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Tolerance development to antimitogenic actions of prostacyclin but not of prostaglandin E_1 in coronary artery smooth muscle cells

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Abstract

This study compares the antimitogenic effects of iloprost and prostaglandin E_1 on platelet-derived growth factor-BB stimulated DNA synthesis ([^{3}H]thymidine incorporation) in bovine coronary artery smooth muscle cells. When added 20–24 h after stimulation with platelet-derived growth factor-BB (20 ng/ml), both iloprost and prostaglandin E_1, concentration-dependently (IC_{50} 3–5 nM) inhibited DNA synthesis. However, when added together with the growth factor (0–24 h), the inhibition of DNA synthesis by iloprost was markedly attenuated, indicating tolerance development. In contrast, no tolerance to antimitogenic effects of prostaglandin E_1 or forskolin were observed. When added to iloprost-tolerant cells, both prostaglandin E_1 and forskolin, still inhibited DNA synthesis. There was no evidence for transcriptional down-regulation of prostacyclin receptor gene by iloprost. The data demonstrate a tolerance development to antimitogenic actions of prostacyclin but not of prostaglandin E_1 and suggest that the receptors, mediating the antiproliferative actions of these prostaglandins, may be different. © 1998 Elsevier Science B.V.

Keywords: Prostacyclin; Prostaglandin E_1; Coronary artery, bovine; Smooth muscle cell; Cell proliferation; Platelet-derived growth factor

1. Introduction

The pathophysiology of restenosis subsequent to vessel injury, for example following percutaneous transluminal coronary angioplasty, is complex. Although chronic recoil may account for progressive luminal narrowing after balloon angioplasty, proliferation of vascular smooth muscle cells is one of several important events in the biology of human restenosis (Schwartz and Reidy, 1996). Therefore, numerous attempts have been undertaken to prevent uncontrolled smooth muscle cell proliferation by appropriate drug treatment, however, so far with limited success (Ross and Fuster, 1996).

Endothelial dysfunction, i.e., a net-loss of tissue-protective endothelium-derived mediators, such as nitric oxide and vasodilatory prostaglandins, has been considered a possible explanation of smooth muscle cell migration, dedifferentiation and mitogenesis (Pomerantz and Hajjar, 1989). Consequently, the action of vasodilatory prostaglandins, such as prostacyclin and prostaglandin E_1, on growth factor-induced mitogenesis was studied in numerous experimental settings. Following the first report (Huttner et al., 1977), several later trials, mainly cell culture studies (Nilsson and Olsson, 1984; Owen, 1985, 1986; Morisaki et al., 1988; Shirotani et al., 1991; Isogaya et al., 1995) confirmed an antiproliferative potential of these compounds. However, the data were not equivocal. For example, different responses were found for prostaglandin E_1 and prostacyclin (Shirotani et al., 1991), there was evidence for a cell cycle-dependent action of prostaglandin E_1 (Owen, 1985, 1986) and negative results were also reported (Willis et al., 1986). Most importantly, two clinical studies, investigating the effect of prostacyclin administration on restenosis after percutaneous transluminal coronary angioplasty, failed to demonstrate prevention of restenosis (Knudtson et al., 1990; Gershlick et al., 1994).

These apparently contradictory findings might be due to

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different experimental setups, for example the presence of non-specified growth factors, such as fetal calf serum, during the measurement of DNA replication. In addition, endogenous prostaglandin production by smooth muscle cells might become a variable itself. Several studies have documented a marked increase in smooth muscle cell-derived prostacyclin after vessel injury (Rimarachin et al., 1994) or after stimulation with growth factors (Hara et al., 1995), probably subsequent to up-regulation of the cyclooxygenase-2 (Wittpoth et al., 1997). This raises the possibility that endogenous prostaglandins might interfere with the exogenously added compounds. Finally, in many studies on antimitogenic actions of prostaglandins, effective concentrations were in the micromolar range (e.g., Huttner et al., 1977; Uehara et al., 1988), i.e., several orders of magnitude above the K_d of these compounds, suggesting a reduced sensitivity in dependency on the experimental conditions.

