Angiotensin II Upregulates Ca_v1.2 Protein Expression in Cultured Arteries via Endothelial H₂O₂ Production

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Key Words

Reactive oxygen species · Ca_v1.2 Ca²⁺ channel · Endothelium · Vascular smooth muscle

Abstract

Background: We previously reported that angiotensin II caused an endothelial-dependent increase in L-type voltage-dependent Ca²⁺ channel (Ca_v1.2) in cultured arteries, but the signaling pathways are not clear. **Methods:** Endothelial damage was generated by brief intra-arterial perfusion with 0.3% CHAPS. Ca_v1.2 expression, function and H₂O₂ were measured by Western blot, tension recording and Amplex Red H₂O₂ assay kit, respectively. **Results:** Angiotensin II dose-dependently upregulated Ca_v1.2 expression in endothelium-intact arteries. The angiotensin II upregulation of Ca_v1.2 expression in endothelium-intact arteries was blocked by NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI), apocynin, a more specific NAD(P)H oxidase inhibitor gp91ds-tat and also by catalase. H₂O₂ similarly upregulated Ca_v1.2 expression in endothelium-intact and endothelium-damaged arteries, and the latter effect was also blocked by DPI and apocynin. Angiotensin II increased H₂O₂ production by endothelium-intact but not by endothelium-damaged arteries, and this effect was blocked by apocynin, catalase and gp91ds-tat. The upregulation of Ca_v1.2 by angiotensin II and H₂O₂ is accompanied by an increased tension

response to KCl and the Ca²⁺ channel activator FPL 64176, and this effect was also attenuated by gp91ds-tat. **Conclusion:** These results suggest that angiotensin II stimulates endothelial NAD(P)H oxidase-produced H₂O₂, which may additionally act through vascular smooth muscle NAD(P)H oxidase, to upregulate vascular Ca_v1.2 protein.

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Introduction

Angiotensin II is a potent vasoconstrictor known to activate various signaling pathways en route to causing release of Ca²⁺ from intracellular stores and increased activity of L-type Ca_v1.2 calcium channels [1, 2]. Upregulation of Ca_v1.2 in arteries has been reported by us in spontaneously hypertensive rats [3] and also by others in angiotensin II-induced hypertensive animals, where the upregulation of Ca_v1.2 is associated with dysfunction of endothelium [4]. Calcium channel blockers are used as effective antihypertensive therapy in patients, implicating the calcium channel in the pathogenesis of hypertension.

We have recently reported an effect of angiotensin II to increase Ca_v1.2 expression in arteries maintained in organ culture for 24 h in an endothelial-dependent but transcription-independent fashion [5]. The goal of this

investigation is to identify some of the principal signaling intermediates in this action of angiotensin II.

Our principal goal was to determine the identity of the substance that the endothelium released which mediated the increase in $\text{Ca}_v1.2$ expression in the vascular smooth muscle cells of the arterial wall. The endothelium is known to release vasoconstrictors such as endothelin [6] and TxA_2 , as well as vasodilators such as nitric oxide and prostacyclin [7]. Angiotensin II actions have been shown to be mediated by many different signaling pathways [1]. Our observation that its action in cultured arteries was dependent on a functional endothelium [5] caused us to look first at those pathways angiotensin II has been shown to affect in the endothelium. Chief among these is an increase in NAD(P)H oxidase [8–11], resulting in an increase in reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2). These increases can be inhibited by NAD(P)H oxidase inhibitors diphenyleneiodonium (DPI) and apocynin [8, 12–15] and a more specific NAD(P)H peptide inhibitor gp91ds-tat [16–18]. Endothelial NAD(P)H oxidase-derived ROS play an important role in endothelial dysfunction, which is implicated in cardiovascular diseases such as hypertension, atherosclerosis, and diabetic vascular hypertrophy [13, 19–21]. It has been reported recently that H_2O_2 is involved in the regulation of $\text{Ca}_v1.2$ in HL-1 cardiomyocytes [22]. These results led us to investigate whether or not angiotensin II-induced ROS such as H_2O_2 are also involved in the regulation of $\text{Ca}_v1.2$ expression in vascular smooth muscle cells and furthermore, if they are, what signaling pathways participate in this action.

