Role of superoxide and thromboxane receptors in acute angiotensin II-induced vasoconstriction of rabbit vessels

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Pfister SL, Nithipatikom K, Campbell WB. Role of superoxide and thromboxane receptors in acute angiotensin II-vasoconstriction of rabbit vessels. Am J Physiol Heart Circ Physiol 300: H2064–H2071, 2011. First published April 11, 2011; doi:10.1152/ajpheart.01135.2010.—This study explored the hypothesis that a portion of angiotensin II-induced constrictions is dependent on superoxide generation and release of a previously unidentified arachidonic acid metabolite that activates vascular smooth muscle thromboxane receptors. Treatment of rabbit aorta or mesentery artery with the thromboxane receptor antagonist SQ29548 (10 μM) reduced angiotensin II-induced contractions (maximal contraction in aorta; control vs. SQ29548: 134 ± 16 vs. 93 ± 10%). A subset of rabbits deficient in vascular thromboxane receptors also displayed decreased contractions to angiotensin II. The superoxide dismutase mimetic Tiron (30 mM) attenuated angiotensin II-induced constrictions only in rabbits with functional vascular thromboxane receptors (maximal contraction in aorta; control vs. Tiron: 105 ± 5 vs. 69 ± 11%). Removal of the endothelium or treatment with a nitric oxide synthase inhibitor, nitro-L-arginine (30 μM) did not alter angiotensin II-induced contractions. Tiron and SQ29548 decreased angiotensin II-constrictions in the denuded aortas by a similar percentage as that observed in intact vessels. The cyclooxygenase inhibitor indomethacin (10 μM) or thromboxane synthase inhibitor dazoxiben (10 μM) had no effect on angiotensin II-induced constrictions indicating that the vasoconstrictor was not thromboxane. Angiotensin II increased the formation of a 15-series isoprostane. Isoprostanes are free radical-derived products of arachidonic acid. The unidentified isoprostane increased when vessels were incubated with the superoxide-generating system xanthine/xanthine oxidase. Pretreatment of rabbit aorta with the isoprostane isolated from aortic incubations enhanced angiotensin II-induced contractions. Results suggest the factor activating thromboxane receptors and contributing to angiotensin II vasoconstriction involves the superoxide-mediated generation of a 15-series isoprostane.

