Activation of NAD(P)H Oxidases by Thromboxane A<sub>2</sub> Receptor Uncouples Endothelial Nitric Oxide Synthase

Miao Zhang, Ping Song, Jian Xu, Ming-Hui Zou

Objective—The thromboxane receptor (TPr) and multiple TPr ligands, including thromboxane A<sub>2</sub> (TxA<sub>2</sub>) and prostaglandin H<sub>2</sub> (PGH<sub>2</sub>), are elevated during vascular and atherothrombotic diseases. How TPr stimulation causes vascular injury remains poorly defined. This study was conducted to investigate the mechanism by which TPr stimulation leads to vascular injury.

Methods and Results—Exposure of bovine aortic endothelial cells to either IBOP or U46619, 2 structurally related TxA<sub>2</sub> mimetics, for 24 hours markedly increased the release of superoxide anions (O<sub>2</sub>•<sup>-</sup>) and peroxynitrite (ONOO<sup>-</sup>) but reduced cyclic GMP, an index of nitric oxide bioactivity. IBOP also significantly suppressed activity of endothelial nitric oxide synthase (eNOS), increased enzyme-inactive eNOS monomers, and reduced levels of tetrahydrobiopterin, an essential eNOS cofactor. IBOP- and U46619-induced increases in O<sub>2</sub>•<sup>-</sup> were accompanied by the membrane translocation of the p67phox subunit of NAD(P)H oxidase. Pharmacological or genetic inhibition of either NAD(P)H oxidase or TPr abolished IBOP-induced O<sub>2</sub>•<sup>-</sup> formation. Furthermore, TPr activation significantly increased protein kinase C-ζ (PKC-ζ) in membrane fractions and PKC-ζ phosphorylation at Thr410. Consistently, PKC-ζ inhibition abolished TPr activation-induced membrane translocation of p67phox and O<sub>2</sub>•<sup>-</sup> production. Finally, exposure of isolated mouse aorta to IBOP markedly increased O<sub>2</sub>•<sup>-</sup> in wild-type but not in those from gp91phox knockout mice.

Conclusion—We conclude that TPr activation via PKC-ζ-mediated NAD(P)H oxidase activation increases both O<sub>2</sub>•<sup>-</sup> and ONOO<sup>-</sup>, resulting in eNOS uncoupling in endothelial cells. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: nitric oxide synthase • peroxynitrite • prostacyclin • signal transduction • thromboxanes
to TPr as a potential target for therapeutic interventions and the development of novel selective antagonists for the treatment of human cardiovascular disease. TPr blockade prevents the formation of occlusive thrombi in models of myocardial infarction.18 Indeed, TPr blockers and TxA2 synthase inhibitors attenuate ischemia/reperfusion-mediated injury in several organs, including the liver19 and heart.20 In addition, pharmacological inhibition or genetic deletion of TPr blocks the development of atherosclerosis in non-diabetic apolipoprotein E knockout mice, indicating that TPr is important for the initiation and progression of atherosclerosis.21,22

Recently, activation of TPr by TxA2 has been found to promote O$_2^-$ production in vascular smooth muscle cells.23–25 However, the mechanism by which TPr activation generates oxidant stress in endothelial cells is unknown. This study was conducted to determine whether TPr stimulation induces oxidative stress in endothelial cells and to investigate the mechanism underlying TPr-induced vascular injury. Our data reveal that TPr stimulation increases endothelial production of O$_2^-$ and peroxynitrite (ONOO$^-$) by increasing protein kinase C-$\xi$ (PKC-$\xi$)-dependent activation of NAD(P)H oxidase. Moreover, they suggest that these NAD(P)H oxidase-derived ROS induce endothelial dysfunction by uncoupling of eNOS activity and increasing tyrosine nitration of the eicosanoid biosynthetic enzyme PG12 synthase (PGIS).

**Materials and Methods**

A full description of materials and methods, including cell culture, animals, localization and quantification of O$_2^-$ in isolated aortae, preparation of membrane fractions, assays of cyclic GMP, cell viability, and cell apoptosis assays can be found in the supplemental materials, available online at http://atvb.ahajournals.org.

