Angiotensin II and Stretch Activate NADPH Oxidase to Destabilize Cardiac Kv4.3 Channel mRNA

Chaoming Zhou, Chandra Ziegler, Lori A. Birder, Alexandre F.R. Stewart, Edwin S. Levitan

Abstract—Pathological and physiological hypertrophy of the heart is associated with decreased expression of the Kv4.3 transient outward current ($I_{\text{to}}$) channel. The downregulation of channel mRNA and protein, which may be proarrhythmic, is recapitulated with cultured neonatal rat ventricular myocytes treated with angiotensin II (Ang II). Here we show that the 4.9 kb 3' untranslated region (3' UTR) of the Kv4.3 channel transcript confers Ang II sensitivity to a promoter-reporter construct. In contrast, Kv4.2 and Kv1.5 3' UTR reporter sequences are insensitive to Ang II. Both Kv4.3 3' UTR reporter mRNA and activity are decreased in Ang II–treated cardiac myocytes, in accordance with a decrease in mRNA stability. This regulation is mediated by Ang II type 1 (AT$_1$) receptors and abolished by NADPH oxidase inhibitors and dominant negative rac. The Ang II effect is also blocked by expression of superoxide dismutase (SOD), but not catalase, showing that superoxide is required. Dominant negative subunits, enzyme inhibitors and hydrogen peroxide experiments show that the apoptosis signal-regulating kinase 1 (ASK1)–p38 kinase pathway mediates downstream signaling from NADPH oxidase.

Key Words: potassium channel □ angiotensin □ stretch □ NADPH oxidase □ ROS □ mRNA stability

Cardiac hypertrophy, caused by congestive heart failure, hypertension, and pregnancy, is associated with a decrease in the transient outward current ($I_{\text{to}}$) in ventricular cardiac myocytes.1,2 The suppression of ventricular $I_{\text{to}}$ is thought to promote calcium influx and myocyte contraction in the early adaptive phase of hypertrophy but may eventually induce calcium overloading of the sarcoplasmic reticulum, leading to arrhythmic firing and sudden death.3,4 The reduction of $I_{\text{to}}$ is usually related to the downregulation of Kv4.3 mRNA and protein that occurs with congestive heart failure in humans and hypertension in animals.5,6 The finding that the latter effect is blocked by angiotensin converting enzyme (ACE) inhibition implicated angiotensin II (Ang II) in the control of cardiac Kv4.3 gene expression.7 Follow-up experiments have shown that Ang II acts directly on cultured neonatal rat ventricular myocytes via Ang II type 1 (AT$_1$) receptors to decrease Kv4.3 mRNA and protein.8 However, the onset of the effect was more rapid than the turnover of the channel mRNA, and Ang II did not act on the Kv4.3 promoter.9 This indirect evidence led to the proposal that Ang II destabilizes Kv4.3 mRNA in cardiac myocytes. In contrast, the $\alpha_1$-adrenergic receptor agonist phenylephrine downregulated Kv4.3 promoter activity,8 suggesting that G$_{\text{i}}$-mediated signaling shared by AT$_1$ receptors and $\alpha_1$-adrenergic receptors might not be involved in this Ang II effect on mRNA stability.

It has been recently demonstrated that AT$_1$ receptors can act via the small G protein rac to stimulate NADPH oxidase in cardiac myocytes.9,10 NADPH oxidase produces superoxide, which serves as a physiological reactive oxygen species (ROS) messenger within the cell. Separate studies have also shown that AT$_1$ receptors, rac and ROS production are activated by mechanical stretch of cardiac myocytes.11-13 Thus, we hypothesized that Ang II and stretch can act via the AT$_1$ receptor–NADPH oxidase pathway to regulate cardiac myocyte Kv4.3 mRNA stability. Here we clone the 3' untranslated region (3' UTR) of the Kv4.3 mRNA and show that Ang II destabilizes reporter construct mRNA containing this sequence. We then use reporter experiments to implicate NADPH oxidase signaling in the control of cardiac channel mRNA stability by Ang II and mechanical stretch.

