ERK activation contributes to regulation of spontaneous contractile tone via superoxide anion in isolated rat aorta of angiotensin II-induced hypertension

Lili Ding, Alexander Chapman, Ryan Boyd, and Hui Di Wang

Department of Community Health Sciences, Faculty of Applied Health Sciences, Brock University, St. Catharines, Ontario, Canada

Submitted 11 April 2006; accepted in final form 2 February 2007

Ding L, Chapman A, Boyd R, Wang HD. ERK activation contributes to regulation of spontaneous contractile tone via superoxide anion in isolated rat aorta of angiotensin II-induced hypertension. Am J Physiol Heart Circ Physiol 292: H2997–H3005, 2007. First published February 16, 2007; doi:10.1152/ajpheart.00388.2006.—Arteries from hypertensive animals and humans have increased spontaneous tone. Increased superoxide anion (superoxide) contributes to elevated blood pressure (BP) and spontaneous tone in hypertension. The association between the extracellular signaling-regulated kinase 1/2 (ERK1/2)-mitogen-activated protein kinase (MAPK) signaling pathway and generation of superoxide and spontaneous tone in isolated aorta was studied in angiotensin II (ANG II) (ANG II)-infused hypertensive (HT) rats. Systolic BP, phosphorylation of ERK, aortic superoxide formation, and aortic spontaneous tone were compared in sham normotensive and HT rats. Infusion of ANG II (0.5 mg·kg⁻¹·day⁻¹ for 6 days) significantly elevated the systolic BP (P < 0.01). The phosphorylation of ERK1/2 vs. total ERK1/2 in thoracic aorta was enhanced, and superoxide was increased in the HT vs. the sham group (P < 0.01). Spontaneous tone developed in the HT group, but not in the normotensive group. MAPK/ERK1/2 (MEK1/2)-ERK1/2 signaling pathway inhibitors, PD-98059 (10 μmol/l), and U-0126 (10 μmol/l), significantly reduced the phosphorylation of ERK1/2, superoxide generation (P < 0.01), and spontaneous tone (P < 0.01) in HT. These findings suggest that ANG II infusion induces the production of superoxide and spontaneous tone and that both are dependent on ERK-MAPK activation. In endothelium-denuded aorta, however, MEK1/2 inhibitors did not inhibit the spontaneous tone, even though they significantly reduced superoxide generation similar to endothelium-intact aorta. These data suggest that inhibition of ERK1/2 signaling pathway, via PD-98059 or U-0126, may regulate spontaneous tone in an endothelium-dependent manner. In conclusion, these findings support the importance of the ERK1/2 signaling pathway in modulating vascular oxidative stress and subsequently mediating spontaneous tone in HT.

ANGIOTENSIN II (ANG II) is a multifunctional peptide that regulates a series of cardiovascular functions, including vascular tone and blood pressure (BP). Superoxide anion in the vasculature appears to play a significant role in ANG II-induced hypertension. Infusion of ANG II increases nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and superoxide anion production in rat aorta (4, 30), and treatment with superoxide dismutase (SOD) or overexpression of human SOD decreases BP in ANG II-infused rats (18) and mice (45). We have reported that mice that lack one of the subunits of NAD(P)H oxidase develop hypertension to a much lesser extent compared with the wild-type animals infused with ANG II (43). These results indicate that increased production of superoxide anion contributes to elevated BP in the ANG II-induced hypertensive model.

Altered vascular reactivity is often associated with elevation of systolic BP. Typically, arteries from hypertensive animals and humans exhibit increased agonist-induced vascular contraction, impaired endothelium-dependent relaxation, and increased spontaneous tone. Spontaneous tone was first defined by Hwa and Bevan (12) as the increase in tension in the vessel in response to stretch and in the absence of any agonists. This abnormal spontaneous tone has been reported in many animal hypertensive models, including ANG II-infused hypertensive rats, deoxycorticosterone acetate (DOCA)-salt hypertensive rats, and spontaneously hypertensive rats (8, 24, 44). Our laboratory has reported previously that spontaneous tone in the aorta of ANG II-infused DOCA-salt hypertension was mediated by L-type calcium channels (8, 44). Furthermore, enhanced production of superoxide anion in hypertensive rats promotes smooth muscle calcium influx and spontaneous tone by directly acting on vascular smooth muscle or indirectly by inactivating endothelium-derived nitric oxide (44).

