Thrombin-Induced Mitogenesis in Coronary Artery Smooth Muscle Cells Is Potentiated by Thromboxane A2 and Involves Upregulation of Thromboxane Receptor mRNA

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Background—Previous studies have shown that thrombin is a potent though slow-acting mitogen for vascular smooth muscle cells (SMC). Because thrombin generation in vivo is accompanied by platelet activation, it has been suggested that platelet-derived factors might enhance thrombin-induced SMC proliferation. No information is available so far on the possible role of thromboxane A2.

Methods and Results—Thrombin (1 U/mL) caused a threefold to fourfold increase of DNA synthesis in cultured bovine coronary artery SMC as assessed from [3H]thymidine incorporation. U 46619, a stable thromboxane A2 mimetic, had only a minor stimulating effect on its own but potentiated the thrombin effect sixfold to sevenfold above control (P < .05). These findings were paralleled by a 52 ± 5% (P < .05) increase in cell number at 48 hours after addition of both mitogens as compared with 24 ± 5% with thrombin alone and no change with U 46619 alone. Thromboxane A2 receptor mRNA was found to be upregulated sixfold 20 minutes after thrombin stimulation. Pretreatment of SMC with thrombin for 4 hours markedly increased U 46619–induced mitogen–activated protein kinase activity, indicating thrombin-induced upregulation of functional thromboxane receptors in SMC.

Conclusions—Thrombin-induced proliferation of SMC is markedly enhanced by thromboxane A2. This might result in an enhancement of SMC proliferation by platelet-derived thromboxane A2 in vivo. (Circulation. 1998;97:589-595.)

Key Words: growth substances • cells • muscle, smooth
appropriate agonists can induce SMC proliferation. In some studies, TXA₂ mimetics, such as U 46619, CTA₂, STA₂, or I-BOP, can stimulate proliferation of vascular SMC,²⁹⁻³² partially doing so by upregulating the synthesis and release of endogenous growth factors such as platelet-derived growth factor (PDGF) or basic fibroblast growth factor (bFGF)³⁰ and by a synergistic action with peptidergic growth factors in intracellular signal transduction via the MAP kinase pathway.¹⁹,²¹ In contrast, other investigators did not detect direct mitogenic effects of TXA₂ on SMC.²²,²³ Part of the explanation may be different culture conditions. This includes the presence of fetal calf serum,¹⁰¹⁹ species differences, as well as the source of SMC, which in many cases are derived from the aorta.

The number of TP receptors in platelets is regulated by hormones (testosterone), receptor agonists (thromboxane mimetics), peptidergic growth factors (PDGF), and disease states such as recurrent myocardial ischemia.²¹⁻²⁶ Indirect evidence suggests that enhanced thrombin generation in blood might also increase the number of TP receptors on the platelet surface.²⁷ An upregulation of TP receptor mRNA by thrombin has also been demonstrated in a megakaryoblastic cell line.²⁸ There is also evidence for functional thromboxane receptors in vascular tissue,²⁹ although their regulation is much less understood. Thus the present data collectively suggest a synergistic interaction between thrombin and TXA₂, possibly occurring at the level of thromboxane formation, intracellular signal transduction, and receptor regulation, respectively.

This study investigates the interactions between thrombin and a chemically stable thromboxane mimetic, U 46619, on proliferation of bovine coronary artery SMC. Evidence is provided that U 46619 potentiates thrombin-induced mitogenesis and that this response involves thrombin-induced expression of TP receptors.

**Methods**

**Cell Culture**

Bovine SMC were isolated enzymatically from the left anterior descending coronary artery of adult female animals, following the method of Fallier-Becker et al.²⁹ The cells were cultivated in an 80% Ham’s F-12/20% Dulbecco’s modified eagle medium (DMEM), supplemented with 15% fetal calf serum (FCS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (all cell culture material was from Gibco or Becton & Dickinson). Cells were grown in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. SMC were characterized microscopically by the typical “hill-and-valley” growth pattern and by indirect immunostaining with a specific monoclonal α-actin antibody (Boehringer Mannheim). The media were exchanged twice a week. Monolayers of the cells were passaged on 150-mm tissue culture dishes once a week with trypsin (0.05%/EDTA (0.5 mmol/L)). Passages 4 to 10 were used for the experiments. Cell viability was assessed by means of trypan blue exclusion test and was found to be >95%.

