TP receptor-mediated vasoconstriction in microperfused afferent arterioles: roles of O$_2^-$ and NO

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Thromboxane A$_2$ (TxA$_2$) produced in the endothelium constricts vascular smooth muscle cells through activation of thromboxane A$_2$/prostaglandin H$_2$ (TP) receptors. In the kidney, TxA$_2$ reduces renal blood flow and glomerular filtration rate through preferential action on the afferent arteriole and glomerulus (3, 15, 25). Some of these effects are mediated via the tubuloglomerular feedback (TGF) response (41), whereas others are apparent in hydropnephrotic kidneys that lack a TGF response and therefore must be due to direct action on afferent arterioles (15,25). Several studies implicate TxA$_2$ to be an important mediator of the renal hemodynamic and blood pressure effects of angiotensin II (ANG II) under normal conditions (42, 43) and in ANG II-dependent forms of hypertension (28). However, the mechanisms by which TP receptor activation causes vasoconstriction remain to be fully elucidated.

Nitric oxide (NO) produced by the endothelium is a ubiquitous vasodilator and modulator of vascular tone. NO buffers the vasoconstrictor action of TxA$_2$ in the isolated aorta (5, 7), coronary artery (37), and pulmonary artery (40). NO also modulates vasoconstriction induced by ANG II and endothelin-1 but not by norepinephrine in the renal afferent arteriole (18, 19). Whether NO plays an important role in modulating vasoconstriction induced by TxA$_2$ in the renal afferent arteriole remains unknown.

Several studies suggest that the oxygen radical superoxide (O$_2^-$) interacts with NO and thus limits its bioavailability. The affinity of NO for O$_2^-$ is so high that its rate of reaction is limited only by diffusion (31). Since O$_2^-$ effectively degrades NO, the biological activity of NO may be determined by the availability of O$_2^-$ (13, 31). NO-mediated vasodilation is impaired in aorta with enhanced generation of O$_2^-$ (12) and can be restored by blockade of TP receptors (2, 33, 38). This led us to the hypothesis that TP receptor activation may be a potent source for the generation of O$_2^-$ and hence for degrading NO in resistance vessels. The objectives of this study were to 1) determine the role of O$_2^-$ in TP receptor activation, 2) to examine the role of NO in TP receptor activation, and 3) to investigate the interaction between NO and O$_2^-$ in TP receptor activation in renal afferent arterioles. The response to the stable TP receptor agonist U-46,619 was studied in rabbit isolated, perfused renal afferent arterioles. The role of NO was assessed from the responses to inhibition of NO synthase with N$^\omega$-nitro-$\omega$-arginine methyl ester (L-NAME). The stable nitroxide 4-hydroxy-[2,2,6,6]-tetramethylpiperidine-1-oxyl (tempol) was used to scavenge O$_2^-$. Tempol is a metal-independent, membrane-permeable superoxide dismutase mimetic that scavenge O$_2^-$. 

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to hydrogen peroxide (H₂O₂) and oxygen. Tempol has been validated as an electron paramagnetic resonance spin-label molecule specifically for O₂⁻ (16, 29) and does not donate NO or scavenge H₂O₂ (16, 29). In vivo studies have shown that tempol reduces damage caused by oxygen radicals in ischemia/reperfusion injury (8), inflammation (21), and radiation (27).