Address for reprint requests and other correspondence: S. L. Pfister, Dept. of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226 (e-mail: spfister@mcw.edu).
mixture. Isometric tension was measured with force-displacement transducers (Grass Instruments) and AD Instruments ETH-400 amplifiers and recorded on a Macintosh computer using MacLab 8e software as previously described (23). Resting tension was adjusted to 2 g, and the vessels equilibrated for 1 h. The KCl concentration of the baths was increased to 40 mM until stable, reproducible contractions were produced. Responses to the TXA₂ mimetic U46619 (10⁻¹⁰–10⁻⁷ M) were obtained. Aortic rings that contracted to KCl but not to U46619 were identified as vTP⁻ (Fig. 1A). Aortic rings that contracted to both KCl and U46619 were vTP⁺. Maximal KCl contractions in vTP⁺ and vTP⁻ were similar (vTP⁺ vs. vTP⁻: 2.4 ± 0.1 vs. 2.7 ± 0.1 g). Mesenteric arterial segments (1.5-mm long) were threaded on two stainless steel wires (40-μm diameter) and mounted on a four-channel wire myograph (model 610M; Danish Myo Technology) as previously described (1). Arteries were equilibrated at 37°C for 30 min in physiological saline solution containing the following (in mM): 119 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 24 NaHCO₃, 1.2 KH₂PO₄, 0.026 EDTA, and 5.5 glucose, bubbled with 95% O₂-5% CO₂. The resting tension was set at 1 mN. Arteries were stimulated two times with KCl (60 mM) plus phenylephrine (10 μM) for 3–5 min at 10-min intervals before the initiation of experimental protocols. Cumulative concentration response curves to isangiogen II (10⁻¹¹–10⁻⁷ M) or 8-isopGF₂α (10⁻¹⁰–10⁻⁶ M) were obtained. Other vessels were pretreated with the TP receptor antagonist SQ29548 (10 μM), the TX synthase inhibitor dazoxiben (10 μM), the cyclooxygenase (COX) inhibitor indomethacin (10 μM), the nitric oxide (NO) synthase inhibitor N⁵-nitro-L-arginine (1-NNa; 30 μM), the SOD mimetic Tiron (30 μM), or vehicle before the administration of angiogen II. Pretreatment time was 10 min for all the inhibitors except Tiron, which was added 30 min before angiogen II. The pretreatment time was based on results from previous studies that showed that both the time period and inhibitor concentration blocked the compounds of interest (4, 40). The aortic endothelium was purposefully removed in some vessels by gently rubbing the intimal surface with a cotton-tipped swab. Relaxation responses to acetylcholine were described above. To determine the effect of angiogen II on arachidonic acid metabolism, strips of aorta were placed in HEPES buffer and incubated at 37°C for 15 min with 14C-arachidonic acid (0.05 μCi, 50 μM) and angiogen II (10 μM) as previously described (26). After incubation, the HEPES buffer was removed, acidified to pH 3.0 with glacial acetic acid, and extracted over ODS extraction columns as described above (26). The isoprostanes were eluted with 6 ml ethyl acetate, evaporated to dryness under N₂, and stored at −40°C until analysis by reverse-phase HPLC. The prostaglandin-like metabolites of arachidonic acid were separated using a reverse-phase HPLC system utilizing a Nucleosil-C18 column. Solvent A was water containing 0.025 M phosphoric acid, and solvent B was acetonitrile. The program consisted of a 40-min isocratic phase with 31% solvent B in solvent A, followed by a 20-min linear gradient to 100% solvent B and a 10-min isocratic phase with 100% solvent B. The flow rate was 1 ml/min. Elution times of radioactive peaks were compared with retention times of known prostaglandin standards.

Isoprostane analysis. Arteries were incubated in HEPES buffer containing vehicle, angiogen II (10⁻⁷ M), or xanthine (100 μM)/xanthine oxidase (0.03 U/ml) for 15 min at 37°C. After incubation the HEPES buffer was removed, the internal standard ([2H₄]8-iso-PGF₂α) was added to the buffer and the buffer was acidified to pH 3.0 with glacial acetic acid and extracted over ODS extraction columns as described above (26). The isoprostanes were eluted with 6 ml ethyl acetate and then were evaporated to dryness under N₂ and stored at −80°C until analysis. Samples were analyzed by using liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS, Agilent 1100 LC/MSD, SL model) using a modification of a method previously described by Nithipatikom et al. (20). The samples were separated on a reverse-phase C18 column (Kromasil, 250 × 2 mm) using water/acetonitrile with 0.1% acetic acid as a mobile phase at the flow rate of 0.3 ml/min. The gradient started at 35% acetonitrile, linearly increased to 100% acetonitrile in 35 min, and held for 10 min. The detection was made in the negative mode. Selective ion monitoring is used for quantitation. The mass-to-charge ratio (m/z) of 353 and 357 is used for 8-iso-PGF₂α and [2H₄]8-iso-PGF₂α, respectively. A standard curve was constructed over the range of 5 to 1,000 pg per injection. The concentration of 8-iso-PGF₂α in the samples was calculated by comparing its ratio of peak area to the standard curve. The results were normalized to the wet weight of the vessel. Studies were repeated three times. To further characterize products, MS/MS analysis was performed by electrospray ionization triple quadrupole mass spectrometer (Waters). The parent ion m/z 353 was fragmented by collision-induced dissociation using argon gas. Only the precursor ion is allowed to pass through the first quadrupole, and the ion is activated with argon in the second quadrupole. Product ion spectra were recorded for the m/z range of 50 to 380. Data were acquired in the profile mode. Results were processed using Masslynx software (Micromass).

