Thromboxane A₂ Regulates Vascular Tone via Its Inhibitory Effect on the Expression of Inducible Nitric Oxide Synthase

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Background—Circulatory failure in sepsis arises from vascular hyporesponsiveness, in which nitric oxide (NO) derived from inducible NO synthase (iNOS) plays a major role. Details of the cross talk between thromboxane (TX) A₂ and the iNOS–NO system, however, remain unknown. We intended to clarify the role of TXA₂ via the cross talk, in vascular hyporesponsiveness.

Methods and Results—We examined cytokine-induced iNOS expression and NO production in cultured vascular smooth muscle cells (VSMCs) and cytokine-induced hyporesponsiveness of the aorta from mice lacking the TXA₂ receptor (TP⁻/⁻ mice). The cytokine-induced iNOS expression and NO production observed in wild-type VSMCs were significantly augmented in TP⁻/⁻ VSMCs, indicating an inhibitory effect of endogenous TXA₂ on iNOS expression. Furthermore, in indomethacin-treated wild-type VSMCs, U-46619, a TP agonist, inhibited cytokine-induced iNOS expression and NO production in a concentration-dependent manner, effects absent from TP⁻/⁻ VSMCs. In an ex vivo system, the cytokine-induced hyporesponsiveness of aortas to phenylephrine was significantly augmented in TP⁻/⁻ aorta but was almost completely canceled by aminoguanidine, an iNOS inhibitor. Accordingly, cytokine-induced NO production was significantly higher in TP⁻/⁻ aorta than in wild-type aorta. Moreover, U-46619 significantly suppressed lipopolysaccharide-induced NO production in vivo only in wild-type mice.

Conclusions—These results suggest that TXA₂ has a protective role against the development of vascular hyporesponsiveness via its inhibitory action on the iNOS–NO system under pathological conditions such as sepsis. (Circulation. 2003;108:2381-2386.)

Key Words: thromboxane ■ prostaglandins ■ nitric oxide ■ nitric oxide synthase ■ vasculature

Nitric oxide (NO) produced in vascular endothelial cells is a major player in the maintenance of vascular tone under physiological conditions.¹ In endotoxemia, however, additional NO production by inducible NO synthase (iNOS) is responsible for vascular hyporesponsiveness.² Interaction of NO and prostanoids, so called “cross talk,” has been shown, and the actions of NO on the cyclooxygenase–prostanoid system have been reported.³ However, there have been few reports on the actions of prostanoids on the iNOS–NO system; these actions include an enhancement of the iNOS–NO system by prostaglandin (PG) E₁ in cultured vascular smooth muscle cells (VSMCs) stimulated with interferon (IFN)-γ.⁴ The effects of PGE₁ in macrophage cell lines are controversial, inhibiting⁵ or stimulating⁶ cytokine- or lipopolysaccharide (LPS)-induced iNOS expression. The details of the cross talk between these 2 major inflammatory systems under pathophysiological conditions thus remain unknown.

VSMCs, when stimulated with cytokines or LPS, show exaggerated NO production because of iNOS upregulation both in vitro and in vivo,⁷⁸ indicating that NO works as a mediator of vascular hyporesponsiveness in endotoxemia, along with resulting shock.¹⁰⁻¹² The cytokines also induce the expression of cyclooxygenase-2, an inducible cyclooxygenase isofrom and a rate-limiting enzyme for prostanoid synthesis.¹³,¹⁴ The role of prostanoids in endotoxemia and septic shock remains unknown, however, although urinary metabolites of PGI₂ and thromboxane (TX) A₂ were elevated in patients suffering from sepsis.¹⁵

TXA₂ is a potent vasoconstrictor and a platelet stimulator.¹⁶,¹⁷ It also shows stimulatory action on proliferation or hypertrophy of VSMCs.¹⁸,¹⁹ Recently, we found that the antagonists for the TXA₂ receptor (TP)²⁰,²¹ augmented iNOS expression and NO production in cultured rat VSMCs stimulated with interleukin (IL)-1β, suggesting a negative regulatory role of TXA₂ on the iNOS–NO system in the vascular system. The present study was designed to clarify further the inhibitory action of TXA₂ on the iNOS–NO system and to evaluate the role of the action in the
regulation of vascular tone using VSMCs and the aorta from mice lacking TP (TP−/− mice). In addition, using a septic model, we tested whether the inhibitory action of TXA2 on the iNOS–NO system also works in vivo.

