Thromboxane A2/Prostaglandin H2 Receptor Activation Mediates Angiotensin II–Induced Postischemic Neovascularization

Frédéric Michel, Jean-Sébastien Silvestre, Ludovic Waeckel, Stefano Corda, Tony Verbeuren, Jean Paul Vilaine, Michel Clergue, Micheline Duriez, Bernard I. Levy

Objective—We analyzed the involvement of thromboxane (TX) A2/prostaglandin (PG) H2 (TP) receptor in ischemia-induced neovascularization in mice.

Methods and Results—Unilateral hindlimb ischemia was induced by right femoral artery ligature in male C57BL/6J mice (n=7 per group). Animals were then treated with or without TP receptor antagonist (S18886, 5 or 10 mg/kg per day; ramatroban, 10 mg/kg per day) or aspirin (30 mg/kg per day) in drinking water for 21 days. Hindlimb ischemia raised plasma level of TXB2, the stable metabolite of TXA2, by 4.7-fold. This increase was blocked by aspirin treatment whereas S18886 (5 or 10 mg/kg per day) had no effect. However, neither S 18886 nor aspirin affected postischemic neovascularization. We next assessed the putative involvement of TXA2 signaling in angiotensin II (Ang II) proangiogenic pathway. Ang II (0.3 mg/kg per day) enhanced TXB2 plasma levels by 2.6-fold over that of control (P<0.01). Ang II-induced TXB2 upregulation was reduced by cotreatment with Ang II type I receptor antagonist (candesartan, 20 mg/kg per day). Angiographic score, capillary number, and foot perfusion were improved by 1.7-, 1.7-, and 1.4-fold, respectively, in Ang II-treated mice compared with controls (P<0.05). Ang II proangiogenic effect was associated with a 1.6-fold increase in VEGF-A protein content (P<0.05) and a 1.4-fold increase in the number of Mac-3–positive cells (ie, macrophages) in ischemic areas (P<0.05). Interestingly, treatments with TP receptor antagonists or aspirin hampered the proangiogenic effects of Ang II.

Conclusion—Endogenous activation of TXA2 receptor by eicosanoids did not modulate spontaneous neovascularization in the setting of ischemia. Conversely, TXA2 signaling is involved in Ang II-induced AT1-dependent vessel growth.

Key Words: angiogenesis ■ angiotensin II ■ ischemia ■ thromboxane A2

Neovascularization occurs in response to local tissue ischemia mainly through inflammation and hypoxia signaling. One mechanism by which hypoxia activates blood vessel growth is the increase in hypoxia-inducible factor (HIF-1α) leading to the expression of key growth factors, including vascular endothelial growth factor (VEGF-A) and fibroblast growth factor (FGF).1 Macrophages and T lymphocytes also promote neovascularization in ischemic areas through the release of proinflammatory cytokines, production of matrix metalloproteinases, and expression of angiogenic factors.2–4 Neovascularization takes place in the context of cross-talk involving numerous factors. Among them, hormones such as angiotensin II (Ang II) have been shown to modulate postischemic neovascularization by activation of Ang II type I receptor (AT1) and upregulation of VEGF-A signaling.5,6 Ang II also enhances macrophage infiltration and subsequently inflammation-dependent vessel growth.6–8

Thromboxane A2 (TXA2) is an unstable metabolite of arachidonic acid formed through the cyclooxygenase pathway. TXA2 is released from activated platelets, monocytes, and damaged vessel wall, and causes platelet aggregation, vasoconstriction, and hypertrophy of vascular smooth muscle.9 Action of TXA2 is mediated by thromboxane A2/PG H2 receptor (TP receptor), which are also able to bind other endogenous ligands such as endoperoxides and isoprostanes. TP receptors are widely expressed in the vasculature and exist as 2 isoforms TPα and TPβ in humans, whereas only TPα is present in rodents.10 As in many vasoconstrictive substances, thromboxane A2 (TXA2) has the potential to participate in the regulation of blood vessel growth, but its effect on angiogenesis remain controversial. The TXA2 mimetic IBOP inhibits migration and proliferation of cultured endothelial cells and formation of vascular-like structure in the matrigel model.11,12 TP receptor agonists block the proangiogenic effects of
Fibronectin (dilution, 1:50) to identify capillaries. Capillary density was then calculated in randomly chosen fields of a definite area using Optimab/Pro software.