**Quantification of O$_2^-$ in Cultured Endothelial Cells**

Dihydroethidium (DHE) is a cell-permeable dye that reacts with superoxide anions specifically to produce a hydrolyzed product, 2-hydroxy-ethidium (HE-OOH), which spontaneously loses water to produce the fluorescent derivative HO-Et$_2^-$ (a product of DHE auto-oxidation) by column C-18.27 The ONOO$^-$ formation was determined by ONOO$^-$-dependent oxidation of dihydroethidium 123 (DHR 123) to rhodamine 123, as described previously.28 Briefly, confluent BAEC were incubated with DHE (5 $\mu$mol/L) for 30 minutes at 37°C, followed by methanol extraction. High-performance liquid chromatography (HPLC) was applied to separate and quantify oxyethidium (a product of DHE and O$_2^-$) and ethidium (a product of DHE auto-oxidation) by column C-18.27 The DHE staining images were analyzed by measuring fluorescent mean intensity using Bioquant image analysis software (Nashville, Tenn).

**HPLC Detection of O$_2^-$ in Isolated Aortae**

Superoxide anions were detected by using HPLC detection of oxyethidium in aortic sections, as described previously.27

**Measurement of ONOO$^-$ Production in Cultured Endothelial Cells**

ONOO$^-$ formation was determined by ONOO$^-$-dependent oxidation of dihydroethidium 123 (DHR 123) to rhodamine 123, as described previously.28 Briefly, confluent BAEC were treated with IBOP (1 $\mu$mol/L) for 4 hours with or without a 30-minute pretreatment with SQ29548. The cells were rinsed with medium and then incubated with DHR 123 (5 $\mu$mol/L) for 60 minutes at 37°C. Rhodamine 123 fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 545 nm using a Synergy HT Multi-Detection Microplate Reader. Results are expressed as a percentage of oxidation in untreated control cultures.

**Measurement of NO Production in Endothelial Cells**

NO levels were determined using the NO-specific fluorescent dye DAF-2 DA, as described previously.29 Briefly, BAEC were serum-starved overnight in phenol red-free EBM medium and then incubated in IBOP (1 $\mu$mol/L) or U46619 (1 $\mu$mol/L) for 24 hours. The cells were then loaded with DAF-2 DA (5 $\mu$mol/L) for 30 minutes, washed with PBS to remove excess probe, and incubated with 10 $\mu$mol/L A23187 and 100 $\mu$mol/L CaCl$_2$ in the dark at 37°C for 15 minutes. The cells were washed twice with PBS, and DAF-2T fluorescence was measured at a 485-nm excitation wavelength and a 545-nm emission wavelength using a spectrophotometer (Synergy HT).

**Measurement of PG12, eNOS Activity, eNOS Dimers/Monomers, and BH4**

The formation of PG12 was assayed by monitoring 6-keto-PGF1-\alpha, the stable PG12 metabolite, in cell culture media after IBOP (1 $\mu$mol/L) treatment for 24 hours in BAEC using a specific EIA kit (Cayman Chemical). To measure eNOS activity, we monitored conversion of $^3$H-L-arginine into $^3$H-L-citrulline, as described previously.30 Levels of eNOS dimers and monomers were determined using low-temperature SDS-PAGE, as detailed previously.30 Intracellular BH4 levels were assayed using HPLC, as previously described.27

**Immunoprecipitation and Western Blotting**

Immunoprecipitation and Western blotting were performed as described previously.30

**Statistical Analyses**

Values are expressed as mean±SEM. Statistical comparisons were performed using a Student t test or 1-way ANOVA with the Bonferroni procedure for post hoc analysis. Values of $P<0.05$ were considered significant.

