three times and incubated with DCFH-DA for 20 min. Cells were then washed with PBS and subjected to trypsin (Gibco) digestion. After centrifugation at 1000 rpm for 5 min, cells were collected and resuspended with PBS at a density of 2×10^6/L for laser scanning.

2.4. Real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen). cDNA was synthesized with SYBR ExScript RT-PCR kit (TOYOBO, Japan) according to the manufacturer's protocol. The PCR primers for rat Kv1.5: 5′-ACA CTG GCC GAT CCA TTC TT-3′ and 5′-ATG GCC ACG ACA TCG ATG AT-3′; Rat SAP97: 5′-AGC AGA GAA GGG CAA GCA TT-3′ and 5′-CAG TGA ACT CCT GCT CCA GTC T-3′; Rat TGF-β_1: 5′-TGC TTC AGC TCC ACA GAG AA-3′ and 5′-TGG TTG TAG AGG GCA AGG AC-3′; and Rat β-actin: 5′-AGG GAA ATC GTG CGT GAC AT-3′ and 5′-GAA CCG CTC ATT GCC GAT AG-3′. Rat β-actin was used as an internal control. Real-time PCR was performed using an ABI PRISM 7500 real-time PCR system (Applied Biosystems, USA) with SYBR green fluorophore (TOYOBO). All of the reactions were performed in triplicate for each sample.

2.5. Western blotting

Western blots were performed using a standard protocol as described previously [20] and probed with the following primary antibodies: anti-Kv1.5 (Santa Cruz Bio technology, USA), -SAP97, -TGF-β_1 (Abcam, UK), -Smad2, -Smad3, -P-Smad2, -P-Smad3, -P-Smad2/3, -P-ERK 1/2, -GAPDH (Cell Signaling Technology, USA), or -Smad7 (R&D Systems, USA) antibodies. Western blot bands were visualized using ECL Plus (Millipore Corporation, USA) and quantified using the Quantity One 1-D analysis software (Bio-Rad, USA).

2.6. Statistical analysis

The data are expressed as the means ± SE. Statistical comparisons were performed by one-way ANOVA followed by Bonferroni