**DNA and Protein Synthesis**

SMC were seeded into 24-well plates (5×10⁴ cells/well) and cultivated for 72 hours under standard conditions until subconfluence was reached. For the following 24 hours, cells were exposed to serum-free medium to allow defined stimulation with agonists. All further incubations were also carried out in serum-free medium, supplemented with 3 μmol/L indomethacin (Luitpold Pharma).

α-Thrombin (1 U/mL), the TXA₂ mimetic U 46619 (Upjohn Diagnostics) (0.3 to 30 μmol/L) and the thromboxane receptor antagonist SQ 29,548 (Bristol-Myers-Squibb Pharmaceuticals) (1 μmol/L) were added to duplicate or triplicate wells. In additional experiments, hirudin (3 ATU/mL) was added 0 to 4 hours subsequent to stimulation with either thrombin alone or thrombin combined with U 46619, respectively.

After 20 hours, [³H]thymidine (0.5 μCi/well) or [¹⁴C]leucine (0.1 μCi/well) (both from DuPont) were added. At the end of the total incubation period of 24 hours, the media were removed and the cells were washed twice with 1 mL ice-cold phosphate-buffered saline (PBS), 0.3 mL ice-cold perchloric acid (0.3 mol/L), and again with cold PBS. The cells were solubilized with 0.3 mL NaOH (0.1 mol/L) for 30 minutes at 37°C. [³H]Thymidine and [¹⁴C]leucine incorporation were quantified by liquid scintillation spectrometry. Protein concentration was measured in 20-μL aliquots from each well with the use of the Bio-Rad colorimetric protein assay¹¹ (Bio-Rad Laboratories).

**Determination of Number of Cells Entering S-Phase**

DNA synthesis was assayed by immunofluorescence labeling of incorporated 5-bromo-2’-deoxyuridine (BrDU) with a cell proliferation kit (BrDU labeling and detection kit I, Boehringer Mannheim). Cells were grown on round glass microscope slides (diameter, 12 mm), serum-deprived, and stimulated with mitogens as described above. Twenty-four hours after addition of the agonist, BrDU incorporation was monitored according to the manufacturer’s instructions. Representative areas of the slides were screened in a standardized way for nuclei stained positive for BrDU. At the time of the BrDU labeling, no differences in cell count (see below) were seen.

**Cell Proliferation Assay**

Cells were grown to ~50% confluence, then made quiescent and stimulated with mitogens as described above. After 24 and 48 hours, respectively, cells were trypsinized and total cell number was assessed by counting the cells per well, using a hemocytometer.

**Myelin Basic Protein Phosphorylation Assay**

MAP kinase activity was assayed by the MBP phosphorylation assay as described by Hawley et al.²² Subconfluent, serum-deprived SMC were treated with indomethacin (3 μmol/L) as described above. Thrombin was added to every second dish for 4 hours. Subsequently, cells were stimulated with U 46619 (3 μmol/L) for 10 minutes. In some experiments, SMC were preincubated with SQ 29,548 (1 μmol/L) for 2 minutes before stimulation with U 46619. After stimulation, SMC were washed with PBS and harvested into kinase buffer (20 mmol/L Tris/HCl, pH 7.5, 1 mmol/L EGTA, 2 mmol/L MgCl₂, 0.1 mol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonylfluoride, 25 μg/mL leupeptin, and 1 μg/mL pepstatin). Harvested cells were briefly sonicated and centrifuged for 5 minutes at 14,000g and 4°C.

Cell extracts (60 μg protein) were incubated for 15 minutes at 30°C in the presence of 20 μg MBP (Sigma), 10 μmol/L ATP, and 4 μCi [³²P]-ATP (Hartmann Analytic). The reaction was terminated by addition of 4× Laemmli buffer and heating of the samples for 5 minutes at 95°C. Proteins were separated by SDS/PAGE (13.5%), gels were fixed in 25% propan-2-ol/10% acetic acid, and bands were visualized by autoradiography.