**Results**

**TxA\textsubscript{2} Mimetics Reduce eNOS Activity in a TPr-Dependent Manner**

Cellular redox status is a key aspect of endothelial biology. Endothelial redox state influences NO production, an essential factor for endothelial function, by altering eNOS activity. To determine whether TPr stimulation alters eNOS activity, we exposed confluent BAEC to varying concentrations of IBOP (1 $\mu$mol/L), a potent TxA\textsubscript{2} mimetic, for 24 hours. IBOP inhibited insulin-stimulated or vascular endothelial growth factor–stimulated eNOS activity (Figure 1A). Exposure of BAEC to U46619 (1 $\mu$mol/L), a TPr agonist structurally related to IBOP, caused a similar reduction in eNOS activity (Figure 1B). IBOP- or U46619-induced reduction in eNOS activity was partially blocked by pretreatment of BAEC with SQ29548 (10 $\mu$mol/L), a potent TPr antagonist (Figure 1A and 1B).

**TxA\textsubscript{2} Mimetics Suppress NO Release and Bioactivity**

Next, we investigated whether the diminished eNOS activity associated with TPr activation was accompanied by a decrease in NO release. Analysis of NO release using the fluorescent dye DAF-2DA revealed that exposure of BAEC to IBOP significantly reduced NO release (Figure 1C). Importantly, the addition of tempol and apocynin, 2 potent antioxidants, attenuated the inhibitory effects of IBOP on NO release (Figure 1C), suggesting an inactivation of NO by O$_2^-$.

NO exerts its biological effects primarily through activation of guanylyl cyclase, which generates cGMP. Thus, intracellular
levels of cGMP are an index of NO bioactivity. As depicted in Figure 1D, cGMP levels were significantly lower in BAEC exposed to IBOP than in cells treated with vehicle. It is of note that the addition of polyethyl glycated superoxide dismutase reduced the effects of IBOP on cGMP (Figure 1D), supporting that reduction of NO bioactivity by IBOP.

**TXA2 Mimetic Increase O2•− Production**

NO bioactivity is determined mainly by the rate of NO production or the rate of NO inactivation by ROS such as O2•−. Recent studies suggest that TPr promotes O2•− formation in vascular smooth muscle cells.32-33 Therefore, we investigated whether TPr alters NO bioactivity by increasing O2•− in endothelial cells.

As shown in Figure 2A, treatment of BAEC with IBOP (1 μmol/L) significantly increased O2•− release.

To determine whether TPr is required for IBOP-induced O2•− production, BAEC were pretreated with SQ29548, a selective TPr antagonist. As shown in Figure 2A, SQ29548 abolished IBOP-induced O2•−. IBOP-induced O2•− was also quenched by pretreatment with tempol, a potent antioxidant (Figure 2A).

To exclude potential off-target effects of SQ29548, TPr expression was genetically suppressed by the transfection of TPr-specific siRNA but not in control siRNA-transfected cells (Figure 2B). As shown in Figure 2C, transfection of neither TPr-specific siRNA nor control siRNA altered the basal levels of O2•− in BAEC. As expected, IBOP significantly increased O2•− release in BAEC transfected with control siRNA, which was inhibited by the addition of tempol (10 μmol/L) (Figure 2C). Conversely, transfection of TPr-specific siRNA ablated IBOP-induced O2•− (Figure 2C).

**Figure 1.** TXA2 mimetics suppress eNOS activity, NO release, and NO bioactivity. A and B, Effects of IBOP and U46619 on the conversion of [3H]-L-arginine into [3H]-citruline in BAEC. Confluent BAEC were incubated with the indicated concentrations of IBOP or U46619 for 24 hours. After the incubation, cells were stimulated with either insulin (100 nmol/L) or vascular endothelial growth factor (VEGF) (50 ng/mL) for 10 minutes. A subset of cultures was preincubated with the TPr antagonist SQ29548 (10 μmol/L); *P<0.05, IBOP or U46619 versus control; †P<0.01, IBOP or U46619 plus SQ29548 versus IBOP alone (n=6). C, Effects of antioxidants on NO production in BAEC. BAEC were treated with IBOP (1 μmol/L) for 24 hours with or without a 30-minute pretreatment with tempol (10 μmol/L) or apocynin (100 μmol/L). NO was assayed by using the fluorescent dye DAF-1 DA. *P<0.05, IBOP versus control. NS indicates no statistical difference (n=4). D, Effects of IBOP on NO bioavailability in human umbilical vein endothelial cells. Human umbilical vein endothelial cells were treated with IBOP (1 μmol/L) for 24 hours, with or without a 30-minute pretreatment with polyethyl glycated superoxide dismutase (PEG-SOD) (10 U/mL) or apocynin (100 μmol/L). Intracellular cGMP contents were assayed as described in Materials and Methods. *P<0.05 versus control. NS indicates no statistical difference (n=4).