Methods

Artery Culture

Isolation of mesenteric arteries from freshly euthanized rats was conducted with protocols approved by our institutional animal care and use committee. The superior mesenteric artery of 300–400 g male Sprague Dawley rats was dissected, cleaned of superficial fat, placed in DMEM containing 0.1% fetal bovine serum in 35-mm diameter culture dishes, and maintained in tissue culture at 37°C and 5% CO_2 with and without angiotensin II, H_2O_2 , catalase or NADPH oxidase inhibitors in DMEM with 0.1% fetal bovine serum and antibiotics for 24 h [5]. After this time, arteries were subjected to one (or more) of the following procedures.

Western Blot

Arterial tissue was minced and homogenized on ice in lysis buffer (50 mM NaCl, 25 mM TrisCl, 0.5% Na deoxycholate, 1% IGEPAL CA-630, 1 mM PMSF, 2 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ leupeptin, pH 7.4) and differentially centrifuged at 13,000 g for 10 min to yield a supernatant containing the channels. Protein con-

centration in the supernatant was determined by a BCA protein assay kit (Pierce) and 20–40 μg protein applied to each lane. Western blots were run using 3–8% Tris Acetate gradient gels (Invitrogen) at 150 V for 1 h, and transferred onto PVDF membranes at 32 V for 1.5 h, blocked with NAP blocker (GBiosciences, Geno Technology Inc.) for 2 h and probed for $\text{Ca}_v1.2$ with a primary antibody (1:1,000) from Alomone versus the II-III linker. Lower molecular weight portions of the blot probed with α -actin antibody (Sigma) served as a control for loading. Western blots were quantitated as previously described [3].

Blood Vessel Tension Recording

Rings (4 mm in length) of the artery were equilibrated in a tissue bath for 1 h prior to measuring tension responses to the L-type Ca^{2+} channel agonist FPL 64176. Tension determinations were performed by cutting 4-mm rings of mesenteric vessels and mounting them on force transducers at their optimal length-tension. Rings were equilibrated for 1 h in Krebs-Henseleit solution (mM): NaCl 118, KCl 5.4, NaH_2PO_4 1.4, MgSO_4 1.2, NaHCO_3 18, glucose 11 bubbled with 95% O_2 and 5% CO_2 (pH 7.4). During the equilibration period, basal tension was adjusted to 1.5 g. Rings were then exposed to KCl (80 mM) or Ca^{2+} channel activator FPL 64176 [5].

Endothelial Damage

Endothelial damage was generated by brief intra-arterial perfusion with Krebs-Henseleit solution containing 0.3% CHAPS for approximately 40 s and endothelial damage was confirmed by the lack of vasodilatory response to acetylcholine, as reported in our previous studies [5]. This procedure does not affect vascular smooth muscle vasoconstrictor responses [5, 23–25].

H_2O_2 Determination

H_2O_2 levels in the culture solution were determined using the Amplex Red H_2O_2 assay kit (Molecular Probes, Invitrogen) [26, 27] according to the manufacturer's instructions. Briefly, the superior mesenteric arteries were dissected, cleaned of superficial fat, and cut into pieces (4 mm in length). The pieces were incubated with Amplex Red (50 μM) and horseradish peroxidase (0.1 μM) at 37°C for 1 h in 0.5 ml Tyrode solution (mM): NaCl 140, KCl 5.4, NaH_2PO_4 0.25, CaCl_2 1, MgCl_2 0.5, HEPES 10, glucose 10, pH 7.4. H_2O_2 released from the arteries was expressed as nanomol per milligram protein extracted from the arteries. In some experiments, H_2O_2 levels were also monitored by H_2O_2 test strips (Thermo Electron Corporation).