**RNA Analysis**

TP receptor mRNA expression was analyzed by RT-PCR, with total RNA from SMC prepared with trizol reagent (Gibco BRL), following standard methods.²₁ First-strand cDNA was synthesized from total
RNA using oligo d(T) and murine leukemia virus reverse transcriptase (Perkin Elmer). The nucleotide sequence for the bovine prostaglandin H2/TXA2 (TP) receptor has been deposited in the EMBL genebank database with the accession numbers U 53484 and U 53485, respectively. PCR primers were designed according to the cDNA sequence corresponding to the putative fourth and seventh transmembrane domain of the receptor (sense: 5’-TGGGGCGCTACACCCTGTCGAGGCAG-3’; antisense: 5’-ATAGACCCAGGGATCCAAGA-3’). A glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) fragment of 238 bp length was amplified in addition to the 410 bp TP-receptor fragment (sense: 5’-TGATACATCAAGAAGGGATCCCA-3’; antisense: 5’-TCTTGGAGCCGATGAGCCCAT-3’). PCRs were carried out using AmpliTaq DNA Polymerase (Perkin Elmer). After a 2-minute predenaturation step at 95°C, the reactions were cycled 30 times in a cycle profile of 1 minute at 95°C, 1.5 minutes at 60°C, and 2 minutes at 72°C, followed by a final extension step for 5 minutes at 72°C. The PCR products were analyzed by automated laser fluorescence with an ALF express sequencing system (Pharmacia Biotech).

**Statistics**

All data are mean ± SEM of n independent experiments, performed in duplicates or triplicates as indicated. Three-way ANOVA followed by linear contrasts and a Bonferroni-Holm procedure for multiple testings or one-way ANOVA followed by Bonferroni multiple comparison tests were used for statistical analysis. Values of *P* < .05 were considered significant.

**Results**

**Potentiation of Thrombin-Induced Stimulation of DNA and Protein Synthesis by U 46619**

Incubation of SMC with the stable TXA2 mimetic U 46619 (0.3 to 30 μmol/L) for 24 hours resulted only in a minor increase of DNA synthesis. Some stimulation (1.5-fold above control) was seen at 3 μmol/L U 46619. Thrombin (1 U/mL) stimulated [3H]thymidine incorporation 3- to 4-fold above control. Coincubation of thrombin (1 U/mL) with U 46619 resulted in a concentration-dependent stimulation of DNA synthesis at increasing concentrations of U 46619. For example, in the presence of 3 μmol/L U 46619, thrombin-induced DNA synthesis was about twice as high as with thrombin alone, indicating a potentiation of thrombin-induced mitogenic reactions by the thromboxane mimetic. Further increase in

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**Figure 1.** Potentiation of thrombin (1 U/mL)-induced [3H]thymidine incorporation by the thromboxane mimetic U 46619 (0.3 to 30 μmol/L). Circles indicate the absence, squares the presence of thrombin. Data are mean ± SEM of n=5 independent experiments (performed in triplicate).

**Figure 2.** Synergistic effects of U 46619 (0.3 to 30 μmol/L) on thrombin (1 U/mL)-induced [14C]leucine incorporation. Circles indicate the absence, squares the presence of thrombin (1 U/mL). Data are mean ± SEM of n=5 independent experiments (performed in duplicate).

U 46619 concentrations resulted in a disappearance of this effect. These data are summarized in Fig 1. A similar potentiation of thrombin-induced DNA synthesis by U 46619 was observed when the incorporation of BrdU was measured (Table).

Similar results as for DNA synthesis were obtained when protein synthesis was measured by determination of [14C]leucine incorporation. U 46619 (0.3 to 30 μmol/L) showed only small, insignificant effects on [14C]leucine incorporation. Thrombin (1 U/mL) 1.5-fold increased [14C]leucine incorporation. However, there was a further increase in protein synthesis when U 46619 and thrombin were combined. As seen with [3H]thymidine, this action of U 46619 was concentration dependent. Maximal effects were observed at 3 μmol/L U 46619. These data are summarized in Fig 2.