Methods

Mice
TP−/− and wild-type mice have a genetic background similar to that of C57BL/6 mice, as reported previously.23 All experiments, which were approved by the Asahikawa Medical College Committee on Animal Research, were performed in 10- to 15-week-old male mice bred in our laboratory.

Culture and Cytokine Treatment of VSMCs
The culture of VSMCs was performed using aorras as reported previously.19 In short, VSMCs in confluence were cultured in serum-free medium for 24 hours and then stimulated with a mixture of cytokines: IL-1β (20 ng/mL), tumor necrosis factor (TNF-α) (20 ng/mL), and IFN-γ (10 ng/mL). For experiments using U-46619, indomethacin (10 μmol/L) was included during the period of serum starvation and cytokine treatment.

Organ Culture and Cytokine Treatment of the Aorta
Isolated annular pieces of thoracic aorta, 10 mm long for examining iNOS expression, NO production, and TXA2 synthesis or 3 mm long for vascular tone evaluation, were cultured in plates filled with 0.5 mL of DMEM-HEPES buffer containing penicillin (50 U/mL), streptomycin (50 μg/mL), and 0.1% BSA under 5% CO2 and 95% air at 37°C. The cytokine mixture was used to stimulate the tissue for 24 hours. In all experiments, vascular endothelium remained functionally intact, on the basis of the comparison of dilatory responses of aortas to acetylcholine before and after each experiment.

Western Blot Analysis of iNOS Expression
The VSMCs or aorta was lysed in a buffer containing 50 mmol/L Tris/HCl, 2% SDS, 10% glycerol, 2% SDS, 10% glycerol, 1 mmol/L EDTA, and 0.5 mmol/L PMSF at pH 6.8. Protein contents were measured with a BCA assay kit (Pierce Chemical). Samples were separated by SDS-PAGE, and then proteins were transferred onto an Immobilon-P membrane (Millipore). After a blocking procedure, the membrane was incubated with an iNOS-specific antibody for 1 hour at room temperature, followed by incubation with a secondary antibody coupled to horseradish peroxidase. After washing, iNOS protein was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). Signals of these blots were scanned and analyzed with computer software (Image Gauge, Fuji Film).

Estimation of NO and TXA2 Production
After VSMCs or aortas were cultured in the presence or absence of cytokines for 24 hours, culture medium was collected, frozen, and stored at −80°C until use. NO production was estimated by measuring levels of NOx, the oxidized derivatives of NO, with an assay kit (Dojindo Laboratory). TXA2 production was estimated by measuring contents of TXB2, a stable metabolite of TXA2, with an EIA kit (Cayman Chemical).

Measurement of Vascular Tension
After 24 hours of incubation with the cytokines, aortic rings were transferred into a bath filled with Krebs-Henseleit solution (composition in mmol/L: NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, and glucose 11, pH 7.4), and were mounted onto holders connected to a transducer with a polygraph recorder (Star Medical). The bathing solution was aerated with 95% O2 and 5% CO2 at 37°C. After an equilibration period, the preparations were precontracted twice with KCl (45.2 mmol/L). Then phenylephrine, an α1-adrenoceptor agonist, or serotonin was added cumulatively. Aminoguanidine (100 μmol/L) and indomethacin (10 μmol/L) were added 30 minutes before the addition of phenylephrine. When the vasodilatory effect of sodium nitroprusside on the aorta precontracted with phenylephrine (10 μmol/L) was examined, it was added cumulatively.