METHODS

Isolation and Microperfusion of Afferent Arterioles

Male New Zealand White rabbits (1.4–1.8 kg) were maintained on tap water and standard chow. Protocols were approved by the Institutional Animal Care and Use Committee of Georgetown University Medical Center and were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health as well as the guidelines of the Animal Welfare Act. Rabbits were anesthetized with xylazine (9 mg/kg im), ketamine (47 mg/kg im), and pentobarbital sodium (11 mg/kg iv) followed by heparin (1,000 USP iv) for anticoagulation. Microdissection and microperfusion of the afferent arteriole were performed as previously described (18, 19). Briefly, the right kidney was extracted via an abdominal incision and immediately placed in ice-cold preservation solution (24). Slices of the kidney were made along the corticomedullary axis and replaced in the preservation solution. A single superficial afferent arteriole with glomerulus attached was microdissected under a stereomicroscope (model SZ40, Olympus) on a temperature-controlled stage maintained at 4°C. The arteriole was transferred to a temperature-regulated chamber mounted on the stage of an inverted microscope (model IX70, Olympus) modified with micromanipulators. The afferent arteriole was cannulated with a series of concentric glass pipettes including holding, perfusion, and exchange pipettes and perfused with alpha modification of minimum essential media (MEMα) at 60 mmHg. The arteriole was superfused at ~1 ml/min with MEMα bubbled with 95% O₂-5% CO₂. The microperfused arteriole was displayed at ×400 magnification (Nomarski optics, Olympus) on a video monitor via a black-and-white camera (model NC 70, Dage-MTI) attached to the inverted microscope and recorded on VHS tape. The area of the afferent arteriole with the consistently most constricted point during an experiment was selected for measurement. Measurements were made with standard vernier calipers (Mitutoyo, Japan).

Protocol

Rabbit microperfused afferent arterioles were gradually warmed to 37°C and allowed to equilibrate for 30 min. Drugs were added to the superfusion solution, and measurements of luminal diameter were made after 10–15 min. To test the viability of the tissue at the completion of the studies, norepinephrine (10⁻⁷ M) was administered. Only those vessels showing a >25% contraction were selected. Figure 1 illustrates an example of an isolated, perfused rabbit afferent arteriole during basal conditions and after norepinephrine (10⁻⁷ M).

Experimental Design

Series 1. The aim of this series was to assess the specificity of tempol as a superoxide dismutase mimetic in the isolated, perfused afferent arteriole. We used the classic quinoline agent paraquat to stimulate intracellular production of O₂⁻ in afferent arterioles. Paraquat redox cycles with cellular di-

![Fig. 1. Example of an isolated, perfused rabbit afferent arteriole (perfusion pressure = 60 mmHg) during basal conditions (A) and after norepinephrine (10⁻⁷ M, B).](image)
activation. The NO synthase inhibitor l-NAME was used at a dose which has previously been shown to block acetylcholine-induced, endothelium-dependent vasodilation of the rabbit afferent arteriole (19). The luminal diameter response to U-46,619 (10^{-10}–10^{-6} M) was determined in afferent arterioles pretreated with l-NAME (10^{-4} M, n = 6).

Series 5. The objective of this series, was to assess whether the effect of tempol on the response to TP receptor activation could be ascribed to potentiation of the effects of NO. The arteriole response to U-46,619 was measured during blockade of NO synthesis and scavenging of O_2^-. The luminal diameter response to graded concentrations of U-46,619 (10^{-10}–10^{-6} M) was measured in vessels pretreated with tempol (10^{-3} M) + l-NAME (10^{-4} M, n = 6).

Drugs and Solutions

U-46,619 (Cayman Chemical) was evaporated under N_2 and reconstituted using 97% ethanol and 55 mM Tris. After further evaporation with nitrogen, aliquots of U-46,619 (10^{-3} M) were made in tissue culture grade H_2O and stored at −20°C until use. All other solutions were prepared fresh daily. Heparin was dissolved in 0.9% NaCl at 1,000 USP/ml. Ifetroban (BMS-180291) was prepared in tissue culture grade H_2O and diluted in superfusion solution before use daily. All other agents including norepinephrine, tempol (4-hydroxy TEMPO), and l-NAME were purchased from Sigma Chemical and prepared similarly. Preservation solution consisted of 150 mM sucrose, 52 mM NaHPO_4 (anhydrous), 16 mM NaH_2PO_4, and 5% BSA which was filtered (0.8 μm), saturated with 95% O_2-5% CO_2 (pH 7.40–7.45), and prepared fresh daily. MEMα solution containing 126.40 mg/l L-arginine and an additional 26 mM NaHCO_3 and 5% BSA for perfusion and 26 mM NaHCO_3 and 0.15% BSA for superfusion were filtered (0.2 μm), saturated with 95% O_2-5% CO_2, and buffered to pH 7.40–7.45 before use daily.