**Antagonism of U 46619–Induced Potentiation of Thrombin-Induced Mitogenesis by Blockade of TP Receptors**

To answer the question of whether the potentiation of thrombin-induced mitogenesis by U 46619 is receptor mediated, thrombin (1 U/mL) and U 46619 (3 μmol/L) were coincubated with the selective thromboxane receptor antagonist SQ 29,548.34 SQ 29,548 (1 μmol/L) alone did not influence [3H]thymidine incorporation in unstimulated cells (data not shown). However, coincubation with SQ 29,548 (1 μmol/L) resulted in a complete prevention of U 46619-mediated amplification of thrombin-induced DNA synthesis.

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**Table**

<table>
<thead>
<tr>
<th>Parameter, % Control</th>
<th>U46619, 3 μmol/L</th>
<th>Thrombin, 1 U/mL</th>
<th>U46619 + Thrombin</th>
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<tbody>
<tr>
<td>BrdU labeling after 24 hours</td>
<td>108±5</td>
<td>124±4*</td>
<td>159±1†</td>
</tr>
<tr>
<td>Cell No. after 24 hours</td>
<td>103±2</td>
<td>102±2</td>
<td>101±2</td>
</tr>
<tr>
<td>Cell No. after 48 hours</td>
<td>103±3</td>
<td>124±5*</td>
<td>152±5†</td>
</tr>
</tbody>
</table>

*P < .05 vs unstimulated cells; †P < .05 vs U46619 or thrombin.
Thrombin Upregulates Vascular Thromboxane Receptors

Expression of TP Receptor mRNA

As a possible mechanism to explain the potentiation of thrombin-induced mitogenic effects by U 46619, we studied the hypothesis that TP receptors are upregulated by thrombin. Stimulation of quiescent SMC with thrombin (1 U/mL) for up to 3 hours resulted in a marked though transient increase in TP receptor mRNA expression. A maximum effect was seen at 20 minutes. At this time, thrombin stimulated TP receptor mRNA expression about sixfold above control. After continuous stimulation with thrombin for 3 hours, TP receptor mRNA expression returned to levels of unstimulated cells. Fig 5 shows representative original tracings of one experiment out of three with similar results.

MAP Kinase Activity

Experiments were performed to determine the effects of thrombin on the expression of TP receptor protein. However, TP receptor could not be detected in membrane preparations from control cells or from thrombin-treated cells by Western blot analysis with polyclonal antibodies raised against an epitope deduced from the cloned bovine TP receptor. Similarly, no receptor binding was detected in ligand-binding studies with [3H]SQ 29,548, [3H]-BOP, and [125I]-BOP, respectively. Since TP receptors could be detected by both, Western blotting and ligand-binding studies in COS-7 cells transiently transfected with TP receptor cDNA (not shown), the lack of detection in SMC is most likely a result of a low level TP receptor expression in SMC.

Therefore, the level of TP receptor expression in SMC was assessed functionally by determination of MAP kinase activity (Fig 6). In control cells, addition of U 46619 did not significantly stimulate MAP kinase activity. These data are consistent with a low basal TP receptor expression and are in line with our findings that U 46619 alone was not capable to stimulate DNA synthesis in SMC. However, when SMC were pretreated with thrombin for 4 hours, a marked stimulation of DNA synthesis was observed that was reflected by an increase in cell number. Again, thrombin-induced SMC proliferation was significantly amplified by U 46619. The data are summarized in the Table.

Cell Proliferation

The effects of thrombin and/or U 46619 on SMC mitogenesis were further studied in a cell proliferation assay. At the time relevant for BrdU labeling (24 hours after stimulation), no differences in the cell count were observed between the groups. In contrast, 48 hours after addition of thrombin, a significant progression of the cell cycle into the M-phase was observed that was reflected by an increase in cell number. This indicates that the potentiation of thrombin-induced DNA synthesis by U 46619 was mediated by TP receptors. These results are shown in Fig 3.

Inhibition of Thrombin-Induced Mitogenesis by Hirudin

SMC mitogenesis requires prolonged exposure to thrombin, and stimulation of DNA synthesis can be inhibited by addition of hirudin from 0 to 8 hours after stimulation with thrombin.13 Therefore, we have studied the effects of a brief exposure to thrombin on U 46619–induced SMC mitogenesis by inhibiting thrombin activity with hirudin from 0 to 4 hours after stimulation. When added up to 4 hours after stimulation, hirudin (3 ATU/mL) completely inhibited thrombin-induced DNA synthesis (Fig 4).

When SMC were stimulated with thrombin and U 46619, immediate neutralization of thrombin with hirudin completely blocked the stimulation of DNA synthesis. Interestingly, when hirudin was added at later time points, a remarkable stimulation of DNA synthesis by U 46619 was observed that was dependent on the duration of thrombin exposure before neutralization with hirudin (Fig 4). Since direct thrombin effects on DNA synthesis were completely inhibited by addition of hirudin within the time window studied, these results suggest a priming effect of thrombin on SMC resulting in an increased mitogenic responsiveness to U 46619.

Figure 3. Potentiation of thrombin (Thr; 1 U/mL)-induced mitogenesis by U 46619 (U46; 3 μmol/L) and its prevention by the selective thromboxane receptor antagonist SQ 29,548 (SQ29; 1 μmol/L). Data are mean ± SEM of n = 4 independent experiments (performed in triplicate). *P < .05 vs Thr, #P < .05 vs Thr + U46.

Figure 4. Inhibition of thrombin-induced mitogenesis by hirudin. Thrombin (Thr, 1 U/mL)-induced mitogenesis was completely blocked by hirudin (Hir, 3 ATU/mL) added from 0 to 4 hours after stimulation. U 46619 (U46, 3 μmol/L) did not stimulate SMC mitogenesis but potentiated the effects of thrombin. When SMC were stimulated with thrombin and U 46619, immediate neutralization (0 min) of thrombin with hirudin also completely blocked the stimulation of DNA synthesis. When hirudin was added at later time points (90 to 240 minutes), a marked stimulation of mitogenesis by U 46619 was observed that was dependent on the duration of thrombin exposure before neutralization with hirudin. Data are mean ± SEM of a representative experiment performed in triplicate. Similar results were obtained in three independent experiments.

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MAP kinase activity by U 46619 was seen (Fig 6). This effect was inhibited by the TP receptor antagonist SQ 29,548. In concert with our findings on TP receptor mRNA, these data provide functional evidence for a thrombin-induced upregulation of TP receptors.

Discussion

The present data indicate that thrombin-induced mitogenesis of coronary artery SMC is markedly enhanced in the presence of a TXA2 mimetic, which, alone, has only minor mitogenic activity. It is also shown that this potentiation of the thrombin-induced response is TP receptor mediated because it is prevented by a selective antagonist. We have demonstrated a marked increase in thromboxane receptor mRNA in SMC and provided functional evidence for TP receptor upregulation by thrombin. These findings may provide a mechanistic basis for the proliferative responses to the two agonists.

Enzymatically active thrombin is a well-known mitogen in cultured vascular SMC.10–12,15,35 However, growth stimulation by thrombin is a slow-onset reaction, showing markedly delayed kinetics in the absence of serum or peptidergic growth factors.4 For example, several days are necessary for thrombin to induce proliferation of bovine aortic SMC.12 On the other hand, activation of transcription factors, such as c-fos or nuclear factor-κB, occurs within a few hours after thrombin stimulation.36 Similarly, phosphorylation of several signal transduction molecules, including MAP kinases, has been observed within minutes after thrombin stimulation.37 Thus despite rapid thrombin receptor activation, the maximal mitogenic effect of thrombin in cultured SMC requires a prolonged exposure to active thrombin.15 This was explained by a progressive thrombin-dependent recruitment of additional cells into the growth fraction. Alternatively, thrombin-induced mitogenesis in vivo might involve the generation or amplification of additional growth promoting signals, present in the thrombus or in the injured vessel wall in vivo but not in cultured SMC.

This study provides evidence for TXA2 as an amplification signal for thrombin-induced SMC proliferation. As noted above, there are conflicting reports as to whether or not TXA2 can stimulate mitogenesis.16–23 We have recently shown that thromboxane mimetics, such as U 46619, induce expression of immediate early genes, such as c-fos or egr-1, and potentiate PDGF-dependent responses in rat aortic SMC.21 Similar data were obtained in SMC prepared from bovine coronary arteries.38 The present study is the first to demonstrate that thrombin-induced mitogenic responses are potentiated by the thromboxane mimetic U 46619 in a receptor-dependent manner. First, U 46619, although a weak mitogen itself, markedly potentiated the mitogenic actions of thrombin. Second, the thromboxane component of this response was blocked by the specific thromboxane receptor antagonist SQ 29,548.

A possible explanation for the synergistic action between thrombin and U 46619 is that thrombin might regulate the expression of thromboxane receptors in SMC. A marked, sixfold increase in TP receptor mRNA levels was seen at 20 minutes. This is much earlier than the autoinduction of thrombin receptor mRNA expression in bovine or rat vascular SMC by thrombin.15,39 An upregulation of TP receptor gene expression by thrombin in a human megakaryoblastic cell line has been previously reported.28
Although TP receptor protein could not be detected by means of Western blotting or ligand binding studies probably because of low receptor protein expression, two lines of evidence support the concept of thrombin-induced upregulation of functional TP receptors in SMC. First, a brief (1.5 to 4 hours) incubation of SMC with thrombin resulted in an increased mitogenic responsiveness to U 46619 that was dependent on the duration of thrombin exposure before neutralization with hirudin. Second, SMC that have been treated with thrombin for 4 hours showed a markedly higher stimulation of MAP kinase by U 46619 compared with nontreated SMC. In concert with the demonstration of increased TP receptor mRNA expression by thrombin, these functional data provide strong evidence for an increased expression of functionally active TP receptors by thrombin. Because of the common pathways of thrombin- and TXA2-induced signaling, including MAP kinase activation,19,21,37 an enhanced expression of thromboxane receptors is probably also translated into growth promoting signals within SMC. Accordingly, we have shown that cell proliferation (cell number 48 hours after stimulation) was potentiated by the combined use of thrombin and U 46619 compared with either agent alone.

Several in vivo studies indicate a synergistic procoagulatory effect between thrombin and TXA2 during formation and resolution of thrombi within coronary arteries. There is also evidence for early upregulation of vascular thrombin receptors in vivo and in SMC tissue culture by peptidic growth factors.28 An increased number of prostaglandin H2/TXA2-receptors has been observed in platelets from patients with active unstable angina and was related to increased thrombin activity.27 Inhibition of thrombin by hirudin in vivo significantly reduced platelet-rich thrombi and neointima proliferation in experimentally injured vessel segments.29 These data suggest that active TXA2 may be generated in platelet-rich thrombi long enough to enhance the mitogenic responses to thrombin. However, thromboxane formation may be limited by autoinactivation of platelet cyclooxygenase.30

To address this possibility, we have measured platelet-derived thromboxane formation in vitro under conditions similar to those associated with clot-related thromboxane formation in vivo (data not shown). In these studies, a marked, aspirin-sensitive thromboxane generation was found, persisting for at least 2 hours after addition of thrombin. This suggests continued cyclooxygenase and thromboxane synthase activity in the platelets over at least this period of time. Clearly, an increased thromboxane receptor number in SMC and associated thromboxane formation will act in concert and might synergize in thrombin-induced mitogenesis not only in vitro but also in vivo.

In summary, we have shown that thrombin-dependent upregulation of vascular TP receptors occurs in thrombin-stimulated SMC and that active thromboxane potentiates proliferative thrombin responses. If these findings are translated into the in vivo situation, they would support the combination of selective thromboxane receptor antagonists in combination with thrombin inhibitors for prevention of SMC proliferation after coronary angioplasty.44–46

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