Liver Kinase B1 Is Required for Thromboxane Receptor-Dependent Nuclear Factor-κB Activation and Inflammatory Responses

Jinlong He, Yanhong Zhou, Junjie Xing, Qilong Wang, Huaiping Zhu, Yi Zhu, Ming-Hui Zou

Objective—Thromboxane A2 receptor (TPr) has been reported to trigger vascular inflammation. Nuclear factor κB (NF-κB) is a known transcription factor. The aims of the present study were to determine the contributions of NF-κB activation to TPr-triggered vascular inflammation and elucidate the mechanism(s) underlying TPr activation of NF-κB.

Approach and Results—The effects of TPr activators, [1S-[1α,2α(Z),3β(1E,3S*), 4α]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (I-BOP) and U46619, on NF-κB activation, phosphorylation of rhoA/rho-associated kinases and liver kinase B1, cell adhesion and migration, proliferation, and endothelium-dependent vasorelaxation were assayed in cultured human umbilical vein endothelial cells, human monocytes, or isolated mouse aortas. Exposure of human umbilical vein endothelial cells to TPr agonists I-BOP and U46619 induced dose-dependent and time-dependent phosphorylation of inhibitor of κB α in parallel with aberrant expression of inflammatory markers cyclooxygenase-2, inducible nitric oxide synthase, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1. Inhibition of NF-κB by pharmacological or genetic means abolished TPr-triggered expression of inflammatory markers. Consistently, exposure of human umbilical vein endothelial cells to either I-BOP or U46619 significantly increased phosphorylation of inhibitor of κB α, IkappaB kinase, rhoA, rho-associated kinases, and liver kinase B1. Pretreatment of human umbilical vein endothelial cells with the TPr antagonist SQ29548 or rho-associated kinases inhibitor Y27632 or silencing of the LKB1 blocked TPr-enhanced phosphorylation of inhibitor of κB α and its upstream kinase, IkappaB kinase. Finally, exposure of isolated mouse aortas to either U46619 or I-BOP enhanced NF-κB activation and vascular inflammation in parallel with reduced endothelium-dependent relaxation in intact vessels.

Conclusions—TPr stimulation instigates aberrant inflammation and endothelial dysfunction via rho-associated kinases/liver kinase B1 kinase-dependent NF-κB activation in vascular endothelial cells.

Key Words: COX-2  endothelial cell  inflammation  LKB1  NF-κB

The inflammatory response is a protective reaction to all acute and chronic infection. However, chronic or aberrant inflammatory response is considered a key pathological event in cardiovascular diseases, including endotoxic shock, hypertension, and coronary heart disease. Nuclear factor κB (NF-κB) is a key transcription factor and is essential to the initiation and development of inflammatory response. In mammals, the NF-κB family consists of 5 members, RelA/ p65, RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2). Under normal conditions, the NF-κB dimers are maintained in inactive form in the cytoplasm in a complex with inhibitor of κB (IκB). After phosphorylation, IκB undergoes ubiquitination and degradation by the proteasome. IκB-free NF-κB dimers translocate into the nucleus and regulate the transcription of downstream genes. Among genes regulated by NF-κB, cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), and cell adhesion molecules, including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, are well known and play significant roles in endothelial cell activation and dysfunction.

Thromboxane A2 (TXA2) is an eicosanoid produced by thromboxane synthase. Local and systemic elevations in TXA2 have been reported in several thrombotic and vascular diseases. TXA2 binds the tTXA2 receptor (TPr), a G protein–linked receptor, which occurs as 2 alternatively spliced subtypes, TPα and TPβ, in humans. TXA2 is expressed in a number of tissues, including platelets, placenta, vascular endothelial cells, and vascular smooth muscle cells.