Biological activity of 8.5-min isoprostane. Aortas from four to eight rabbits were incubated as before with angiogen II. Identical control (cell free) incubations without tissue were carried out in parallel. Following incubation and extraction, the samples were chromatographed on the LC as described above. Fractions eluting with the 8.5-min peak were collected, extracted with cyclohexane/ethyl ace-
tate, dried down under a stream of N2 and stored under nitrogen at −40°C until vascular reactivity studies were performed. The biological sample (or cell-free control) was suspended in 100 µl of ethanol (10 µl per 6-ml bath was the maximal concentration administered) and tested for vasoconstrictor activity under basal tone and before the administration of increasing concentrations of angiotensin II (10⁻¹¹–10⁻⁷ M) or phenylephrine (10⁻⁹–10⁻⁵ M). Results were compared with vessels treated with a submaximal concentration of 8-iso-PGF₂α (5 nM) added before the vasoconstrictor.

Materials. U46619, SQ29548, 8-iso-PGE₂, 8-iso-PGF₂α, [²H₄]-8-iso-PGF₂α, and TXB₂ were from Cayman Chemical (Ann Arbor, MI). ¹⁴C-arachidonic acid was obtained from New England Nuclear. Angiotensin II was from Peninsula Labs (San Carlos, CA). Indomethacin, phenylephrine, Tiron, and 1-NNA were from Sigma (St. Louis, MO); dazoxiben was from Pfizer.

Statistical analysis. The vascular reactivity data were expressed as the means ± SE. Statistical evaluation of the vascular reactivity data was performed by using a repeated-measures two-way ANOVA followed Bonferroni posttest when significant differences were present. A value of P < 0.05 was considered statistically significant.

RESULTS

Pretreatment of vTP+ aortas with the TP receptor antagonist SQ29548 resulted in a rightward shift of the concentration-response curve to angiotensin II (Fig. 2A). The maximal contraction to angiotensin II was reduced from 134 ± 16 to 93 ± 10% with SQ29548 (P < 0.05). The contractile response of aortic rings from vTP+ and vTP− rabbits to angiotensin II is shown in Fig. 2B. Angiotensin II produced a concentration-dependent contractile response in both vTP+ and vTP− rabbits but the contraction was greater in vTP+ rabbits compared with vTP− rabbits (maximal response; vTP− vs. vTP+: 69 ± 6 vs. 132 ± 11%; P < 0.01). The log EC₅₀ values for angiotensin II in the vTP+ and vTP− aortas were not different (1.36 and 1.63 nM, respectively). SQ29548 had no effect on angiotensin II-induced contractions of vTP− aortas (data not shown). Segments of vessels from vTP+ aortas were incubated with ¹⁴C-arachidonic acid in the presence and absence of angiotensin II (10 µM), and the ¹⁴C metabolites were resolved by reverse-phase HPLC. Radioactive products comigrating with 6-keto-PGF₁α, PGE₂, and PGF₂α were detected. There was no evidence that rabbit aortas produced TXB₂, the stable metabolite of TXA₂, either under basal conditions or when stimulated with angiotensin II (Fig. 4).

Removal of the endothelium (data not shown) or prior treatment with the NO synthase inhibitor 1-NNA (data not shown) had no effect on angiotensin II-induced contractions. However, if denuded vessels were pretreated with the TP receptor antagonist, angiotensin II contractions were reduced (Fig. 5A). Because reactive oxygen species are increased by angiotensin II in vascular smooth muscle cells, an additional study evaluated angiotensin II-induced contractions in vTP+ rabbits in the presence of the SOD mimetic Tiron. Inhibition of superoxide in both intact and denuded vessels attenuated angiotensin II-induced contractions.
giotensin II-induced contractions (Fig. 5, B and C). To compare responses in denuded vessels with intact vessels and to control for variations in responses to angiotensin II that occur between rabbits, the Tiron and SQ29548 results were expressed as percent inhibition from control responses in both the intact and denuded vessels. Results are shown in Table 1 and indicated that a similar inhibitory effect is seen when intact and denuded vessel responses are compared.