Materials and Methods
Cloning of Channel 3'-UTR Sequences and Generation of Reporter Constructs

The cDNA fragments corresponding to the entire 3' UTR of Kv4.3 were amplified by RT-PCR from 2 µg of total RNA extracted from mouse heart. The first-stand cDNA was obtained by reverse tran-
scription with oligo dT and random hexamer primer using reverse-transcription reactions (20 μL) containing 15 mmol/L (NH₄)₂SO₄, 20 mmol/L Tris-HCl (pH 8.8), 2 mmol/L MgCl₂, 0.05% Tween-20, 500 μmol/L dNTPs (Roche), 0.2 mmol/L dithiothreitol (DTT), 10 μmol/L random hexamer primer (Invitrogen), 1 U of DNase I (Roche), 1 U of RNasin (Invitrogen), and 200 U of Superscript II reverse transcriptase (Invitrogen). Reactions were incubated at 42°C for 50 minutes. The primers for RT-PCR were designed from mouse Kv4.3 coding region and mouse expressed sequence tags (ESTs) for 5′ and 3′ ends. For confirmation, the PCR product was ligated into Zero Blunt TOPO PCR Cloning Kit (Invitrogen). The PCR product was confirmed by DNA sequencing. The PCR products were cut with XbaI and SalI sites and ligated into the PG4.3 reporter plasmid. These clones and a commercial cDNA clone (catalog nos. Clone ID 4527103 and Clone ID 30356567; Open Biosystems) were used as a control. For mechanical stretch experiments, transfection with empty vectors was used as a control. For dominant negative subunit experiments, transfection with empty vectors was used as a control. For mechanical stretch experiments, myocytes

**Figure 1. Effect of Ang II on Kv4.3 3′-UTR reporter constructs.** A, Reporter constructs of various lengths of Kv4.3 3′-UTR. A linearized schematic of the PGL3 vector with luciferase coding sequence (yellow) and the SV40 polyadenylation signal (green) is shown at top. Kv4.3 3′-UTR sequence is indicated by orange. Note that PG4.3B used the PGL3 vector, SV40 late polyadenylation site. B, Basal reporter activity in neonatal ventricular cardiomyocytes. Reporter activity of Kv4.3 3′-UTR constructs are compared with PGL3 in the absence of Ang II (see Materials and Methods for details). C, Effect of Ang II treatment on reporter activity normalized to cotransfected Renilla luciferase (filled bars). For each construct, we normalized the Ang II data to the normalized activities in vehicle-treated myocytes (open bars). Note that the 3′-UTR constructs derived from the Kv1.5 (PG1.5) and Kv4.2 (PG4.2) were, like PGL3, insensitive to Ang II, whereas constructs containing most of the Kv4.3 3′-UTR were sensitive to Ang II (n=3 to 9; *P<0.05, **P<0.01). D, Lack of an Ang II effect on PG4.3B expressed in cardiac fibroblasts (n=3).

**Rat Neonatal Cardiac Myocyte Culture and Treatments**

Neonatal rat ventricular cardiac myocytes were isolated from collagenase-treated hearts from 1-day-old Sprague-Dawley rats, as described previously. Following dissociation and preplating for 2 hours to remove nonmyocytes, cardiac myocytes were cultured at low density (0.5×10⁶ viable cells per well) in 6-well plates containing in minimal essential medium (MEM) supplemented with 5% calf serum (Gibco) and bromodeoxyuridine (100 μmol/L) to prevent nonmyocyte proliferation. Twenty hours after plating, the cardiac myocytes were transfected using lipofectamine 2000 at a 1:1 ratio for DNA to lipofection reagent for 4 hours with serum-containing medium. Typically, 3 μg of DNA per well was used. However, in experiments comparing various lengths of the Kv4.3 3′-UTR (ie, Figure 1), molar concentration of reporter plasmid was kept constant (1.5 to 3 μg of DNA per well was used). Twenty hours after transfection, the medium was replaced with serum-free MEM supplemented with human insulin (10 μg/mL, Sigma), human transferrin (10 μg/mL, Sigma), and BSA (1 mg/mL, Sigma). For fibroblast cultures, nonmyocytes isolated in the preplating step were retained and cultured as described above, except that bromodeoxyuridine was omitted from the medium.