NO Production in a Septic Model
Blood was collected by cardiac puncture 20 hours after injection of LPS (30 mg/kg IP) alone or LPS plus U-46619 (100 μg/kg IV) and was centrifuged at 1500 g for 15 minutes in the presence of EDTA (1 mg/mL). The NOx level in plasma was then measured with a kit (Dojindo Laboratory).

Reagents
Phenylephrine, serotonin, sodium nitroprusside, indomethacin, PMSF, and LPS (026:B6) were purchased from Sigma Chemical. U-46619 was from Cayman Chemical. Aminoguanidine was purchased from Calbiochem. Penicillin, streptomycin, and DMEM were purchased from Gibco-BRL, and BSA from Seikagaku Kogyo. Murine IL-1β and IFN-γ were obtained from Peprotech, and TNF-α from Genzyme/Technie. Anti-iNOS monoclonal antibody was from Transduction Laboratories. Anti-murine IgG and anti-rabbit IgG were from Amersham Pharmacia Biotech.

Statistical Analysis
All data are expressed as mean±SEM. Statistical analyses were performed with Student’s t test or 2-way ANOVA followed by Dunnett’s test. Analysis and graphing of the data were performed with Prism III (GraphPad Software). The symbol n shows the number of independent experiments performed in samples prepared from different animals. Probability values of P<0.05 were considered to be significant.

Results

Effects of Cytokines on iNOS Expression in Wild-Type VSMCs
We first examined the effect of cytokines on iNOS expression in cultured VSMCs. We used a cytokine mixture of IL-1β, TNF-α, and IFN-γ to stimulate VSMCs because these are the typical inflammatory cytokines released abundantly during acute-phase responses. In wild-type VSMCs, the cytokines induced iNOS expression in a time-dependent manner (Figure 1A), whereas its expression was not detected in the absence of cytokines (data not shown). The expression of iNOS protein was apparent at 8 hours of incubation with cytokines and reached a plateau after 24 hours.

Endogenous TXA2 Inhibits iNOS Expression and NO Production in Cytokine-Stimulated VSMCs
To examine the effect of endogenous TXA2 on cytokine-induced iNOS expression, we compared levels of iNOS expression and NO production between wild-type and TP−/− VSMCs. The expression level of iNOS was significantly greater in TP−/− VSMCs than in wild-type VSMCs (Figure 1B). In addition, NO production was significantly increased in TP−/− VSMCs over that in wild-type VSMCs (Figure 1C), indicating an inhibitory effect of endogenous TXA2 on the iNOS–NO system. TXA2 production was similar between wild-type and TP−/− VSMCs; these were 3.5±1.4 and 7.3±3.5 pg/mg of protein, respectively (P=0.30, n=3 to 4).

U-46619 Has a Potent Inhibitory Effect on the iNOS–NO System
To evaluate the effect of TXA2 on iNOS expression without any influence of other prostanoids, we used U-46619, a TP
agonist, and indomethacin. In wild-type VSMCs, U-46619 inhibited the cytokine-induced iNOS expression in a concentration-dependent manner, with the inhibition reaching 34% of control (Figure 2, A and B). The inhibitory effect was not observed at all in TP−/− VSMCs, indicating that TP mediates the effect of U-46619 (Figure 2A). In agreement with this result, U-46619 inhibited cytokine-induced NO production in a concentration-dependent manner in wild-type VSMCs (Figure 2C); in addition, the inhibitory effect of U-46619 disappeared from TP−/− VSMCs (data not shown).

These results clearly show that both endogenous TXA2 and an exogenous TP agonist inhibit the iNOS–NO system in cultured VSMCs via TP.

