Prostacyclin Induces Apoptosis of Vascular Smooth Muscle Cells by a cAMP-Mediated Inhibition of Extracellular Signal-Regulated Kinase Activity and Can Counteract the Mitogenic Activity of Endothelin-1 or Basic Fibroblast Growth Factor

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Abstract—Prostanoids can suppress vascular smooth muscle cell (VSMC) proliferation, but the mechanism through which this is mediated has not been identified. In this study, we show rat aortic VSMCs to express the EP₁, EP₂, EP₃, EP₄, and IP receptors. The EP₄ receptor–specific agonist, 11-deoxy-PGE₁, induced a time-dependent phosphorylation of protein kinase C and extracellular signal-regulated kinase (ERK) 1/2 in serum-depleted (0.1%) VSMCs, whereas the EP₂ receptor agonist, butaprost, was without effect. PGI₂ or iloprost at the IP receptor inhibited basal ERK phosphorylation with IC₅₀ values of ≈10 nmol/L. Iloprost also attenuated the sustained activation of ERK induced by endothelin-1 or basic fibroblast growth factor (bFGF). Endothelin-1 or bFGF significantly increased the number of VSMCs counted 24 hours later compared with basal, and both responses were blocked by the MEK inhibitor, U0126, or iloprost. Under basal conditions, U0126 or iloprost reduced the number of viable cells and increased caspase-3 activity, which could be reversed by coapplication with endothelin-1, bFGF, or the adenylate cyclase inhibitor, SQ22536. Endothelin-1, bFGF, or SQ22536 prevented the depression to below basal levels of ERK phosphorylation induced by iloprost. Forskolin activated caspase-3 and attenuated basal ERK phosphorylation, which were prevented by SQ22536, endothelin-1, or bFGF. These data suggest that iloprost induces apoptosis via a cAMP-mediated suppression of ERK activity. In turn, this apoptotic response can be blocked by a mitogenic stimulus that re-establishes ERK activity back to basal levels, but at the expense of any concomitant proliferative activity. However, ERK stimulation by a selective EP₄ receptor agonist, suggests that prostanoids may have diverse and complex roles in VSMC physiology. (Circ Res. 2004;94:759-767.)

Key Words: prostacyclin • apoptosis • vascular smooth muscle cells • endothelin • mitogen-activated protein kinase

The medial layer of human arteries is composed of vascular smooth muscle cells (VSMCs) interspersed between elastic lamellae. Endothelial cells and a small number of myointimal cells form the intimal layer. The medial VSMCs are the primary regulators of vascular tone, and are usually quiescent in vivo. In certain situations, however, such as after balloon angioplasty, surgery, or in atherosclerosis, they proliferate and migrate into the intima and eventually become the predominant cell type in vascular lesions. Under physiological conditions, it has been suggested that prostanoids secreted by the endothelial cells can prevent the proliferation of VSMCs. Endothelial cells synthesize and release several vasodilators and vasoconstrictors, which inhibit or stimulate the contraction and proliferation of the underlying smooth muscle, respectively. Vasodilators include endothelium-derived nitric oxide and PGI₂, whereas endothelin and thromboxane A₁ (TXA₁) are known vasoconstrictors. In situations of endothelial dysfunction the normal antiproliferative drive for the smooth muscle cell population may be impaired. However, the mechanism through which proliferation is inhibited has yet to be elucidated.

Prostanoids are a diverse family of oxygenated fatty acids derived from arachidonic acid. The major source of free arachidonic acid is the phospholipids of the cell membrane, from which it is largely liberated by the actions of phospholipases. Cyclooxygenase (COX) converts arachidonic acid to PGH₂, which is then used to synthesize the individual prostanoids, including PGE₂, PGD₂, PGF₂α, TXA₂, and PGI₂. The first prostanoid receptor to be cloned was the TXA₂ (TP) receptor, and since then, homology screening has been used to clone prostanoid receptors from several species. Because it is well known that TXA₂ and PGI₂ exhibit aggregatory and
nonaggregatory effects on platelets, respectively. It is clear that different members of this G protein–coupled receptor family can evoke different responses in the same system. On the basis of their activities, the eight prostanoid receptors can be subdivided into three categories. The relaxant receptors, including the DP, EP₂, EP₄, and IP receptors, generally cause increases in intracellular cAMP and mediate vasodilatation. The EP₁, FP, and TP receptors can be grouped as contractile receptors that are coupled to increases in intracellular Ca²⁺. Lastly, the EP₃ receptor is reported to be an inhibitory receptor, often inducing decreases in intracellular cAMP. However, details of the signaling mechanisms utilized by the individual prostanoid receptor types, particularly with respect to controlling VSMC survival are presently unavailable.