Recently, several cellular signaling molecules, including extracellular signaling-regulated kinase 1/2 (ERK1/2)-mitogen-activated protein kinase (MAPK), have been shown to play important roles in the regulation of smooth muscle contraction and relaxation. ERK is one of three major members of the MAPK family and is an important step in signal transduction of several growth factors, mitogens, neurotransmitters, and hormones (13). The ERK1/2-MAPK signaling pathway is activated by ANG II and is involved in oxidative stress and vasoconstriction (17, 28, 36, 48, 49).

The present study investigated the possible association of ERK1/2-MAPK signaling pathway in spontaneous tone, a specific form of altered reactivity of blood vessels in hypertension. We hypothesized that the spontaneous tone in aorta from rats infused with ANG II for 6 days is mediated by oxidative stress via the ERK1/2-MAPK pathway.

MATERIALS AND METHODS

Animal Model

All animal protocols were approved and conducted according to the recommendations of the Research Sub-Committee of Brock University on Animal Care and Use and the Canadian Council on Animal Care. Male Sprague-Dawley rats (12–14 wk of age, 350–450 g body wt) were anesthetized by continuous inhalation of 2–3% isoflurane. An incision was made in the midscapular region under sterile conditions. Osmotic minipumps (Alzet, Alza) containing ANG II were then...
implanted with a delivery rate of 0.5 mg·kg⁻¹·day⁻¹. Sham-operated animals underwent an identical surgical procedure, except that the osmotic minipumps contained 0.15 mol/l NaCl and 0.01% acetic acid. The procedures have previously been described (44). Systolic BP was determined before and at the end of the 6 days of infusion by tail-cuff plethysmography. Ten repeated values were averaged at each determination. The BP measured by the tail-cuff method is well correlated with intra-arterial measurements in normal and hypertensive animals (15).

**Western Blotting of ERK Phosphorylation**

The thoracic aorta from ANG II-infused hypertensive and sham normotensive rats were cleaned of adherent fat in cold HEPES buffer containing (mmol/l) 137 NaCl, 5.4 KCl, 1.0 CaCl₂, 0.5 MgCl₂, and 5.0 HEPES. After equilibration for 10 min, aortic rings were given either PD-98059 (10 μmol/l), U-0126 (10 μmol/l), SOD (250 U/ml), or apocynin (10 μmol/l) for 45 min at 37°C and pH 7.4, and then quickly frozen in liquid nitrogen to be crushed and solubilized in lysis buffer (20 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerolphosphate, 1 mmol/l Na₃VO₄, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mmol/l PMSF) on ice. Homogenates were centrifuged (11,000 g for 15 min, 4°C), and supernatant protein levels were assayed (Bradford method). Twenty micrograms of each sample were separated on a 10% SDS-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes for standard Western blot analyses using monoclonal antibodies specific for total ERK, as well as phosphorylated ERK (1:1,000, Cell Signaling Technology, Beverly, MA).

**Superoxide Anion Generation in Aorta**

The details for measuring superoxide anion generation have been described previously (44). Briefly, the thoracic aorta was cleaned and cut into rings 5 mm in length in cold HEPES buffer. After equilibration for 10 min at 37°C and pH 7.4, aortic rings were given various treatments for 30–45 min. The aortic rings were then transferred to test tubes containing 250 μl HEPES buffer and 5 μmol/l lucigenin for 10 min at 37°C. The luminometer was set to report arbitrary units of light emitted and integrated over a 30-s interval. Ten repeated measures were collected over 5 min and averaged. Tiron (10 mmol/l), a nonenzymatic scavenger of superoxide anion, was then added to quench the superoxide anion-dependent chemiluminescence. After 5 min, readings from the last 90 s of an additional 5-min period were averaged. The difference between the initial set of readings and the readings following tiron treatment was taken to calculate superoxide anion production. The vessels were blotted dry and weighed. The results