Two days later, cultures were subjected to treatment with vehicle, 100 mmol/L Ang II, or stretch. For experiments using chemical inhibitors, cells were preincubated with inhibitors or vehicle for 30 minutes before treatment (ie, Ang II or stretch). For dominant negative subunit experiments, transfection with empty vectors was used as a control. For mechanical stretch experiments, myocytes
Results

The Kv4.3 3′ UTR Confers Sensitivity to Ang II

We hypothesized that the 3′ UTR mediates downregulation of cardiac Kv4.3 mRNA by Ang II. Analysis of the Kv4.3 channel coding sequence, promoter, and mRNA size\(^6\,\)\(^15\) led us to conclude that the 3′ UTR was \(\approx\)5 kb in length. We aligned EST database clones with the genomic mouse Kv4.3 sequence to design primers and then used RT-PCR with mouse heart mRNA to clone the first 4868 bases of the 3′ UTR. We then used a commercial EST clone, which includes a polyadenylation site and a polyA tail, to provide the last 29 bases. Sequence analysis showed that the mouse cardiac RT-PCR products are 99% identical to sequences in the mouse EST database (GI nos. 29356835, 19547440, 24533457, 17962295, 13151612, 3283901, and 50926030), some of which were posted after our cloning.

To test whether the 3′ UTR was responsible for the response to Ang II, we expressed luciferase reporter con-
structs containing Kv4.3 3′-UTR sequences in neonatal cardiac myocytes (Figure 1A). The construct of PG4.3A, which contains the complete native Kv4.3 3′ UTR, with a putative polyadenylation site and a polyA tail, produced low expression compared with the PGL3 vector (Figure 1B). Because this might be attributable to the noncanonical polyadenylation sequence in the channel construct, we substituted the 3′ 600 bases with the SV40 polyadenylation site from the PGL3 vector to generate the PG4.3B construct (Figure 1A). This increased expression 5-fold compared with the PG-4.3A construct (Figure 1B). We then expressed these constructs in neonatal ventricular cardiac myocytes and tested for sensitivity to Ang II. Specifically, we normalized the channel 3′-UTR firefly luciferase reporter activity to a cotransfected constitutively active Renilla luciferase reporter to eliminate variations in transfection and global nonspecific effects. Our measurements showed that treatment with 100 nmol/L Ang II for 7 hours, a period that produces a nearly peak effect on native Kv4.3 channel mRNA, did not affect normalized reporter expression induced by the control vector (PGL3), a construct containing the 3′ 800 bases of the channel 3′ UTR (PG4.3C), and constructs containing 3′-UTR sequences derived from Kv1.5 (PG1.5) or Kv4.2 (PG4.2). However, Ang II reduced reporter activity in the 2 constructs that contain the first 4.3 kb of the Kv4.3 3′ UTR (Figure 1C). Finally, we determined that the Ang II regulation of the Kv4.3 3′-UTR reporter does not occur in cultured neonatal cardiac fibroblasts (Figure 1D). Thus, the 3′ UTR of the Kv4.3 mRNA specifically confers sensitivity Ang II in cardiac myocytes.

**Ang II–Induced Destabilization of the Kv4.3 3′ UTR**

The decrease in reporter activity induced by the channel 3′ UTR could reflect inhibition of luciferase translation or destabilization of the mRNA encoding luciferase. An effect on translation would not affect reporter mRNA concentration, but mRNA destabilization would lower luciferase mRNA levels. In fact, with destabilization, the effect on luciferase mRNA should be larger than the change in luciferase activity because luciferase protein must turnover (t_{1/2} ≈ 4 hours) (data not shown) to report mRNA downregulation as a decrease in enzyme activity.