One of these variables might be a changed prostaglandin receptor sensitivity. Interestingly, no study on prostaglandin-related control of mitogenesis appears to have considered a possible variation of prostaglandin receptor density or affinity in smooth muscle cells. Several of the biological actions of prostacyclin, including inhibition of platelet aggregation (Alt et al., 1986) and vasodilation (Vermue and Houwertjes, 1985) were found to be reduced by agonist-induced receptor down-regulation. We have previously demonstrated that treatment of endothelial cells with the prostacyclin mimetic iloprost may completely down-regulate endothelial prostacyclin receptors (Schröder and Schrör, 1993). Others have provided evidence for additional involvement of more distal signal transduction pathways, for example adenylate cyclase (Edwards et al., 1987; Jaschonek et al., 1988). Thus, the question arises whether prostacyclin-related control of smooth muscle cell growth involves modulation of prostacyclin receptors or their coupling to intracellular signal transduction pathways.

This study investigates the antiproliferative effects of the stable prostacyclin mimetic iloprost in cultured bovine coronary artery smooth muscle cells under well defined in vitro conditions. Specifically, any contribution of endogenous prostaglandins was excluded by indomethacin pretreatment and platelet-derived growth factor-BB was the only mitogen that was added to stimulate DNA replication. The following questions were asked: (i) Does iloprost exert an antimitogenic effect, (ii) Is there any evidence for tolerance to the antimitogenic actions of iloprost and does this occur at the receptor level or more distal parts of the signal transduction cascade such as the mitogen-activated protein kinase (MAP-kinase), and (iii) Does prostaglandin E_1, which is not an arachidonic acid metabolite and is not a physiologically generated prostaglandin in vascular smooth muscle cells, behave similar to the prostacyclin mimetic iloprost. A preliminary report of part of the results was presented previously (Grosser et al., 1995).

2. Materials and methods

2.1. Coronary artery smooth muscle cell culture

Bovine coronary artery smooth muscle cells were isolated enzymatically (Fallier-Becker et al., 1990) from the media of the left anterior descending coronary artery of adult female animals as previously described (Grosser et al., 1997). In brief, the cells were cultured in HAM's F12/Dulbecco's modified Eagle medium (8:2, v/v), supplemented with 15% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C on 150-mm Petri dishes. Smooth muscle cells were identified by phase contrast microscopy, showing the typical hill and valley structure and by indirect immunostaining with a specific monoclonal α-actin antibody. The media were exchanged twice a week and subconfluent monolayers were split 1:4 once a week using a trypsin–EDTA (0.05%/0.5 mM) solution.

2.2. Protocol

Smooth muscle cells of passages 4–10 were seeded in 24-well plates (5×10^4 cells/well) and allowed to grow for 72 h. During the subsequent 24 h, cells were kept in serum-free medium to achieve growth arrest and synchronization of the cell cycle at the G_0/G_1 boundary. After washing twice with phosphate-buffered saline, containing indomethacin (3 μM), cells were exposed to fresh serum-free media, again containing indomethacin. Platelet-derived growth factor-BB (20 ng/ml) was added for 24 h. The compounds to be studied, iloprost (1–100 nM), prostaglandin E_1 (1–100 nM), forskolin (30 nM), or the vehicle were added either simultaneously with platelet-derived growth factor (time 0 h) for the total incubation period (0–24 h) or only for the last 4 h of the incubation period (20–24 h) according to the specific protocol. In the cross-tolerance experiments, fresh prostaglandin-containing medium was added for the last 4 h (20–24 h). [^{3}H]Thymidine was added in all experiments at 20–24 h (0.5 μCi/well).

2.3. [^{3}H]Thymidine incorporation

At the end of the 24-h incubation period, media were removed and the cell monolayers were washed sequentially twice with 1 ml ice-cold phosphate-buffered saline, 0.3 ml perchloric acid (0.3 M) for 1 min, and again phosphate-buffered saline. The cells were then solubilized by addition of 0.1 M NaOH (0.3 ml) for 30 min at 37°C. An 0.2 ml aliquot was added to 5 ml of scintillation fluid. [^{3}H]Thymidine incorporation was quantified by liquid scintillation spectrometry. Total cell-protein was measured in aliquots from each well using the Bio-Rad colorimetric protein assay (Bradford, 1976). Cell viability was assessed by trypan blue exclusion and was found to be higher than 95% in all experimental conditions.