---

**Fig. 1. Extracellular signaling-regulated kinase (ERK) 1/2 activation by angiotensin (ANG) II-infusion in endothelium-intact aorta.**

A and B: effects of 6 days of ANG II infusion on activation of ERK1/2. C and D: effects of antioxidants on activation of ERK1/2. The aortic rings from sham-normotensive (sham) or ANG II-infused hypertensive rats (ANG II-infusion) were incubated with either MAPK/ERK (MEK) 1/2 inhibitors, PD-98059 (10 μmol/l) and U-0126 (10 μmol/l) (A), or NADPH oxidase inhibitor apocynin (10 μmol/l) or superoxide dismutase (SOD) (300 U/ml) (B) for 45 min. The phosphorylated and total ERK1/2 in the aorta were evaluated by Western blot analysis. The ratio of phosphorylated vs. total ERK1/2 was used as an indicator for ERK1/2 activity and calculated as the percentage of the sham group. Values are expressed as means ± SE. Increased ERK activation by ANG II-infusion was blunted by MEK1/2 inhibitors but not by apocynin or SOD. *P < 0.01 compared with the sham group. †P < 0.05 compared with PD-98059. Each group contains aortic rings from 3–4 different rats.
were expressed as units of light generated per minute per milligram of tissue.

**Measurement of Spontaneous Tone**

The thoracic aorta was cleaned of adherent fat and cut into rings 5 mm long in ice-cold physiological buffer containing (mmol/l) 118.3 NaCl, 4.7 KCl, 0.6 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, 25 NaHCO3, 0.026 Na2-EDTA, and 5.5 glucose. The rings were then mounted on the triangular hooks and suspended in organ chambers containing 10 ml buffer maintained at 37°C and pH 7.4 by gassing with 95% O2–5% CO2. Rings were stretched in a stepwise fashion over 1 h to optimal tension (5 g). Sodium nitroprusside (SNP, 100 nmol/l) was introduced for the last 30 min to allow adjustment under passive conditions. After washout of nitroprusside, aortic rings from ANG II-infused hypertensive rats, but not from sham-operated normotensive rats, contracted spontaneously (8, 44). Spontaneous tone was expressed as a percentage of the contraction induced by 120 mmol/l KCl.

The role of the ERK signaling pathway in mediating the spontaneous tone was determined by incubating rings with specific inhibitors of MEK1/2 (an upstream mediator of ERK1/2), PD-98059 (10 μmol/l), or U-0126 (10 μmol/l). Their inactive analogs SB-202474 (10 μmol/l) and U-0124 (10 μmol/l) were used as negative controls, respectively. Each treatment was given in the last 30 min during the stretching of the rings to its optimal tension. At the end of the experiments, the rings were contracted by 120 mmol/l KCl. The spontaneous tone in rings from hypertensive rats was expressed as a percentage of the contraction evoked by 120 mmol/l KCl.

**Endothelium was removed by gently rubbing the lumen of the aorta. Lack of relaxation to acetylcholine (10−6 mol/l) was used to demonstrate that the endothelium had been effectively denuded.**

**Drugs**

A stock solution of ANG II was made in 0.9% NaCl and 0.01% acetic acid. Stock solutions of PD-98059, SB-202474, U-0126, and U-0124 were made in DMSO. All stock solutions were stored in −20°C. Apocynin was dissolved in DMSO before use. Other drugs were made freshly in deionized water just before each experiment. ANG II, acetylcholine chloride, lucigenin, SNP, and tiron were purchased from Sigma Chemical (Oakville, ON). PD-98059, SB-202474, U-0126, and U-0124 were purchased from CalBiochem (San Diego, CA).