We measured cardiac myocyte cell luciferase transcript levels by real-time PCR. Ang II did not affect the real-time PCR curves (Δ fluorescence versus cycle number) for luciferase mRNA expression induced by the control PGL3 vector (Figure 2A). Likewise, 18S ribosomal RNA levels were similar in vehicle and Ang II–treated cells (Figure 2A and 2B). However, the PG4.3B curves were right shifted in Ang II–treated cells (Figure 2B). In these assays, a rightward shift of 1 cycle is indicative of a halving in concentration of the reporter mRNA. On average, Kv4.3B luciferase mRNA concentration was reduced 3-fold by Ang II (Figure 2C). This change is double the effect on luciferase enzyme activity (Figure 1C) and so is indicative of destabilization of the channel 3′ UTR. Furthermore, because this response is comparable to the effect on native channel mRNA (which is too fast to be transcriptional), destabilization of the 3′ UTR

**Figure 3.** Ang II acts via AT1 receptors, NADPH oxidase, and Rac. The effect of 100 nmol/L Ang II on the PG4.3B reporter construct was blocked by 100 nmol/L candesartan, an AT1 receptor antagonist (A), the NADPH oxidase inhibitors 100 μmol/L apocynin (Apo) (B), and 10 μmol/L diphenylene iodonium (DPI) (C) or expression of Rac1 T17N, a dominant negative Rac subunit. n=3; ***P<0.001, **P<0.01. CON indicates control.
fully accounts for Ang II downregulation of Kv4.3 mRNA in neonatal cardiac myocytes.

**AT1 Receptor–Activated NADPH Oxidase Mediates the Ang II–Induced mRNA Destabilization**

Initial experiments eliminated Ca²⁺ and protein kinase C as viable messengers in the Ang II effect on endogenous Kv4.3 mRNA (data not shown). Therefore, we began our signaling studies on the PG4.3B reporter by testing whether AT1 receptors mediate the Ang II effect on the 3' UTR and then examined the role of NADPH oxidase. Figure 3A shows that the Ang II effect was blocked by 100 nmol/L candesartan, a specific AT1 receptor antagonist. In contrast, candesartan did not affect reporter activity induced by the control vector PGL3, PG4.3B, or the Kv1.5 3' UTR reporter construct PG1.5 in the absence of Ang II (supplemental Figure IA through IC). Thus, we conclude that the candesartan effect is specific for the Ang II effect on the Kv4.3 3' UTR. To investigate whether cardiac myocytes use NADPH oxidase to downregulate the channel mRNA, we incubated cells with 100 μmol/L apocynin or 10 μmol/L diphenylene iodonium (DPI). These structurally and mechanistically distinct inhibitors of NADPH oxidase each abolished the effect of Ang II (Figure 3B and 3C). Thus, we conclude that the apocynin effect is specific for the Ang II effect on the Kv4.3 3' UTR. To investigate whether cardiac myocytes use NADPH oxidase to downregulate the channel mRNA, we incubated cells with 100 μmol/L apocynin or 10 μmol/L diphenylene iodonium (DPI). These structurally and mechanistically distinct inhibitors of NADPH oxidase each abolished the effect of Ang II (Figure 3B and 3C). Again, controls with PGL3, PG4.3B, and PG1.5 and apocynin in the absence of Ang II show that this action is specific (supplemental Figure IA through IC). Finally, we examined the effect of a dominant negative rac subunit (Rac1 T17N), because the rac G protein mediates activation of NADPH oxidase by Ang II in cardiac myocytes. As can be seen in Figure 3D, Rac1 T17N also inhibited the Ang II destabilization effect. Hence, NADPH oxidase mediates the AT1 receptor–induced destabilization of the Kv4.3 3' UTR.