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Activation of TPr by TXA₂ is reported to inhibit endothelial cell migration, intercellular communication, and vascular tube formation. Moreover, activation of TPr induces apoptosis in endothelial cells through inhibition of Akt phosphorylation. TXA₂ can also attenuate endothelial insulin signaling through the rho-associated kinase (ROCK)/liver kinase B (LKB)1/ phosphatase and tensin homolog pathway. TPr has also been reported to increase the expression of ICAM-1 and VCAM-1 in human endothelial cells through induction of NF-κB activation. However, how TPr activates NF-κB remains poorly understood. In this study, we investigated the mechanism underlying TPr activation of NF-κB in vascular cells. Our results demonstrate that LKB1 is required for TPr-dependent NF-κB activation.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

U46619 and I-BOP Induce Expression of COX-2 in HUVECs

COX-2 plays a key role in the process of inflammation. To determine whether TPr agonists alter COX-2 expression, human umbilical vein endothelial cells (HUVECs) were starved overnight and treated with TPr agonist U46619 (1 μmol/L) for different periods of time. COX-2 expression was significantly increased 1 hour after U46619 treatment; expression peaked at 4 hours and decreased slightly after prolonged (>4 hours) incubation (Figure 1A and 1B). U46619-induced COX-2 expression was also dose-dependent. Figure 1C and 1D show dose-dependent increase in COX-2 expression 4 hours after U46619 treatment, with a significant increase seen after treatment with 1 μmol/L U46619. Similarly, [1S-[1alpha,2alpha(Z),3beta(1E,3S*)], 4alpha]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (I-BOP), a TPr agonist that is structurally related to U46619, increased COX-2 expression in HUVECs in a time-dependent and dose-dependent manner (data not shown).

Inflammatory proteins such as cell adhesion molecules and iNOS are well-characterized markers of NF-κB activation and play an important role in inflammation in endothelial cells. To elucidate the important roles of TPr in vascular inflammation, we examined the effects of the TPr agonists, U46619 and I-BOP, on levels of ICAM-1, VCAM-1, and iNOS mRNA and protein in endothelial cells. Treatment of HUVECs with either U46619 or I-BOP for 4 hours enhanced expression of all 3 proteins (Figure 1E). Quantitative real-time polymerase chain reaction analysis consistently showed significant upregulation of ICAM-1, VCAM-1, iNOS, and COX-2 mRNAs by U46619 or I-BOP (Figure 1F).

U46619- and I-BOP–Enhanced Inflammation Is TPr Mediated

We next investigated whether TPr was required for U46619-triggered or I-BOP–triggered inflammatory responses. To this end, the TPr-specific antagonist, SQ29548 (10 μmol/L), was added to HUVECs 30 minutes before the addition of U46619 or I-BOP. Whereas SQ29548 alone had no effect on basal levels of COX-2, iNOS, ICAM-1, and VCAM-1, SQ29548 reduced the levels of inflammatory protein (Figure 1 in the online-only Data Supplement), and mRNA enhanced by U46619 (Figure IB–ID in the online-only Data Supplement) and I-BOP (data not shown). These data provide strong evidence that TPr activation promotes inflammatory response in HUVECs. Because the effects of I-BOP and U46619 on TPr-enhanced expression of proinflammatory genes were highly comparable, we examined the effects of TPr activation by interchangeably using I-BOP or U46619.
TPr Activation Activates the NF-κB Pathway

ICAM-1, VCAM-1, iNOS, and COX-2 are well-characterized downstream targets of NF-κB. IkBα phosphorylation by upstream kinases is a central event in NF-κB activation.26–28 Thus, we investigated whether TPr alters the phosphorylation of IkBα. I-BOP (1 μmol/L) increased the phosphorylation of IkBα in a time-dependent manner (Figure 2A), with a significant increase seen after 30 minutes of treatment (Figure 2B). I-BOP consistently lowered levels of total IkBα (Figure 2A), suggesting that IkBα was degraded after being phosphorylated.26–28 Similarly, I-BOP increased levels of p-IkBα in a dose-dependent manner (Figure 2C), with significant changes observed 30 minutes after treatment with a dose of at least 0.01 μmol/L I-BOP (Figure 2D).

TPr Activation of NF-κB Is TPr Dependent

To further confirm the effect of TPr activation on NF-κB activation, HUVECs were treated with SQ29548 (10 μmol/L), a TPr-specific antagonist, 30 minutes before treatment with TPr agonist (I-BOP). As expected, SQ29548 alone had no effect on phosphorylation of IkBα; however, the antagonist abolished the increase in p-IkBα induced by TPr agonists (Figure 2E and 2F).