Effect of Endogenous TXA2 on NO Production in the Aorta Ex Vivo
To confirm that the inhibitory effect of endogenous TXA2 on iNOS expression also works in the aorta, we examined NO production in cytokine-stimulated aortas (Figure 3A). In both wild-type and TP−/− aortas, cytokines increased NO production significantly over that in respective control aortas. The increase, however, was significantly higher in TP−/− aorta than in wild-type aorta, indicating that the inhibitory effect of endogenous TXA2 on NO production also works ex vivo. In control and cytokine-treated wild-type aortas, the contents of TXB2 were 217±44 and 308±35 pg/mg of protein, respectively (P=0.15, n=4 to 6). In TP−/− aortas, these were 201±37 and 302±37 pg/mg of protein, respectively (P=0.10, n=4 to 6). Thus, there were no significant differences in TXA2 production between wild-type and TP−/− aortas under both control and cytokine-treated conditions.

Effect of Endogenous TXA2 on the Vascular Tone
We next examined whether TXA2 plays a role in the control of vascular tone ex vivo. Phenylephrine elicited contractile responses in both wild-type and TP−/− control aortas, and the maximum tensions were 553±17 and 525±17 mg (n=10), respectively, indicating that there was no difference in contractility between wild-type and TP−/− aortas. Aminoguanidine, indomethacin, or both did not affect the phenylephrine-induced contractile responses in the control aortas (data not shown).

In wild-type aorta, cytokine treatment significantly attenuated the phenylephrine-induced contractile response; the maximum tension was significantly decreased, and the EC50
value of the response was significantly increased (Figure 3B and the Table). The decrease in the maximum tension was restored almost completely by pretreatment with aminoguanidine, a selective iNOS inhibitor, whereas the recovery of the potency of phenylephrine was partial. Conversely, indomethacin also restored the cytokine-induced hyporesponsiveness and indomethacin in cytokine-induced decrease in maximum tension of phenylephrine-contracted aortas. Values are percentage of maximum tension obtained in respective cytokine-untreated (control) aorta. \( P \leq 0.05 \) vs respective aorta from wild-type mice. \( P \leq 0.05 \) vs respective control aorta from wild-type mice.

Figure 3. Roles of endogenous TXA\(_2\) in cytokine-induced NO production and hyporesponsiveness of aorta ex vivo. A, Increased production of NO in cytokine-stimulated aorta from TP\(^{-/-}\) mice. Aortas were treated with vehicle or cytokines for 24 hours. \( P \leq 0.05 \) vs respective control aorta. \( P \leq 0.05 \) vs cytokine-treated aorta from wild-type mice. \( n = 5 \). B, Effects of aminoguanidine and indomethacin on cytokine-induced decrease in maximum tension of phenylephrine-contracted aortas. Aminoguanidine: \( n = 5 \) to 14.

Role of TXA\(_2\) in a Septic Model In Vivo

Finally, using a septic model in which LPS was injected intraperitoneally, we examined whether the inhibitory action of TXA\(_2\) on the iNOS–NO system works in vivo. When we examined the aortic responsiveness to phenylephrine 20 hours after LPS injection, there was no change in responsiveness in either wild-type or TP\(^{-/-}\) mice, suggesting that the production of inflammatory cytokines was insufficient to induce aortic hyporesponsiveness, at least in the present murine model. However, mice showed septic hypotension several hours after LPS injection, suggesting that the vascular hyporesponsiveness took place at peripheral blood vessels. In agreement with this result, LPS treatment induced a marked increase in NO production (Figure 4). Although there was no difference in the degree of NO production between wild-type and TP\(^{-/-}\) mice treated with LPS alone, U-46619 suppressed LPS-induced NO production significantly, by 56%, in wild-type mice, which effect was absent in TP\(^{-/-}\) mice (Figure 4). These results suggest that TXA\(_2\) is able to inhibit the iNOS–NO system under a systemic inflammatory condition in vivo.