Reagents

DMEM culture medium was purchased from GIBCO. All other chemicals were purchased from Sigma. The NAD(P)H oxidase peptide inhibitor gp91ds-tat ([H]RKKRRQRRR-CSTRIRRL-[NH₂]) and a scrambled peptide gp91ds-tat ([H]RKKRRQRRR-CLRITRQSR[NH₂]) were used as described previously [16, 17] and were synthesized by Biosynthesis Inc. This peptide inhibitor is considered to be a more specific NAD(P)H oxidase inhibitor [28, 29].

Statistical Analysis

Data are presented as mean $\pm$ SEM, and statistical significance was determined by Student's t test between two groups and analysis of variance followed by post hoc test as appropriate between multiple groups. A p value <0.05 was considered significant.

Fig. 1. a Angiotensin II dose-dependently upregulated expression of Cav1.2 protein. Western blots of rat mesenteric arteries maintained in organ culture 24 h in 0.1% serum with and without angiotensin II. α -Actin was utilized as a loading control. **b** Quantitation of the Western results in **a**. The number of different experiments performed is indicated in each column of the bar graph. * $p < 0.05$ compared with control.

Results

Angiotensin II Upregulation of Cav1.2 Protein Expression

Angiotensin II (0.05–5 μ M) dose-dependently upregulated the expression of Cav1.2 protein in cultured mesenteric arteries as shown in figure 1.

Involvement of NAD(P)H Oxidase in the Upregulation of Cav1.2 Protein Expression by Angiotensin II

Our initial attempts to determine signaling pathway intermediates involved in this effect involved screening of inhibitors of different enzymes. We observed that DPI and apocynin, two chemically unrelated inhibitors of the NAD(P)H oxidase enzyme complex [30–33], both blocked the angiotensin II-induced upregulation of Cav1.2 protein (fig. 2). At the concentration used here, neither DPI nor apocynin alone significantly affected basal Cav1.2 protein expression (not shown). Combined with our previous report [5], which showed that angiotensin II caused an endothelium-dependent upregulation of Cav1.2 expression, these results suggested to us that angiotensin II was activating endothelial NAD(P)H oxidase, much as had been reported in vivo by Mollnau et al. [34] and Higashi et al. [12]. We presumed the inhibitory effects of DPI and apocynin were mediated, at least in part, at the level of the endothelium. The involvement of endothelial NAD(P)H oxidase in the upregulation of Cav1.2 expression was further confirmed by a more specific NAD(P)H oxidase inhibitor, gp91ds-tat [28, 29], as shown in figure 3.

H₂O₂ Involvement in the Upregulation of Cav1.2 Protein Expression

These results suggested that endothelial superoxide generated by NAD(P)H oxidase was involved in the signaling pathway. We next turned to address the identity of the signaling molecule, generated and released by the endothelium of the intima, which signals the vascular smooth muscle of the media to increase expression of Cav1.2. Superoxide is highly reactive and not particularly membrane permeable [35], but it can be converted to H₂O₂ by superoxide dismutase. H₂O₂ is a much more stable molecule than superoxide and is cell membrane permeable [21, 32, 35, 36]. In the following experiments we tested the effects of H₂O₂. As shown in figure 4, H₂O₂ also dose-dependently upregulated expression of Cav1.2 in cultured arteries (fig. 4). Additionally, the angiotensin II-induced increase in Cav1.2 was abrogated by culture in catalase, which converts peroxide to water (fig. 4) [36]. Incubation of endothelial intact arteries in angiotensin II resulted in an increased production of H₂O₂, which was inhibited by both NAD(P)H oxidase inhibitors apocynin and catalase (fig. 5a), and also by gp91ds-tat (fig. 5b). After endothelial damage the angiotensin II-induced H₂O₂ production was greatly reduced (fig. 5a). Finally, arteries with endothelium damaged by brief perfusion with CHAPS prior to culture still responded to H₂O₂ (fig. 6). These results suggest that peroxide is the likely endothelially derived mediator for the angiotensin II effect on Cav1.2 protein expression.

Fig. 2. Angiotensin II upregulation of Cav1.2 protein was strongly depressed by two inhibitors of NAD(P)H oxidase, diphenyleneiodonium chloride (DPI, **a**) and apocynin (**b**). **c, d** Quantification of the Western results in **a** and **b**, respectively. The number of different experiments performed is indicated in each column of the bar graph. * $p < 0.05$ compared with control. ⁺ $p < 0.05$ compared with angiotensin II.