**TxA2 Mimetic IBOP Increases ONOO• Formation**

O2•− rapidly reacts with NO to form ONOO•, a powerful oxidant. At physiological pH, ONOO• has a half-life less than 1 second and stably modifies proteins with 3-nitrotyrosine (3-NT), a modification that is considered to be a footprint for reactive nitrogen species, including ONOO•, in cultured endothelial cells.31 We found that a 24-hour exposure of BAEC to IBOP increased 3-NT-positive proteins (Figure 3A), implying that TPr activation increased ONOO• formation in BAEC. In line with this result, IBOP also significantly enhanced ONOO•-dependent oxidation of DHR 123 in BAEC (Figure 3B). Importantly, preadministration of SQ29548 abolished IBOP-induced DHR 123 oxidation, suggesting that TPrs are required for IBOP-induced ONOO• formation. Furthermore, blocking the sources of ONOO• formation by pretreatment with either tempol (scavenging O2•−) or l-NAME (a nonselective inhibitor for nitric oxide synthase), also inhibited DHR 123 oxidation (Figure 3B).

**The TxA2 Mimetic IBOP Increases PGIS Nitration and Decreases PGI2 Formation**

To determine whether TPr-derived ROS may have detrimental effects other than NO inactivation, we analyzed PGIS nitration and PGI2 formation in IBOP-exposed BAEC. We have previously demonstrated that ONOO• inactivates PGIS by increasing tyrosine nitration at its active site.32,33 In addition, PGIS nitration triggers TPr-mediated vasospasms in vessels exposed to hypoxia or endotoxins. Western blot analysis of 3-NT immunoprecipitates using an anti-PGIS antibody or PGIS immunoprecipitates using anti-3-NT antibody revealed that a 24-hour exposure of BAEC to IBOP significantly increased the amount of nitrated PGIS (Figure 3C and Supplemental Figure II). IBOP also significantly suppressed the levels of 6-keto-PGF1α, a stable hydrolyzed metabolite of...
we investigated whether increased ONOO⁻ formation associated with TPr activation increases levels of inactive eNOS monomers in BAEC. Low-temperature SDS-PAGE revealed that exposure of BAEC to IBOP (1 μmol/L) for 24 hours significantly increased eNOS monomers, and adenoviral overexpression of the dominant negative mutant of either p47phox or p67phox, 2 essential components of active NAD(P)H oxidase, inhibited IBOP-induced reduction of eNOS dimers and the increase of eNOS monomers (Figure 4A). Consistent with this result, pretreatment with antioxidants (apocynin, tempol, or polyethyl glycated superoxide dismutase) all lowered IBOP-induced NO reduction (Figure 1B) and cGMP reduction (Figure 1C). We also found that a 24-hour exposure of BAEC to either IBOP or U46619 significantly decreased levels of BH4 (Figure 4B), a cofactor that is essential for eNOS activity, which was prevented by the addition of apocynin (Figure 4B). Similarly, adenoviral overexpression of p67phox dominant negative, which selectively inhibited NAD(P)H oxidase, also reversed TPr-induced BH4 reduction (Figure 4C).