TPr Activation Increases the Expression of Inflammatory Genes via NF-κB

1x kinase (IKK)α/β are well-known upstream kinases of IkB phosphorylation. Phosphorylation of these kinases at Ser 177 and Ser 181 is linked to their activation.4 Thus, we reasoned that TPr stimulation with I-BOP might activate NF-κB by phosphorylating IKK. I-BOP increased levels of p-IKKβ in a dose-dependent and time-dependent manner (Figure 3A and 3B). To further explore whether the gene expression mediated by TPr agonists was NF-κB mediated, HUVECs were pre-treated with NF-κB-specific inhibitor for 30 minutes before the addition of U46619 or I-BOP. Although the inhibitor alone had no effect, it significantly attenuated TPr-enhanced inflammatory gene expression in HUVECs (Figure 3C).

Translocation of NF-κB subunit p65 into nuclei is a pivotal step in NF-κB activation. Thus, p65-specific small interfering RNA (siRNA) was used to suppress the activity of NF-κB. p65 was significantly decreased by p65-specific siRNA, compared with control siRNA (Figure 3D). As expected, TPr agonist treatment increased the expression of iNOS, COX-2, VCAM-1, and ICAM-1 in cells treated with control siRNA, but not in those treated with p65 siRNA, indicating that NF-κB activation is required for TPr upregulation of COX-2, VCAM-1, ICAM-1, and iNOS expression in endothelial cells.

LKB1 Phosphorylation Is Required for TPr-Induced NF-κB Activation

We previously reported that TPr stimulation increases the phosphorylation of LKB1 at serines 428 and 30723 in vascular smooth muscle cells.19,20 Thus, we investigated whether TPr stimulation alters the phosphorylation of LKB1 at Ser 428 in HUVECs. U46619 increased the level of p-LKB1 in these cells in a time-dependent manner (Figure 4A). Increased LKB1 phosphorylation was detected as early as 5 minutes and peaked

Figure 2. Thromboxane A2 receptor (TPr) agonists activated the nuclear factor κB pathway. A and B, TPr activation increased the phosphorylation of inhibitor of κB (IkBα) in a time-dependent manner. Human umbilical vein endothelial cells (HUVECs) were treated with I-BOP (1 μmol/L) for the indicated times. Western analysis of levels of phosphorylated and total IkBα (A) and quantitation of levels of phosphorylated IkBα (B) were performed. Data are means±SEM. (n=3; *P<0.05 vs 0 hour). C and D, HUVECs were treated with the indicated concentrations of I-BOP for 30 minutes. Western analysis of levels of phosphorylated and total IkBα (C) and quantitation of levels of phosphorylated IkBα (D) were performed. Data are means±SEM (n=3; *P<0.05 vs 0 μmol/L). E and F, HUVECs were preincubated in the presence or absence of SQ29548 for 30 minutes and treated with I-BOP or U46619 (1 μmol/L) for another 30 minutes. Western analysis of levels of phosphorylated and total IkBα (E) and quantitation of levels phosphorylated IkBα (F) were performed. Data are means±SEM (n=3; *P<0.05 vs control; #P<0.05 vs I-BOP alone; $P<0.05 vs U46619 alone).
at 120 minutes, after U46619 treatment. I-BOP also increased the phosphorylation of LKB1 in HUVECs (data not shown).

Next, we assayed whether genetic suppression of LKB1 alters TPr-induced NF-κB activation. As expected, transfection of LKB1-defective mutant (LKB1-S428A, in which serine 428 is replaced with alanine) altered TPr-induced phosphorylation of IKK and consequent NF-κB activation. Because endothelial cells are difficult to transfect with plasmids, we performed the experiments using a human monocytic cell line, human acute monocytic leukemia cell line (THP-1), which were transfected with control small interfering RNA (siRNA) or NF-κB-P65 siRNA or LKB1-D194A (in which Asp 194 is replaced with alanine), or of plasmids encoding AMP-activated protein kinase (AMPK)α1 or AMPKα2, increases NF-κB activation in THP-1 cells.