Statistics

All values are reported as means ± SE. Overall significance between dose responses was determined from repeated measures analysis of variance and the Scheffe post hoc test where appropriate. A Student’s t-test was used to determine significance between groups. P < 0.05 was determined to be significant.

RESULTS

Series 1

Figure 2 shows the luminal diameter response to paraquat (10^{-7}–10^{-3} M) in vehicle pretreated vessels and in vessels pretreated with tempol (10^{-3} M). From a baseline of 18.41 ± 1.37 μm, paraquat caused dose-dependent reductions in luminal diameter (ED_{50} = 8.6 ± 3.1 μM) of microperfused afferent arterioles that were abolished by tempol (P < 0.05). Superfusion of tempol alone (10^{-3} M) had no effect on basal luminal diameter (15.42 ± 1.98 μm) after 15 (15.50 ± 1.62 μm) or 60 (15.74 ± 1.43 μm) min.

Series 2

Figure 3 illustrates the luminal diameter response to TP receptor activation with U-46,619 in microperfused afferent arterioles in the presence and absence of ifetroban. From a baseline of 15.63 ± 0.89 μm, U-46,619 (10^{-10}–10^{-6} M) caused graded reductions in luminal diameter (ED_{50} = 7.5 ± 5.0 nM). Ifetroban (10^{-6} M) pretreatment had no significant effect on basal luminal diameter (17.83 ± 1.18 to 19.26 ± 1.59 μm) of microperfused afferent arterioles but abolished the response to U-46,619 (P < 0.001). An additional series was undertaken to contrast the luminal diameter response to U-46,619 when given in the bath + lumen compared with in the bath alone. U-46,619 (10^{-10}–10^{-6} M) given in the bath + lumen had no significant effect on the luminal diameter (18.23 ± 1.98 to 19.86 ± 1.56 μm). Furthermore, U-46,619 (10^{-10}–10^{-6} M) given in the bath during sham exchange through the lumen also had no significant effect on luminal diameter (17.77 ± 1.01 to 20.48 ± 3.15 μm). Therefore, all further series were conducted using U-46,619 given in the bath only.

Series 3

Figure 4 illustrates the luminal diameter response to U-46,619 (10^{-10}–10^{-6} M) in microperfused afferent arterioles in the presence and absence of the membrane-permeable superoxide dismutase mimetic tempol. Tempol (10^{-3} M) abolished the contractile response to U-46,619 across the dose range studied (P < 0.001). Basal luminal diameter was 16.57 ± 0.77 μm and remained unchanged in response to tempol + U-46,619 (17.25 ± 0.38 μm). At the end of the experiments, luminal diameter responses to norepinephrine (10^{-7} M) were not significantly different in vessels treated with U-46,619 alone or with U-46,619 + tempol (−54 ± 7% vs. −36 ± 16%).

Series 4

Figure 5 illustrates the luminal diameter response to low (10^{-10} M) and high (10^{-6} M) doses of U-46,619 in the presence or absence of NO synthesis blockade with l-NAME. Pretreatment with l-NAME (10^{-4} M) significantly (P < 0.001) reduced basal afferent arteriolar
luminal diameter by 20 ± 1% (from 15.30 ± 1.22 to 12.23 ± 0.50 μm) and significantly \( P < 0.05 \) enhanced the vasoconstrictor response to U-46,619 \( (10^{-10} - 10^{-6} \text{ M}) \). L-NAME significantly \( P < 0.05 \) increased the sensitivity and maximal responses of afferent arterioles to U-46,619. Additional studies were conducted to examine the luminal diameter response to L-NAME \( (10^{-4} \text{ M}) \) given in the bath + lumen. This reduced the luminal diameter by 22 ± 2%, which is not significantly different compared with the bath alone as indicated above or compared with Ito et al. (19).