Laser Doppler Perfusion Imaging
We gathered functional evidence for ischemia-induced change in mouse hindlimb vascularization. Blood perfusion in the paw was assessed through laser Doppler imaging, as previously described.3 For each animal, perfusion was measured in both the ischemic and nonischemic paw and the ratio was calculated. Results were expressed as the mean ratio of ischemic to nonischemic values for each experimental group.

Determination of VEGF-A Expression
VEGF-A protein expression was determined by Western blot in ischemic and nonischemic legs, as previously described.3 Gastrocnemius samples were homogenized in a lysis buffer supplemented with protease inhibitor. Samples were loaded on an SDS-PAGE gel (10%) and protein transferred to a nitrocellulose sheet (Hybond enhanced chemiluminescence [ECL]; Amersham). Blots were incubated with antibodies against VEGF-A (Santa Cruz Biotechnology; dilution of 1:2,000). As a protein loading control, membranes were stripped, incubated with a goat polyclonal antibody directed against total actin (Santa Cruz Biotechnology; dilution of 1:5,000). Specific protein was detected by chemiluminescence reaction (ECL; Amersham).

Determination of Plasma TXB2 Concentration and Tissue Ang II Levels
At time of euthanization, blood was collected and plasma samples were recovered by centrifugation without clot and stored at −20°C until analysis. Plasma TXB2 concentration was measured using a commercially available enzyme immunoassay kit (Cayman Chemical). Ang II tissue content was measured in ischemic and nonischemic gastrocnemius using Ang II enzyme immunoassay kit, according to manufacturer instructions (Spi Bio).

Evaluation of Macrophage Number (Mac-3–Positive Macrophages)
Frozen tissue sections (7 μm) were incubated with rat polyclonal antibody directed against Mac-3 (1:50; BD Pharmingen). After incubation with a biotinylated anti-rat IgG, immunostains were visualized by using avidin-biotin horseradish peroxidase visualization systems (Vectorstain ABC kit elite; Vector Laboratories).

Statistical Analysis
Results are expressed as mean±SEM. One-way analysis of variance ANOVA was used to compare each parameter. Post hoc Bonferroni t-test comparisons were then performed to identify which group differences account for the significant overall ANOVA. A value of P<0.05 was considered significant.

Results
Ischemia Increases Ang II and TXA2 Plasma Levels
Plasma levels of TXB2, the stable metabolite of TXA2, were assayed by enzyme-linked immunosorbent assay (ELISA). Hindlimb ischemia raised TXB2 plasma levels by 4.7-fold compared with sham nonischemic animals (P<0.01; Figure 1A). As expected, treatment of ischemic mice with S 18886 at 5 or 10 mg/kg per day did not significantly affect TXB2...
TXA2 signaling is not involved in the spontaneous neovascularization (Figure 1B) and foot perfusion ratio (Figure 1C). Therefore, inhibition with aspirin (30 mg/kg per day) was the negative control. However, S 18886 or ramatroban administration did not affect the ischemic to nonischemic angiographic score compared with Ang II-treated animals; P<0.05. Administration of AT1 receptor blocker reduced TXB2 to untreated levels (5.4±1.1 ng/mL versus 12.8±2.2 ng/mL in Ang II and candesartan-treated mice compared with Ang II-treated animals; P<0.05). Treatment with S 18886 did not affect the Ang II-induced increase in TXB2 plasma levels (12.2±1.7 ng/mL). Aspirin administration hampered the increase in TXB2 plasma levels observed after Ang II treatment (6.8±1.0 ng/mL versus 12.8±2.2 ng/mL in Ang II-treated and aspirin-treated mice compared with Ang II-treated animals; P<0.05). Taken together, these results suggest that Ang II upregulated TXA2 levels through AT1-dependent mechanism.