As expected, U46619 markedly increased the level of phosphorylated forms of IKK, p65, and IκB in THP-1 cells transfected with wild-type LKB1 (Figure 4F). Interestingly, U49919-enhanced phosphorylation of IKK, p65, and IκB was absent in cells overexpressing LKB1 mutants or AMPK (Figure 4F). Taken together, these results further confirm that TPr, via LKB1 phosphorylation at Ser 428, is required for AMPK-independent NF-κB activation.

Antioxidants Do Not Affect TPr-Induced NF-κB Activation

We had previously reported that TPr increased reactive oxygen species (ROS) in both endothelial cells and vascular smooth muscle cells. As expected, incubation of U46619 significantly increased ROS production in HUVECs (data not shown). To establish whether ROS was involved in TPr-enhanced NF-κB–mediated COX-2 expression, HUVECs were preincubated with tempol or mitotempol, which alone slightly increased COX-2 expression, caused a further enhancement of COX-2 in I-BOP–treated HUVECs. Consistently, tempol or mitotempol could not change the increase of p-1xB mediated by I-BOP. As depicted in Figure IIA in the online-only Data Supplement, tempol or mitotempol, which alone slightly increased COX-2 expression, caused a further enhancement of COX-2 in I-BOP–treated HUVECs. Consistently, tempol or mitotempol could not change the increase of p-1xB mediated by I-BOP (Figure IIB in the online-only Data Supplement), indicating that ROS was not required for TPr-increased NF-κB activation.

Silencing of Atypical Protein Kinase C-ζ Does Not Alter TPr-Induced NF-κB Activation

Atypical protein kinase C-ζ (PKC-ζ) is reported to be responsible for LKB1-dependent AMPK activation. To
Phosphorylation of liver kinase B (LKB1) at Ser 428 is required for thromboxane A2 receptor (TPr)–induced nuclear factor κB (NF-κB) activation. A, TPr activation increased phosphorylation of LKB1 in a time-dependent manner. Human umbilical vein endothelial cells (HUVECs) were treated with U46691 (1 μmol/L) for the indicated times, and levels of phosphorylated and total LKB1 were determined by Western analysis. B–E, Silencing of LKB1 blocks TPr agonist–induced NF-κB activation. HUVECs were transfected with control small interfering RNA (siRNA) or LKB1 siRNA for 48 hours and treated with I-BOP or U46619 (1 μmol/L) for 4 hours. Western analysis of levels of phosphorylated and total inhibitor of κB (IκB)α and LKB1 (B) and quantitation of levels of phosphorylated IκBα (C) were performed. Data are means±SEM (n=3; *P<0.05 vs I-BOP alone; $P<0.05 vs U46619 alone). D, Silencing of LKB1 reduces TPr–induced IkappaB kinase (IKK) phosphorylation. Levels of phosphorylated and total IKKα were determined by Western analysis. E, Silencing of LKB1 abolishes TPr–enhanced expression of inducible nitric oxide synthase (iNOS), vascular cell adhesion molecule (VCAM)-1, and intercellular adhesion molecule (ICAM)-1. F, LKB1, but not AMP-activated protein kinase (AMPK)α, is required for TPr–induced NF-κB activation. Human THP-1 cells were transfected with plasmids encoding LacZ, LKB1, but not AMP-activated protein kinase (AMPK)α and β-actin.

ROCK Is the Upstream Regulator of LKB1

Because the results described above refuted the involvement of ROS/PKCζ in TPr-induced NF-κB activation and we had previously found that TPr alters ROCK activity,18 we reasoned that ROCK might be important for LKB1 phosphorylation and consequent induction of inflammatory genes in HUVECs. As shown in Figure 5A, U46619 activated Rho protein in a TPr-dependent manner. As expected, Rho/ROCK inhibitor Y27632 abolished U46619-enhanced phosphorylation of LKB1 (Figure 5B), suggesting that ROCK might be an upstream regulator of LKB1. We next investigated whether Y27632 alters TPr-induced NF-κB activation in HUVECs. Y27632 totally blocked the increase in phosphorylation of IκBα and the decrease in total IκBα induced by U46619 (Figure 5C and 5D), which suggests that the Rho/ROCK pathway is the upstream regulator of NF-κB.