2.4. MAP kinase assay

MAP kinase activity was assessed in terms of myelin basic protein phosphorylation. Subconfluent smooth muscle cells in dishes (diameter: 100 mm) were exposed to serum-free medium for 24 h. Then, the medium was replaced by fresh serum-free medium, supplemented with indomethacin (3 μM). The time-protocol for iloprost (100 nM) desensitization was exactly the same as for the [^{3}H]thymidine incorporation experiments: smooth muscle cells were preincubated with iloprost or vehicle for 20 h. According to previous data the maximum activation of MAP kinase occurred at 10 min after stimulation with platelet-derived growth factor (Grosser et al., 1997; Sachinidis et al., 1995). Thus, iloprost (100 nM) was administered immediately (2 min) before MAP kinase activation was initiated by platelet-derived growth factor (20 ng/ml for 10 min) to both groups (iloprost-tolerant and non-tolerant cells, respectively). The same procedure was used for forskolin (30 nM). Thereafter, cells were washed with phosphate-buffered saline and scraped into 0.4 ml of kinase buffer of the following composition (mM): Tris–HCl, pH 7.5, 20; EGTA 1; $MnCl_2$ 2; sodium orthovanadate 0.1; phenylmethane sulfonylfluoride 1; leupeptin 0.025. The cells were briefly sonicated and centrifuged for 5 min at $14{,}000 \times g$. An aliquot of the supernatant was used for protein determination. The phosphorylation assay was carried out as described (Huwiler et al., 1995). In brief, aliquots of the cell extract (60 μg protein) were incubated for 15 min at 30°C with 20 μg myelin basic protein, 10 μM ATP and 4 μCi of γ-[^{32}P]-ATP. The reaction was terminated by addition of Laemmli buffer and heating for 5 min at 95°C. The proteins were separated by sodium dodecyl sulfate (SDS, 13.5%) polyacrylamide gel electrophoresis. After fixation in 25% propan-2-ol/10% acetic acid the gels were dried and exposed on an X-ray hyperfilm at -80°C.

2.5. Northern blot analysis of smooth muscle cell RNA

Smooth muscle cells in serum-free medium were treated with iloprost (100 nM) for 2 h and 20 h, respectively. Incubations were performed in the presence of platelet-derived growth factor-BB (20 ng/ml). Total RNA was prepared using the single step Trizol method according to the recommendations of the producer (Gibco BRL, Eggenstein, Germany). Poly A^+-fractions were prepared from total RNA with paramagnetic particles (Poly ATtract, Promega, Madison, WI, USA), subsequently electrophoresed through agarose gels (1%) containing formaldehyde, and transferred onto a Nylon membrane (Hybond N, Amersham, Braunschweig, Germany). A [^{32}P]-labelled RNA probe was generated using a 600-bp genomic fragment from the 3′-end of the bovine vascular smooth muscle cell prostacyclin-receptor coding sequence (accession #Z93039 and #Z933040, EMBL databank), cloned in our laboratory (Hasse et al., 1997). The fragment was inserted into the pKS bluescript vector (Stratagene, Heidelberg, Germany). Hybridization was carried out at 45°C in 50% formamide, 5 × standard saline phosphate EDTA (SSPE), 0.2% SDS, 5 × Denhardt's, 100 μg/ml herring sperm DNA, 4×10^6 cpm/ml of the radiolabelled prostacyclin receptor probe and, as a reference, 5×10^5 cpm/ml of a random-primed rat glyceraldehyde-3-phosphate dehydrogenase fragment (1.4 kb). After stringent washing at 72°C in 2 × SSPE, 0.2% SDS (2 × 10 min) and 0.2% SSPE, 0.2% SDS (2 × 15 min) the membrane was exposed to a Kodak XOmat AR film for 15 h.

2.6. Materials

The following compounds were gifts: Iloprost from Schering (Berlin, Germany), prostaglandin E_1 from Schwarz-Pharma (Monheim, Germany) and indomethacin from Luitpold-Pharma (München, Germany). Platelet-derived growth factor-BB and the monoclonal α-actin antibody were purchased from Boehringer Mannheim (Mannheim, Germany). Methyl-[^{3}H]thymidine was purchased from DuPont NEN (Bad Homburg, Germany), γ-[^{32}P]ATP from Hartmann Analytik (Braunschweig, Germany). Scintillant (Rotiszint eco-plus) was obtained from Carl Roth (Karlsruhe, Germany). Myelin basic protein, collagenase, elastase and trypsin inhibitor for the enzymatic smooth muscle cell isolation were purchased from Sigma (Deisenhofen, Germany). All the other cell culture media and reagents were from Gibco-Life Technologies (Eggenstein, Germany).

2.7. Statistics

The data are mean ± S.E.M. of n independent measurements, performed in duplicate or triplicate as indicated. Group differences were analysed by the two-tailed Student's t-test for unpaired data or one-way analysis of variance and post-hoc Scheffé test as appropriate. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Inhibition of [^{3}H]thymidine incorporation by iloprost

Incubation of smooth muscle cells with platelet-derived growth factor-BB for 24 h stimulated [^{3}H]thymidine incorporation 3–4-fold above control. Pulse-labelling experiments further indicated that [^{3}H]thymidine incorporation became stimulated at about 12–14 h after addition of the mitogen and approached a maximum at about 20 h (data not shown). These findings were similar to data obtained in other cultured smooth muscle cell preparations (Nilsson and Olsson, 1984; Owen, 1986; Morisaki et al., 1988; Bachhuber et al., 1995).

The addition of iloprost (1–100 nM) during the last 4 h of the incubation period (20–24 h) reduced the platelet-derived growth factor-induced DNA synthesis in a concentration-dependent manner, by 50–60% (Fig. 1). The IC_{50} amounted to 3–5 nM, a maximum effect was obtained at 10 nM. A completely different picture was seen, when iloprost was added simultaneously with the mitogen, i.e., at time 0–24 h. There was still an inhibition of [^{3}H]thymidine incorporation. However, the maximum inhibition was only half of that seen with administration of the compound at time 20–24 h after addition of the growth factor and was further reduced at higher concentrations of iloprost. No inhibition at all was seen at 100 nM (Fig. 1). These data demonstrate a potent antimitogenic effect of iloprost if the compound is added during the synthesis phase of the cell cycle but not if the compound is already added to resting cells.

Degradation of iloprost was excluded by the demonstration that supernatants of cells from the total 24 h incubation period were equally potent inhibitors of ADP-induced platelet aggregation as were samples taken from the 4-h incubation supernatant or samples drawn shortly after addition of iloprost to the culture medium (not shown). Moreover, addition of fresh iloprost (100 nM, 20–24 h) to iloprost (100 nM, 0–20 h)-treated cells did not result in an inhibition of DNA synthesis (see 3.3.), indicating tolerance development towards the antimitogenic actions of iloprost.

3.2. Inhibition of [^{3}H]thymidine incorporation by prostaglandin E_1

The addition of prostaglandin E_1 at time 20–24 h after addition of the mitogen resulted in an inhibition of DNA synthesis similar to that seen with iloprost. The IC_{50} was slightly lower than that of iloprost, amounting to approximately 3 nM, maximum inhibition was observed at 10 nM. However, in contrast to iloprost, there was no reduced efficacy if prostaglandin E_1 was added to quiescent cells simultaneously with platelet-derived growth factor (0–24 h). These data are shown in Fig. 2 and suggest that the antimitogenic effects of iloprost and prostaglandin E_1 might be mediated by different pathways. Similarly to iloprost, there was no degradation of prostaglandin E_1 as assessed by its inhibitory actions on platelet aggregation (see above).

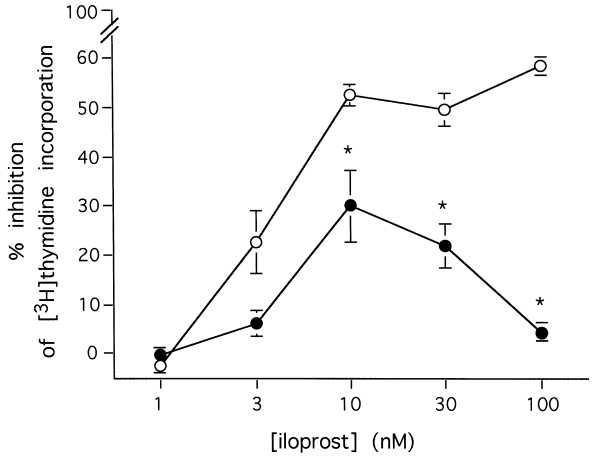

Fig. 1. The inhibition of platelet-derived growth factor (20 ng/ml)-stimulated [^{3}H]thymidine incorporation in smooth muscle cells by iloprost (1–100 nM). Iloprost was added to quiescent cells together with platelet-derived growth factor for the total incubation period (0–24 h, closed circles) or only for the last 4 h of incubation (20–24 h, open circles). Data are % inhibition of the growth factor-stimulated [^{3}H]thymidine incorporation. The data are mean ± S.E.M. of $n = 3$ independent experiments (duplicates). ∗: $P < 0.05$ 0–24 h vs. 20–24 h incubation at the same concentrations of iloprost.

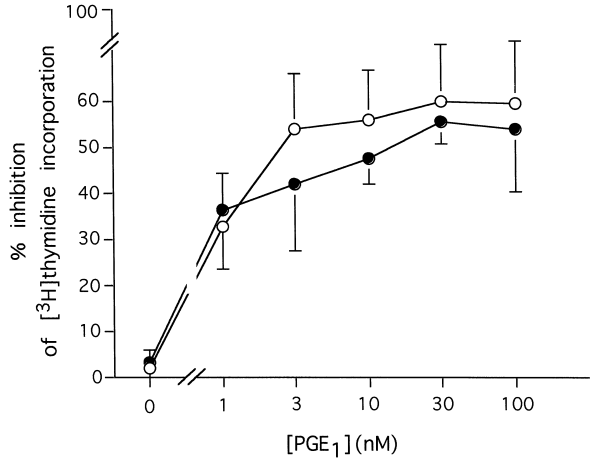

Fig. 2. The inhibition of platelet-derived growth factor (20 ng/ml)-stimulated [^{3}H]thymidine incorporation in smooth muscle cells by prostaglandin E_1 (1–100 nM). Prostaglandin E_1 was added to quiescent cells together with platelet-derived growth factor for the total incubation period (0–24 h, closed circles) or only for the last 4 h of incubation (20–24 h, open circles). Data are % inhibition of the growth factor-stimulated [^{3}H]thymidine incorporation. The data are mean ± S.E.M. of $n = 3$ independent experiments (duplicates). ∗: $P < 0.05$ 0–24 h vs. 20–24 h incubation at the same concentrations of prostaglandin E_1.

3.3. Cross incubation of iloprost-tolerant cells with prostaglandin E_1

To evaluate whether the antiproliferative actions of iloprost and prostaglandin E_1 might be mediated by different receptors, a pharmacological approach was taken, i.e., determination of possible cross desensitization by the two agonists. In smooth muscle cells made tolerant to iloprost, the inhibition of [^{3}H]thymidine incorporation by iloprost was abolished, whereas that by prostaglandin E_1 was unchanged (Fig. 3). This experiment suggests different receptors for the antimitogenic action of the compounds and additionally shows that iloprost does not down-regulate the antimitogenic action of prostaglandin E_1.

3.4. Inhibition of [^{3}H]thymidine incorporation by forskolin

In order to elucidate if the tolerance towards the antimitogenic effects of iloprost occurs at the level of adenylate cyclase, experiments were carried out with the direct stim-

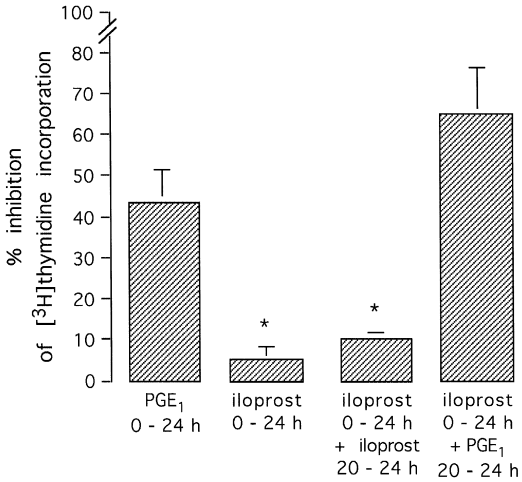

Fig. 3. The absence of cross tolerance between iloprost (100 nM) and prostaglandin E_1 (PGE_1, 10 nM) in iloprost-tolerant cells. The inhibition of platelet-derived growth factor-stimulated [^{3}H]thymidine incorporation in smooth muscle cells was determined in the absence (not shown) and continuous presence of prostaglandin E_1 or iloprost for 0–24 h as indicated. Both compounds were also applied again for the last 4 h of incubation (20–24 h) to iloprost-tolerant samples. Data are % inhibition of platelet-derived growth factor-stimulated [^{3}H]thymidine incorporation and are mean ± S.E.M. of $n = 4$ independent experiments (triplicates). *: $P < 0.05$ (iloprost alone vs. prostaglandin E_1).

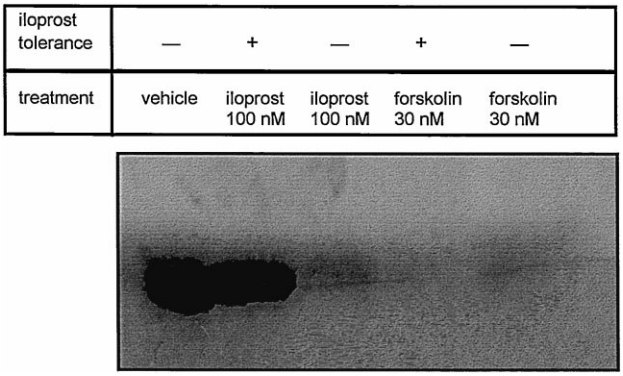

Fig. 4. The inhibition of platelet-derived growth factor-BB-induced MAP kinase activity by iloprost (100 nM) and forskolin (30 nM) in iloprost-tolerant and non-tolerant cells. Platelet-derived growth factor causes a significant increase in myelin basic protein phosphorylation which was largely prevented by treatment with iloprost. This inhibitory action of iloprost was not seen, when cells were made tolerant by a 20-h treatment with iloprost and exposed to fresh medium, containing iloprost at the same concentration. Forskolin (30 nM) exerted a complete inhibition of MAP kinase activation in cells in non-tolerant and iloprost-tolerant cells. An original autoradiograph, representative for $n = 3$ similar experiments, is shown.

ulator of adenylate cyclase, forskolin. The inhibition by forskolin (30 nM) of [^{3}H]thymidine incorporation, added together with platelet-derived growth factor (0–24 h), was similar to the maximum inhibition with the prostaglandins. Table 1 compares the antimitogenic actions of forskolin and iloprost. Forskolin inhibited [^{3}H]thymidine incorporation to a similar extent when added simultaneously with the growth factor (0–20 h) or 20 h after addition of the growth factor (20–24 h). Thus, there was no tolerance development towards forskolin. In addition, forskolin inhibited DNA synthesis to a comparable extent when added to cells made tolerant to iloprost (see above) as compared to non-tolerant cells (not shown). This further suggests the membrane receptor and/or its coupling to G-proteins but not the adenylate cyclase as a possible target of tolerance development to iloprost.

Table 1
The effects of iloprost (100 nM) and forskolin (30 nM) on platelet-derived growth factor-stimulated [^{3}H]thymidine incorporation (10^3 dpm/mg protein) in smooth muscle cells

Time of incubation with iloprost or forskolin	Control	Iloprost	Forskolin
0–24 h	27.2 ± 5.4 (0%)	24.8 ± 4.2 (9%)	12.1 ± 3.4[a] (55%)
20–24 h	–	12.9 ± 3.0[a] (53%)	11.6 ± 2.6[a] (57%)

[a] $P < 0.05$ (platelet-derived growth factor + iloprost or forskolin vs. control).

Iloprost or forskolin were added to the incubation media either together with the growth factor for the total incubation period of 0–24 h or only for the last 4 h (20–24 h) after growth factor stimulation. The data are mean ± S.E.M. of $n = 8$ independent experiments (triplicates). The % inhibition as referred to platelet-derived growth factor stimulated preparations in the absence of prostaglandins (control) is indicated in parentheses.

3.5. MAP kinase phosphorylation assay

In order to evaluate whether tolerance to the antimitogenic actions of iloprost also affects cellular signal transduction pathways that are involved in growth control, the activity of MAP kinase was determined. Platelet-derived growth factor alone caused a marked increase in myelin basic protein phosphorylation which was completely prevented by iloprost (100 nM) and forskolin (30 nM), respectively. If the same experiments were conducted with cells that were tolerant to iloprost, this inhibition was not further detected, while the inhibitory response to forskolin remained unchanged. Fig. 4 shows one representative experiment out of three with similar results.