**Statistical Analysis**

Data are expressed as means ± SE. Statistical comparisons were made by ANOVA. Significance was accepted when *P* was <0.05.

**RESULTS**

**Effect of ANG II on Systolic BP**

ANG II infusion for 6 days increased systolic BP from 127 ± 25 to 180 ± 15 mmHg (*n* = 8, *P* < 0.01). In contrast, infusion of the vehicle did not affect BP in sham-operated rats (from 125 ± 12 to 128 ± 4 mmHg, *n* = 8, *P* > 0.05). At the
end of 6 days, BP in ANG II-infused rats was significantly higher than that in sham-operated rats (P < 0.01).

**ERK1/2-MAPK Activation by ANG II**

The activation of ERK1/2 was estimated by the extent of phosphorylation of ERK1/2 vs. total ERK1/2 by Western blot analysis. Figure 1, A and B, shows that the extent of phosphorylation of ERK1/2 was significantly increased in the endothelium-intact aorta of ANG II-infused hypertensive rats compared with the aorta of sham rats (n = 4, P < 0.01), while the total amount of ERK1/2 was the same in the two groups (n = 4, P > 0.05). The ratio of phosphorylated vs. total ERK1/2 was increased significantly in aorta of ANG II-infused hypertensive rats compared with sham-operated rats (n = 4, P < 0.01).

The effect of two structurally different MEK1/2 inhibitors, PD-98059 and U-0126, on ANG II-induced activation of ERK1/2 is also shown in Fig. 1, A and B. The phosphorylated ERK1/2 in aortic rings from ANG II-infused rats was significantly increased compared with rings from sham control rats, and this increase was blunted by both MEK1/2 inhibitors. The inhibition with U-0126 was much greater than that with PD-98059 (n = 4, P < 0.01). In contrast, the inactive analogs, SB-202474 and U-0124, had no effects on the phosphorylation of ERK1/2 in rings from ANG II-infused rats (n = 3, P > 0.05, data not shown).

The effect of NADPH oxidase and vascular superoxide anion on phosphorylation of ERK1/2 is shown in Fig. 1, C and D. Acute treatment of aorta with apocynin and SOD did not inhibit the phosphorylation of ERK1/2 induced by the ANG II infusion (n = 3, P > 0.05).

Figure 2 shows the ERK1/2 and phospho-ERK1/2 responses in endothelium-denuded aorta in the presence and absence of superoxide scavenger Tempol and NADPH oxidase inhibitor apocynin. We found that neither endothelium denudation nor superoxide scavenger (P > 0.05) and NADPH oxidase inhibitor have any effects on the level of ERK1/2 phosphorylation.

**ANG II-Induced Spontaneous Tone**

*Endothelium-intact aorta*. Aortic rings from ANG II-infused hypertensive rats (Fig. 3B), but not those from sham-operated normotensive rats (Fig. 3A), developed spontaneous tension. The spontaneous tone was characterized by two components: a tonic contraction and rhythmic oscillatory spikes that were superimposed on the tonic contraction. The frequency of rhythmic oscillatory spikes was 4.7 ± 0.3 times per minute. SNP at 1 μmol/l abolished spontaneous tone, and tone was reestablished following washout of SNP. In aortic rings from ANG II-infused hypertensive rats, both the tonic and oscillatory components developed 1.1 ± 0.1 g of tension each (n = 8), which represented 20.3 ± 2.6 and 19.7 ± 0.1% of the contraction evoked by 120 mmol/l KCl (5.4 ± 0.3 g), respectively. The sum of the two components was 2.2 ± 0.2 g, which represented 41.1 ± 3.7% of the contraction evoked by 120 mmol/l KCl.

The effect of the two MEK1/2 inhibitors on spontaneous tone was examined (Fig. 4). In aortic rings from ANG II-infused hypertensive rats, PD-98059 and U-0126, when tested independently, reduced the magnitude of the tonic contractions (Fig. 4B), the rhythmic oscillatory contractions (Fig. 4C), and the sum of two components (Fig. 4D) (n = 8, P < 0.01). The inhibitory effect of U-0126 was greater than that of PD-98059 (P < 0.01).