Chelation of Exogenous Calcium With EDTA Abolishes TPr Agonist–Induced NF-κB Activation

Increase of intracellular calcium is a central event in TPr signaling.16,17 Thus, it was important to investigate whether calcium chelation inhibited TPr-induced NF-κB activation. As depicted in Figure IID in the online-only Data Supplement, EDTA, a calcium chelator, abolished the phosphorylation of IκB, indicating that TPr-induced calcium increase was required for NF-κB activation in endothelial cells.
Inhibition of ROCK Blocks TPr-Enhanced Vascular Inflammation

To determine whether ROCK/LKB1 serves as an intermediate component of the TXA2-TPr signaling pathway regulating the inflammatory response, a specific inhibitor of ROCK, Y27632, was added to HUVECs before the addition of TPr agonists. Y27632 ablated TPr-enhanced expression of ICAM-1, VCAM-1, iNOS, and COX-2 (Figure 5E). Downregulation of ICAM-1, VCAM-1, and COX-2 by Y27632 was further confirmed by real-time polymerase chain reaction (Figure 5F).

TPr Activation Impairs Cell Migration and Increased THP-1 Adhesion Through NF-κB Activation

We next investigated whether TPr agonists affect endothelial cell migration in a Boyden chamber assay. The TPr agonist U46619 significantly impaired cell migration (Figure IIIA in the online-only Data Supplement). To further confirm whether this impairment is attributable to TPr-enhanced NF-κB activation, HUVECs were pretreated with SQ29548, Y27632, or NF-κB activation inhibitor before treatment with U46619. SQ29548, Y27632, or NF-κB activation inhibitor alone abolished U46619-impaired migration (Figure IIIA in the online-only Data Supplement), suggesting that NF-κB activation by ROCK is required for U46619 impairment of endothelial cell migration.

Next, we investigated the effects of TPr agonists on cell adhesion. HUVECs were treated with U46619 overnight and incubated with stained THP-1 cells for 30 minutes. Adhesion of THP-1 cells increased in U46619-stimulated HUVECs, and U46619-enhanced adhesion was blocked in cells pretreated with SQ29548, Y27632, or NF-κB inhibitor (Figure IIIB in the online-only Data Supplement). Taken together, these results support the essential role of ROCK-mediated NF-κB activation in endothelial adhesion activity.

TPr Activation Triggered Inflammatory Response in Mice Through NF-κB Activation

Finally, we determined whether TPr agonists triggered a whole-body inflammatory response via NF-κB activation. C57BL/6 mice that had been pretreated with NF-κB inhibitor or vehicle alone were administered U46619. Serum was collected, and cytokines were determined. U46619 significantly increased the levels of interleukin-1β, interleukin-6, and necrosis factorα (Figure IV in the online-only Data Supplement). Importantly, pretreatment with NF-κB inhibitor ablated U46619-induced elevation of the cytokines, compared with pretreatment with vehicle alone.

TPr-Induced NF-κB Activation Impaired Endothelium-Dependent Relaxation

We thought it would be interesting to determine whether TPr-induced NF-κB–mediated expression of the inflammatory genes ICAM-1, VCAM-1, and COX-2 occurs in intact vascular explants and whether overexpression of inflammatory molecules impairs endothelium-dependent relaxation after U46619 treatment. To this end, mouse aortic rings were prepared and assayed for inflammatory markers and acetylcholine-induced endothelium-dependent relaxation. U46619 markedly increased the levels of VCAM-1 and COX-2 and, importantly, pretreatment with NF-κB inhibitor abolished the U46619-induced overexpression (Figure 6A).

We next investigated whether U46619-enhanced NF-κB activation results in abnormal endothelium-dependent relaxation. U46619 caused a 30% reduction in endothelium-dependent vasorelaxation compared with vehicle-treated aortic rings (Figure 6B). U46619 treatment had no effect on endothelium-independent relaxation (data not shown). Unlike U46619, exposure of vessels to phenylephrine, a selective α1-adrenergic receptor agonist, did not alter acetylcholine-induced vasorelaxation (data not shown). Furthermore, pretreatment with SQ29548 or NF-κB inhibitor abolished U46619-impaired vasorelaxation (Figure 6B), suggesting that TPr-mediated NF-κB activation is responsible for impaired relaxation.