G protein–coupled receptors stimulate mitogenesis, in part, via the extracellular signal-regulated kinases (ERKs), which are members of the mitogen-activated protein (MAP) kinase family. In turn, MAP kinases phosphorylate and regulate the activity of key enzymes and nuclear proteins, which ultimately modulate the expression of genes essential for proliferation. In addition, G protein–coupled receptors can also activate other members of the MAP kinase family, which regulate apoptosis. An antiproliferative activity of G protein–coupled receptors may thus result as a consequence of inducing the cell death program.

In this study, we have identified the prostanoid receptor complement expressed in rat aortic VSMCs by using receptor-selective antibodies. In addition, changes in the activity status of key signaling effectors known to play an important role in mediating proliferation have been identified in response to prostanoid receptor-selective agonists. The antiproliferative activity of prostanoids has been determined by monitoring any effect on the increase in VSMC numbers induced by either endothelin-1 (ET-1) or basic fibroblast growth factor (bFGF), both of which have been shown to promote VSMC proliferation in atherosclerosis. Any antiproliferative activity detected has been assessed to determine if it is through the induction of apoptosis.

Materials and Methods

Materials
Antibodies to ERK1/2, protein kinase C (PKC), CREB, MEK1/2, SAPK, and p38 were from Cell Signaling Technology. The anti-cytochrome c antibody was from Pharmigen, and the antibody to the active form of caspase-3 was from Promega. Enzymes for the primary preparation of VSMCs were from Worthington. ET-1 and bFGF were provided by Sigma. EP receptor agonists and the receptor antibodies were supplied by Cayman Chemicals. Iloprost and PGI₂ were from Amersham. SQ22536, U0126, and forskolin were from Calbiochem.

Cell Culture and Trypan Blue Exclusive Assay
Rat aortic VSMCs were isolated by the enzyme dispersion method and routinely cultured in DMEM/Ham’s F12 (1:1) containing 10% serum. They were used between passages 6 to 15. To determine the number of viable cells, a single cell suspension in the presence of Trypan Blue was counted using a hemocytometer.

Deglycosylation of Receptor Protein
N-glycosidase F is able to release all common classes of N-glycans from the protein backbone. Membrane fractions prepared from

Figure 1. EP and IP receptor expression in VSMCs and the effect of selective agonists on the activity status of ERK. A, Membrane protein from VSMCs incubated with (B) or without N-glycosidase F was analyzed by Western blotting using antibodies specific for the EP₁, EP₂, EP₄, and IP receptors. G, VSMCs incubated for 30 minutes with the appropriate vehicle (CON), prostaglandin E₂ (PGE₂), butaprost (BUT), GR63799 (GR), 11-deoxy-PGE₁ (11D), iloprost (ILO) (all at 1 μmol/L), or 10% serum (FCS) were analyzed by Western detection using antibodies specific for ERK (ERK) and phosphorylated ERK (ERK-P). Labeling at the bottom of the panel denotes receptor selectivity for each agonist (1, 2, 3, and 4 correspond to the EP receptors).
VSMC monolayers were incubated for 2 hours at 37°C with 2.5 U N-glycosidase F (Roche) per 100 μg membrane protein.

Western Blotting Analysis
Cells were incubated in medium containing 0.1% serum, 4 hours before each experiment. Analysis of cell cycle parameters showed the VSMCs to be in a proliferative phase. Experiments were terminated by applying SDS PAGE sample buffer. Equivalent amounts of protein per sample were electrophoretically resolved on 10% polyacrylamide gels. After electrophoretic transfer, the nitrocellulose membrane was blocked overnight at 4°C with 5% dried milk. All antibody incubations were for 1 hour at 22°C. Immuno-complexes were visualized using enhanced chemiluminescence detection (Amersham) and each Western blot shown is a representative of at least three separate experiments.

Caspase Assay
Cells were incubated for 8 hours at 37°C in the presence of the appropriate agents. Homogeneous caspase-3/7 reagent (Promega) was added and incubated at room temperature for 30 minutes.

Immunofluorescent Staining
Cells were fixed in 2% formaldehyde for 30 minutes at 4°C and permeabilized in Tris-buffered saline with Triton X-100 (0.1%) and Tween 20 (0.1%). After blocking with goat serum (0.1%), the cells were incubated overnight at 4°C with the appropriate primary antibody. Secondary antibody incubations were for 1 hour at 22°C, and images were captured using a Leica TCS-SP-MP confocal microscope. Images shown are representative of three separate experiments.

Time-Lapse Confocal Microscopy
Cells attached to glass coverslips were transferred to a chamber on the confocal microscope stage that was regulated to 37°C. Annexin V-FLUOS was added to the chamber according to Roche’s instructions. Propidium iodide was added to a final concentration of 500 nmol/L. The cells were visualized using an infinity corrected, ×63 water immersion, UV and infrared transmissible, 1.2 NA, plan apochromat lens. Annexin V-FLUOS and propidium iodide were both excited using the 488-nm laser line. The cell permeant nuclear dye, Hoechst, was visualized by two-photon excitation, using a solid state Millenium V pumped Tsunami Ti:sapphire laser tuned to 780 nm. Images were captured every 4 minutes throughout the experimental period.

Statistical Analysis
All data are expressed as mean±SEM (n=4, three replicates per test group). Statistical significance was determined by Student’s t test.

Results
EP and IP Receptor Complement and Effect of Deglycosylation
Western analysis of VSMC membrane protein showed strong immunoreactive products for the EP1, EP2, EP4, and IP receptor types and a faint signal for the EP3 receptor (Figure 2). Changes in the phosphorylation status of ERK, PKC, and CREB by EP4 and IP receptor–selective agonists. Whole-cell extracts were prepared from VSMCs after treatment with incomplete media (0.1% FCS, Control) or that containing 11-deoxy-PGE1 (1 μmol/L) (A) or iloprost (1 μmol/L) (B) for the times shown (minutes). Activity changes were demonstrated by Western detection with phosphospecific antibodies (-P). Total levels of the kinases were unaffected (ERK).

Figure 2. Changes in the phosphorylation status of ERK, PKC, and CREB by EP4 and IP receptor–selective agonists. Whole-cell extracts were prepared from VSMCs after treatment with incomplete media (0.1% FCS, Control) or that containing 11-deoxy-PGE1 (1 μmol/L) (A) or iloprost (1 μmol/L) (B) for the times shown (minutes). Activity changes were demonstrated by Western detection with phosphospecific antibodies (-P). Total levels of the kinases were unaffected (ERK).
The apparent lower molecular mass (41 kDa).

**Effect of EP and IP Receptor Stimulation on the Phosphorylation Status of ERK**

Western analysis using phosphospecific antibodies was used to determine changes in the activity status of ERK on application of prostanoid receptor-selective agonists to VSMCs (Figure 1C). Treatment with PGE$_2$ (1 µmol/L) for 30 minutes caused a reduction in basal ERK activity, whereas the EP$_4$ receptor–selective agonist, 11-deoxy-PGE$_1$ (1 µmol/L), induced ERK phosphorylation. The EP$_3$ receptor agonist, GR63799 (1 µmol/L), inhibited basal ERK phosphorylation as did the IP receptor-selective analogue, iloprost (1 µmol/L). In contrast, the EP$_1$ receptor agonist, butaprost (1 µmol/L), was without effect.

The inhibitory effect of iloprost on basal ERK levels was dose-dependent (Figure 1D), and a decrease in ERK phosphorylation was apparent using subnanomolar concentrations. The inhibitory effect of PGI$_2$, however, was not as marked as iloprost at low concentrations (100 pmol/L), which may reflect the instability of PGI$_2$ compared with the synthetic mimetic. Interestingly, only a short pulse of PGI$_2$ or iloprost was sufficient to induce ERK inhibition. For example, application of PGI$_2$ or iloprost (10 nmol/L and 1 µmol/L) for 15 minutes followed by incubation for 45 minutes in the presence of minimal growth media induced a decrease in ERK phosphorylation that was comparable to that obtained after incubation for 1 hour in the continued presence of agonist (Figure 1E).