**Downstream Signaling From NADPH Oxidase**

NADPH oxidase generates superoxide that can then be converted into hydrogen peroxide. This conversion is catalyzed by SOD, whereas hydrogen peroxide is broken down by catalase. Therefore, to test for the involvement of superoxide and hydrogen peroxide in the Ang II effect, cardiac myocytes were transfected with expression vectors for SOD and catalase. If hydrogen peroxide is the physiological mediator, SOD should enhance the response and catalase should inhibit the response. However, we found that catalase left the Ang II effect intact and SOD abolished the Ang II effect on the Kv4.3 3' UTR (Figure 4A). SOD did not affect baseline signals from PGL3, PGKv4.3B, and PGKv1.5 (supplemental Figure I). Thus, there were no nonspecific effects or interference with the reporter assay. These results imply that superoxide, not hydrogen peroxide, acts as the physiological mediator of the Ang II effect.

ROS can activate the mitogen-activated protein (MAP) kinase kinase kinase ASK1. To test for involvement of ASK1, cardiac myocytes were transfected with a dominant negative ASK1 (ASK1DN) expression plasmid. Figure 4B shows that ASK1DN blocks the Ang II effect showing...
that ASK1 is necessary for this regulation. In contrast, controls were unaffected by ASK1DN (supplemental Figure I). We then tested whether we could bypass upstream Ang II signaling by using hydrogen peroxide to directly activate ASK1. As can be seen in the PG4.3B results of Figure 4C, bath application of 1 μmol/L hydrogen peroxide mimicked the Ang II response and 10 μmol/L produced a larger effect. In contrast, hydrogen peroxide did not decrease signals from PGL3 and PG1.5 (Figure 4C).

Finally, the effects of hydrogen peroxide and Ang II on the Kv4.3 3′UTR were not additive (n=2) (supplemental Figure IIA), suggesting that both agents activate the same pathway. Together, these results suggest that ASK1 is necessary and sufficient for a specific destabilization of the Kv4.3 3′ UTR.

ASK1 is known to induce activation of p38 kinase, a MAP kinase that is activated in response to cellular stress. Therefore, to test whether p38 kinase is involved in the Ang II–induced destabilization of Kv4.3 mRNA, we first tested for an effect of a chemical inhibitor of p38 kinase on the 3′UTR reporter. Figure 5A shows that 20 μmol/L SB239063 abolished the Ang II effect. We also found that SB239063 did not affect control constructs (supplemental Figure I), suggesting that the inhibitor acted specifically. To independently implicate p38 kinase in the Ang II effect, cardiac myocytes were cotransfected with a dominant negative p38 kinase subunit (p38DN) (B). Therefore, we conclude that the Ang II acts via the ASK1-p38 pathway to destabilize Kv4.3 mRNA.

**Stretch-Induced Downregulation of Cardiac Kv4.3 mRNA**

Mechanical stretch of cardiac myocytes is known to activate rac and to induce generation of ROS. Recently, it has also been found that stretch directly activates cardiac myocyte AT1 receptors, even in the absence of Ang II. Because all of these stretch targets are involved in the signaling described here, we explored whether stretch acted on the Kv4.3 3′ UTR. Cardiac myocytes were plated on collagen I-coated flexible plates and then subjected to repetitive stretch for 6.5 hours. Figure 6A (left) shows that stretch decreased 3′UTR PGKv4.3B reporter activity. In contrast, mechanical stretch did not affect reporter activity in the absence of Ang II. In contrast, Figure 6B (right) shows that stretch decreased 3′UTR PGKv4.3B reporter activity. In contrast, mechanical stretch did not affect reporter activity in the absence of Ang II. In contrast, Figure 6B (right) shows that stretch decreased 3′UTR PGKv4.3B reporter activity. In contrast, mechanical stretch did not affect reporter activity in the absence of Ang II. In contrast, Figure 6B (right) shows that stretch decreased 3′UTR PGKv4.3B reporter activity. In contrast, mechanical stretch did not affect reporter activity in the absence of Ang II. In contrast, Figure 6B (right) shows that stretch decreased 3′UTR PGKv4.3B reporter activity. In contrast, mechanical stretch did not affect reporter activity in the absence of Ang II. In contrast, Figure 6B (right) shows that stretch decreased 3′UTR PGKv4.3B reporter activity. In contrast, mechanical stretch did not affect reporter activity in the absence of Ang II.
Discussion