Because TPr agonists markedly increased the expression of COX-2, we investigated whether inhibition of COX-2 by NS398, a selective COX-2 inhibitor, has an effect on endothelium-dependent relaxation. As expected, the addition of NS398 significantly improved acetylcholine-induced vasorelaxation (Figure 6C), suggesting that COX-2–derived factors might contribute to impaired endothelium-dependent vasorelaxation in intact vessels.

Discussion

Increasing evidence indicates that aberrant activation of TPr contributes to the initiation and progression of cardiovascular diseases, including atherosclerosis, hypertension, and diabetes mellitus. The present study has unveiled a novel mechanism, whereby TPr, via ROCK-mediated LKB1-dependent NF-κB activation, triggers aberrant vascular inflammation accompanied by impaired vasorelaxation in vivo. Consistently, inhibition of ROCK or LKB1 blocked TPr agonist–mediated NF-κB activation. Finally, inhibition of COX-2, a well-characterized downstream effector of NF-κB, normalized endothelium-dependent relaxation. Overall, our results suggest that ROCK-mediated, but LKB1-dependent, NF-κB activation by TPr might be responsible for aberrant inflammation and vascular dysfunction often seen in cardiovascular diseases (Figure 6D).

The most important finding of this study is the identification of LKB1-dependent NF-κB activation and consequent inflammation in vascular endothelial cells. Overexpression of LKB1, but not AMPK, increased the phosphorylation of IKK and IKBr with the expression of NF-κB effector genes, including ICAM-1, iNOS, and COX-2. Consequently, LKB1 knockdown decreased the phosphorylation of IKKβ mediated by TPr agonists. Interestingly, the effects of LKB1 on NF-κB activation seem to be dependent on ROCK-induced LKB1 phosphorylation. First, we found that ROS-activated PKC-ζ pathway, which is important in LKB1-dependent AMPK activation, was not involved in TPr-induced NF-κB activation, suggesting that neither PKC-ζ nor AMPK are required; Second, inhibition of ROCK with Y27632 blocked TPr-induced NF-κB activation and induction of NF-κB target genes. Finally, the most conclusive evidence, supporting an essential role of LKB1 phosphorylation in TPr-induced NF-κB activation, is that overexpression of a phosphorylation-defective LKB1 mutant (LKB1-S428A) blocked NF-κB activation.
activation. Indeed, we previously reported that TPr agonists increase LKB1 phosphorylation at Ser 428, and that TPr-activated, Rho-mediated LKB1 phosphorylation attenuates insulin signaling in endothelium.18 Because inflammation is a known risk factor for insulin resistance, it is highly anticipated that NF-κB–mediated inflammation might be involved in TPr-induced insulin resistance.

LKB1-enhanced NF-κB activation seems to be mediated by increased IKK phosphorylation. Whether IKK is phosphorylated by LKB1 or another of the LKB1 family kinases remains unclear, and this warrants further investigation. AMPK is one of 14 downstream targets of LKB1,33 and the role of AMPK in NF-κB signaling is highly controversial. For example, AMPK has been reported to directly phosphorylate IKKβ.34 However, our published work and the work of others indicate that AMPK activation inhibited NF-κB activation in endothelial cells, and mice deficient in AMPKκα2 exhibited excessive activation of NF-κB.35 Although the roles of AMPK in the NF-κB pathway remain to be determined, our results suggest that LKB1-dependent IKK phosphorylation is independent of AMPK. Further investigation is warranted.

Endothelium can be considered as an initial pathogenic factor in many diseases, and endothelial cell activation and dysfunction impair cell function or cause vascular inflammation.36,37 Another important finding of this study is that aberrant NF-κB activation by TPr activation causes impairment of endothelium-dependent relaxation in vivo. We have further reported that selective inhibition of COX-2 normalizes vascular relaxation in aortic rings, supporting the idea that NF-κB–mediated induction of COX is operational in intact vascular explants. Importantly, small doses of TPr trigger whole-body inflammation, as evidenced by increased serum cytokine levels in mice in vivo. These results suggest that TPr induces systemic inflammation, and that TPr-induced inflammation extends beyond vascular walls. Thus, our results support the concept that overproduction of TPr is a potent inducer of both vascular and systemic inflammation. As a result, TPr antagonists, ROCK inhibitors, or inhibition of LKB1 are potential key targets to inhibit TXA2-induced inflammation in relevant diseases, such as diabetes mellitus, in which increased TXA2 release is evident.38

In conclusion, this study provides evidence that LKB1 is required for TPr-instigated systemic inflammation, and that inhibition of LKB1 or ROCK might be effective in preventing TPr-induced vascular dysfunction and related disorders.