**Series 5**

Figure 6 shows the luminal diameter response to U-46,619 \( (10^{-10} - 10^{-6} \text{ M}) \) in microperfused afferent arterioles pretreated with tempol \( (10^{-3} \text{ M}) \) in the presence or absence of NO synthesis blockade with L-NAME \( (10^{-4} \text{ M}) \). L-NAME significantly \( P < 0.05 \) restored a vasoconstrictor response to U-46,619 in tempol pretreated arterioles. Whereas U-46,619 had no effect in vessels pretreated with tempol \( (16.57 ± 0.77 \text{ to } 17.25 ± 0.38 \mu m) \), U-46,619 significantly decreased luminal diameter by 32% in vessels pretreated with tempol + L-NAME \( (12.23 ± 0.51 \text{ to } 8.27 ± 1.04 \mu m) \). Clearly, the inhibitory action of tempol during U-46,619-induced vasoconstriction was blunted by L-NAME.

**DISCUSSION**

The stable TP receptor agonist U-46,619 vasoconstricts isolated rabbit microperfused afferent arterioles. This response can be attributed to activation of TP receptors because it is blocked by ifetroban. These
results are similar to those previously reported for TP receptor-mediated vasoconstriction of isolated aorta (7), pulmonary arteries (40), and afferent arterioles of the hydronephrotic rat kidney preparation (25). The new findings in the present study are that the vasoconstrictor response to U-46,619 is abolished by a membrane-permeable superoxide dismutase mimetic and is enhanced by NO synthesis blockade. We conclude that TP receptor activation leads to generation of O$_2^-$, which is permissive in the vasoconstriction, and to NO, which buffers the vasoconstriction. L-NAME restored a vasoconstrictor response to U-46,619 in vessels pretreated with tempol. This suggests an important role for the interaction between NO and O$_2^-$ in TP receptor-mediated vasoconstriction in the afferent arteriole.

NO is a powerful endothelium-derived vasodilator that maintains basal vascular tone and modulates the vasoconstrictor actions of several agonists within the kidney. Ito et al. demonstrated that NO blunts the vasoconstriction caused by ANG II (19) and endothelin-1 (18) of isolated, microperfused afferent arterioles. The present study confirms that blockade of NO synthesis significantly reduces basal luminal diameter, indicating that NO is produced tonically in rabbit isolated, perfused afferent arterioles (19). The data show, for the first time, that TP receptor activation stimulates NO, which buffers the vasoconstrictor action of U-46,619 in afferent arterioles.

The mechanism by which U-46,619 stimulates NO in afferent arterioles remains unknown. One possible mechanism may involve a TP receptor located on the endothelium. Kent et al. (22) identified a TP receptor on isolated human endothelial cells that stimulates increases in intracellular calcium, which can activate endothelial-derived NO synthase. The importance of the endothelium in modulating the vasoconstrictor actions of TP receptor activation depends on the vascular site. For example, removal of the endothelium or blockade of NO synthesis in the aorta (7) and coronary artery (37) enhances U-46,619-induced vasoconstriction. However, blockade of NO synthase in the pulmonary vasculature either enhances (40) or has no effect (20) on U-46,619-induced vasoconstriction. Endothelial NO synthase may also be activated indirectly by a rise in intracellular calcium generated in response to a primary action of TP receptors on vascular smooth muscle cells where TP receptors are readily expressed. Dora et al. (6) have shown such electromechanical coupling between vascular smooth muscle and endothelial cells of the hamster cheek pouch. Activation of TP receptors in afferent arterioles of the hydronephrotic kidney increases intracellular calcium (25). However, the role of electromechanical coupling or endothelial TP receptors in mediating the stimulation of NO in the afferent arteriole remains to be elucidated.

O$_2^-$ is scavenged extracellularly by copper-zinc superoxide dismutase (CuZnSOD) and intracellularly by CuZnSOD and manganese SOD. Mehta et al. (26) showed that inhibition of Tx$\alpha$$_2$ synthesis decreases the production of O$_2^-$ in activated human neutrophils. They suggested that Tx$\alpha$$_2$ stimulates the production of O$_2^-$.