Fig. 3. Upregulation of Cav1.2 protein in rat mesenteric arteries by angiotensin II was inhibited by a more specific NAD(P)H oxidase inhibitor gp91ds-tat, and was not inhibited by the scrambled-tat. **a** One of the representative Western blots in 3 different ex-

periments. **b** Quantitation of the Western blots in **a**. The number of different experiments performed is indicated in each column of the bar graph. * $p < 0.05$ compared with control. ⁺ $p < 0.05$ compared with angiotensin II.

Fig. 4. a Hydrogen peroxide upregulates Cav1.2 protein, and catalase, a peroxide scavenger, inhibits the upregulation of Cav1.2 protein by angiotensin II. **b** Quantification of the Western results in **a**. The number of different experiments performed is indicated in each column of the bar graph. * $p < 0.05$ compared with control. ⁺ $p < 0.05$ compared with Angiotensin II.

Fig. 5. H₂O₂ production by mesenteric arteries under different conditions. a Endothelial (E) damage with 0.3% CHAPS prevented the angiotensin II- (0.5 μM) stimulated increase in H₂O₂ production. Both apocynin (100 μM) and catalase (2,000 U/ml) inhibited angiotensin II-induced elevation of H₂O₂ production. **b** Angiotensin II- (0.5 μM) stimulated increase of H₂O₂ produc-

tion was inhibited by NAD(P)H oxidase inhibitor gp91ds-tat (10 μM), but not by scrambled-tat (10 μM). The number of different experiments performed is indicated in each column of the bar graph. Apocynin alone had no significant effect on H₂O₂ production. * $p < 0.001$ compared with control. ⁺ $p < 0.001$ compared with angiotensin II.

Fig. 6. a Hydrogen peroxide increases expression of Cav1.2 protein even in the presence of a damaged endothelium (E), and the increases are inhibited by the NAD(P)H oxidase inhibitors DPI and apocynin. **b** Quantification of the Western results in **a**, with three experiments in each group. * $p < 0.05$ compared with control. ⁺ $p < 0.05$ compared with H₂O₂.

Since H₂O₂ has been reported to stimulate a non-phagocytic NAD(P)H oxidase in vascular smooth muscle cells [37], the effects of NAD(P)H oxidase inhibition on the increase in Cav1.2 expression in arteries by H₂O₂ was tested. As shown in figure 6, NAD(P)H oxidase inhibitors DPI and apocynin both inhibited the upregulation of Cav1.2 in vascular smooth muscle by H₂O₂ after endothelial damage.

Upregulation of Cav1.2 Function by H₂O₂

Our previous study reported that upregulation of Cav1.2 by angiotensin II is accompanied by an upregulation of functional Cav1.2 activity, assessed using the Ca²⁺ channel activator FPL 64176 [5]. Similarly, the upregulation of Cav1.2 by H₂O₂ is also accompanied by an increased tension response to both KCl (which depolarizes smooth muscle cells and opens the voltage-dependent Cav1.2 channel) and FPL 64176 (fig. 7). These effects were blocked by the calcium channel blocker nifedipine.

Attenuation of Angiotensin II-Induced Upregulation of Cav1.2 Function by gp91ds-tat

Angiotensin II induced an upregulation of Cav1.2 activity as evidenced by an increased tension response to KCl and the Ca²⁺ channel activator FPL 64176. That the effects of KCl and FPL 64176 involved Cav1.2 activity was confirmed by their inhibition by the Ca²⁺ channel blocker nifedipine. The angiotensin II-induced increased ten-

sion responses to KCl and FPL 64176 were attenuated by NADPH oxidase inhibitor gp91ds-tat, as shown in figure 8. After endothelial damage, angiotensin II no longer induced an upregulation of Cav1.2 activity, as shown by the lack of increased tension responses to KCl and FPL 64176 (fig. 9).