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None.

Disclosures

None.

References


Significance

Overwhelming evidence suggest that aberrant activation of Thromboxane A_2 receptor (TPr) contributes to the initiation and progression of cardiovascular diseases, including atherosclerosis, hypertension, and diabetes mellitus. By using a combination of gain-of-function and loss-of-function approaches with pharmacological or genetic means, the present study has unveiled a novel mechanism, whereby TPr, via rho-kinase-associated kinase inhibitors, or inhibition of liver kinase B1 are potential key targets to inhibit thromboxane A_2–induced inflammation in relevant diseases, such as diabetes mellitus, in which increased thromboxane A_2 release is evident.
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Supplement Material

Liver kinase B1 is required for thromboxane receptor-dependent NF-κB activation and inflammatory responses

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Supplemental Figure  I

U46619- and I-BOP-enhanced inflammation is mediated by TPr. A-D. HUVECs were treated with 10 μM TPr antagonist SQ29548 30 min prior to treatment with I-BOP (1μM) or U46619 (1μM) for 4 h. Expression of inflammatory genes was detected by western analysis (A) and real-time PCR (B-D). Data are means ± SEM. (n=4; *, p<0.05 versus control).
Supplemental Figure Ⅱ

Effects of antioxidants, calcium chelation, and PKC-ζ inhibition on TPr-induced NF-κB activation in HUVECs. A. HUVECs were treated with I-BOP(1μM) for 4 hours in the presence or absence of Tempol(10μM) or mitotempol(10μM). HUVECs were pre-incubated with Tempol or mitotempol for 30 min prior to the addition of I-BOP or U46619. Expression of COX-2 and β-actin were detected by western blots. B. HUVECs were pre-incubated with or without Tempol(10μM) or mitotempol(10μM) for 30 min prior to treat with I-BOP(1μM) for 30 min. Expression of p-IκBα, IκBα and β-actin were detected as described in Methods and Materials. C. HUVECs were transfected with PKC-ζ siRNA or control siRNA for 48 h. After the transfection, cells were treated with I-BOP or U46619 (1 μM) for 4h. Expression of COX-2 and PKC-ζ were detected by western blots. D. HUVECs were incubated with EDTA(100mM) prior to treat with I-BOP or U46619(1μM) for 30 min. Expression of p-IκBα, IκBα and β-actin were detected by western blots.
Supplemental Figure III

TPαr agonist impaired cell migration and increased THP-1 adhesion through NF-κB activation. HUVECs were pretreated in the presence or absence of SQ29548, Y27632, and NF-κB inhibitor and treated with or without U46619 overnight. **A.** HUVEC migration was assessed using Boyden chamber assay and migrated cells were counted. Data are means ± SEM. (n=4; *, p<0.01 versus control; #, p<0.05 versus U46619 alone) **B.** THP-1 cells were stained and incubated with HUVECs for 30 min. Nonadherent cells were removed by washing and adherent cells were counted. Data are means ± SEM. (n=3; *, p<0.01 versus control; #, p<0.05 versus U46619 alone).
Supplemental Figure IV

TPr agonist induced inflammatory response in mice through NF-κB activation. Mice were pretreated with or without NF-κB inhibitor and treated with U46619 overnight, and levels of indicated serum cytokines were determined. Data are means ± SEM. (n=5–7; *, p<0.05 versus control; #, p<0.05 versus U46619 alone).
Supplemental Table 1

Primers used in this study.