In contrast, the tonic contractions, the rhythmic oscillatory contractions, and the sum of two components were...
not significantly affected by their inactive analogs, SB-202474 and U-0124 (P > 0.05).

**Endothelium-denuded aorta.** The role of endothelium in the modulation of spontaneous tone was examined in endothelium-denuded rings. Endothelium denudation failed to develop significant spontaneous tone in aortic rings from sham-operated normotensive rats. In aortic rings from ANG II-infused hypertensive rats, however, endothelium denudation increased the tonic contractions and decreased the magnitude of rhythmic oscillatory contractions compared with endothelium-intact rings from ANG II-infused hypertensive rat (P < 0.01). However, the total of the two components of spontaneous tone was not affected by endothelium denudation.

The effect of MEK1/2 inhibitors on spontaneous tone in endothelium-denuded rings was examined (Fig. 5). In contrast to their inhibitory role in endothelium-intact rings, PD-98059 and U-0126 had no significant inhibitory effects on the tonic contractions (Fig. 5B) or the magnitude of rhythmic oscillatory contractions (Fig. 5C) in endothelium-denuded aortic rings (n = 6–7, P > 0.05). Consequently, the sum of two components (Fig. 5D) was also not affected by the treatments (P > 0.05).

**Vascular Superoxide Anion Production**

**Endothelium-intact aorta.** The effect of ANG II infusion and MEK inhibitors on aortic superoxide anion production is displayed in Fig. 6A. Lucigenin chemiluminescence was 4.0 ± 3.6 mU·mg⁻¹·min⁻¹ (n = 8) in aortic rings from sham-operated normotensive rats, and it was significantly increased in the rings from ANG II-infused hypertensive rats (110 ± 10 mU·mg⁻¹·min⁻¹, n = 9, P < 0.01).

Pretreatment with PD-98059 or U-0126 significantly decreased tiron-quenchable lucigenin chemiluminescence in the rings from ANG II-infused hypertensive rats (n = 6, P > 0.05). The inhibition of superoxide anion production by U-0126 was greater than that of PD-98059. The two inactive analogs, SB-202474 and U-0124, had no effect on superoxide anion production (P > 0.05).

**Endothelium-denuded aorta.** In endothelium-denuded aorta (Fig. 6B), the production of superoxide anion was similar to that in endothelium-intact rings. Superoxide anion production in endothelium-denuded rings from sham-operated rats was 3.8 ± 3.6 mU·mg⁻¹·min⁻¹ (n = 9, P > 0.05 compared with that in endothelium-intact rings). Superoxide anion production in endothelium-denuded rings from ANG II-infused rats was 120 ± 13 mU·mg⁻¹·min⁻¹ (n = 10, P > 0.05 compared with endothelium-intact rings).

Pretreatment with PD-98059 and U-0126 had no effect on tiron-quenchable lucigenin chemiluminescence in endothelium-denuded rings from sham-operated rats (n = 6, P > 0.05). Similar to endothelium-intact rings, tiron-quenchable

![Fig. 4. Effects of MEK1/2 inhibitors on spontaneous tone from endothelium-intact aortic rings of ANG II-infused rat. A: trace “Vehicle” represents the development of spontaneous tone from an endothelium-intact aortic ring treated by vehicle (0.1% DMSO). Trace “PD98059” shows a MEK1/2-ERK signaling pathway inhibitor, PD-98059 (10 μmol/l), which decreased the development of spontaneous tone. Trace “U0126” shows the inhibitory effect of another MEK1/2-ERK signaling pathway blocker, U-0126 (10 μmol/l), on the development of spontaneous tone. B, C, and D: responses of tonic, oscillatory, and sum of spontaneous tone, respectively. The values are expressed as a percentage of the contraction evoked by 120 mmol/l KCl. Values are means ± SE. *P < 0.01 compared with control and negative analog group. †P < 0.01 compared with PD-98059. Each group contains samples from 4–10 rats.](http://ajpheart.physiology.org/)**
chemiluminescence was significantly decreased by PD-98059 and U-0126 to 63 ± 17 and 59 ± 5.9 mU·mg⁻¹·min⁻¹ (n = 8–9, P < 0.01) in endothelium-denuded rings from ANG II-infused hypertensive rats. These two inactive analogs had no effect on superoxide anion production (P > 0.05).