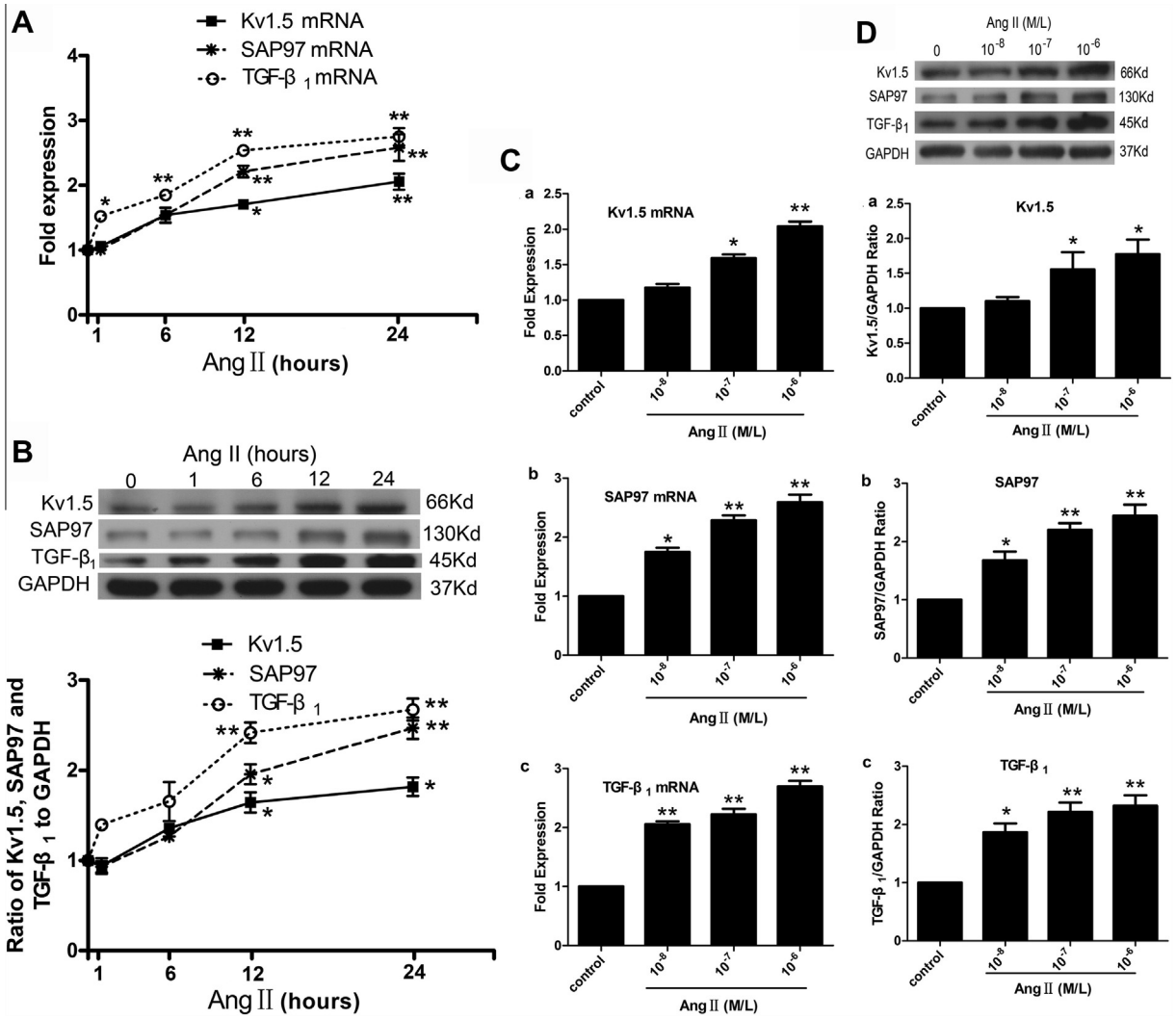

Fig. 1. Effect of Ang II on Kv1.5, SAP97 and TGF-β_1 expression in vitro. (A and B) Neonatal rat atrial myocytes were treated with Ang II for different time periods (0–24 h) and then harvested. (C and D) Neonatal rat atrial myocytes were treated with different concentrations of Ang II (0–10^{-6} M) for 24 h and then harvested. (A and C) After RNA extraction, mRNA levels of Kv1.5, SAP97 and TGF-β_1 were quantified using real-time PCR ($n = 5$). (B and D) Western blotting was performed to determine the protein levels of Kv1.5, SAP97 and TGF-β_1 ($n = 5$). $^*P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control.

post hoc test (SPSS 17.0). P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Ang II upregulates the expression of Kv1.5, SAP97 and TGF-β_1 in vitro

In the present study, we first explored if Ang II could promote Kv1.5, SAP97 and TGF-β_1 expression in neonatal rat atrial myocytes. As shown in Fig. 1, the treatment of atrial myocytes with Ang II caused a significant increase in the Kv1.5, SAP97 and TGF-β_1 expression levels. Ang II initiated TGF-β_1 mRNA expression as early as 1 h after the onset of incubation, and this expression continued to increase after 24 h (Fig. 1A). Similarly, the Kv1.5 and SAP97 mRNA expression levels were increased by Ang II at 12 h after initiation of treatment, and these levels continued to increase after 24 h (Fig. 1A). Cells incubated with Ang II for 12 and 24 h had increased Kv1.5, SAP97 and TGF-β_1 protein expression levels (Fig. 1B). Ang II was shown to upregulate the mRNA and protein expression of Kv1.5, SAP97 and TGF-β_1 in a concentration-dependent manner (10^{-8} M to 10^{-6} M, Fig. 1C and D).

3.2. Involvement of TGF-β_1 and ERK 1/2 in Ang II-induced upregulation of Kv1.5, SAP97 and Smad2/3 in Vitro

We examined if either TGF-β_1 or ERK 1/2 are involved in Ang II-induced expression of Kv1.5 and SAP97. As shown in Fig. 2, Western blot analysis revealed that either an anti-TGF-β_1 antibody or the ERK inhibitor PD98059 significantly inhibited Ang II-induced expression of Kv1.5 (Fig. 2B), SAP97 (Fig. 2C) and P-Smad2/3 (Fig. 2D) in atrial myocytes. The AT_1 receptor antagonist losartan reversed the Ang II-induced upregulation of Kv1.5 ($P < 0.05$; Fig. 2B), SAP97 ($P < 0.05$; Fig. 2C) and P-Smad2/3 ($P < 0.05$; Fig. 2D). However, the AT_2 receptor antagonist PD123319 did not reverse these Ang II-induced changes ($P > 0.05$), suggesting that an AT_1 receptor-specific mechanism is involved in the Ang II-mediated expression of Kv1.5 and SAP97 and the activation of the TGF-β_1/Smad2/3 signaling.