Cardiac hypertrophy is associated with decreased Kv4.3 channel expression, which is thought to be proarrhythmic. In vivo angiotensin converting enzyme (ACE) inhibitor effects led to the demonstration that Ang II can act directly on cardiac myocytes to decrease Kv4.3 mRNA.7,8 However, the kinetics of the Ang II effect along with studies of the Kv4.3 promoter raised the possibility that the channel mRNA was destabilized by AT1 receptors. Here, studies based on generating reporter constructs with the cloned cardiac mRNA sequences show that Ang II does indeed destabilize the Kv4.3 mRNA and localizes this regulation to the 3′ UTR. Furthermore, we demonstrate that NADPH oxidase generated–superoxide acts via ASK1 and p38 kinase to mediate this effect. We also show that mechanical stretch of cardiac myocytes downregulates Kv4.3 mRNA by the same pathway (Figure 7). Finally, we established that this regulation is specific because Kv1.5 and Kv4.2 3′ UTRs are insensitive to Ang II. Thus, we have implicated physiological ROS signaling in long-term control of Kv4.3 expression and hence cardiac excitability.

These findings have broad implications for function of the cardiovascular system. First, the AT1 receptor–stretch regulation of cardiac Kv4.3 expression could be activated in response to physiological and pathological changes in blood pressure. Second, because ASK1 is activated by a variety of ROS,16,19 different ROS sources could trigger destabilization of Kv4.3 mRNA. For example, cardiac mitochondria generate ROS after reperfusion following ischemia. In addition, ROS generated by other cell types (ie, endothelial and inflammatory cells) could diffuse into myocytes to affect the channel message. Therefore, the regulation described here could also be induced by ROS generated independently of AT1 receptors. Third, NADPH oxidase regulation of Kv4.3 may occur in other cell types. We did not detect this effect in neonatal cardiac fibroblasts, but these cells do not normally express Kv4.3. However, the rac–NADPH oxidase–p38 pathway has been found in angiotensin-sensitive neurons in the brain and vascular smooth muscle,20–23 and, likewise, Kv4.3 channels are found in brain and smooth muscle.15,24 Thus, NADPH oxidase could act in vascular smooth muscle cells and hypothalamic angiotensin-sensitive neurons to suppress channel Kv4.3 expression. In each of these cases, this would be depolarizing and thus would promote hypertrophy. Finally, NADPH oxidase–induced mRNA destabilization may not be limited to the Kv4.3 transcript. We found that this regulatory mechanism does not affect the 3′ UTRs of Kv4.2 and Kv1.5. Because the former channel gene is regulated during cardiac hypertrophy in rodents (although not present in human ventricle), NADPH oxidase must not regulate stability of all transcripts associated with hypertrophy. Still, further study may reveal that Ang II–ROS–p38 signaling regulates 3′-UTR sequences of other mRNAs in the cardiovascular system.

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References


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Supplemental Figures:

**Fig. S1.** Effects of agents on (A) PGL3, (B) PG4.3B and (C) PG1.5 in the absence of Ang II. Inhibitors of the Ang II effect on the Kv4.3 3’ UTR, including Candesartan (Candes), Apocynin (Apo), Superoxide dismutase (SOD), dominant negative ASK1 (ASK1DN), and the p38 kinase inhibitor SB239063 (SB), do not affect the baseline activities of the three reporter constructs. Catalase (Cat) causes an increase in the baseline of PG4.3B, but not PGL3. However, since Cat does not significantly affect the % change induced by Ang II (Fig. 4A), this does not alter our conclusions. In A and B, n = 3 or 4. In C, each point represents the results from a separate subculture well from the same day, and the lines indicate the mean from 3 replicate wells.
Fig. S2. A. Hydrogen peroxide and Ang II effects on PG4.3B are not additive. B. PGL3 and PG4.2 are insensitive to mechanical stretch of cardiac myocytes. For both A and B, the mean results from two independent experiments are shown.