**DISCUSSION**

The major finding in this study is that spontaneous tone in isolated aortic rings of ANG II-infused hypertensive rats is dependent on increased activity of the ERK signaling pathway via an increase of superoxide anion production. We found that the activity of ERK1/2-MAPK is increased in the aorta of 6-day ANG II-infused hypertensive rats, and that this increased activation of ERK1/2-MAPK-enhanced superoxide anion production. To the best of our knowledge, this is the first study that demonstrates the involvement of ERK1/2-MAPK in the development of spontaneous tone in the aorta of ANG II-induced hypertensive rats.

This study showed that there was development of spontaneous tension in aortic rings isolated from ANG II-infused hypertensive rats, but not in normotensive rats, agreeing with the previous finding in the same hypertensive model (44), as well as other models, such as the spontaneous hypertensive rats (38), aortic coarctation-induced hypertension (33), and DOCA-salt hypertensive rats (8). Spontaneous tone was observed after the aorta was stretched to the optimal resting tension for contraction, and the tone had two components: tonic contraction and rhythmic oscillatory contractions. The degree of the tone was directly proportional to the preload and intraluminal pressure (8). The spontaneous active tone in the aorta of hypertensive rats has previously been shown to be a Ca²⁺-dependent phenomenon (44). Moreover, it was found that the influx of Ca²⁺ was mediated via L-type channels, as nifedipine prevented the development of the tone (25, 33, 44). The impaired function of ATP-sensitive K⁺ (KATP) channels may contribute to the spontaneous tone (6).

Considerable evidence indicates that oxidative stress contributes to enhanced vascular contraction. Our laboratory reported previously that spontaneous tone in the aorta of hypertension is mediated by vascular NADPH oxidase-derived superoxide anion (44). It has also been shown that superoxide anion generation is increased in aorta of ANG II-infused rats by activation of NADPH oxidase (3, 4, 30, 46). Treatment of aorta with superoxide anion scavengers, Tempol and SOD, or a specific NAD(P)H oxidase inhibitor, apocynin, decreases superoxide anion levels, as well as inhibiting spontaneous tone. These findings are consistent with the hypothesis that increased superoxide anion generation inactivates nitric oxide (16, 47). Moreover, superoxide anion can generate H₂O₂, evoking a loss of endothelium (42), thereby contributing to exaggerated vasoconstrictor responses (37, 47).

The development of tonic spontaneous tone appears to be independent of the endothelium as tonic tone developed in both endothelium-intact and denuded aorta. On the contrary, endothelium denudation increased the tonic contraction, a result that may be due to the removal of endothelial-derived nitric oxide, which has been demonstrated to play an important role in limiting spontaneous tone (8, 44). In contrast, the magnitude of oscillatory contractions likely depends on endothelium-derived nitric oxide, because the oscillatory tone was significantly decreased by PD-98059 and U-0126 (404/11006 17 and 59/11006 5.9 mU/18528 1 mg/11002 1 min/18528 1 n/11005 8 –9, P/11021 0.01) in endothelium-denuded rings from ANG II-infused hypertensive rats. These two inactive analogs had no effect on superoxide anion production (P > 0.05).
inhibited by endothelium denudation and N\textsuperscript{G}-nitro-L-arginine methyl ester treatment (6, 44). In disagreement with our finding, Jin et al. (14) recently reported that the development of spontaneous tone was only observed in endothelium-denuded but not endothelium-intact aortic rings from ANG II-infused rats. The discrepancy is likely due to the different degree of endothelial dysfunction, which is severe in the present study. The results of this study demonstrated that activation of the ERK1/2-MAPK mediates spontaneous tone via increasing superoxide anion production in ANG II-infused hypertensive rats. Recently, it has been reported that the activation of NADPH oxidase in ANG II-infused hypertensive rats is dependent on ERK1/2-MAPK activation (17). The ERK1/2-MAPK signaling pathway is also involved in activation of the NADPH oxidase by ANG II in human neutrophils (10). Stimulation of NADPH oxidase by ANG II in human neutrophils is mediated by ERK, p38 MAPK, and cytosolic phospholipase A\textsubscript{2} (10). In agreement with those results, we found that phospho-