3.3. Involvement of Smad2 and Smad3 in Ang II-induced expression of Kv1.5 and SAP97 in vitro

Because Ang II upregulates Kv1.5 and SAP97 in parallel to increased P-Smad2/3, we further examined if Smad2, Smad3 or

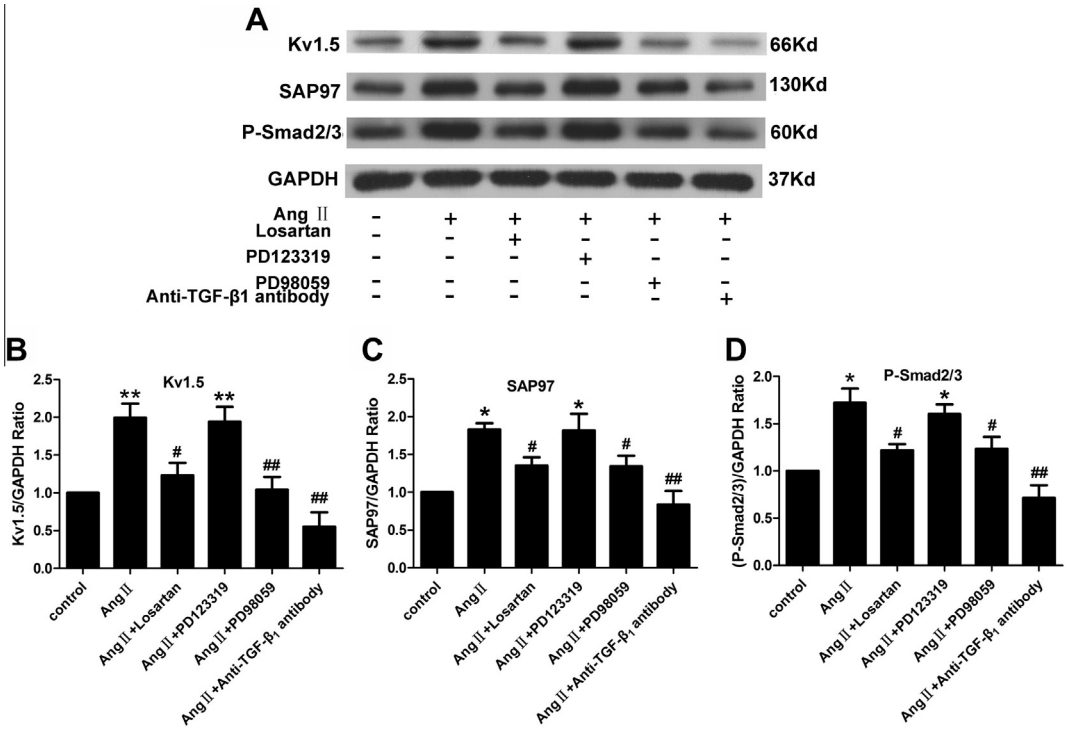

Fig. 2. Effect of the AT_1 and AT_2 receptor antagonists, ERK inhibitor and anti-TGF-β_1 antibody on Ang II-induced expression of Kv1.5, SAP97 and Smad2/3 in vitro. Atrial myocytes were treated with vehicle control or 10^{-6} M Ang II in the presence or absence of 10 μM losartan, 100 μM PD123319, 10 μM PD98059 or 2 μg/mL anti-TGF-β_1 antibody for 24 h. (A) Representative Western blot gel depicts the protein expression of Kv1.5, SAP97, and P-Smad2/3. (B–D) The protein levels of Kv1.5 (B), SAP97 (C), and P-Smad2/3 (D) were determined by Western blot ($n = 6$). $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. Ang II; $^{\#\#}P < 0.01$ vs. Ang II.