The hypothesis was explored that the isoprostane 8-iso-PGF$_2\alpha$ may contribute to angiotensin II-induced contractions in the rabbit arteries. The 8-iso-PGF$_2\alpha$ elicited concentration-dependent contractions in vTP/aortas. The effect was dependent on an interaction with the TP receptor since no contractions were observed in the vTP/aortas or when vTP/aortas were treated with SQ29548 (Fig. 1B). The production of 8-iso-PGF$_2\alpha$ is evident in isolated aortas of vTP/aortas but there was no change in 8-iso-PGF$_2\alpha$ production following angiotensin II treatment (vTP/aortas: 0.58 ± 0.15 vs. 0.47 ± 0.06 pg/mg; vTP/aortas: 0.53 ± 0.02 vs. 0.41 ± 0.14 pg/mg; n = 4). Similar results were observed in vTP/aortas (basal vs. angiotensin II: 0.59 ± 1.7 vs. 1.2 ± 1.7; n = 3). However, the LC-ESI-MS method used to quantify 8-iso-PGF$_2\alpha$ indicated that there were a number of other peaks in which the most abundant molecular ion was m/z 353, the mass that is indicative of a F$_2$-isoprostane structure derived from arachidonic acid. The ion chromatograms of the mass spectra data from the control and angiotensin II-treated samples were overlayed, and a single product that migrated at 8.5 min was increased in the angiotensin II-treated aortas (Fig. 6A). A similar product was observed if mesenteric vessels (Fig. 6B) were used or if the aortic vessels were pretreated with the superoxide-generating system xanthine/xanthine oxidase (Fig. 6C). When denuded vessels were incubated with angiotensin II, the production of the 8.5-min peak was similar to that observed in intact vessels (data not shown). Pretreatment of

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<th>Treatment</th>
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<tr>
<td>Intact + SQ29548</td>
<td>26.7 ± 6.7</td>
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<tr>
<td>Denuded + SQ29548</td>
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<td>Intact + Tiron</td>
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Values are means ± SE for n = 6–12. %Inhibition was calculated as [%contraction of control − %contraction with inhibitor] ÷ [%contraction of control] × 100 for both intact and denuded aortas. See Figs. 2 and 5.
The 8.5-min peak was isolated from aortas incubated with angiotensin II and tested for activity on isolated rings of rabbit aorta. The 8.5-min peak to control: 39 ± 5% vs. 64 ± 8%; P < 0.05; Fig. 8A]. This effect was also seen with 8-iso-PGF2α (5 nM) using isolated rings of rabbit aorta [contraction to angiotensin II (5 × 10⁻⁸ M); control vs. 8-iso-PGF2α: 27 ± 9 vs. 56 ± 11%; P < 0.05; Fig. 8B]. This effect of the 8.5-min peak or 8-iso-PGF2α also occurred if phenylephrine was used to contract the blood vessels [data not shown; contraction to phenylephrine (10⁻⁸ M); control vs. 8.5-min peak: 50 ± 4 vs. 60 ± 4%; control vs. 8-iso-PGF2α: 56 ± 3 vs. 70 ± 5%; P < 0.05]. In vTP− rabbits, there was no effect of 8-iso-PGF2α or the 8.5-min peak to enhance angiotensin II- or phenylephrine-induced contractions (data not shown).

**DISCUSSION**

This study explored the hypothesis that a portion of angiotensin II-induced contractions is dependent on superoxide generation and the release of a mediator that activates vascular smooth muscle cell TP receptors. The major findings confirmed that a TP receptor antagonist attenuates angiotensin II-induced contractions in rabbit aorta and mesentery artery. If rabbits lack vascular TP receptors, there are also reduced contractile responses to angiotensin II. Neither TXA2 nor PGH₂ contribute to angiotensin II-induced contractions, but an inhibitor of superoxide reduces angiotensin II-induced contractions. Furthermore, angiotensin II increases the production of a F₂-isoprostane that is not 8-isoPGF₂α, and this F₂-isoprostane can potentiate angiotensin II-induced contractions through a mechanism that involves the vascular TP receptor.