3.6. Prostacyclin receptor expression

The possible down-regulation of prostacyclin receptors in smooth muscle cells in the continuous presence of iloprost was studied by Northern hybridization. Cells were treated with iloprost (100 nM) for 2 and 20 h, respectively. Fig. 5 demonstrates the results from an original experiment. Prostacyclin receptor mRNA was detectable but unchanged by iloprost treatment. This observation does not support the notion that down-regulation of prostacyclin

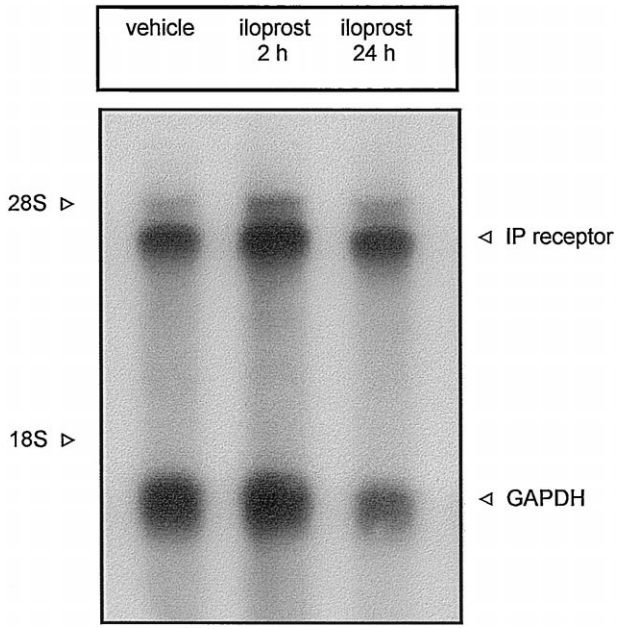

Fig. 5. The effects of iloprost on prostacyclin receptor (IP receptor) gene expression. Smooth muscle cells were treated either with vehicle or with iloprost (100 nM) for 2 h or 20 h in the presence of platelet-derived growth factor-BB (20 ng/ml). A labelled fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was cohybridized and used as an internal standard for mRNA quantity. No differences in the expression of the mRNA for the prostacyclin receptor (IP) were detected. An original blot, representative for $n = 3$ similar experiments, is shown.

receptor gene expression is a possible mechanism of tolerance development.

4. Discussion

This study confirms and extends previous findings on antimitogenic actions of iloprost and prostaglandin E_1. Both compounds were potent inhibitors of platelet-derived growth factor-induced mitogenesis at low (≤ 10 nM) concentrations as assessed in terms of [^{3}H]thymidine incorporation. Both compounds also exerted a similar maximum inhibitory effect, resulting in an approximately 50% inhibition of total [^{3}H]thymidine incorporation. However, there were marked differences between iloprost and prostaglandin E_1 with respect to the duration of prostaglandin exposition: Treatment with iloprost, when started together with addition of platelet-derived growth factor (0–24 h), resulted in a significantly reduced inhibition of mitogenesis as opposed to the effects seen with addition of the compound at 20–24 h after growth factor stimulation. There was even a complete tolerance at higher concentrations (100 nM) of iloprost when these were present throughout the total incubation period (0–24 h) which was also seen at the level of MAP kinase activation. These data suggest concentration-dependent development of tolerance to the antimitogenic actions of iloprost.

Vasodilatory prostaglandins, such as prostacyclin and prostaglandin E_1, are supposed to exert their biological actions, including modulation of mitogenesis, via receptors at the cell membrane (for review, see the work of Coleman et al., 1994; Schrör, 1997). Thus, the strength and duration of a prostaglandin-mediated event is critically dependent upon the number of accessible receptors that are coupled to cellular signal transduction pathways. Earlier in vitro studies have shown that the vasodilatory (Vermue and Houwertjes, 1985) and antiplatelet (Edwards et al., 1987; Jaschonek et al., 1988) actions of iloprost are subject to heterologous desensitization, possibly taking place at the level of G_s/adenylate cyclase coupling. We have previously shown that prostacyclin receptors on bovine aortic endothelial cells undergo an agonist-induced down-regulation after prolonged exposure to the prostacyclin mimetic iloprost (Schröder and Schrör, 1993). The present data agree with this finding, suggesting that the prostacyclin receptors in vascular smooth muscle cells behave similar. Since prostacyclin is the major cyclooxygenase product, synthesized by vascular smooth muscle cells (Uehara et al., 1988), this may explain why other investigators failed to detect the inhibition of growth responses in smooth muscle cells, treated with prostacyclin for 1–4 days (Willis et al., 1986). Alternatively, inhibition of endogenous prostacyclin generation by indomethacin might have resulted in a hypersensitivity to the agonist. In this context, the low IC_{50}, amounting to < 5 nM, is remarkable as opposed to concentrations of > 1 μM which had to be used by others to obtain an antimitogenic effect (Morisaki et al., 1988; Uehara et al., 1988). Whether this is due to the presence of additional growth factors in serum, species differences and/or stimulated endogenous prostaglandin formation, remains to be determined.

To establish that the desensitization occurred at the receptor level, we have determined the antimitogenic actions of forskolin. There was no time-dependent attenuation of antimitogenic actions of forskolin. In addition, there was a comparable inhibition of DNA synthesis by forskolin in iloprost-tolerant and non-tolerant cells, respectively, suggesting that it is the receptor availability or receptor/G-protein coupling but not more distal signal transduction pathways that are involved in tolerance toward iloprost.

After cloning of the bovine prostacyclin receptor (Hasse et al., 1997), we have studied the possible transcriptional regulation of this receptor by Northern hybridization. There was no evidence for a down-regulation of the receptor mRNA by iloprost, suggesting that tolerance development did not occur at the level of receptor gene regulation. However, an iloprost-dependent change in mRNA stability remains to be investigated. In addition, the Northern hybridizations represent the mRNA of all cells. However, measuring bromodeoxyuridine incorporation using immunohistochemistry, revealed that only about one third of the smooth muscle cells were released into S-phase after

stimulation with platelet-derived growth factor. Thus, Northern hybridization may not be sensitive enough to detect changes in prostacyclin receptor mRNA.

We have also tried to quantify the number of receptors and to determine their affinity by ligand-binding studies, using [^{3}H]iloprost. These experiments were not successful under a variety of experimental conditions. Possible explanations are a low density of the receptors, possibly associated with a high non-specific binding. Unfortunately, there are no high-affinity antagonists available. However, the unchanged antimitogenic response to forskolin in terms of [^{3}H]thymidine incorporation and MAP kinase activity in iloprost-tolerant cells strongly suggests the receptor or receptor/G-protein coupling as putative levels of desensitization.

In contrast to iloprost, prostaglandin E_1 at an equieffective antimitogenic potency did not show any tolerance development. There was also no cross desensitization as seen from an unchanged antimitogenic effect of prostaglandin E_1 in iloprost-tolerant smooth muscle cells. This adds further evidence to the concept of different receptors, mediating the antimitogenic actions of iloprost and prostaglandin E_1.

Endogenous prostaglandins contribute to regulation of several functions in vascular smooth muscle cells: Control of local perfusion, for example after stimulation by shear–stress (Alshihabi et al., 1996) and control of excessive growth after stimulation by cytokines or mitogens (Pomerantz and Hajjar, 1989). Clearly, high local prostacyclin formation at an area of vessel injury should not result in a complete inhibition of mitogenesis, i.e., wound healing. There is a marked stimulation of prostacyclin biosynthesis after induction of cyclooxygenase-2 by growth factors in rat aortic smooth muscle cells (Rimarachin et al., 1994). In our preparations, there was a transient 4–5-fold increase in prostacyclin production in the absence of indomethacin after stimulation with platelet-derived growth factor (unpublished results). Thus, in addition to cell cycle-dependent actions of prostacyclin (Nilsson and Olsson, 1984; Owen, 1986), tolerance development at the receptor level might be an important regulatory event which is not shared by prostaglandin E_1 because this non-arachidonic acid metabolite is not generated in vascular cells.

High endogenous prostacyclin production, for example in acute myocardial ischaemia, is associated with a marked platelet desensitization to iloprost (Jaschonek et al., 1986). Chronic administration of prostacyclin to patients with advanced atherosclerosis was found to result in a 3-fold decrease of platelet sensitivity against prostacyclin after 3 days of infusion (Sinzinger et al., 1981). Finally, there was no inhibition of ADP-induced platelet aggregation and no inhibition of late restenosis in patients, treated with intravenous infusions of prostacyclin for 36 h subsequent to percutaneous transluminal coronary angioplasty (Knudtson et al., 1990; Gershlick et al., 1994). It will be interesting to see whether prostaglandin E_1, which is not generated in significant amounts within the cardiovascular system and, according to the data of the present study, may act on receptors that are different from prostacyclin receptors, might be a more useful alternative.

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