PKC-ζ Is Required for TPr-Mediated Activation of NAD(P)H Oxidase
PKC-ζ is an atypical PKC that is found in many vascular cell types, including BAEC. We have previously found that PKC-ζ is required for hypochlorite (HOCl)-induced activation of NAD(P)H oxidase in endothelial cells. This led us to investigate whether PKC-ζ is also required for TPr-mediated increases in O₂⁻ and ONOO⁻ in BAEC. Exposure of BAEC to U46619 significantly increased the amount of PKC-ζ in membrane fractions, an index of PKC-ζ activation (Figure 5A). Pharmacological inhibition of PKC-ζ with PKC-ζ-specific myristoylated pseudosubstrate (PKC-ζ-PS) abolished U46619-induced translocation of PKC-ζ from cytosol to membrane fractions (Figure 5A). Similarly, exposure of BAEC to IBOP increased the phosphorylation of PKC-ζ at Thr410 (Figure 5B).

Next, we investigated the role of PKC-ζ in NAD(P)H oxidase activation. NAD(P)H oxidase activation requires the translocation of p67phox from the cytoplasm to the cell membrane. Western blot analysis of membrane fractions revealed that IBOP or U46619 markedly increased membrane locations of p67phox (Figure 5C). Notably, the increase of membrane p67phox was markedly attenuated by the addition of PKC-ζ pseudosubstrate (PKC-ζ-PS), a selective PKC-ζ inhibitor (Figure 5C).

To exclude potential off-target effect of PKC-ζ-PS, we further tested whether genetic inhibition of PKC-ζ affected the effects of TPr in endothelial cells. As depicted in Figure 5D, transfection of PKC-ζ siRNA but not control siRNA markedly lowered the levels of PKC-ζ in BAEC. Moreover, like TPr-specific siRNA, PKC-ζ-specific siRNA significantly reduced IBOP-induced O₂⁻ formation (Figure 5E). Finally, adenoviral overexpression of PKC-ζ dominant negative but not adenoviruses encoding green fluorescent protein (GFP) blocked IBOP-increased formation of eNOS monomer (Figure 5F).

IBOP Increases O₂⁻, ONOO⁻, and eNOS Monomers in Isolated Aortae
We next determined whether TPr activation increases O₂⁻ in isolated aortae ex vivo. Mouse aortae isolated from C57BL/6J

PGL₃ (Figure 3D). As cyclooxygenase is the rate-limiting enzyme for PGL₂ formation, a small reduction of PGIS activity might be due to increased availability of its substrate, cyclooxygenase-derived prostaglandin H₂, to active PGIS.

**TPr Activation Uncouples eNOS**
All 3 NOS are dimeric enzymes comprised of 2 identical subunits with a zinc tetrathiolate (ZnS₄) cluster. The zinc-thiolate structure is essential in maintaining both eNOS dimer structure and activity. We had previously shown that ONOO⁻, formed by O₂⁻ and NO at a diffusion-controlled rate, oxidizes the zinc-thiolate cluster of eNOS and uncouples the enzyme (causing it to form O₂⁻ instead of NO). Thus,
mice were exposed to IBOP for 30 minutes, and \( \text{O}_2^- \) production was assayed by 2 independent methods. Consistent with the findings in cultured BAEC, exposure of mouse aortae to IBOP for 30 minutes significantly increased \( \text{O}_2^- \) production, as measured by both fluorescence imaging and HPLC (Figure 6A and 6B). Coadministration of L-NAME, a noncompetitive NOS inhibitor, markedly attenuated IBOP-induced \( \text{O}_2^- \) formation, suggesting that uncoupled NOS contributes to \( \text{O}_2^- \) production (Figure 6C).

**NAD(P)H Is Required for IBOP and Increases \( \text{O}_2^- \), ONOO\(^-\), and eNOS Monomers in Isolated Aortae**

To further confirm that IBOP increased \( \text{O}_2^- \) production is through NAD(P)H oxidase activation, aortae isolated from gp91phox knockout mice were used. As shown in Figure 6A and 6B, IBOP treatment for 30 minutes increased \( \text{O}_2^- \) production in aortae of wild-type (WT) mice but not gp91phox knockout mice. IBOP also increased ONOO\(^-\) formation in aortae of wild-type mice but not gp91phox knockout mice.
WT aortae by immunohistochemical stain for 3-NT positive proteins, whereas IBOP did not increase 3-NT staining in gp91phox knockout mice aortae (Figure 6D). Finally, exposure of mouse aortae to IBOP increased monomer of eNOS in WT but not in gp91phox knockout mice (Figure 6E).