Discussion

The present communication describes portions of the signaling pathway involved in the increase in Cav1.2 protein in cultured mesenteric artery induced by angiotensin II. Although angiotensin II has also been reported to increase expression of the same channel in HL-1 cardiomyocytes [22], the effect reported here is significantly different. Endothelial involvement is manifest in this vascular effect but not in the cardiac effect. While both effects involve NAD(P)H oxidase, the cardiac effect involves the cAMP signaling pathway and is transcriptional [20], whereas the vascular effect examined here is posttranscriptional [5]. The differences in transcriptional regulation should not be surprising, given that the cardiac and vascular forms of the channel are regulated by two separate promoters [38–40].

Even though the endothelium is clearly involved in this angiotensin II effect, we can be reasonably certain that the increased Cav1.2 expression is not due to increas-

Fig. 7. Mesenteric arteries incubated with H_2O_2 (200 μ M, **b**) for 24 h showed an increased tension response to KCl (80 mM, **b**) and Ca^{2+} channel activator FPL 64176 (0.1–3 μ M, **e**) compared with its control, respectively (**a**, **d**). The effects of KCl and FPL 64176 were further confirmed by the calcium channel blocker nifedipine (Nif, 1 μ M). Similar results were seen in 4 and 6 different experiments for KCl and FPL 64176, respectively. Summary data for KCl

and FPL 64176 are shown in **c** and **f**, respectively. * $p < 0.05$ compared with control. ** $p < 0.001$ compared with tension induced by FPL 64176 (3 μ M) in control and H_2O_2 groups. Because nifedipine almost completely inhibited FPL 64176- (3 μ M) induced tension, note that the value is very close to zero, and it is difficult to see the bar in the figure.

es of the protein in the endothelium. $Ca_v1.2$ is not generally believed to be expressed in endothelium [41], despite occasional reports to the contrary [42]. Furthermore, expression there would not lead to increased vasoconstriction by a Ca^{2+} channel activator such as that observed in our previous studies [5] and in this study. Finally, after endothelial damage, administration of H_2O_2 still upregulated $Ca_v1.2$ expression, excluding the possibility that the upregulation of $Ca_v1.2$ expression is endothelial.

The cultured artery preparation enabled us to determine that the signaling mediator released by endothelium in response to angiotensin II stimulation and causing increases in $Ca_v1.2$ expression is H_2O_2 , one of the mem-

brane-permeable ROS [21, 32, 35, 36]. ROS produced by NAD(P)H oxidase in response to angiotensin II stimulation have been described in the endothelium by many authors [8, 19, 20, 43, 44]. H_2O_2 has been reported to be an endothelial-dependent constricting factor [45] and is known to constrict many different vascular beds at similar concentrations used here [46–48], although the contraction is often transient and followed by relaxation [47, 49]. Our H_2O_2 determination (fig. 5) showed that after endothelial damage angiotensin II had no effect on H_2O_2 production. We also observed that catalase partially blocked angiotensin II-induced tension increases in mesenteric arteries with intact endothelium, but had no ac-

Fig. 8. Mesenteric arteries with intact endothelium incubated with angiotensin II displayed an increased tension response to KCl (80 mM, upper panels) and Ca²⁺ channel activator FPL 64176 (FPL 3 μM, lower panels), and these effects of angiotensin II were attenuated by incubation together with NAD(P)H oxidase inhibitor gp91ds-tat (10 μM) and not by scrambled-tat (10 μM). KCl and FPL 64176 stimulated Ca_v1.2 activity, as confirmed by inhibition by the Ca²⁺ channel blocker nifedipine (Nif, 1 μM). Summary data from four different experiments for KCl and FPL 64176 are shown on the right. * p < 0.01 vs. control. + p < 0.05 vs. angiotensin II.

tion in arteries with damaged endothelium (not shown). Therefore, the increase in H₂O₂ production by angiotensin II is primarily derived from endothelium. Inhibition of angiotensin II-induced vasoconstriction by catalase was also reported by another group [50]. Our results which show that angiotensin II causes an endothelium-dependent increase in ROS production is also supported by other reports [12, 51]. For example, after endothelium denudation, the increased ROS generation induced by angiotensin II in rat aorta was absent [12], and ROS produced by endothelial NAD(P)H oxidase is a major source of oxygen radical generation in the arterial wall [51].