ERK1/2 in the aorta was significantly increased after 6 days of ANG II infusion in vivo. Importantly, we found that acute treatment with either MEK1/2 inhibitors, PD-98059 or U-0126, significantly decreased the generation of superoxide anion in the aorta of ANG II-infused hypertensive rats in vitro, but not their inactive analogs. These results support the notion that ANG II infusion induced oxidative stress and is dependent on ERK1/2-MAPK activation.

The ERK1/2 signaling pathway not only may play a role in the development of spontaneous tone but also may mediate agonist-induced contraction. It has been demonstrated that 5-hydroxytryptamine can potentiate endothelin-1 and noradrenalin-induced rat tail artery contraction, and this potentiation is mediated by activation of ERK signaling pathway. However, it has also been demonstrated that ERK1/2 had no effect on KCl, thromboxane A\textsubscript{2} receptor (39), and ANG II-induced contraction (L. Ding and H. D. Wang, unpublished observation).

**Fig. 6.** Effects of MEK1/2 inhibitors on aortic superoxide anion. The endothelium-intact (A) or denuded (B) aortic rings from sham-normotensive (sham) or ANG II-infused hypertensive (ANG II-infusion) rats were incubated with either MEK1/2 inhibitors, PD-98059 (10 \mu mol/l) and U-0126 (10 \mu mol/l), or their inactive analogs, SB-202474 (10 \mu mol/l) and U-0124 (10 \mu mol/l), for 45 min. The aortic superoxide anion levels were measured by 5 \mu mol/l lucigenin. Values are expressed as means \pm SE. Lucigenin chemiluminescence was markedly increased in both endothelium-intact and denuded rings from ANG II-infused hypertensive rats, which was significantly inhibited by MEK1/2 inhibitors. *P < 0.01 compared with sham group. †P < 0.01 compared with ANG II group and their own inactive analogs. ‡P < 0.01 compared with PD-98059. Each group contains aortic rings from 7–10 different rats.
The activation of ERK1/2-MAPK seems to be indirectly related to the development of spontaneous tone in ANG II-infused hypertensive rats. It has been reported that ERK1/2-MAPK can mediate vascular contraction by modulation of intracellular Ca\(^{2+}\) concentration (40) or of contractile regulating proteins such as caldesmon and calponin, resulting in changed actin myosin interaction (19, 20, 23). Activation of ERK1/2-MAPK is involved in stretch-mediated bovine coronary artery contraction (28) and peroxide-induced pulmonary arterial contraction (27). It has also been reported that PD-98059 prevented ANG II-induced contraction in resistance arteries in vitro (5), and chronic treatment with PD-98059 prevented the development of hypertension in ANG II-infused rats (17). In our study, both MEK1/2 inhibitors, PD-98059 and U-0126, inhibited tonic spontaneous tone, as well as rhythmic oscillatory contraction in aorta, suggesting a role for the ERK1/2-MAPK signaling pathway in mediating the aortic tone. Furthermore, we found that the ERK1/2-MAPK signaling pathway mediates the effect indirectly through decreasing vascular superoxide anion generation, which could subsequently improve the bioavailability of nitric oxide. This is supported by the evidence that, in endothelium-denuded aorta, MEK1/2 inhibitors did not inhibit either tonic or oscillatory spontaneous tone, although their effect on superoxide anion generation remained similar to that in endothelium-intact aorta.