**IBOP Increases Endothelial Cell Apoptosis and Decreases Cell Viability**
There is abundant evidence that ROS contribute to vascular dysfunction through oxidative damage by impairing endothelial cell growth and causing apoptosis. Therefore, we next determined whether TPr activation affects endothelial cell viability and induces apoptosis. Exposure of BAEC to IBOP for 24 hours significantly decreased cell viability (Supplemental Figure IA) and increased apoptosis (Supplemental Figure IB), which was blocked by TPr antagonist SQ29548.

**Discussion**
In this study, we have provided evidence that TPr stimulation increases the production of ROS by increasing PKC-ζ-dependent NAD(P)H oxidase activation and that constant TPr stimulation causes endothelial dysfunction by uncoupling eNOS activity and increasing tyrosine nitration of PGIS, all of which result in excessive endothelial cell apoptosis.

One of the most important findings of the present study is that TPr stimulation via ONOO− leads to eNOS uncoupling. Recent studies reveal that eNOS uncoupling might play a causal role in cardiovascular diseases, including hypertension, diabetes, and ischemic diseases. In this study, we further advance this concept by demonstrating that eNOS uncoupling arises from TPr stimulation. TPr stimulation not only decreased eNOS activity by increasing inactive eNOS monomers and reducing BH4 levels, but it also decreased NO bioavailability (as measured by cGMP). Notably, L-NAME was less effective than apocynin in suppressing TPr-initiated O2•− formation in isolated aortae. Further evidence for a NAD(P)H oxidase-mediated eNOS uncoupling comes from the study with gp91phox−/− mice. IBOP treatment for 30 minutes increased O2•− and ONOO− (3-NT staining) in the aortae of WT mice but not in those of gp91phox knockout mice. Consistently, exposure of mouse aortae to IBOP increased monomer of eNOS in WT mice but not in gp91phox knockout mice.

Taken together, our data suggest that O2•− and ONOO− derived from TPr-activated NAD(P)H oxidase function as “kindling” oxidants, acting to uncouple eNOS in endothelial cells.

Our earlier studies33,35 have demonstrated that submicromolar levels of ONOO− inhibit PGIS through PGIS nitration and, as a consequence, trigger TPr-mediated vasospasm and endothelial apoptosis. Here, we show that TPr in turn increases PGIS nitration. As we have shown that endogenous ONOO− induces PGIS nitration and TxA2 activation in several disease conditions (eg, in atherosclerotic vessels, hypoxia-reperfusion injury, diabetes, and hypertension),38 the ONOO−-PGIS nitration-TPr activation-ONOOC− pathway might form a feed-forward loop to increase vascular injury and endothelial dysfunction via eNOS uncoupling. This potential pathway is important, as TPr expression and serum levels of multiple TPr ligands are elevated, both locally and...
systemically, in patients undergoing angioplasty and in patients with several vascular and thrombotic conditions, including ischemia, unstable angina, myocardial infarction, and reocclusion after coronary thrombolysis. Increased formation of 3-NT, a marker of ONOO\(^{-}\)/H\(_{2}\)O\(_{2}\), has been detected in many diseases. Because there is overwhelming evidence that ROS play a causal role in the development of cardiovascular diseases and diabetes, TPr-initiated ROS production might contribute to excessive oxidant stress observed in these diseases, and ROS formation might serve as the common pathway for TPr-induced vascular pathways. This observation might also be applicable to other cell types. Our recent report shows that the TXA\(_{2}\) analogs IBOP and U46619 promote the formation of O\(_{2}\)/H\(_{2}\)O\(_{2}\) in vascular smooth muscle cells. PKC-\(\zeta\) is required for TPr-initiated O\(_{2}\)/H\(_{2}\)O\(_{2}\) release and NAD(P)H oxidase activation in endothelial cells. Indeed, we have previously shown that HOCl increases O\(_{2}\)/H\(_{2}\)O\(_{2}\) and ONOO\(^{-}\) generation via PKC-\(\zeta\)-dependent activation of NAD(P)H oxidase. Activation of PKC-\(\zeta\) has also been shown to modulate TPr-mediated apoptosis in cardiac myocytes. How TPr stimulation leads to PKC-\(\zeta\) activation remains unknown and warrants further investigation. In summary, the data presented here reveal that TPr stimulation is a potent instigator of vascular endothelial dysfunction by its ability to enhance NAD(P)H oxidase assembly and translocation to the plasma membrane of the endothelial cell, probably via activation of PKC-\(\zeta\). NAD(P)H oxidase-generated O\(_{2}\)/H\(_{2}\)O\(_{2}\) and ONOO\(^{-}\), in turn, function as kindling oxidants, resulting in eNOS uncoupling, PGIS nitration, and endothelial dysfunction. Our results likely have broad implications in a variety of vascular diseases, including atherosclerosis, diabetes, hypertension, and ischemic injury.