Smad7, the inhibitory Smad, are involved in Ang II-induced expression of Kv1.5 and SAP97. Transfection with siRNAs targeting Smad2, Smad3, or Smad7 significantly blocked the protein expression level of Smad2, Smad3, or Smad7 (>90%) as measured by Western blot (Fig. 3A, a–c). Fig. 3B shows that knockdown of Smad2, Smad3, or Smad7 had no significant effect on the basal protein expression levels of either Kv1.5 or SAP97 ($P > 0.05$). As shown in Fig. 3C, knockdown of Smad7 did not inhibit Ang II-induced expression of either Kv1.5 or SAP97 ($P > 0.05$, compared with Non-transf + Ang II group). However, knockdown of either Smad2 or Smad3 almost completely blocked Ang II-induced expression of Kv1.5 and SAP97 ($P < 0.05$; Fig. 3C), indicating that Smad2 and Smad3 are involved in Ang II-induced expression of Kv1.5 and SAP97. Because Ang II was shown to increase the expression of TGF-β_1 (Fig. 1) and P-Smad2/3 (Fig. 2D), these results indicate that Ang II could upregulate Kv1.5 and SAP97 expression by activating the TGF-β_1/P-Smad2/3 signaling.

3.4. AT_1 receptor and TGF-β_1 are responsible for Ang II-induced ROS production, and the antioxidants diphenyleneiodonium (DPI) and N-acetyl cysteine (NAC) block Ang II-induced ROS production and Ang II-induced expression of P-ERK 1/2, Kv1.5, SAP97 and P-Smad2/3 in vitro

We sought to determine if AT_1 receptor and TGF-β_1 are involved in Ang II-induced ROS generation and if ROS participate in Ang II-induced expression of P-ERK 1/2, Kv1.5, SAP97 and P-Smad2/3. As shown in Fig. 4A, incubation with Ang II for 24 h markedly increased ROS production in atrial myocytes (398.0 ± 22.5% of the control group, $P < 0.01$), whereas pretreatment with AT_1 receptor antagonist losartan, anti-TGF-β1 antibody, DPI (the NAD(P)H oxidase inhibitor) or NAC (a glutathione precursor and H_2O_2 scavenger) significantly diminished Ang II-induced ROS production (109 ± 10.1%, 143 ± 12.8%, 84.6 ± 1.8% and 42.8 ± 2.6% of the control group, respectively; $P < 0.01$). Western blotting revealed that pretreatment with either DPI or NAC inhibited Ang II-induced expression of P-ERK 1/2 (Fig. 4B, a), Kv1.5 (Fig. 4B, b), SAP97 (Fig. 4B, c) and P-Smad2/3 (Fig. 4B, d). These results indicate that AT_1 receptor and TGF-β_1 are reponsible for Ang II-induced ROS production. Meanwhile, these data suggest that ROS play a critical role in Ang II-induced expression of P-ERK 1/2, Kv1.5, SAP97 and P-Smad2/3 and that ROS could upregulate P-Smad2/3, which is involved in Ang II-induced expression of Kv1.5 and SAP97.

4. Discussion

Kv1.5. is expressed in cardiac tissue, where it carries the I_{Kur} current that modulates atrial repolarization, and in the pulmonary vasculature, where it regulates vessel tone in response to oxidative changes [3,7,8]. Although Ang II has been shown to induce Kv1.5 expression in parallel with a marked increase in ROS generation in HUVECs [10], the effect of Ang II on cardiac Kv1.5 expression remains to be elucidated. Because Ang II, the effector molecule of the RAS, triggers a great amount of ROS in myocardium [9], it is likely that Kv1.5 is regulated by Ang II-mediated signalings.

In the present study, we demonstrate that Ang II upregulates Kv1.5 and its anchoring partner SAP97 in neonatal rat atrial myocytes and that the mechanisms involved are activation of the ROS-dependent Ang II/AT_1 receptor/TGF-β_1/P-Smad2/3 and Ang II/ERK 1/2 signalings.