While activation of ERK1/2-MAPK could increase NADPH oxidase activity (17, 28), it is also reported that reactive oxygen species, including superoxide, could regulate the ERK1/2-MAPK signaling pathway (21). Therefore, the possibility that superoxide anion may mediate spontaneous tone by increasing the activity of the ERK1/2-MAPK signaling pathway merits consideration. However, our data show that SOD and apocynin failed to decrease ERK phosphorylation. This suggests that the increased activity of ERK1/2-MAPK was the result of 6 days of ANG II infusion and was not dependent on the increase of oxidative stress in the hypertensive rats.

All types of vascular cells can generate superoxide anion. The major superoxide anion-generating enzyme in the vasculature, NADPH oxidase, is expressed in endothelial cells, vascular smooth muscle cells, and cells in the adventitial layer (1, 29, 41, 44). NADPH oxidase is also expressed by infiltrating neutrophils and monocytes (2, 10).

In addition to oxidative stress, other mechanisms have been reported to be involved in spontaneous tone development, including phosphatidylinositol 3-kinase (25, 26), RhoA/Rho kinase (14), cyclooxygenase (COX) product (8), and K\(_{ATP}\) channel (6). Several studies have shown that phosphatidylinositol 3-kinase is involved in the upstream regulation of MAPK (9, 22). In contrast, it is likely that RhoA/Rho kinase and ERK1/2 are two parallel signaling pathways. While the effect of RhoA/Rho kinase in smooth muscle cell contraction is via myosin light chain phosphorylation, the ERK1/2 modulates intracellular Ca\(^{2+}\) concentration (40) or contractile regulating proteins (19, 20). Although it has been reported that induction of COX-2 expression is dependent on ERK activation in keratinocytes (11), it is not known how the ERK1/2 pathway relates to COX product and K\(_{ATP}\) channel in spontaneous tone development.

The importance of aorta in vascular development and pulse pressure has been reviewed recently (34). In particular, spontaneous tone in aorta and other large arteries may decrease compliance and disturb the blood flow to smaller vessels, thereby contributing to the pathophysiology of hypertension. Spontaneous tone was not observed until 5 or 6 days of ANG II infusion. In addition, spontaneous tone exists in several hypertensive animal models (7, 26, 35, 44), suggesting that it is more likely a hypertension-related response than a receptor-mediated response. The results obtained from this study, as well as other studies, suggest that substances that prevent spontaneous tone may be possible therapeutic treatments of hypertension or hypertension-related diseases.

Admittedly, extrapolations of findings in aorta to the small resistance vessels should be avoided. Indeed, spontaneous tone has been observed in resistant arteries of DOCA rats (25). Investigation in small vessels in ANG II-induced hypertension would be important future avenues of investigation.

In summary, we found that the activity of ERK1/2-MAPK signaling pathway was enhanced in ANG II-infused rats. MEK1/2 inhibitors, PD-98059 and U-0126, but not their inactive analogs, SB-202474 and U-0124, significantly reduced spontaneous tone. This reduction in spontaneous tone was also accompanied by a reduction in superoxide anion levels in aortas from ANG II-infused rats. Inhibition of the ERK1/2-MAPK signaling pathway failed to block spontaneous tone in endothelium-denuded aorta from the same ANG II-infused rats, even though it decreased vascular superoxide anion production. These findings support the importance of the ERK1/2-MAPK signaling pathway in modulating vascular oxidative stress and subsequently mediating spontaneous tone in ANG II-infused hypertension.

ACKNOWLEDGMENTS

We thank Dayle Belme for animal care support. We thank the Department of Biology at Brock University, and Dr. Vincenzo De Luca for permitting the use of some facilities and equipment.

GRANTS

This work was financially supported by the Faculty of Applied Health Sciences at Brock University and Canadian Institute for Health Research. H. D. Wang was supported by a New Investigator Award from Heart and Stroke Foundation of Canada.

REFERENCES

ERK ACTIVATION MEDIATES SPONTANEOUS TONE IN HYPERTENSION