### Table: Effects of Aminoguanidine and Indomethacin on EC\(_{50}\) Values in Phenylephrine-Induced Contractions of Cytokine-Treated Aortas

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EC(_{50}), nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta from wild-type mice</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>54 ± 5</td>
</tr>
<tr>
<td>Cytokines</td>
<td>298 ± 51*</td>
</tr>
<tr>
<td>+ Aminoguanidine</td>
<td>94 ± 20*</td>
</tr>
<tr>
<td>+ Indomethacin</td>
<td>182 ± 44*</td>
</tr>
<tr>
<td>+ Aminoguanidine and indomethacin</td>
<td>99 ± 18*</td>
</tr>
<tr>
<td>TP(^{-/-}) mice</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>62 ± 7</td>
</tr>
<tr>
<td>Cytokines</td>
<td>483 ± 66*†</td>
</tr>
<tr>
<td>+ Aminoguanidine</td>
<td>248 ± 43*†</td>
</tr>
<tr>
<td>+ Indomethacin</td>
<td>274 ± 36*†</td>
</tr>
<tr>
<td>+ Aminoguanidine and indomethacin</td>
<td>121 ± 27*†</td>
</tr>
</tbody>
</table>

\( n = 5 \) to 14.

\( * P \leq 0.05 \) vs respective control aorta.

\( † P \leq 0.05 \) vs respective aorta from wild-type mice.
Discussion

We previously showed an inhibitory effect of TXA2 on cytokine-induced iNOS expression in cultured rat VSMCs, using TP antagonists. However, these antagonists had some limitations in evaluating the net contribution of TXA2 because of their low specificities, which could lead to the activation of receptor(s) other than TP, such as EP2,25,26 and of their simultaneous inhibition of TXA2 synthase.22 In the present study, we used TP−/− mice to clarify the role of TXA2 in the regulation of vascular tone.

We have shown that inflammatory cytokines induced time-dependent iNOS expression in murine VSMCs as reported in other species, indicating that NO participates in the regulation of vascular function under inflammatory conditions in mice also. To examine the effect of endogenous TXA2 on the cytokine-induced expression of iNOS, we compared iNOS expression between wild-type and TP−/− VSMCs. Indeed, endogenous TXA2 inhibited iNOS expression and subsequent NO production in cytokine-stimulated VSMCs, showing clearly that TP mediates the inhibitory effect of TXA2 on the iNOS−NO system.

Because endogenous prostanooids other than TP, such as PGE2 and PGI2 may increase iNOS expression7 and thereby antagonize TXA2 function, we examined the effect of an exogenous TP agonist, U-46619, on iNOS expression in cultured VSMCs in the presence of indomethacin, which would abrogate production of endogenous prostanooids. The result was that U-46619, via TP, inhibited the cytokine-induced iNOS expression and subsequent NO production.

On the basis of the effects of TXA2 on cultured VSMCs, we next determined whether TXA2 works as a regulator of vascular tone ex vivo. In wild-type aorta, the cytokines induced hyporesponsiveness to phenylephrine, which was significantly restored by pretreatment with aminoguanidine, suggesting a major role of the iNOS−NO system in the hyporesponsive phenomenon. In TP−/− aorta, the hyporesponsiveness to phenylephrine was significantly aggravated but was abolished by pretreatment with aminoguanidine to an extent similar to that found in wild-type aorta, suggesting augmented expression of iNOS in cytokine-treated TP−/− aorta. In support of this result, NO production was significantly increased in cytokine-treated TP−/− aorta over that in cytokine-treated wild-type aorta. These results clearly show that TXA2 plays a role in the control of vascular tone.

Pretreatment of aortas with aminoguanidine restored the maximum tension elicited by phenylephrine almost completely in both wild-type and TP−/− aorta. The increases in EC50 values in phenylephrine-induced contraction, however, were only partially restored by pretreatment with aminoguanidine in both wild-type and TP−/− aortas, suggesting the participation of mediator(s) other than NO in hyporesponsiveness. In the wild-type aorta, indomethacin significantly restored the cytokine-induced hyporesponsiveness to a level comparable to that seen with pretreatment with aminoguanidine, suggesting the vasorelaxant prostaglandins, such as PGI2, to be other major players in the hyporesponsive phenomenon, and their participation in concert with NO. In TP−/− aorta, however, the restoration of maximum tension by pretreatment with indomethacin was significantly lower than that with aminoguanidine, suggesting that the iNOS−NO system had become a major and dominant modulator of vascular tone in cytokine-treated TP−/− aorta because of the lack of inhibition of iNOS expression by TXA2. As expected, combined pretreatment with both aminoguanidine and indomethacin further restored the hyporesponsiveness in both wild-type and TP−/− aorta to a similar extent. However, after the combined pretreatment, the EC50 values remained somewhat higher in both cytokine-stimulated wild-type and TP−/− aortas than those in respective control aortas, suggesting the participation of factor(s) other than NO or prostanooids in the hyporesponsiveness.