The first series of experiments showed that angiotensin II caused a concentration-related contractile response that was partially inhibited by pretreatment with SQ29548. Previous studies (17, 37) by others indicated that in certain blood vessels angiotensin II increased TXA₂ synthesis. There is no evidence that the rabbit aorta produces TXA₂ (24), and if vessels were pretreated with dazoxiben, a specific inhibitor of TX synthase, there was no difference in angiotensin II-induced contractions compared with the controls. Some studies (2, 27) reported that the endoperoxide metabolite PGH₂ mediates endothelium-dependent contractions via an interaction with the TP receptor. However, in the present study, pretreatment of aortas with the COX inhibitor indomethacin also had no effect on the response to angiotensin II eliminating the COX metabolites PGH₂ or TXA₂ as mediators. Finally, to support that a portion of angiotensin II-induced contractions required an interaction with the vascular TP receptor, we used the vascular TP-receptor-deficient rabbits. These rabbits have been well characterized in previous studies (5, 25) and have been shown to contract similarly to agonists like KCl, endothelin, and norepinephrine. This is the first study to evaluate angiotensin II responses, and results showed that contractions were decreased in vTP− compared with the vTP+ rabbits.

To explain the mechanistic interaction between angiotensin II and TP receptors, it is hypothesized that angiotensin II increases superoxide production and that superoxide contrib-
utes to contractions via a mechanism that involves the TP receptor. A scavenger of superoxide, Tiron, inhibited a portion of the contractions to angiotensin II in the vTP+/H11001 rabbit aorta. In vTP− aortas, Tiron had no effect on angiotensin II-induced contractions supporting a role for the TP receptor in the response. In the isolated rabbit aorta, vascular cells other than the endothelial cell are the major source of superoxide. Pagano and coworkers (21, 22) showed that mechanical removal of the endothelium had no effect on the production of superoxide when vessels were incubated with the SOD inhibitor diethyl-dithiocarbamate. The present studies showed that angiotensin II-induced contractions were similar in both the intact and denuded vessels. Furthermore, a portion of the angiotensin II-induced contractions in the denuded vessels was still dependent on superoxide and TP receptor activation, as both Tiron and SQ29548 attenuated the response. The present study did not examine a model of angiotensin II-induced hypertension but instead investigated whether the acute vasoconstrictor response to angiotensin II available and enhance angiotensin II contractions. However, in rabbit aorta, L-NNA did not alter angiotensin II-induced contractions.

Numerous reports support an interaction between TXA2 and angiotensin II (7, 13, 17, 38, 39). In mice with a targeted disruption of the TP receptor gene and subjected to chronic angiotensin II infusion, the absence of TP receptors blocked the development of hypertension (7, 12). Wilcox and coworkers (33, 36) studied rabbits following acute low dose (60 ng·kg⁻¹·min⁻¹), or high dose (200 ng·kg⁻¹·min⁻¹) angiotensin II infusion. High-dose but not low-dose angiotensin II increased blood pressure and markers of oxidative stress in the renal afferent arterioles. Isolated renal afferent arterioles from both groups had enhanced angiotensin II-mediated contractions compared with the sham infusion. Blockade of TP receptors decreased the contractions. With high-dose angiotensin II infusion, there was an even greater increase in angiotensin II contractions that was inhibited by both a TP receptor antagonist and SOD. The present study did not examine a model of angiotensin II-induced hypertension but instead investigated whether the acute vasoconstrictor response to angiotensin II...
was also dependent on the activation of the TP receptor. In both a conduit vessel (aorta) and resistance-sized vessel (mesenteric artery), a portion of the response to angiotensin II was inhibited by a TP receptor antagonist.