**TXA2 and Ang II Proangiogenic Effect**

The angiographic score, capillary number, and paw perfusion was improved by 1.7-, 1.7-, and 1.4-fold, respectively in Ang II-treated mice when compared with controls (P<0.05; Figure 2). S 18886, ramatroban, and aspirin treatments hampered the Ang II-induced increase in the neovascularization process (P<0.05 versus Ang II-treated mice). AT1 receptor blockade also reduced Ang II-induced activation of postischemic neovascularization (Figure 2).

**Ang II Neovascularization**

In the absence of ischemia, Ang II alone increases TXB2 plasma levels by 1.5-fold compared with untreated mice (1.9±0.3 ng/mL versus 1.3±0.2 ng/mL in Ang II-treated mice versus untreated animals, respectively; P<0.05, n=5). VEGF-A protein levels were raised Ang II tissue levels by 1.4-fold 7 days after the onset of ischemia (18.7±2.5 pg/mg proteins versus 13.4±2.1 pg/mg proteins in ischemic gastrocnemius versus non ischemic gastrocnemius; P<0.05, n=6). Exogenous administration of Ang II, by osmotic minipump, enhances Ang II tissue contents by 2.9-fold (54.2±14.2 pg/mg proteins; P<0.05 versus untreated ischemic gastrocnemius). We therefore analyzed the putative involvement of TXA2 in Ang II proangiogenic effect in the setting of ischemia.

**TXA2 Synthesis and Ang II**

In the absence of ischemia, Ang II further enhanced TXB2 plasma levels by 2.6-fold compared with untreated ischemic mice (12.8±2.2 ng/mL versus 4.9±0.6 ng/mL in Ang II-treated mice compared with untreated animals, respectively; P<0.01). Administration of AT1 receptor blocker reduced TXB2 to untreated levels (5.4±1.1 ng/mL versus 12.8±2.2 ng/mL in Ang II and candesartan-treated mice compared with Ang II-treated animals; P<0.05). Treatment with S 18886 did not affect the Ang II-induced increase in TXB2 plasma levels (12.2±1.7 ng/mL). Aspirin administration hampered the increase in TXB2 plasma levels observed after Ang II treatment (6.8±1.0 ng/mL versus 12.8±2.2 ng/mL in Ang II-treated and aspirin-treated mice compared with Ang II-treated animals; P<0.05). Taken together, these results suggest that Ang II upregulated TXA2 levels through AT1-dependent mechanism.

**TXA2 and Ang II Signaling**

The angiographic score, capillary number, and paw perfusion was improved by 1.7-, 1.7-, and 1.4-fold, respectively in Ang II-treated mice when compared with controls (P<0.05; Figure 2). S 18886, ramatroban, and aspirin treatments hampered the Ang II-induced increase in the neovascularization process (P<0.05 versus Ang II-treated mice). AT1 receptor blockade also reduced Ang II-induced activation of postischemic neovascularization (Figure 2).
Discussion

The main results of this study are that TXA2 does not modulate spontaneous neovascularization reaction after hindlimb ischemia. Conversely, TXA2 signaling is involved in Ang II-induced AT1-dependent vessel growth in ischemic areas.

The role of TXA2 signaling in angiogenesis remains controversial. TP receptor activation has been shown to either activate or inhibit endothelial cell proliferation and angiogenesis in different experimental models.17,18 This apparent opposite action is likely related to the existence of 2 TP isoforms, α and β, with opposite effects. TPβ expression and subsequent signaling result in inhibition of angiogenesis, whereas TPα activates the angiogenic phenotype.15,14 Animal models lack TPβ, whereas cultured endothelial human cells express both isoforms and, in these cells, TPβ-related effects overcome that of TPα, resulting in inhibition of the angiogenic reaction.10

In this study, we hypothesized that the TXA2-related pathway may affect the proangiogenic effect of exogenously added angiogenic factors, such as Ang II. In absence of ischemia, Ang II improves TXB2 plasma levels but is unable to modulate capillary number. Similarly, normal arteries are known to be totally immune against exogenous growth factors likely because growth factor receptors are rapidly downregulated.26,27 In contrast, in the setting of ischemia, we showed that Ang II further increases TXB2 plasma level through AT1-dependent mechanism and that TP blockers administration hamper Ang II-induced vessel growth. In addition, TP receptor antagonists totally abrogate the Ang II-induced VEGF-A upregulation suggesting that TXA2 is an upstream regulator of VEGF-A protein levels. S 18886 and ramatroban also reduce the number of Mac-3–positive cells in ischemic areas, demonstrating that TP receptor activation is involved in Ang II proinflammatory effect. Similarly, a specific involvement of prostanoids has been demonstrated in IL-1β-induced angiogenesis.24 These results are also in line

