Regulation of Thrombomodulin Expression in Human Vascular Smooth Muscle Cells by COX-2–Derived Prostaglandins

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Abstract—There is concern that cyclooxygenase (COX)-2 inhibitors may promote atherothrombosis by inhibiting vascular formation of prostacyclin (PGI₂) and an increased thrombotic risk of COX-2 inhibitors has been reported. It is widely accepted that the prothrombotic effects of COX-2 inhibitors can be explained by the removal of platelet-inhibitory PGI₂. Using microarray chip technology, we have previously demonstrated that thrombomodulin (TM) mRNA is upregulated in cultured human coronary artery smooth muscle cells by the stable prostacyclin mimetic iloprost. This study is the first to demonstrate a stimulation of the expression of functionally active thrombomodulin in human smooth muscle cells by prostaglandins, endogenously formed via the COX-2 pathway. Because TM is an important inhibitor of blood coagulation, these findings provide a novel platelet-independent mechanism to explain the prothrombotic effects of COX-2 inhibitors. The full text of this article is available online at http://circres.ahajournals.org.

Key Words: cyclooxygenase • prostaglandins • thrombomodulin • smooth muscle cells • thrombosis

Selective cyclooxygenase (COX)-2 inhibitors were developed to reduce gastrointestinal toxicity of nonsteroidal antiinflammatory drugs (NSAID). However, it has been proposed that COX-2 is the predominant source of vascular prostacyclin (PGI₂) formation. Thus, there is concern that COX-2 inhibitors may promote atherothrombosis by inhibiting vascular formation of PGI₂. In fact, an increased thrombotic risk of COX-2 inhibitors has been reported. Very recently, rofecoxib (Vioxx) was voluntarily withdrawn from the market because of an increased risk of serious cardiovascular events, including myocardial infarction and stroke.8,9

It is widely accepted that COX-2 inhibitors may promote atherothrombosis by inhibiting formation of PGI₂, which is an important inhibitor of platelet aggregation.10 This is particularly important under conditions with increased COX-2 expression, such as atherosclerosis.10,12 In addition, selective COX-2 inhibitors fail to inhibit platelet thromboxane A₂ (TXA₂) formation.13 Thus, the increased thrombotic risk may result from unopposed thromboxane A₂ actions on platelets.14

However, via their effects on gene expression in vascular cells, prostaglandins may also inhibit thrombosis by mechanisms independent of platelet inhibition. Using microarray chip technology, we have previously demonstrated that thrombomodulin (TM) mRNA is upregulated in cultured human coronary artery smooth muscle cells (SMCs) by the stable prostacyclin mimetic iloprost.15

TM serves as a cell surface receptor for thrombin. Thrombin bound to TM can activate protein C (PC). Activated protein C (aPC) is an important inhibitor of blood coagulation by neutralizing the feedback-loop of thrombin generation via factors Va and VIIIa, respectively.17

The present study demonstrates that prostacyclin mimetics stimulate the expression of functionally active TM protein in cultured human SMCs. More importantly, evidence is presented for a regulation of TM expression and activity by prostaglandins, endogenously formed via the COX-2 pathway. In immunohistochemical studies on atherectomy specimens from human carotid arteries, COX-2 and TM were found to be colocalized in SMC from atherosclerotic lesions. These findings provide a novel, platelet-independent mechanism to explain the prothrombotic effects of COX-2 inhibitors.

Materials and Methods

Iloprost and cicaprost were kindly provided by Schering, Butaprost by Dr P. Gardiner (Bayer, Middlesex, UK), MB28797 by Rhône-Poulenc Rorer, ONO-AE1–329 by ONO Pharmaceuticals. Etoricoxib was synthesized by Laboratorien Berlin-Adlershof GmbH.
Hirudin was from RheinBiotec. Human Protein C was from Enzyme Research Laboratories.

**Cell Culture**

Human coronary artery SMCs were from Cambrex (Verviers, Belgium). Cells were grown in SMC Growth Medium 2 (C-22062) according to the supplier’s protocol. Human smooth muscle cells were isolated from coronary arteries, vena saphena, or arteria mammaria by the explant technique and cultured as previously described. Before the experiments, cells were synchronized by serum withdrawal for 48 hours.