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Materials and Methods

Cells and reagents

Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC (Manassas, VA). I-BOP ([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-heptenoic acid), U46619, and SQ29548 were obtained from Cayman Chemical (Ann Arbor, MI). Antibodies against PKC-ζ, phospho-IκBα, phospho-LKB1 (Ser 428), COX-2, and iNOS were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against β-actin, LKB1, IκBα, ICAM-1, and VCAM-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). NF-κB inhibitor(NF-κB Activation Inhibitor II, JSH-23), tempol and mitotempol were obtained from Santa Cruz Biotechnology. Y27632 was obtained from Calbiochem (Billerica, MA). The Rho activation assay kit was purchased from Thermo Fisher Scientific, Inc. (Rockford, IL). siRNA targeting LKB1, IκBα, PKCζ and control siRNA were purchased from Santa Cruz Biotechnology. The siRNA delivery agent Lipofectamine™ RNAi MAX was from Invitrogen (Carlsbad, CA). All other reagents were acquired from Sigma (St Louis, MO), unless otherwise indicated.

Animals

C57BL/6 wild-type mice, 8-12 weeks of age, were housed in temperature-controlled cages under a 12-h light-dark cycle with free access to water and regular rodent diet, as described previously 1, 2. Mice were injected with 150 μL of 20 mM NF-κB activation inhibitor or phosphate-buffered saline (PBS) once daily for 3 days before injection with 15 μL of 1 mM U46619 or PBS. Mice were euthanized by inhalation of isoflurane, followed by collection of blood and removal of aortas. The animal protocol was approved by the University of Oklahoma Health Science Center Institutional Animal Care and Use Committee.

Cell culture

HUVECs were cultured in endothelial cell basal medium (EBM) supplemented with EGM™ SingleQuot from LONZA (Walkersville, MD), 5% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin as previously described 3. Cells between passages 3 and 8 were used in all experiments. Cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C and grown to 70%-80% confluence before treatment with the indicated agents.

Western analysis
Western analysis was performed as described previously \(^3\). The integrated intensity of individual bands was quantified using densitometry and AlphaEase FC software (Alpha Innotech, San Leandro, CA).

**Real-time polymerase chain reaction (PCR)**

Total mRNA was isolated from HUVECs with the QIAGEN\textsuperscript{®} RNeasy\textsuperscript{®} Mini kit, and mRNA levels of target genes were quantified by real-time PCR as described previously \(^4\). \(\beta\)-actin was used as endogenous control for the statistical analysis. Nucleotide sequences of the primers are shown in Table I.

**siRNA-mediated gene silencing**

Cells were cultured to 70% confluence and transfected with gene target-specific siRNA or control siRNA for 6 h using the delivery agent Lipofectamine\textsuperscript{TM} RNAi MAX according to the manufacturer’s instructions. The medium was replaced with fresh medium, and cells were cultured for another 36 h. Cells were harvested after treatment with the indicated agents.

**Assays of cell migration and adhesion**

Endothelial cell migration assay was conducted using a Boyden chamber purchased from BD Biosciences (San Jose, CA) according to the manufacturer’s instructions. Assays of THP-1 cell adhesion to HUVECs were performed as described previously\(^5\).

**Assays of endothelium-dependent and endothelium-independent vasorelaxation**

Endothelium-dependent and -independent vasorelaxation were assayed in an organ chamber, as described previously \(^1\). Aortic rings approximately 4 mm in length were isolated from C57BL/6 wild-type mice and incubated in EBM supplemented with 7% FBS, 100 IU/mL penicillin, 100 ug/mL streptomycin, and endothelial cell growth factors. Aortic rings were pretreated with SQ29548 or NF-\(\kappa B\) inhibitor for 30 min and treated with U46619 overnight. After treatment, rings were transferred to the organ chamber. Acetylcholine and sodium nitroprusside were used to induce endothelium-dependent and endothelium-independent relaxation, respectively.

**Immunohistochemistry and cytokine assays**

Immunohistochemistry was performed on aortas and serum cytokine levels were determined as described previously, with minor modifications \(^1\).

**Statistical analysis**
All quantitative data were expressed as mean ± standard error of the mean (SEM). Differences between individual groups were analyzed using a one-way, repeated measures analysis of variance with Student's t tests. P<0.05 was considered statistically significant. For nonquantitative data, results were representative of at least three independent experiments.

References:


