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Thromboxane A_2 induces cell signaling but requires platelet-derived growth factor to act as a mitogen

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Abstract

This study investigates thromboxane A_2-induced cell signaling and mitogenesis of bovine coronary artery smooth muscle cells. The thromboxane mimetic U 46619 [(15*S*)-hydroxy-11,9-(epoxymethano)prosta-5*Z*,13*E*-dienoic acid] (10 μM) stimulated $[Ca^{2+}]_i$ signals, phosphorylation of MAP kinase (mitogen-activated protein kinase), and expression of *c-fos* mRNA in smooth muscle cells. In contrast, no stimulation of DNA synthesis or cell proliferation by U 46619 was observed. However, platelet-derived growth factor-BB (20 ng/ml)-induced mitogenesis was potentiated by U 46619. Similar results were obtained with I-BOP [1*S*-(1α,2β(5Z),3α(1*E*,3*R**),4α)]-7-[3-(3-hydroxy-4-(4′-iodophenoxy)-1-butenyl)-7-oxabicyclo[2.2.1]heptan-2-yl]-5-heptenoic acid]. These potentiating effects were abrogated by a specific thromboxane receptor antagonist, suggesting that the potentiation of platelet-derived growth factor-BB-induced smooth muscle cell mitogenesis by U 46619 and I-BOP was mediated by thromboxane receptors. It is concluded that thromboxane A_2 generated by blood platelets at the site of vessel injury induces cell signaling in smooth muscle cells but acts as a mitogen only in the presence of growth factor(s).

Keywords: Coronary artery smooth muscle cell; Mitogenesis; Thromboxane A_2; PDGF (platelet-derived growth factor)

1. Introduction

Activated blood platelets are the dominating source of thromboxane A_2 in the cardiovascular system. Blood platelets accumulate at the site of vessel lesion and directly release their activation products such as platelet-derived growth factor or thromboxane A_2 to the subendothelial layer of the vessel wall (Harker, 1987). Thus, the production of thromboxane A_2 at sites of vascular injury may contribute to lesion formation characteristic of restenosis and atherosclerosis (Ross, 1993).

Previous studies have shown that thromboxane A_2 mimetics, such as U 46619 [(15*S*)-hydroxy-11,9-(epoxymethano)prosta-5*Z*,13*E*-dienoic acid)], STA_2 [(9,11-epithio-11,22-methano-thromboxane A_2)] or I-BOP [1*S*-(1α,2β(5*Z*),3α(1*E*,3*R**),4α)]-7-[3-(3-hydroxy-4-(4′-iodophenoxy)-1-butenyl)-7-oxabicyclo[2.2.1]heptan-2-yl]-5-heptenoic acid] stimulate smooth muscle cell mitogenesis (Uehara et al., 1988; Morinelli et al., 1994; Sachinidis et al., 1995). In contrast, other investigators did not detect direct mitogenic effects of thromboxane A_2 mimetics on vascular smooth muscle cells (Ali et al., 1993; Crowley et al., 1994; Jones et al., 1995). However, thromboxane A_2 mimetics have been found to increase smooth muscle cell mitogenesis in the presence of other growth stimuli such as thrombin (Zucker et al., 1995), insulin (Hanasaki et al., 1990) or serum (Akopov et al., 1988; Nagata et al., 1992).

This study was undertaken to investigate the possible mitogenic effects of thromboxane A_2, using two structurally different thromboxane A_2 mimetics, namely U 46619 and I-BOP, and their interaction with platelet-derived growth factor-induced mitogenesis in bovine coronary artery smooth muscle cells.

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2. Materials and methods

2.1. Cell culture

Bovine coronary artery smooth muscle cells were isolated enzymatically from the left anterior descending coronary artery as described by Fallier-Becker et al. (1990). Smooth muscle cells were grown in Ham's F12 medium supplemented with Dulbecco's modified Eagle medium (20%), fetal calf serum (15%), penicillin (100 U/ml), and streptomycin (0.1 mg/ml) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Smooth muscle cells were characterized microscopically by the typical 'hill and valley' growth pattern and by immunostaining with a monoclonal α-actin antibody (Boehringer Mannheim, Germany). The cells were passaged by harvesting with trypsin/EDTA (0.05%/0.5 mM). Passages 4–6 were used for the experiments.

2.2. Measurement of $[Ca^{2+}]_i$

Measurement of $[Ca^{2+}]_i$ was performed as previously described (Sachinidis et al., 1995). Briefly, smooth muscle cells were cultured on round glass microscopic slides (diameter 12 mm) and loaded with 2 μM fura-2-penta-acetoxymethyl ester for 20 min at 37°C. The slides were positioned diagonally in a cuvette and fluorescence was measured in a spectrofluorometer (Perkin-Elmer LS 50, Überlingen, Germany) at excitation wavelengths of 340 and 380 nm and at an emission wavelength of 505 nm. Maximum (R_{max}) and minimum (R_{min}) fluorescence was determined by addition of digitonin (30 μM), followed by Tris/EGTA (0.1 M-25 mM). Fluorescence was corrected for cellular autofluorescence.

2.3. MAP kinase phosphorylation

Analysis of MAP kinase (mitogen-activated protein kinase) phosphorylation was performed by Western blotting as previously described (Sachinidis et al., 1995). Briefly, smooth muscle cells were seeded in 24-well culture plates (4×10^5 cells/well) and grown until subconfluency as described above. The medium was then replaced by serum-free medium for 24 h and cells were stimulated for the indicated times. Proteins were separated by SDS (sodium dodecyl sulfate) (7.5%) polyacrylamide gel electrophoresis, electrotransferred to polyvinyldiene difluoride membranes (Millipore, Eschborn, Germany), and immunoblotted with anti-rat MAP kinase R2 IgG (immune globulin G) antibodies. Phosphorylation of the 42-kDa MAP-II kinase isoform was monitored by the shift in electrophoretic mobility of the phosphorylated MAP-II kinase ($p42^{mapk}$).

2.4. Expression of c-fos mRNA

RNA extraction and Northern blotting was performed as previously described (Sachinidis et al., 1995). Briefly, smooth muscle cells were grown in 75-cm^2 culture flasks, serum-starved for 24 h, and stimulated for the indicated times. Total RNA was extracted by the guanidinium isothiocyanate/CsCl procedure according to Chirgwin et al. (1979). RNA was then separated by electrophoresis in a 6% formaldehyde-1.2% agarose gel, blotted on Hybond N^+ membranes, and probed with a [^{32}P]-labeled 1.0-kb *v-fos* cDNA which hybridized to the 2.2-kb mRNA of *c-fos*. The same membranes were rehybridized with a 0.77-kb cDNA probe for β-actin.

2.5. DNA synthesis and cell proliferation

Smooth muscle cells were seeded into 24-well plates (5×10^4 cells/well) and grown until subconfluency. In order to allow defined stimulation, the cells were incubated in serum-free medium for 24 h. Subsequently, the stimuli were added to triplicate wells at the indicated concentrations. Indomethacin (3 μM) was added in all experiments in order to avoid interactions with endogenously synthesized prostaglandins. After 20 h, [^{3}H]thymidine (1.0 μCi/ml) was added for additional 4 h. At the end of the total incubation period of 24 h, the media were removed and cells were washed sequentially with cold phosphate-buffered saline, trichloroacetic acid (10%), ethanol/ether (1:1), and again with phosphate-buffered saline. The cells were solubilized by addition of 0.1 M NaOH for 20 min at 37°C and [^{3}H]thymidine incorporation was quantified by liquid scintillation spectrometry. Cell protein was determined using the Bio-Rad assay (Bio-Rad, München, Germany) according to Bradford (1976).

DNA synthesis was also assayed by measuring the incorporation of 5-bromo-2′-deoxyuridine (BrdU) using a cell proliferation kit (BrdU labeling and detection kit III, Boehringer, Mannheim, Germany). Cells were stimulated as described above and BrdU incorporation was monitored according to the manufacturer's instructions.

For the measurement of cell proliferation, smooth muscle cells were grown to about 50% confluence, then serum-deprived and stimulated as described above. After 24 and 48 h, respectively, cells were trypsinized and total cell number was assessed by counting the cells per well using a hemocytometer.

2.6. Materials

Enzymes for cell isolation were purchased from Sigma, Deisenhofen (Germany). Cell culture media were obtained from Gibco-Life Technologies (Eggenstein, Germany). Bovine recombinant platelet-derived growth factor-BB was obtained from Boehringer Mannheim (Germany). The thromboxane A_2-mimetic I-BOP was generously provided by Dr. P.V. Halushka (MUSC, Charleston, SC, USA), the thromboxane A_2-receptor antagonist SQ 29,548 ({1s-[1,2(5z),3,4]}-7-{3[(2-phenylaminocarbonyl)hydrazino]-methyl}-7-oxabicyclo(2.2.1)hept-2-yl)-5-heptenoic acid) by

Dr M. Ogletreee (Squibb Pharmaceuticals, Princeton, NJ, USA), and indomethacin by Dr Raake (Luitpold Pharma, München, Germany). The thromboxane A_2-mimetic U 46619 was from Upjohn Diagnostics (Kalamazoo, MI, USA), [^{3}H]thymidine was from DuPont NEN (Dreieich, Germany). All other chemicals were of reagent grade and purchased from Merck (Darmstadt, Germany).

2.7. Statistics

The data are means ± S.E.M. of n independent observations. Statistical analysis was performed by two-tailed t-test. P levels of < 0.05 were considered significant.

3. Results

3.1. Intracellular $[Ca^{2+}]_i$ signals

The changes in $[Ca^{2+}]_i$ subsequent to addition of U 46619 at a concentration of 10 μM are exemplifed in Fig. 1. In the presence of extracellular Ca^{2+} (1 mM), U 46619 induced a rapid elevation in $[Ca^{2+}]_i$ that declined towards basal values within one min. Evaluation of three separate experiments was performed by calculating the maximum increase in $[Ca^{2+}]_i$ at 15 s. U 46619 caused an increase in $[Ca^{2+}]_i$ from 68 ± 8 nM (basal value) to 132 ± 9 nM ($P < 0.05$). In the absence of extracellular Ca^{2+}, U 46619 induced an increase in $[Ca^{2+}]_i$ from 45 ± 4 nM (basal value) to 120 ± 12 nM ($P < 0.05$). Pretreatment of the cells with SQ 29,548 (10 μM) for 2.5 min completely inhibited U 46619-induced $[Ca^{2+}]_i$ signals.

Co-incubation of platelet-derived growth factor-BB (20 ng/ml) with U 46619 (10 μM) resulted in similar changes as seen with platelet-derived growth factor-BB alone and there was also no detectable change in this response when the cells were pretreated with the thromboxane receptor antagonist SQ 29,548 (not shown).

3.2. MAP kinase phosphorylation

Fig. 2 demonstrates the shift in mobility of the 42-kDa MAP-kinase ($p42^{mapk}$) band after stimulation with U 46619 (10 μM). U 46619 caused a time-dependent shift of the 42-kDa band with a maximum between 5 and 10 min (Fig. 2). Densitometric analysis ($n = 3$) showed that approximately 30% of the 42-kDa band was shifted at 5 min. When cells were stimulated with platelet-derived growth factor-BB (20 ng/ml), the 42-kDa band was maximally shifted (100%) at 5 min.

3.3. c-fos mRNA expression

The time course of the 3.4 kb *c-fos* mRNA expression induced after stimulation with U 46619 (10 μM) is demonstrated in Fig. 3. U 46619 stimulated a maximal

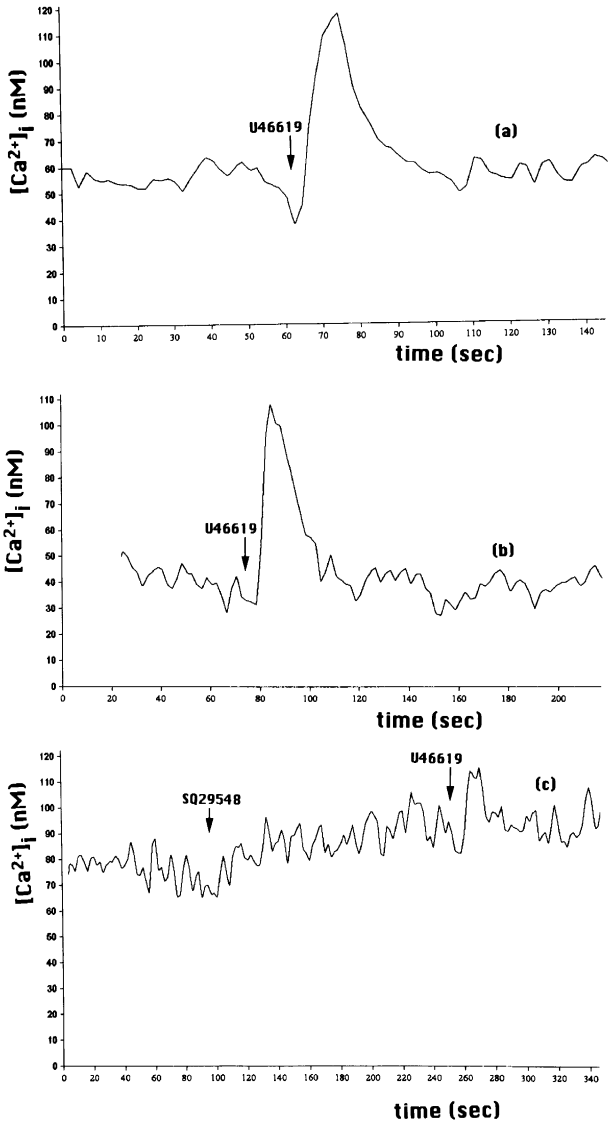

Fig. 1. Changes in $[Ca^{2+}]_i$ induced by U 46619 (10 μM) in the presence (A) and in the absence of extracellular Ca^{2+} (B). Pretreatment of the cells with SQ 29,548 (10 μM) for 2.5 min caused a complete inhibition of $[Ca^{2+}]_i$ signals (C). (Original tracings representative for $n = 3$ experiments).

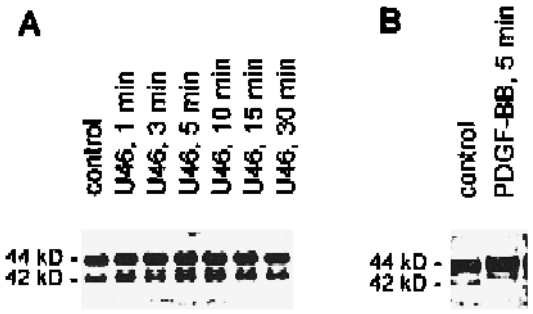

Fig. 2. Time-course for the mobility shift of the 42-kDa MAP-II kinase band after stimulation with U 46619 (10 μM) (A) and platelet-derived growth factor-BB (20 ng/ml) (B).

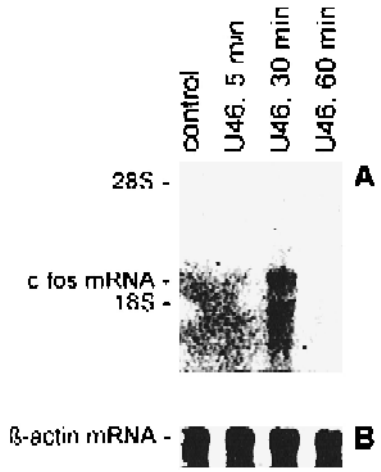

Fig. 3. Time course of the *c-fos* mRNA expression after stimulation with U 46619 (10 μM) (A). The same membranes were rehybridized with a probe for β-actin (B).

induction of *c-fos* mRNA at 30 min. After 60 min, the values returned towards control.

3.4. DNA synthesis

Incubation of smooth muscle cells with platelet-derived growth factor-BB (20 ng/ml) induced a 4–5-fold increase in DNA synthesis above control. In contrast, neither I-BOP (30 nM) nor U 46619 (10 μM) increased DNA synthesis in smooth muscle cells. Increasing the concentrations of U 46619 to 100 μM and of I-BOP to 100 nM did also not result in any stimulation of DNA-synthesis (not shown). However, either of these thromboxane mimetics caused a marked additional stimulation of [^{3}H]thymidine incorporation by platelet-derived growth factor-BB. This potentiation of platelet-derived growth factor-BB-induced DNA synthesis by the thromboxane mimetics was receptor-mediated as seen from the inhibition of this effect by the thromboxane receptor antagonist SQ 29,548 (10 μM). The data are summarized in Fig. 4 and Fig. 5.

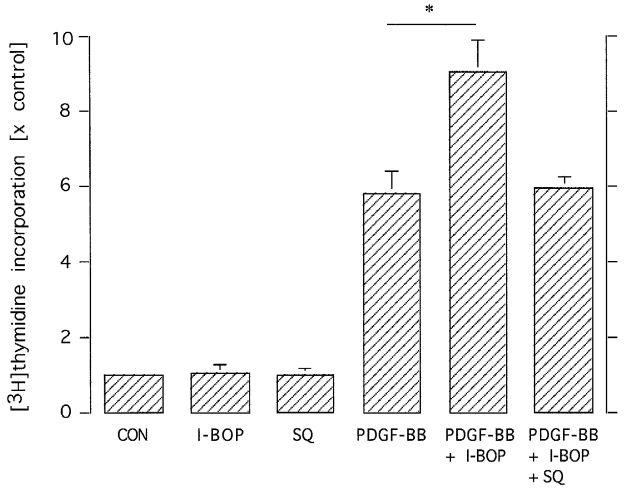

Fig. 4. Effects of I-BOP (30 nM), SQ 29,548 (SQ, 10 μM), platelet-derived growth factor-BB (PDGF-BB, 20 ng/ml) alone and in coincubation experiments on [^{3}H]thymidine incorporation in smooth muscle cells. Data are normalized to incorporation under non-stimulated control conditions (CON = 1). Data are means ± S.E.M. of $n = 3$ experiments performed in quadruplicate. * $P < 0.05$ vs. PDGF-BB.

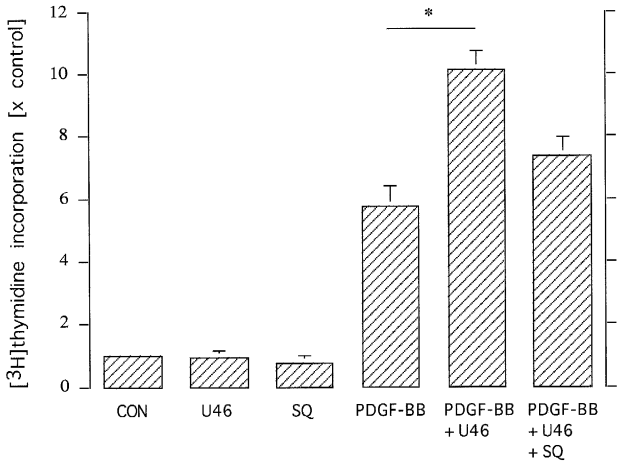

Fig. 5. Effects of U 46619 (U46, 10 μM), SQ 29,548 (SQ, 10 μM), platelet-derived growth factor-BB (PDGF-BB, 20 ng/ml) alone and in coincubation experiments on [^{3}H]thymidine incorporation in smooth muscle cells. Data are normalized to incorporation under non-stimulated control conditions (CON = 1). Data are means ± S.E.M. of $n = 3$ experiments performed in quadruplicate. * $P < 0.05$ vs. PDGF-BB.

In addition to the measurement of [^{3}H]thymidine incorporation, DNA synthesis was also assessed by the measurement of 5-bromo-2′-deoxyuridine incorporation using a cell proliferation kit (BrdU labeling and detection kit III, Boehringer Mannheim, Germany). Results obtained with this method were conclusive with the data obtained with the measurement of [^{3}H]thymidine incorporation (not shown).

3.5. Cell proliferation

The effects of platelet-derived growth factor-BB and the thromboxane mimetic U 46619 were further studied in a cell proliferation assay. 48 h after the addition of platelet-

Table 1
Potentiation of platelet-derived growth factor-BB (PDGF-BB, 20 ng/ml)-induced smooth muscle cell proliferation by the thromboxane mimetic U 46619 (10 μM) and its inhibition by SQ 29,548 (10 μM)

	Cell number (% control)	
	After 24 h	After 48 h
Unstimulated cells	103 ± 4	108 ± 7
PDGF-BB	111 ± 5	145 ± 10 [a]
U 46619	107 ± 3	124 ± 4
PDGF-BB + U 46619	110 ± 3	186 ± 12 [a,b]
PDGF-BB + U 46619 + SQ 29,548	104 ± 4	151 ± 11 [a]

The data are expressed as percentage control (cell number at the time of stimulation = 100%). Data are means ± S.E.M. of $n = 4$ independent experiments; [a] $P < 0.05$ vs. unstimulated cells, [b] $P < 0.05$ vs. PDGF-BB.

derived growth factor-BB (20 ng/ml), a significant progression of the cell cycle into the M-phase was observed that was reflected by an increase in cell number. No significant proliferatory response was seen with U 46619 (10 μM). However, similar to the effects on DNA synthesis, a significant potentiation of platelet-derived growth factor-BB-induced mitogenesis by U 46619 was seen that was abrogated by SQ 29,548 (10 μM). The data are summarized in Table 1.

4. Discussion

The present study provides evidence that platelet-derived growth factor-induced mitogenesis in bovine coronary artery smooth muscle cells is markedly potentiated by thromboxane A_2 which, by its own, stimulated several cellular signaling events but did not show mitogenic activity.

Blood platelets accumulate at a site of vessel lesion and directly release their activation products such as platelet-derived growth factor or thromboxane A_2 to the subendothelial layer of the vessel wall (Harker, 1987). Several lines of evidence indicate that thrombus-derived factors may contribute to neointima formation in injured vessels (Bauters et al., 1995). Using a pig model of vessel injury, Schwartz et al. (1992) demonstrated that the size of coronary thrombus determines the formation of neointima after percutaneous coronary angioplasty. The importance of luminal thrombi for neointima formation is further supported by clinical findings showing that patients with intracoronary thrombi had a significantly higher risk to develop restenosis after coronary angioplasty (Bauters et al., 1995). Similarly, when angioplasty was performed in patients with unstable angina pectoris, a situation where intraluminal thrombus plays a major role, the risk of restenosis was higher than that observed when coronary angioplasty was performed in patiens with stable angina (De Groote et al., 1995). In addition to the release of platelet-derived growth factors, significant amounts of thromboxane A_2 are generated in platelet-containing clots for at least 2 h (Glusa, personal communication). Thus, production of thromboxane A_2 at sites of vascular injury may contribute to lesion formation characteristic of restenosis and atherosclerosis (Ross, 1993).

Several earlier studies have addressed the question of whether or not thromboxane A_2 or appropriate agonists can stimulate smooth muscle cell proliferation. The data were conflicting. In some studies, thromboxane A_2 mimetics, such as U 46619, STA_2, or I-BOP, stimulated smooth muscle cell mitogenesis (Uehara et al., 1988; Morinelli et al., 1994; Sachinidis et al., 1995). In contrast, other investigators did not detect direct mitogenic effects of thomboxane A_2 mimetics on vascular smooth muscle cells (Ali et al., 1993; Crowley et al., 1994; Jones et al., 1995). However, thromboxane A_2 mimetics have been found to increase smooth muscle cell mitogenesis in the presence of other growth stimuli such as insulin (Hanasaki et al., 1990), thrombin (Zucker et al., 1995), or serum (Akopov et al., 1988; Nagata et al., 1992). Part of the explanation may be different tissue and culture conditions. This includes the presence of fetal calf serum with numerous growth factors at 0.1% (Morinelli et al., 1994) or 10% concentration (Nagata et al., 1992) in smooth muscle cells prepared from young animals, several weeks of age. Another explanation may be related to species differences as well as to the source of smooth muscle cells which in many cases were derived from the aorta. The present study appears to be the first using coronary artery smooth muscle cells prepared from adult animals. In addition, all previous studies were performed in the absence of inhibition of cyclooxygenase product formation by indomethacin or any related compound. We have found in separate experiments that our coronary artery smooth muscle cells produce prostacyclin as a major cyclooxygenase product which is a potent inhibitor of smooth muscle cell proliferation (Grosser et al., 1995). Therefore, in the present study, all of our experiments were performed in the presence of indomethacin in order to exclude any possible contribution of smooth muscle cell-derived prostaglandins to the overall mitogenic response.

Several signal transduction pathways, such as $[Ca^{2+}]_i$ transients (Dorn and Becker, 1993; Sachinidis et al., 1995), MAP kinase activation (Morinelli et al., 1994), and expression of immediate-early gene mRNA (Dorn et al., 1992; Sachinidis et al., 1995), have been found to be stimulated by thromboxane A_2 in vascular smooth muscle cells. However, negative results have also been reported (see in Schrör and Schröder, 1994). In this study, thromboxane A_2 stimulated $[Ca^{2+}]_i$ signals and this was independent of external Ca^{2+}, confirming earlier findings of Dorn and Becker (1993) in rat aortic smooth muscle cells. Moreover, thromboxane A_2 also stimulated MAP kinase phosphorylation and induction of *c-fos* mRNA. These signaling events, however, were not sufficient to stimulate mitogenesis in bovine coronary artery smooth muscle cells. This finding is in line with the previous observations demonstrating that the induction of early response genes by platelet-derived growth factor-AA was not sufficient to stimulate DNA synthesis in rat smooth muscle cells (Sachinidis et al., 1993). Similarly, Inui et al. (1994) have shown, that platelet-derived growth factor-AA increased protein synthesis but had no mitogenic activity in smooth muscle cells from spontaneously hypertensive rats.

Interestingly, even in the absence of direct mitogenic effects, a marked potentiation of platelet-derived growth factor-BB-induced smooth muscle cell proliferation was observed in our study. These potentiation effects were abrogated by SQ 29,548, a thromboxane A_2 receptor antagonist, suggesting that the potentiation of platelet-derived growth factor-BB-induced mitogenesis by U 46619 was mediated by thromboxane receptors.

Activated platelets release both, platelet-derived growth factor and thromboxane A_2, to the subendothelial layer of the vessel wall. Thus the synergistic effects of thromboxane A_2 and platelet-derived growth factor on the proliferation of smooth muscle cells might be of importance for both, the neointima formation in atherosclerotic vessels as well as for wound healing after vessel injury.

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