SAP97 is a member of the membrane-associated guanylate kinase superfamily, which plays a major role in the spatial localization and clustering of ion channels [5]. Previous studies have shown that in cardiomyocytes, SAP97 colocalizes with Kv1.5 channel polypeptides, and the overexpression of SAP97 augments the I_{Kur} current [6]. In our study, we found that Ang II upregulated the expression of Kv1.5 and its anchoring protein SAP97, which is

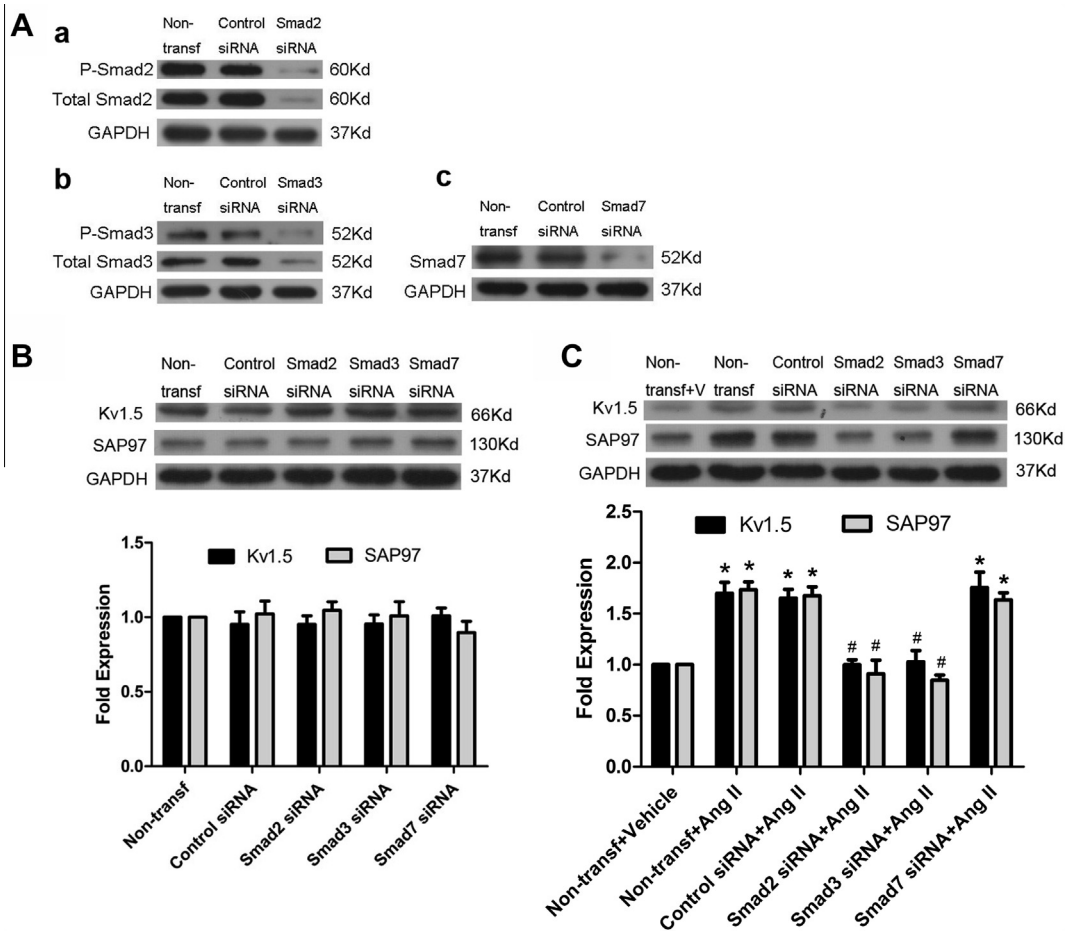

Fig. 3. Effect of Smad2, Smad3 or Smad7 mRNA knockdown on the basal and Ang II-induced expression of Kv1.5 and SAP97 in vitro. (A) Atrial myocytes were transfected with either 10 nM control siRNA or 10 nM siRNAs targeted to Smad2, Smad3 and Smad7. The representative Western blot gel depicts the protein expression levels of Smad2 (a), Smad3 (b) and Smad7 (c) in untransfected cells (non-transf) and in cells transfected with control siRNA or the targeting siRNA. Smad2, Smad3 and Smad7 were successfully knocked down. (B) Effect of Smad2, Smad3 or Smad7 knockdown on the basal expression levels of Kv1.5 and SAP97 in atrial myocytes as measured by Western blot ($n = 5$). (C) Effect of Smad2, Smad3 or Smad7 knockdown on Ang II-induced Kv1.5 and SAP97 expression in atrial myocytes as measured by Western blot ($n = 5$). $^{*}P < 0.05$ vs. non-transf + vehicle; $^{\#}P < 0.05$ vs. non-transf + Ang II.

consistent with the close interaction between SAP97 and Kv1.5. Because SAP97 is the anchoring partner of Kv1.5, Ang II-induced upregulation of SAP97 might be an automatic adaptation to the increased Kv1.5 expression level induced by Ang II in neonatal rat atrial myocytes.