Although our standard treatment times were 24 h in culture, generation of H₂O₂ induced by angiotensin II may start much more rapidly, as shown by our H₂O₂ measurement results (fig. 5). We have observed rapid contraction of artery rings in response to angiotensin II, another effect partially inhibited by catalase (not shown), but also

observed by others [50]. On the other hand, the effects of H₂O₂ may last longer, since it was reported that only a 5-min exposure of ventricular myocytes to 30 μM H₂O₂ increased Ca_v1.2-mediated current which persisted for at least 9 h [52]. We also observed that the concentration of H₂O₂ in culture medium was not stable and degraded very quickly, for example, 250 μM H₂O₂ was degraded to approximately 5 μM after 30 min as measured by H₂O₂ test strips from Thermo Electron Corporation (not shown). Thus, even a short exposure to H₂O₂ may initiate much later and longer lasting events like the upregulation of Ca_v1.2 protein expression. Considerably more time is likely required for effects on Ca_v1.2 protein levels to be manifest.

A typical mesenteric artery preparation in our experiment has a wet weight of approximately 20 mg (roughly equal to 20 μl), from which approximately 0.5 mg protein was extracted. Each artery was cultured in a volume of

Fig. 9. Mesenteric arteries with damaged endothelium incubated with angiotensin II displayed a similar tension response to KCl (80 mM, upper panels) and Ca^{2+} channel activator FPL 64176 (FPL 3 μM , lower panels). KCl and FPL 64176 stimulated $\text{Ca}_v1.2$ activity, as confirmed by inhibition by the Ca^{2+} channel blocker nifedipine (Nif, 1 μM). Summary data from three different experiments are shown on the right. No significant differences in response to KCl and FPL 64176 were found between control and angiotensin II groups.

0.5 ml. The observed increase in H_2O_2 in media of approximately 4 nmol/mg protein would therefore be equivalent to an increase of 2 nmol/0.5 ml or 4 μM , and would translate to a concentration increase of 100 μM if restricted to the approximately 20- μl volume of the arterial wall. Since our sampling may have missed the peak H_2O_2 level as well as some H_2O_2 bound in the artery, H_2O_2 could have risen to even higher levels to affect $\text{Ca}_v1.2$ expression. Therefore, the concentrations of H_2O_2 used in this study (5–250 μM) may coincide with the angiotensin II (0.5 μM)-produced H_2O_2 level in arteries. Although most of the time the concentration of angiotensin II (0.5 μM) we used was higher than that in the in vivo condition, in figure 1 we found that even in the nanomolar range (0.05 μM) angiotensin II still upregulated $\text{Ca}_v1.2$ expression. In order to test the mechanism more easily, we chose the middle concentration (0.5 μM) in following experiments. On the other hand, the metabolism and working process

of angiotensin II in vitro and in vivo may be different. In in vitro experiments in our system, angiotensin II was administered only once for 24 h and may have been degraded during 24-h culture, while in vivo, although the concentration of the angiotensin II is much lower, it is synthesized/delivered and works continuously on $\text{Ca}_v1.2$ expression for a much longer time. Consequently, the final effect may be similar to the in vitro results shown here.

Although NAD(P)H oxidase inhibitors DPI and apocynin exert some nonspecific effects [53–55], it is still very likely that the inhibition of H_2O_2 generation in response to angiotensin II by these inhibitors is involved in the reduced angiotensin II-induced $\text{Ca}_v1.2$ upregulation. First, apocynin has been reported to inhibit O_2^- generation in vascular endothelial cells [8] and vascular O_2^- generation from endothelial cells in the arterial wall [12]. Second, DPI and apocynin, two chemically unrelated NAD(P)H

oxidase inhibitors, both blocked angiotensin II-induced $\text{Ca}_v1.2$ upregulation, and apocynin also inhibited angiotensin II-induced H_2O_2 generation (fig. 5). Third, H_2O_2 , which can be generated from superoxide produced by endothelial NAD(P)H oxidase, upregulated $\text{Ca}_v1.2$ expression in both endothelium-intact arteries (fig. 3) and arteries with damaged endothelium (fig. 6). Finally, the effects of DPI and apocynin were further confirmed by the inhibitory actions of a more specific NAD(P)H oxidase inhibitor, gp91ds-tat [16, 17, 28, 29], on both $\text{Ca}_v1.2$ expression and H_2O_2 production.