Sources of Funding
This study was supported by NIH Grants (HL079584, HL080499, HL074399, HL085920, HL096032, and HL105157), a Grant-in-Aid from the Juvenile Diabetes Research Foundation, a Research Award...
from the Oklahoma Center for the Advancement of Science and Technology, a Research Award from the American Diabetes Association, and funds from the Travis Endowed Chair of the University of Oklahoma Health Science Center (all to M.-H.Z.).

**Disclosures**

None.

**References**


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Arterioscler Thromb Vasc Biol. published online October 14, 2010;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2010/10/14/ATVBAHA.110.207712.citation

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Supplement Material

Materials

IBOP ([1S-(1 alpha,2 beta(5Z),3 alpha(1E,3R),4 alpha)]-7-[3- (3-hydroxy-4- (4'-iodophenoxy) -1-butenyl)-7-oxabicyclo-[2.2.1]heptan-2-yl]-5-h eptenoic acid), U46619, SQ29548, L-nitro-arginine methyl ester (L-NAME), dihydrorhodamine 123, anti-thromboxane synthase antibody, and enzyme-linked immunoassay kits for 6-keto-PGF1α and cyclic GMP (cGMP) were obtained from Cayman Chemicals (Ann Arbor, MI). MTT cell proliferation assay kit and cell death detection ELISA kit were purchased from Roche (Indianapolis, IN). Protein A-sepharose was purchased from GE Healthcare (Piscataway, NJ).

Dihydroethidium (DHE) and 4,5-Diaminofluorescein diacetate (DAF-2 DA) were purchased from Calbiochem (Gibbstown, NJ). Anti-PKC-ζ, anti-p67phox, and PKC-ζ siRNA were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). TPr siRNA was from Ambion (Austin, TX). Monoclonal antibody against 3-NT was purchased from Upstate Biotechnology Incorporated (Waltham, MA). Rabbit anti-PGIS antibody was from Oxford Biomedical Research, Inc (Oxford, MI). Antibody against eNOS was obtained from Cell Signaling Technology (Boston, MA). Texas red-conjugated anti-rabbit IgG was from Jackson ImmunoResearch Laboratories, Inc. All other reagents were acquired from Sigma (St Louis, MI), unless otherwise indicated.

Cell Culture
Bovine aortic endothelial cells (BAECs) were grown in endothelial basal medium (EBM) containing 2% serum, 100 units/ml penicillin, 100 μg/ml streptomycin and growth factors. Human umbilical vein endothelial cells (HUVECs) were grown in Medium 200 containing 2% serum, 100 units/ml penicillin, 100 μg/ml streptomycin and endothelial cell supplement. All cells were maintained at 37°C under 5% CO₂ and were between the third and tenth passages.

**Animals**

C57BL/6J (WT) mice and gp91phox knockout mice at 2 – 3 months of age were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in temperature-controlled cages under a 12-h light-dark cycle and given free access to water and normal chow. Mice were euthanized with inhaled isoflurane. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Oklahoma University.

