Activation of NAD(P)H Oxidases by Thromboxane A₂ 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 A₂ (TxA₂) and prostaglandin H₂ (PGH₂), 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 [1S-(1α,2β(5Z),3α(1E,3R),4α]-7-[3-(3-hydroxy-4-(d’-iodophenox)-1-butenyl)-7-oxabicyclo[2.2.1]heptan-2-yl]-5’-heptenoic acid (IBOP) or U46619, 2 structurally related TxA₂ mimetics, for 24 hours markedly increased the release of superoxide anions (O₂⁻⁻) and peroxynitrite (ONOO⁻) 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 O₂⁻⁻ 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 O₂⁻⁻ 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 O₂⁻⁻ production. Finally, exposure of isolated mouse aortae to IBOP markedly increased O₂⁻⁻ 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 O₂⁻⁻ and ONOO⁻, resulting in eNOS uncoupling in endothelial cells. (Arterioscler Thromb Vasc Biol. 2011;31:125-132.)

Key Words: nitric oxide synthase • peroxynitrite • prostacyclin • signal transduction • thromboxanes

Increased formation of reactive oxygen species (ROS)/reactive nitrogen species is associated with several cardiovascular disease states.1-3 Multiple oxidase systems contribute to increased levels of ROS/reactive nitrogen species, notably NAD(P)H oxidase.2,4,5 Endothelial nitric oxide synthase (eNOS) itself can generate superoxide anions (O₂⁻⁻) rather than nitric oxide (NO), a phenomenon known as eNOS “uncoupling.”6 This transformation of eNOS from a protective enzyme to a contributor of oxidative stress has been observed in an in vitro model,7 in animal models of cardiovascular diseases,8-10 and in patients with cardiovascular risk factors.11,12 eNOS uncoupling, which is associated with diseases such as diabetes, hypertension, and atherosclerosis, has been ascribed to several mechanisms, including inadequate concentrations of l-arginine and tetrahydrobiopterins (BH4), as well as disruption of the zinc-thiolate center of eNOS.7-12

Eicosanoids, derived from arachidonic acid, are present in a wide variety of human tissues and participate in diverse processes, including inflammation and immunity, hemostasis, wound healing, kidney function, and vessel tone.13 The biosynthesis of eicosanoids involves the formation of thromboxane A₂ (TxA₂), prostacyclin (PGI₂), and prostaglandins. Eicosanoid biosynthetic pathways require 2 cyclooxygenase isoforms (cyclooxygenase 1 and 2), to catalyze the conversion of arachidonic acid to prostaglandin H₂, the central metabolite in the pathway. Most tissues express one or both cyclooxygenase enzymes and produce a variety of eicosanoids.14,15 Several endogenously produced eicosanoids, such as prostaglandin H₂, TxA₂, HETEs, and isoprostanes, exert their physiological effects by binding to the thromboxane receptor (TPr). TPr is a 7-membrane-spanning, G-protein-coupled receptor that on activation initiates many processes important in the pathophysiology of multiple diseases and their sequelae.13 For example, TPr stimulation activates human monocytes and facilitates tumor necrosis factor-α and interleukin-1β production.16 The prothrombotic properties of TPr result from its promotion of platelet activation, aggregation, and degradation. TxA₂ is a potent stimulus for neo intima formation in coronary arteries and promotes smooth muscle cell proliferation and migration.15,16
TPR expression and serum levels of multiple TPR ligands are elevated, both locally and systemically, in patients in several vascular and thrombotic diseases. TPR density is increased in atherosclerotic coronary arteries and in vessels with severe intimal hyperplasia. These observations point 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. Indeed, TPR blockers and TxA2 synthase inhibitors attenuate ischemia/reperfusion-mediated injury in several organs, including the liver and heart. In addition, pharmacological inhibition or genetic deletion of TPR blocks the development of atherosclerosis in nondiabetic apolipoprotein E knockout mice, indicating that TPR is important for the initiation and progression of atherosclerosis.

Recently, activation of TPR by TxA2 has been found to promote O2− formation in vascular smooth muscle cells. 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 O2− and peroxynitrite (ONOO−) by increasing protein kinase C-ζ (PKC-ζ)-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 PGI2 synthase (PGIS).

Materials and Methods
A full description of materials and methods, including cell culture, animals, localization and quantification of O2− in isolated aortae, preparation of membrane fractions, assay 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 O2− in Cultured Endothelial Cells
Dihydrorhodamine (DHE) is a cell-permeable dye that reacts with superoxide anions specifically to produce a hydroxylated product, 2-hydroxy-ethidium (HE-OOH), which spontaneously loses water to produce the fluorescent derivative HO-Et by increasing protein kinase C-ζ (PKC-ζ)-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 PGI2 synthase (PGIS).

HPLC Detection of O2− in Isolated Aortae
Superoxide anions were detected by using HPLC detection of oxyethidium in aortic sections, as described previously.