**Semiquantitative RT-PCR**

Total RNA was prepared with Tri Reagent (Sigma-Aldrich, Taufkirchen, Germany). RT-PCR was performed using 250 ng total RNA with the OneStep RT-PCR kit QIAGEN (Qiagen) following the manufacturer’s manual. A 502-bp thrombomodulin (TM) fragment was amplified using the following primers: sense, CAT-TCGGGCTTGCTCATAGGC; antisense, GAAGGCTGCGACCAATAACG (20 pmol each; Invitrogen). For semiquantitative analysis, a 238-bp GAPDH fragment was coamplified with the following primers (15 pmol each): sense, TCTTTGAGGCCCATGTAGGCC-CAT; antisense, TCTTTGAGGCCCATGTAGGCC-CAT. After reverse transcription for 30 minutes at 50°C and a denaturation step for 15 minutes at 95°C, the following thermal profile was used: 1 minute 95°C, 1 minute 60°C, 1 minute 72°C (30 cycles), and a final elongation step at 72°C for 15 minutes.

**Western Blot Analysis**

Cell lysis and Western blotting was performed as previously described. Membranes were probed with the following monoclonal antibodies: anti-thrombomodulin (Santa Cruz Biotechnology; 1:140), anti-human COX-2 (Cayman; 1:1000).

**Analysis of Human Atherosclerotic Lesions**

Atherectomy specimens (n=19) from the internal carotid artery were collected retrospectively from the files of the Institute of Pathology, Universitätsklinikum Düsseldorf, Germany. All cases had symptomatic occlusive disease of one of the internal carotid arteries. The specimens were fixed in 4% buffered formaldehyde, cut transversely, and embedded in paraffin. TM was detected using a polyclonal TM antibody (1:250, American Diagnostica). In addition, SM-actin (monoclonal, HHF 35, 1:100, DAKO) and COX-2 (polyclonal, 1:150, Cayman Chemical Company) were stained in consecutive sections. The primary antibody against TM was detected by streptavidin/horseradish peroxidase–coupled secondary antibody and diaminobenzidine (DAKO) as a chromogen. The primary antibodies against SM-actin and COX-2 were detected by an alkaline phosphatase-conjugated secondary antibody and the reaction was developed using Fast Red (DAKO) as chromogen. Finally, the slides were counterstained with hemalaun. The staining patterns were evaluated by a senior pathologist (M. Sarbia).

**Cell-Based Protein C Activation Assay**

SMCs cultured in 96-well plates were washed twice with PBS and incubated in serum-free medium containing 20 mmol/L HEPES, 200 μg/mL bovine serum albumin, 400 nmol/L protein C, and 10 nmol/L protein C with the IP receptor agonist iloprost (ilo, 100 nmol/L) on TM mRNA expression in human coronary artery SMCs (data are mean±SEM from n=6 independent experiments; *P<0.05).

**Figure 1.** Effects of the IP receptor agonist iloprost (ilo, 100 nmol/L) on TM mRNA expression in human coronary artery SMCs (data are mean±SEM from n=6 independent experiments; *P<0.05).

**Figure 2.** Effects of various prostaglandin receptor agonists on the expression of TM protein in human coronary artery SMCs. A, B, and C, Western blots, representative for n=3 to 4 independent experiments, demonstrating the stimulation of TM expression by the IP receptor agonists iloprost (ilo 0.1 to 100 nmol/L), cicaprost (cica, 0.1 to 10 nmol/L), the EP receptor agonist PGE2 (100 nmol/L), the EP2 receptor agonist butaprost (buta, 1 μmol/L), and the EP3 receptor agonist ONO-AE1–329 (ONO, 100 nmol/L) as well as the inhibitory effects of the EP3 receptor agonist MB28797 (M&B, 100 nmol/L). D, Western blot demonstrating the electrophoretic mobility of TM protein under reducing and nonreducing conditions, respectively. E, Cell-based protein C activation assay demonstrating the stimulatory effects of ilo (data are mean±SEM from n=4 independent experiments; *P<0.05).
thrombin. Cells were incubated at 37°C for 80 minutes, the supernatants were removed and added to an equal volume of a solution of 20 mmol/L tris(hydroxymethyl)aminomethane hydrochloride, pH 7.5, 150 nmol/L NaCl, 1 mg/mL bovine serum albumin, and 10 U/mL hirudin. After 5 minutes, the activity of aPC was measured using a chromogenic substrate (S-2366, Chomogenix) according to the manufacturer’s protocol.