**Localization and Quantification of O₂⁻ in Isolated Aortae**

The descending thoracic aorta was removed from C57BL/6J and gp91phox knockout mice, cut into 5 mm-long segments, and suspended in Kreb’s buffer (118.3 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄, 2.5 mmol/L CaCl₂, 25.0 mmol/L NaHCO₃, 0.026 mmol/L EDTA, and 11.0 mmol/L glucose) that was maintained at 37°C. The aortic segments were incubated for 30 min with IBOP (1 μmol/L). The aortic rings were then immersed in tissue-
freezing medium, snap frozen in liquid nitrogen, and cryosectioned into 8-μm thick slices. The slices were then incubated with DHE (10 μmol/L) in a light-protected humidified chamber at 37°C for 30 min. The DHE staining was captured under microscope and analyzed by the same method as the cellular culture staining mentioned above.

**Preparation of Membrane Fractions**

Cells were washed with ice-cold PBS and incubated on ice for 20 min in hypotonic lysis buffer (10 mmol/L Tris, pH 7.4; 0.5 mmol/L EDTA; 1 mmol/L sodium orthovanadate; 1 mmol/L phenylmethylsulfonyl fluoride; 10 µg/ml leupeptin; 10 µg/ml aprotinin). The lysates were centrifuged at 400,000 g for 30 min. The resulting supernatant was saved (cytosolic fraction), and the pellet was resuspended in hypotonic buffer containing 1% Triton X-100. This mixture was re-centrifuged at 400,000 g for 30 min, and the supernatant (membrane fraction) was collected.

**Assay of Cyclic GMP**

Confluent BAECs were treated with 1 μmol/L IBOP for 24 h. After being washed with PBS, cells were stimulated for 15 min with 10 μmol/L of the calcium ionophore, A23187. Cellular cGMP content was determined using an enzyme-linked immunoassay kit according to the manufacturer’s instructions.
Cell Viability and Cell Apoptosis Assay

Cell viability was evaluated using an MTT assay kit and cell apoptosis was detected using a cell death detection ELISA kit according to the manufacturer's instructions.

Immunofluorescence

Thoracic aorta was dissected, fixed in 4% paraformaldehyde for 24 h, and embedded with paraffin. Sections (5 µm) were microwaved in citrate buffer for antigen retrieval. Sections were incubated with protein block buffer then with rabbit anit-3-NT primary antibody for overnight at 4°C. Texas red-conjugated anti-rabbit IgG were used to detect 3-NT. Images were captured by a fluorescence microscope under identical parameters of imaging. The 3-NT staining intensity was quantified using Bioquant Image analysis software (Bioquant/TRW, Nashville TN).
**Supplemental Figure I**

**TxA₂ mimetic IBOP decreases cell viability and increases endothelial cell apoptosis.** A. Cell viability, as determined by MTT assay, in BAECs exposed to 1 µmol/L IBOP for 24 h in the presence or absence of 1 µmol/L SQ29548. *p < 0.05 IBOP treated versus untreated control cells and †p < 0.05 IBOP plus SQ29548 versus IBOP treated cells (n=4). B. Cell apoptosis, as determined by cell death detection ELISA kit, in BAECs exposed to 1 µmol/L IBOP for 24 h in the presence or absence of 1 µmol/L SQ29548. *p < 0.05 IBOP treated versus untreated control cells and †p < 0.05 IBOP plus SQ29548 versus IBOP treated cells (n=4).

**Supplemental Figure II**

**TxA₂ mimetic increases PGIS nitration.** PGIS nitration was analyzed by immunoprecipitation with PGIS and western blot with 3-NT in BAECs treated with 0.1 µmol/L or 1 µmol/L IBOP for 24 h.
A. Cell Viability (% of control)

- control
- IBOP
- SQ29548
- SQ29548 + IBOP

B. Cell Apoptosis (% of control)

- control
- IBOP
- SQ29548
- SQ29548 + IBOP

Zhang et al., Supplemental Figure I