**ERK and PKC Activation Induced by the EP$_4$ Receptor**

The EP$_4$ receptor–selective agonist, 11-deoxy-PGE$_1$ (1 µmol/L), induced a time-dependent phosphorylation of PKC and ERK in serum-depleted VSMCs (Figure 2A). However, the kinetic profiles for the activation were distinct. ERK phosphorylation was rapid in onset and had declined to basal levels by 1 hour, whereas an increase in PKC activity was not observed over the first 5 minutes of agonist treatment but remained elevated over basal throughout the remainder of the time course examined (4 hours).

**IP Receptor–Mediated Phosphorylation of CREB**

Changes in the phosphorylation status of the PKA substrate, CREB, were used to monitor cAMP generation. There was no change in the phosphorylation of CREB or the related protein, ATF-1, under basal conditions over the time course investigated (Figure 2A). Application of iloprost (1 µmol/L) increased CREB but not ATF-1 phosphorylation that was maximal by 30 minutes and which remained sustained over the remainder of the investigation period (Figure 2B). In contrast, changes in CREB phosphorylation were not observed after treatment with 11-deoxy-PGE$_1$ (1 µmol/L) (Figure 2A).

**IP Receptor–Mediated Inhibition of ERK and MEK Activity Induced by ET-1 or bFGF**

In addition to investigating the inhibitory effect of iloprost on basal ERK activity, we also looked at the effect on mitogen-induced phosphorylation. Under basal conditions, the activity of ERK remained unchanged with respect to time (Figure...
Addition of iloprost (1 μmol/L) had no effect on basal ERK phosphorylation, 15 minutes after application. However, throughout the remainder of the time course, iloprost inhibited basal levels. In contrast, basal levels of PKC phosphorylation were unaffected.

Activation of ERK by ET-1 (100 nmol/L) was rapid and remained elevated over basal throughout the time course examined (Figure 3A). The initial activation of ERK induced by ET-1 (over 30 minutes) was unaffected by coapplication with iloprost. However, iloprost inhibited the sustained phase of ET-1-induced ERK phosphorylation and the level obtained after 1 hour fell to basal levels. ET-1 also stimulated PKC activity but this was unaffected by iloprost (Figure 3A).

Similar findings were obtained after coapplication of iloprost on the evoked phosphorylation of ERK by bFGF (10 ng/mL) (Figure 3B). The growth factor induced a time-dependent increase in the activity of ERK1 and ERK2 and also a third isoform that is possibly the splice variant of ERK1 (ERK1/β). The increased activity of the ERKs was not apparent over the initial 5 minutes after bFGF application, but reached a peak by 15 minutes and remained elevated over basal throughout the remainder of the time course. Iloprost had little effect on the initial peak of bFGF-induced activity (up to 15 minutes) but attenuated the subsequent sustained phosphorylation of ERK. PKC activity was also increased by bFGF application, but this was unaffected by iloprost (Figure 3B).

Confirmation of the inhibitory effect on ET-1– and bFGF-induced ERK phosphorylation by iloprost was obtained by immunofluorescent labeling (Figure 3C). After 1-hour incubation in the presence of bFGF, most of the phosphorylated ERK appeared in the nuclei. This increase in ERK activity was reduced by iloprost, which also decreased basal ERK activity. Similar inhibitory effects with iloprost were observed after treatment of ET-1 for 30 minutes (data not shown).

To determine if iloprost could exert a similar inhibitory effect on the activity of the upstream kinases to ERK1/2, the phosphorylation status of MEK1/2 was determined over time. Application of bFGF induced a rapid increase in the activity of MEK1/2, which remained elevated over basal throughout the time course examined (Figure 4A). The activity induced by bFGF over the initial 15 minutes after application was unaffected by iloprost. However, the sustained MEK activity was reduced to basal levels by treatment with iloprost. Basal MEK phosphorylation was inhibited 15 minutes after iloprost treatment without the presence of the growth factor (data not shown). The same patterns were repeated by the application of ET-1 (data not shown).

Iloprost-Mediated Effects on the Activity Status of the SAPK or p38 Kinases

Analysis of basal SAPK and p38 activities showed the phosphorylation status of these kinases remained constant over a period of 4 hours in the presence of 0.1% serum (Figure 4B). Several phosphorylated isoforms of the SAPK family were evident although their levels were low except for the p56 isoform. Application of iloprost (1 μmol/L) to VSMCs had no effect on the basal activity of the SAPKs or p38 at any time point examined.