Statistics
Data are mean±SEM of n independent experiments. Statistical analysis was performed by one-way ANOVA followed by Bonferroni Multiple Comparisons test using GraphPad InStat version 3.01 for Windows 95 (GraphPad Software). Levels of P<0.05 were considered significant.

Results
Induction of Functional TM in SMCs by Prostaglandins
First, the upregulation of TM in cultured human coronary artery SMCs by the PGI₂ mimetic iloprost, as initially described in the microarray experiments, was verified by RT-PCR (Figure 1) and Western blotting (Figure 2A), respectively. In addition to iloprost, the more specific IP receptor agonist cicaprost as well as the EP agonist PGE₂ also stimulated TM expression (Figure 2B). Low nanomolar concentrations of cicaprost were effective in inducing TM protein expression. In separate experiments, TM expression was also stimulated by the EP₂ agonist butaprost (1 μmol/L) and the EP₄ agonist ONO-AE1–329 (100 nmol/L), whereas the EP₃ agonist MB28767 (100 nmol/L) prevented the stimulatory effects of these agonists (Figure 2C). Thus, several Gₛ-coupled prostaglandin receptors are capable of mediating the upregulation of TM. The molecular identity of the TM protein was verified by the demonstration of its different electrophoretic mobility under reducing and nonreducing conditions, respectively (Figure 2D). The functional properties of TM in SMCs were assessed by the cell-based measurement of protein C activation. These experiments demonstrated that the induction of TM protein expression by

Figure 5. TM immunostaining of atherectomy specimens from human internal carotid arteries (A). SM actin (B) and COX-2 immunostaining (C) of the corresponding areas in consecutive sections; original magnifications 400×.
iloprost resulted in an increase in TM activity (Figure 2E), thus indicating that active TM was induced at the surface of iloprost-treated SMCs. TM expression was induced by forskolin (10 μmol/L) and db-cAMP (1 mmol/L) and prevented by H89 (100 nmol/L) (Figure 3), indicating the possible involvement of the cAMP/protein kinase A signaling pathway. Finally, the stimulation of TM protein expression by iloprost was also demonstrated in cultured SMCs isolated from vena saphena and arteria mammaria, respectively (Figure 4).

Localization of TM and COX-2 in Human Atherosclerotic Lesions
To investigate the possible significance of these findings in human atherosclerosis, atherectomy specimens were stained for TM, SM actin, and COX-2, respectively. All analyzed specimens contained multiple areas in the neointima that were SMC-rich and were strongly positive for TM. Frequently, the SMCs expressed also COX-2 at the TM-positive sites (Figure 5). Furthermore, SM actin–negative macrophages in the neointima were also positive for TM and COX-2 (Figure 6).

Regulation of TM Expression by COX-2–Derived Prostaglandins
To study the possible regulation of TM expression by endogenous prostaglandins, COX-2 was induced by phorbol 12-myristate,13-acetate (PMA, 100 nmol/L) in cultured human coronary artery SMCs (Figure 7A). PMA also markedly induced TM protein expression in these cells (Figure 7B). The relative role of endogenous prostaglandins in the PMA-induced TM expression was studied using the nonspecific COX inhibitor diclofenac (1.5 μmol/L) and the COX-2-selective inhibitor etoricoxib (10 μmol/L), respectively. Both COX inhibitors significantly (P<0.05) reduced the PMA-induced TM mRNA (Figure 8A) and protein (Figure 8B) by ≈30% to 40%, indicating that the stimulatory effect of PMA was partially mediated by COX-2–derived prostaglandins. In control experiments, no inhibitory effects of the COX inhibitors on COX-2 expression were observed (Figure 8C). Finally, the effects of the COX-derived prostaglandins on TM function were studied in a cell-based protein C activation assay. These experiments demonstrate that inhibition of COX-2 resulted in a significant reduction of aPC formation in PMA-stimulated SMC (Figure 9), indicating that COX-2–derived prostaglandins are involved in the upregulation of functionally active TM.