Isoprostanes represent a unique series of PG-like compounds that were identified as products of the peroxidation of arachidonic acid catalyzed by oxygen free radicals (3, 6, 30). At least two isomers of the isoprostanes, 8-iso-PGF$_{2\alpha}$ and 8-iso-PGE$_2$, possess significant biological activity (35) acting as vasoconstrictors through a TP receptor mechanism (10, 14, 16, 32). Since the arachidonic acid inhibitor studies indicated that neither TXA$_2$ nor PGH$_2$ contributed to angiotensin II-induced contractions in the rabbit aorta, we explored the possibility that angiotensin II released an isoprostane, like 8-iso-PGF$_{2\alpha}$, that may then contribute to angiotensin II-induced contractions. While we were able to measure 8 iso-PGF$_{2\alpha}$ in the rabbit vessels, angiotensin II did not increase its production. Based on the mechanism of isoprostane synthesis, four endoperoxide regioisomers are formed and then further reduced to the isoprostanes. The four regioisomers are denoted as either 5-(type VI), 12-(type VI), 8-(type IV), or 15-(type III) series depending on the carbon atom to which the side chain hydroxyl is attached. For example, 8-iso-PGF$_{2\alpha}$ is also called 15-F$_2$T-Iso P or 8P$_{2\alpha}$-III. As an indication of the large number of potential isoprostanes, each of the four regioisomers can theoretically be comprised of eight racemic diastereomers. Therefore, the possibility exists for the formation of 64 different F$_2$-isoprostanes. There is direct evidence for the formation of each of the four classes of regioisomers from both in vitro and in vivo studies (15). Very few of these regioisomers have been studied for effects on vascular tone. In the present study, the LC-ESI-MS results showed that angiotensin II increased a unique isoprostane from blood vessels and that this production was not dependent on the endothelium. LC/MS/MS analysis of this metabolite revealed a molecular weight of 354 that is indicative of the F$_2$-isoprostanes. The ions observed at $m/z$ 309 and $m/z$ 193 ions further evidenced that the isoprostane increased by angiotensin II is of the F series (19). Further characterization studies are ongoing to determine the stereochemistry of the hydroxyl and alkyl groups and should provide more definitive structural identification of the isoprostane isomer.

Finally, it was important to show that the F$_2$-isoprostane had biological activity if it is a mediator of enhanced angiotensin II-induced vasoconstriction. The F$_2$-isoprostane had no vasoconstrictor activity on basal tone. A possible explanation may relate to the amount of the unknown metabolite that is isolated from the rabbit aorta and subsequently tested for biological activity. Because we are isolating an unknown factor, it is difficult to quantify its production, and therefore, it may be that we are not adding a large enough concentration to see a direct constrictor effect. Several extraction and HPLC steps are required for isolation, which would also contribute to a loss of mass of the unknown product. However, the F$_2$-isoprostane did enhance angiotensin II-mediated contractions. A similar enhancement was observed if a subthreshold concentration of 8-iso-PGF$_{2\alpha}$ was tested. The effect is dependent on the TP receptor since there was no enhanced effect in vTP$^{-}$ rabbits. The ability of isoprostanes to amplify responses is not restricted to angiotensin, as both 8-iso-PGF$_{2\alpha}$ and the newly identified F$_2$-isoprostane also increased phenylephrine-induced contractions through a TP receptor-mediated mechanism. The results suggest that isoprostanes increase sensitivity to vasoconstrictor agonists, a potentially detrimental effect in conditions like atherosclerosis, diabetes, and hypertension. This mechanism may be especially important in diseases in which oxidative stress, leads to increased generation of isoprostanes. Isoprostanes are well recognized as biomarkers of oxidative stress but these compounds also display important biological effects. Much of the available data on isoprostane biological effects are related to known synthetic derivatives, like 8-isoPGF$_{2\alpha}$ and 8-iso-PGE$_2$. This study describes novel findings concerning previously unidentified, biologically derived isoprostanes that are potentially linked to the TP receptor. Further characterization of this pathway has the potential to advance our knowledge in the possible causes of vascular diseases, ultimately leading to better therapeutic treatments.