Figure 2. Quantitative evaluation of microangiography (A), capillary density (B, capillary appears in white, arrows indicating representative examples of fibronectin-positive capillaries), and paw perfusion (C) 21 days after femoral artery occlusion. Values are mean±SEM. n=7 per group. *P<0.05 vs control ischemic mice. †P<0.05 vs Ang II-treated (0.3 mg/kg per day) ischemic mice. Cont indicates untreated mice with hindlimb ischemia; Ang II, mice with hindlimb ischemia treated with Ang II; Ang II+S18886, mice with hindlimb ischemia treated with Ang II and S18886 at 10 mg/kg per day; Ang II+Ram, mice with hindlimb ischemia treated with Ang II and ramatroban at 10 mg/kg per day; Ang II+Asp, mice with hindlimb ischemia treated with Ang II and aspirin (30 mg/kg per day); Ang II+Cand, mice with hindlimb ischemia treated with Ang II and the AT1 receptor blocker candesartan (20 mg/kg per day); Isch, ischemic leg; N.Isch, nonischemic legs.
with previous studies showing that TXA₂ mediates Ang II-related effects. TXA₂ triggers Ang II-dependent vasoconstriction in the kidney vasculature and in rat hindlimb. TXA₂ is also involved in Ang II-induced vascular smooth muscle cell (VSMC) proliferation. Aspirin treatment abrogates Ang II-induced neovascularization, it is therefore likely that other TP receptor ligands, isoprostanes or HETE, do not interfere in this process. Taken together, our results underline that TXA₂/TP signaling mediates Ang II proangiogenic effect in ischemic tissue.

However, TP receptor inhibition, in absence of exogenous addition of Ang II, does not modulate spontaneous neovascularization. Similarly, aspirin administration does not affect vessel growth, as previously described. The reason for this discrepancy is unclear. One can first hypothesize that ischemia-induced Ang II upregulation may increase TXA₂ tissue contents to levels that are insufficient to modulate new vessel growth in ischemic areas. In support of this view, exogenous administration of Ang II markedly enhances TXB₂ plasma levels compared with untreated ischemic mice. In this context, Ang II-induced TXA₂ upregulation participates to post-ischemic neovascularization. Second, numerous pathways and cell types are involved in spontaneous vessel growth in vivo. Exogenous FGF enhances neovascularization in animal models of peripheral arterial occlusion, yet, the angiogenic and arteriogenic processes are unaffected in mice lacking endogenous FGF-2. Hence, S18886 treatment alone may underestimate the importance of endogenous TXA₂ because of some sort of compensatory response by other proangiogenic pathways. Finally, TXA₂ may play a permissive role in the proangiogenic effect of Ang II and subsequently may modulate vessel growth in pathological situations associated with Ang II level upregulation.

In conclusion, endogenous activation of TXA₂ receptor by eicosanoids did not modulate spontaneous neovascularization in the setting of ischemia. In contrast, TP receptor activation is involved in Ang II proangiogenic effect. This study also highlights the concept that TP receptor inhibition might be of interest in the treatment of diseases associated with Ang II overproduction, such as diabetic retinopathy.
References

Thromboxane A2/Prostaglandin H2 Receptor Activation Mediates Angiotensin II–Induced Postischemic Neovascularization
Frédéric Michel, Jean-Sébastien Silvestre, Ludovic Waeckel, Stefano Corda, Tony Verbeuren, Jean Paul Vilaine, Michel Clergue, Micheline Duriez and Bernard I. Levy

Arterioscler Thromb Vasc Biol. 2006;26:488-493; originally published online December 29, 2005;
doi: 10.1161/01.ATV.0000201969.93348.74
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
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/26/3/488

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/