These results may relate to changes in blood pressure in vivo. Interruption of the NAD(P)H oxidase signaling pathway has been shown to reduce blood pressure [17, 33, 56]. Selective knock out [56] or inhibition of gp91^{phox} [17], a phagocytic NAD(P)H oxidase subunit which is only expressed in endothelium but not in vascular smooth muscle cells [51], decreased both basal blood pressure [56] and angiotensin II-induced blood pressure elevation [17]. The NAD(P)H oxidase inhibitor apocynin has also been shown to reduce blood pressure in angiotensin II-infused animals [33]. In principle, the acute vasoconstrictive effects of angiotensin II could contribute to its elevation of blood pressure. However, angiotensin II effects on isolated vessels tend to desensitize [57, 58], whereas its effects on blood pressure in vivo tend to be maintained as long as the exposure continues [4]. An increase in $\text{Ca}_v1.2$ protein may help compensate for any desensitization of the tissue toward the acute pressor action of angiotensin II. An increase in L-type Ca channel activity has been associated with angiotensin II infusion in rats [4]. Therefore, an increase in L-type Ca^{2+} channel expression (and activity) might contribute to blood pressure increases associ-

ated with elevated angiotensin II levels under pathophysiological conditions.

Although the involvement of isoform specificity of NADPH oxidase was not studied directly in this study, we assume that it is very likely that the phagocytic isoform of gp91^{phox} (also known as nox2) was involved in the upregulation of $\text{Ca}_v1.2$ expression. First, our studies showed that this action of angiotensin II required an intact endothelium, and gp91^{phox} is only expressed in endothelium [51]. Second, gp91ds-tat is an NAD(P)H oxidase inhibitor which mainly inhibits the gp91^{phox} isoform.

H_2O_2 is also known to stimulate the nonphagocytic NAD(P)H oxidase in vascular smooth muscle cells [37] and to stimulate multiple signaling pathways in vascular smooth muscle [59]. Our results clearly indicate that inhibitors of NAD(P)H oxidase act on the endothelial enzyme complex to reduce H_2O_2 generation (fig. 5), but they also block the downstream effect of H_2O_2 in the vascular smooth muscle (fig. 6).

We endorse the notion that H_2O_2 should be added to the list of endothelially derived substances which affect vascular smooth muscle as a paracrine mediator [60] under pathophysiological conditions at least. Our results suggest that angiotensin II-stimulated H_2O_2 generation by endothelium contributes to the upregulation of $\text{Ca}_v1.2$ channels in vascular smooth muscle in cultured arteries.

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Erratum

In *J Vasc Res* 2011;48:67–78, a p value for the black column (4) in figure 5b is missing. The corrected figure and legend should be as follows:

Fig. 5. H₂O₂ production by mesenteric arteries under different conditions. **a** Endothelial (E) damage with 0.3% CHAPS prevented the angiotensin II- (0.5 μM) stimulated increase in H₂O₂ production. Both apocynin (100 μM) and catalase (2,000 U/ml) inhibited angiotensin II-induced elevation of H₂O₂ production. **b** Angiotensin II- (0.5 μM) stimulated increase of H₂O₂ produc-

tion was inhibited by NAD(P)H oxidase inhibitor gp91ds-tat (10 μM), but not by scrambled-tat (10 μM). The number of different experiments performed is indicated in each column of the bar graph. Apocynin alone had no significant effect on H₂O₂ production. * p < 0.001, # p < 0.05 compared with control. + p < 0.001 compared with angiotensin II.