It was unclear if Ang II could affect Kv1.5 expression in atrial myocytes. Here, we demonstrated that Ang II could induce Kv1.5 and SAP97 expression in a time- and concentration-dependent manner in neonatal rat atrial myocytes. This finding is consistent with the observation in HUVECs, where Ang II has been shown to increase Kv1.5 expression with similar temporal and concentration profiles [10]. Regarding the transcriptional regulation of Kv1.5 expression, Mia et al. reported that Kv1.5 is downregulated by AMP-activated protein kinase in Xenopus oocytes [21]. Archer et al. found that normoxic HIF-1α activation decreases Kv1.5 expression in PASMCs [8]. In this study, we show that Ang II increases Kv1.5 and SAP97 expression by activating the TGF-β_1/P-Smad2/3 signaling in neonatal rat atrial myocytes. Our results revealed that the AT_1 receptor antagonist losartan (but not the AT_2 receptor antagonist PD123319) reversed the Ang II-induced upregulation of Kv1.5 and SAP97, suggesting that an AT_1 receptor-specific mechanism is involved. Furthermore, we found that the ERK inhibitor PD98059 and the anti-TGF-β_1 antibody inhibited Ang II-induced expression of Kv1.5, SAP97 and P-Smad2/3. There are crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-β_1 [22]. TGF-β_1 has been shown to activate ERK 1/2 in many cell types, and ERK 1/2 has been reported to phosphorylate Smad [22–24]. Thus, the ERK 1/2 and TGF-β_1/P-Smad2/3 signalings might interact with each other in Ang II-induced Kv1.5 and SAP97 expression.

Phosphorylation of Smad2/3 allows for the translocation of this protein into the cell nucleus to activate target gene transcription [22]. The present study showed that knockdown of either Smad2 or Smad3 inhibited Ang II-induced expression of Kv1.5 and SAP97. However, knockdown of either Smad2 or Smad3 has no significant effect on the basal expression of Kv1.5 or SAP97 in neonatal rat atrial myocytes. Knockdown of Smad7 had no significant effect on either the basal or Ang II-induced expression of Kv1.5 and SAP97. These results indicate that Smad2 and Smad3, but not Smad7, are involved in Ang II-induced expression of Kv1.5 and SAP97. This finding implicates that gene therapies targeting Smad2 or Smad3 might have the potential to alter Kv1.5 expression in pathophysiological conditions such as AF when the Ang II levels are high in the cardiac tissues.

In this study, we found that AT_1 receptor and TGF-β_1 are responsible for Ang II-induced ROS production. We showed that the antioxidants DPI and NAC diminished Ang II-induced ROS production and Ang II-mediated Smad2/3 phosphorylation, resulting in