We finally examined the regulatory role of TXA2 on the iNOS−NO system in vivo using a septic model. LPS stimulates the secretion of several kinds of inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and IFN-γ, and reproduces a septic condition. In this model, however, we could not detect aortic hyporesponsiveness to phenylephrine in either wild-type or TP−/− mice. This was in contrast to cytokine-induced aortic hyporesponsiveness ex vivo, suggesting an insufficient production of inflammatory cytokines to induce iNOS expression in the aorta under the present conditions. After the injection of LPS, symptoms of septic shock appeared gradually, and the blood pressure fell beyond the limit of measurement (systolic blood pressure <60 mm Hg) in both wild-type and TP−/− mice, suggesting the establishment of hyporesponsiveness at the level of peripheral blood vessels. In accordance with the development of hypotension, NO production increased significantly more than 20-fold above basal values in both wild-type and TP−/− mice. There was no difference, however, in the degree of LPS-induced NO production between wild-type and TP−/− mice, suggesting that the treatment with LPS alone could not stimulate the production of TXA2 sufficiently to inhibit iNOS expression. Therefore, to test this possibility, we examined the effect of U-46619 administered exogenously on NO production in the septic model. Intravenous administration of U-46619 has been used as a model of sudden death caused by systemic thromboembolism. In the present experiment, we decreased the dosage of U-46619 to avoid a massive thromboembolism and sudden death (mortality <20%). In this condition, U-46619 showed a potent inhibitory effect on LPS-induced NO production in wild-type mice, indicating that the activation of TP indeed inhibits LPS-induced NO production in vivo. These results
indicate that TXA₂ may play an important supporting role in the maintenance of the vascular responsiveness under septic conditions, when large amounts of TXA₂ are produced by activated platelets, as seen in disseminated intravascular coagulation accompanying the septic condition. It is noteworthy, however, that PGH₂, isoprostanes, or other lipid peroxidation products might participate in the inhibition of the iNOS–NO system, because these agents could activate TP under systemic inflammatory conditions.

TP is expressed abundantly in VSMCs, and TXA₂ is consequently one of the most potent vasoconstrictors, along with other mediators such as angiotensin II and endothelin-1. Interestingly, the inhibitory effects of angiotensin II and endothelin-1 on cytokine-induced iNOS expression have also been reported in cultured rat VSMCs, although the pathophysiological roles of these effects in the control of vascular tone are not known. The receptors corresponding to these vasoconstrictors all belong to the family of G protein–coupled receptor and activate primarily Go, suggesting that the inhibitory effect on iNOS expression is a common feature of the vasoconstrictors activating Go and that this effect would maintain vascular tone in a coordinating manner. In this way, the interaction of TXA₂ and the iNOS–NO system apparently may not be a specific event.

However, considering a coordinated TXA₂ production and iNOS NO system apparently may not be a specific and important phenomenon, as shown in this study. In contrast, some investigators have reported that the prostaglandins activating Go, such as PGE₂ and PGH₂, have a stimulatory effect on iNOS expression, however, remains to be clarified.

In conclusion, the present study clearly showed that TXA₂ downregulates iNOS expression in VSMCs stimulated with the cytokines both in vitro and ex vivo and that the action is mediated by TP. Moreover, we showed that the stimulation of TP inhibits NO production in an inflammatory model in vivo. These findings would contribute to better understanding of the mechanism underlying the hyporesponsiveness of the vascular tissues under systemic inflammatory conditions.

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References

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