Discussion
This study is the first to demonstrate a stimulation of the expression of functionally active thrombomodulin in human SMCs by prostaglandins, endogenously formed via the COX-2 pathway.

Thrombomodulin (TM) is a monomeric, type-1 transmembrane protein that serves as a cell surface receptor for thrombin. Thrombin binds to TM at high affinity (kDa 0.5 to 5 nmol/L), resulting in a conformational change of the thrombin molecule and an altered substrate specificity to activate protein C (PC).16,17 Activated protein C (aPC) proteolytically degrades factors Va and VIIa, thereby inhibiting further thrombin generation. In addition to its role in the regulation of plasmatic coagulation, TM modulates mitogenic signaling and thrombin-induced cell proliferation.21,22
TM is predominantly localized on endothelial cells. However, endothelial TM has been shown to be markedly downregulated in atherosclerotic coronary arteries. Thus, under certain pathological conditions, such as in advanced atherosclerosis, SMCs may become a functionally relevant source of TM. Interestingly, in nondiseased human aortas, only endothelial cells but not SMCs are positive for TM, whereas in atherosclerotic vessels, both intimal and medial SMCs express TM.

TM is an important regulator of thrombosis. A local overexpression of TM prevented atherothrombosis in a stasis/injury model of arterial thrombosis in rabbits. In a rabbit vein graft model, an early loss of TM expression was observed, which significantly impaired vein graft thrombore-sistance and resulted in an enhanced thrombin generation. In clinical studies, soluble TM was found to be inversely associated with the risk of coronary heart disease.

A functional cAMP responsive element is present in the 3’-untranslated region of the human thrombomodulin gene. Accordingly, in cell culture experiments, TM was found to be upregulated by cAMP in SMCs and in embryonal carcinoma cells. Importantly, prostacyclin infusion was able to increase soluble TM levels in patients with pulmonary arterial hypertension. This was confirmed in the present study. Several agonists acting at G-coupled prostaglandin receptors, such as iloprost or PGE2, were shown to stimulate TM expression in cultured SMCs obtained from human coronary arteries, mammary arteries, and saphenous veins, respectively. In contrast, agonists at Gi-coupled receptors, such as MB28767, prevented the stimulatory effects of iloprost. Thus, different prostaglandins are likely to modulate TM expression in SMCs.

In immunohistochemical studies on human atherectomy tissues, we have demonstrated that TM is expressed in SMCs. Importantly, SMCs expressed also COX-2 at the TM-positive sites. Thus, we have hypothesized that prostaglandins endogenously formed via the COX-2 pathway might act in an autocrine or paracrine fashion to stimulate TM expression. To study this hypothesis, COX-2 was induced in coronary artery SMCs by PMA. PMA also markedly stimulated TM expression in this model. The relative role of endogenous prostaglandins in the PMA-induced TM expression was studied using the nonspecific COX inhibitor diclofenac and the COX-2–selective inhibitor etoricoxib, respectively.

Both COX inhibitors significantly reduced the PMA-induced TM expression by ~30% to 40%, indicating that the stimulatory effect of PMA was at least partially mediated by COX-2–derived prostaglandins. In control experiments, no inhibitory effects of the COX inhibitors on COX-2 expression were observed. Accordingly, TM-mediated activation of protein C was also significantly inhibited by the COX inhibitors.

Taken together, this study demonstrates that COX-2–derived prostaglandins regulate the expression of functionally active TM. Because TM is an important inhibitor of blood
coagulation, these findings provide a novel, platelet-independent mechanism to explain the prothrombotic effects of COX-2 inhibitors.

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