Measurement of ONOO− Production in Cultured Endothelial Cells
ONOO− formation was determined by ONOO−-dependent oxidation of dihydrorhodamine 123 (DHR 123) to rhodamine 123, as described previously. Briefly, confluent BAEC were treated with IBOP (1 μmol/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 μmol/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 previously. Briefly, BAEC were serum-starved overnight in phenol red-free endothelial cell basal medium (Ebm) medium and then incubated in IBOP (1 μmol/L) or U46619 (1 μmol/L) for 24 hours. The cells were then loaded with DAF-2 DA (5 μmol/L) for 30 minutes, washed with PBS to remove excess probe, and incubated with 10 μmol/L A23187 and 100 μmol/L CaCl2 in the dark at 37°C for 15 minutes. The cells were washed twice with PBS, and DAF-2 fluorescence was measured at a 485-nm excitation wavelength and a 545-nm emission wavelength using a spectrofluorometer (Synergy HT).

Immunoprecipitation and Western Blotting
Immunoprecipitation and Western blotting were performed as described previously.

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
TxA2 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 μmol/L), a potent TxA2 mimetic, for 24 hours. IBOP inhibited insulin-stimulated or vascular endothelial growth factor–stimulated eNOS activity (Figure 1A). Exposure of BAEC to U46619 (1 μmol/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 μmol/L), a potent TPR antagonist (Figure 1A and 1B).

TxA2 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 signifi-
As shown in Figure 2A, treatment of BAEC with IBOP decreased NO release in BAEC. As expected, IBOP significantly increased release of 3-NT 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 3-NT (Figure 2C).

**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 the effects of IBOP on cGMP (Figure 1D), supporting that reduction of NO bioactivity in BAEC.

**The TXA2 Mimetic IBOP Increases PGIS Nitration and Decreases PGI2 Formation**

To determine whether TP receptor activation increased ONOO− formation in BAEC, we first evaluated the conversion of 3H-L-arginine into 3H-citrulline 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 nM) or vascular endothelial growth factor (VEGF) (50 ng/mL) for 10 minutes. A subset of cultures was preincubated with the TP receptor antagonist SQ29548 (10 μmol/L). A P<0.05 versus control; †P<0.01, IBOP or U46619 versus control; ‡P<0.05, IBOP or U46619 versus control; †‡P<0.05, 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. A P<0.05 versus control. NS indicates no statistical difference (n=4).

Western blot analysis confirmed that TP receptor levels were reduced in BAEC transfected with TP receptor-specific siRNA but not in control siRNA-transfected cells (Figure 2B). As shown in Figure 2C, transfection of neither TP receptor-specific siRNA nor control siRNA altered the basal levels of O2− in BAEC. As expected, IBOP significantly increased release of O2− in BAEC transfected with control siRNA, which was inhibited by the addition of tempol (10 μmol/L) (Figure 2C). Conversely, transfection of TP receptor-specific siRNA ablated IBOP-induced O2− release (Figure 2C).

To determine whether TP receptor is required for IBOP-induced O2− production, BAEC were pretreated with SQ29548, a selective TP receptor antagonist. As shown in Figure 2A, treatment of BAEC with IBOP (1 μmol/L) significantly increased O2− release.

To exclude potential off-target effects of SQ29548, TP receptor expression was genetically suppressed by the transfection of TP receptor-specific small interfering RNA (siRNA). Western blot analysis confirmed that TP receptor levels were reduced in BAEC transfected with TP receptor-specific siRNA but not in control siRNA-transfected cells (Figure 2B). As shown in Figure 2C, transfection of neither TP receptor-specific siRNA nor control siRNA altered the basal levels of O2− in BAEC. As expected, IBOP significantly increased release of O2− in BAEC transfected with control siRNA, which was inhibited by the addition of tempol (10 μmol/L) (Figure 2C). Conversely, transfection of TP receptor-specific siRNA ablated IBOP-induced O2− release (Figure 2C).
TPr Activation Uncouples eNOS

All 3 NOS are dimeric enzymes comprised of 2 identical subunits with a zinc thiolate cluster (ZnS₄) structure and activity.³⁴ 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).³² Thus, 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 adenosinoverexpression of the dominant negative mutant of either p47phox or p67 phox.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 1C) and cGMP reduction (Figure 1D). 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, adenosinoverexpression 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.²⁷ 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, adenosinoverexpression of PKC-ζ dominant negative but not adenoviruses encoding...
green fluorescent protein (GFP) blocked IBOP-increased formation of eNOS monomer (Figure 5F).

**IBOP Increases O$_2^{−−}$, ONOO$^{−}$, and eNOS Monomers in Isolated Aortae**

We next determined whether TPr activation increases O$_2^{−−}$ in isolated aortae ex vivo. Mouse aortae isolated from C57BL/6J mice were exposed to IBOP for 30 minutes, and 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 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 O$_2^{−−}$ formation (Figure 6C), suggesting that uncoupled NOS contributes to O$_2^{−−}$ production.