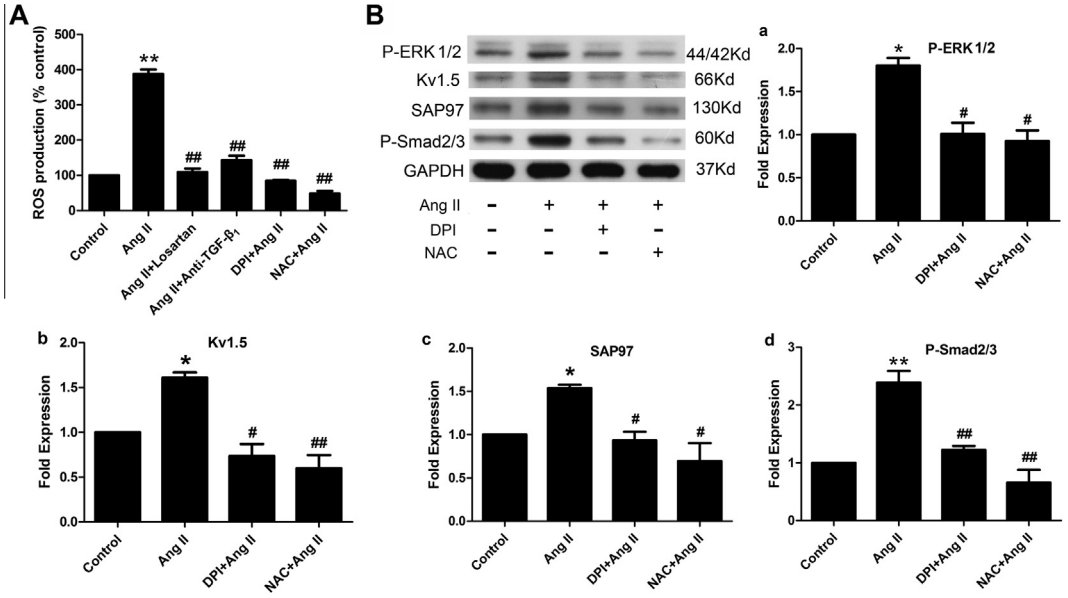

Fig. 4. Effect of the AT_1 receptor antagonist losartan, anti-TGF-β_1 antibody and antioxidants DPI and NAC on Ang II-induced ROS production and effect of DPI and NAC on Ang II-induced expression of P-ERK 1/2, Kv1.5, SAP97 and P-Smad2/3 in vitro. (A) Neonatal rat atrial myocytes were pretreated with losartan (10 μM), anti-TGF-β_1 antibody (2 μg/mL), DPI (the NAD(P)H oxidase inhibitor, 10 μM) or NAC (a glutathione precursor and H_2O_2 scavenger, 10 mM) for 1 h, treated with Ang II (10^{-6} M) for 24 h and harvested. The intracellular ROS levels were detected by flow cytometry using DCFH-DA as an ROS-sensitive fluorescence probe ($n = 5$). (B) Atrial myocytes were pretreated with DPI (10 μM) or NAC (10 mM) for 1 h, treated with Ang II (10^{-6} M) for 24 h and harvested. Western blotting was performed to measure the protein levels of P-ERK 1/2 (a), Kv1.5 (b), SAP97 (c) and P-Smad2/3 (d) ($n = 5$). $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control; $^{\#}P < 0.01$ vs. Ang II; $^{\#\#}P < 0.01$ vs. Ang II.

decreased Kv1.5 and SAP97 protein expression levels. Ang II triggers the secretion of TGF-β_1, which can exert its effects through the ROS-dependent phosphorylation of Smad2/3 [17]. Because the anti-TGF-β_1 antibody was shown to significantly inhibit Ang II-mediated Smad2/3 phosphorylation (see Fig. 2D) and Ang II-induced expression of Kv1.5 and SAP97 (see Fig. 2B and C), these results indicate that the ROS-dependent AT_1 receptor/TGF-β_1/P-Smad2/3 signaling serves as an important mechanism in Ang II-induced expression of Kv1.5 and SAP97. Meanwhile, our results show that ROS participate in ERK 1/2 activation by mediating Ang II-induced ERK 1/2 phosphorylation (see Fig. 4B, a). Because the ERK inhibitor PD98059 decreased Ang II-induced Kv1.5 and SAP97 expression (see Fig. 2B and C), these data suggest that ROS-dependent ERK 1/2 phosphorylation is involved in Ang II-induced Kv1.5 and SAP97 expression. In the present study, we demonstrate that ROS participate in cardiac Kv1.5 regulation, and that the antioxidants DPI and NAC can inhibit Kv1.5 expression. Since oxidative stress has been implicated as a major underlying pathology that promotes AF [9], this finding suggests that antioxidant therapy could potentially inhibit Kv1.5 expression and I_{Kur} density, contributing to the treatment of AF.

In conclusion, our results elucidate the regulatory mechanisms involved in atrial Kv1.5 and SAP97 expression. We demonstrate that Ang II upregulates Kv1.5 and SAP97 expression in neonatal rat atrial myocytes, and the mechanisms of this upregulation involve the activation of the ROS-dependent Ang II/AT_1 receptor/TGF-β_1/P-Smad2/3 and Ang II/ERK 1/2 signalings (Supplementary Fig. S1). Further, this study provides new evidence supporting the application of antioxidants in AF treatment because these agents could inhibit Ang II-mediated Kv1.5 expression.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org./10.1016/j.bbrc.2014.10.088.

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