Griendling et al. (10) provided direct evidence that ANG II stimulates O$_2^-$ production in vascular smooth muscle cells via activation of NADPH oxidase. We selected the nitroxide tempol in our studies. It is a stable, membrane-permeable, metal-independent SOD mimetic (16, 29). U-46,619 caused a dose-dependent vasoconstriction of afferent arterioles. This response was completely prevented in vessels pretreated with tempol. We conclude that U-46,619 stimulates the production of O$_2^-$, which is permissive for TP receptor-induced vasoconstriction of the afferent arteriole.

The source of O$_2^-$ production in the renal afferent arteriole remains unknown. O$_2^-$ is produced by cellular electron transport chains such as those in mitochondria and endoplasmic reticulum (14), NO synthase (4, 32), cyclooxygenase (23), lipoxygenase (23), xanthine oxidase (12), and NADPH oxidase (10). All of these enzymes are expressed in the kidney. NO synthase and cyclooxygenase are known to be expressed in the afferent arteriole. The importance of these enzymes in mediating U-46,619-induced stimulation of O$_2^-$ in the afferent arteriole remains to be determined.

Our data suggest that one mechanism whereby O$_2^-$ mediates U-46,619-induced vasoconstriction is through interaction with NO. Gryglewski et al. (13) first showed in vascular endothelial cells that O$_2^-$ is involved in the inactivation of NO. Since then, several studies have demonstrated that scavenging of O$_2^-$ increases the release of bioactive NO in the vasculature in situ (39) and in cultured vascular endothelial cells (12). In the present study, we show that blockade of the U-46,619-induced vasoconstriction by tempol is largely prevented by inhibition of NO synthesis with L-NAME. This data suggests that production of O$_2^-$ after U-46,619 decreases bioactivity of stimulated NO and that this promotes the vasoconstriction of afferent arterioles. However, additional mechanisms must be involved since L-NAME augmented the response to U-46,619 in the absence of tempol. Superoxide can also stimulate inositol 1,4,5 triphosphate (IP$_3$) formation and thus increase intracellular calcium in vascular smooth muscle cells (44). Therefore, tempol may reduce superoxide-mediated increases in intracellular calcium and thus block the vasoconstrictor response to U-46,619.

Tempol has been evaluated extensively as a scavenger of O$_2^-$ in vitro and in vivo. We evaluated the specificity of tempol’s actions in the rabbit isolated perfused afferent arteriole. Tempol given alone for 60 min did not alter the diameter of the vessel. This indicates that tempol does not have nonspecific effects on afferent arteriole tone. Vessels treated with paraquat, which is a O$_2^-$-generating quinoline, showed graded but reversible vasoconstriction consistent with the contractile effects of O$_2^-$ on blood vessels (34). Vessels pretreated with tempol were protected fully from paraquat-induced contractions, consistent with tempol’s proposed mechanism of actions as a superoxide dismutase mimetic. Furthermore, we have previously reported (35)
that tempol given in vivo reduces a marker for oxygen radical production. This confirms its ability to scavenge superoxide radical. The afferent arteriole responses to norepinephrine were unaffected by tempol (−69 ± 17% vs. −67 ± 13%, unpublished observations), indicating that G protein coupled signal transduction was intact in vessels treated with tempol. However, we have not performed direct studies to exclude the possibility that tempol impaired the vasoconstrictor response to U-46,619 by uncoupling G proteins. We have recently reported that the elevated blood pressure and renal vasoconstriction in the spontaneously hypertensive rat (SHR) are normalized by tempol (35, 36); however, TP receptor antagonism is not effective in this model (11). This suggests that tempol is not a TP receptor antagonist. We found that the antihypertensive and renal receptor antagonism is not effective in this model (11). We have recently performed direct studies to exclude the possibility that G protein coupled signal transduction was intact in vessels treated with tempol. However, we have not investigated.

In conclusion, this study provides evidence that generation of O₂ contributes to the contractile response to activation of TP receptors in afferent arterioles. TP receptor activation also stimulates NO, which buffers the contraction. The balance between O₂ and NO is important in the vasoconstrictor response. These results suggest a role for oxygen radical therapy in conditions of endothelial dysfunction associated with oxidative stress and increased TxA₂ production in the kidney such as hypertension, diabetes, and renal failure.

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