**NAD(P)H Oxidase IsRequired for IBOP Induced O$_2^{−−}$, ONOO$^{−}$, and eNOS Monomers in Isolated Aortae**

To further confirm that IBOP increased O$_2^{−−}$ production is through NAD(P)H oxidase activation, aortae isolated from

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**Figure 3.** TxA$_2$ mimetics increase ONOO$^{−}$ and PGIS nitration and decrease the formation of PGI$_2$. A, Western blot analysis of 3-NT in BAEC treated with IBOP (1 μmol/L) for 24 hours. Band density is expressed in arbitrary units as a percent of control band density. ▲P<0.05, IBOP versus control (n=4). B, Increased ONOO$^{−}$ formation. BAEC were exposed to IBOP (1 μmol/L) for 4 hours with or without a 30-minute pretreatment with SQ29548 (4 μmol/L), tempol (10 μmol/L), or L-NAME (100 μmol/L). ONOO$^{−}$ was measured by using DHR 123 oxidation as described in Materials and Methods. ▲P<0.05, IBOP versus control; NS indicates no statistical difference (n=4). C, Immunoprecipitation (IP)/Western blot (WB) analysis of PGIS nitration in BAEC treated with IBOP (0.1 to 1 μmol/L) for 24 hours. ▲P<0.05 versus control (n=3). D, Effects of IBOP on PGI$_2$ formation. BAEC were treated with IBOP (1 μmol/L) for 24 hours, and PGIS activity was assayed by measuring the level of stable PGI$_2$ metabolite 6-keto-PGF1α in cell culture supernatants. Data are expressed as mean±SEM. ▲P<0.05, IBOP versus control (n=5).

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**Figure 4.** Identification of NAD(P)H oxidase as the source of O$_2^{−−}$ resulting in eNOS uncoupling. A, Effects of NAD(P)H oxidase inhibition on IBOP-induced eNOS oxidation. BAEC were exposed to IBOP (1 μmol/L) for 24 hours after being infected with adenoviruses encoding GFP (ad-GFP), the dominant negative mutants of p47$^{phox}$ (ad-p47$^{phox}$ DN), or the dominant negative mutants of p67$^{phox}$ (ad-p67$^{phox}$ DN), or the dominant negative mutants of p67$^{phox}$ (ad-p67$^{phox}$ DN), eNOS monomers and eNOS dimers were assayed as described in Methods and Materials. ▲P<0.05, IBOP-treated GFP-infected cells versus untreated cells GFP alone; †P<0.05, IBOP-treated cells versus untreated cells after ad-GFP transfection (monomer) (n=3). B, Effects of IBOP and U46619 on BH4 levels in BAEC. BAEC were treated with IBOP (1 μmol/L) or U46619 (1 μmol/L) for 24 hours in the presence or absence of 100 μmol/L apocynin. BH4 was assayed as described in Materials and Methods (n=3). ▲P<0.05, IBOP or U46619 versus untreated cells. NS indicates no statistical difference. C, Effects of NAD(P)H oxidase inhibition on the levels of BH4 and BH2+other biopterins levels in BAEC. Confluent BAEC were treated with IBOP (1 μmol/L) for 24 hours after ad-GFP or ad-p67$^{phox}$ DN transfection. ▲P<0.05 versus untreated GFP-transfected cells; NS indicates no statistical difference (n=3).
gp91phox knockout mice were used. As shown in Figure 6A and 6B, IBOP treatment for 30 minutes increased O$_2^-$ production in aortae of wild-type (WT) mice but not gp91phox knockout mice. IBOP also increased ONOO$^-$ formation in WT aortae by immunohistochemical stain for 3-NT positive proteins, whereas IBOP did not increase 3-NT staining in gp91phox knockout mice (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 O$_2^-$ 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 O$_2^-$ 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 O$_2^-$ and ONOO$^-$ derived from TPr-activated NAD(P)H oxidase function as “kindling” oxidants, acting to uncouple eNOS in endothelial cells.

Our earlier studies$^{33,35}$ 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 TxA$2$ activation in several disease conditions (eg, in atherosclerotic vessels, hypoxia-reperfusion injury, diabetes, and hypertension),$^{33}$ the ONOO$^-$--PGIS nitration-TPr activation-ONOO$^-$ 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\(^{-}\), 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\(_2\)^{-} in vascular smooth muscle cells.

PKC-\(\zeta\) is required for TPr-initiated O\(_2\)^{-} release and NAD(P)H oxidase activation in endothelial cells. Indeed, we have previously shown that HOCl increases 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\(_2\)^{-} 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, HL089920, 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. 2011;31:125-132; originally published online October 14, 2010; doi: 10.1161/ATVBAHA.110.207712

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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 epentenoic 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