Effect of IP Receptor Stimulation and MEK Inhibition on Cell Proliferation Induced by bFGF or ET-1

We assessed the ability of iloprost to modulate the proliferative activity of bFGF or ET-1 by directly counting viable cells. Application of bFGF (10 ng/mL) or ET-1 (100 nmol/L) significantly increased the number of VSMCs counted 24 hours later...
compared with controls (Figure 5A). These increases were abolished by iloprost (1 μmol/L) or a submaximal concentration of the MEK inhibitor, UO126 (5 μmol/L). In addition, UO126 or iloprost were shown to decrease the number of viable cells in the absence of bFGF or ET-1.

To substantiate if the decreases observed in cell number after PGI₂, iloprost, or UO126 treatment were due to an antiproliferative effect or the induction of cell death, we examined the activity of a terminal effector of the apoptotic pathway, caspase-3. Iloprost (Figure 5B) and PGI₂ (Figure 5C) were found to induce a concentration-dependent increase in caspase-3 activity with EC₅₀ values in the low nanomolar range. ET-1 or bFGF had no effect on basal levels of caspase-3 activity but prevented any increase evoked by iloprost (Figure 5B) or PGI₂ (Figure 5C). In addition, ET-1 could also block the induction of caspase-3 after UO126 treatment.

Treatment of VSMCs for 30 minutes with UO126 or iloprost reduced basal ERK activity as determined by Western analysis (Figure 5D). The induced phosphorylation of ERK by bFGF was also decreased back to basal levels by cotreatment with iloprost or UO126 (Figure 5D).

**Induction of Apoptosis by cAMP Production and Its Effect on ERK Activity**

To evaluate the effect of cAMP production on caspase-3 activity and to determine its influence on ERK phosphorylation, we used forskolin (100 μmol/L) to activate the adenylate cyclases and SQ22536 (300 μmol/L) to inhibit them. Forskolin significantly activated caspase-3 in control cells, whereas SQ22536 or bFGF (10 ng/mL) had no effect (Figure 6A). However, SQ22536 or bFGF could abolish the forskolin-induced caspase-3 activity. In combination with SQ22536, bFGF slightly but significantly inhibited basal caspase-3 activity. Caspase-3 activation with either iloprost (1 μmol/L) or UO126 (5 μmol/L) were both prevented by SQ22536 (Figure 6A). Cells serum-starved for 24 hours showed effects on caspase-3 activity that were comparable to cells that had been depleted of serum (0.1%) for 4 hours (Figure 6B).

Forskolin inhibited, whereas SQ22536 stimulated, basal ERK phosphorylation (Figure 6C). These effects appeared to counteract one another on coapplication of the drugs.
SQ22536 enhanced the phosphorylation of ERK induced by bFGF and blocked the depression of basal ERK activity caused by iloprost or UO126 application.

**Induction of Apoptosis by Iloprost and Rescue by ET-1**

Changes in caspase-3 activity were also examined by immunostaining. VSMCs treated with ET-1 (100 nmol/L) for 8 hours showed no detectable staining using an antibody to the active form of caspase-3 (Figure 7A). These cells also showed strong cytoplasmic staining for cytochrome c. In contrast, cells treated with iloprost (1 μmol/L) showed a marked decrease in cytochrome c compared with basal, and staining was detected for the active form of caspase-3. Cells cotreated with iloprost and ET-1 showed little staining for active caspase-3 and the pattern of cytochrome c distribution was similar to that for control cells.

To confirm the induction of apoptosis by iloprost, cells were also examined over time using confocal microscopy. Cells were incubated in the presence of annexin V to determine phosphatidylserine flip and propidium iodide as a marker of membrane integrity. Nuclear condensation, as determined by an increase in blue staining, was apparent 3 hours after iloprost application (Figure 7B). By 8 hours, cytoplasmic blebbing was evident and many cells showed staining for annexin V with no propidium iodide uptake. Throughout the 18-hour time period, the cells had retained some degree of normal morphology, and there was little evidence of detachment from the coverslip. VSMCs incubated for 17 hours with iloprost and ET-1 showed no propidium iodide uptake and little staining by annexin V (Figure 7C).

**Discussion**

Atherogenesis is a dynamic process involving inflammation, oxidant stress, and endothelial dysfunction, leading to the migration of medial VSMCs into the intima where they proliferate. An increase in prostanoid biosynthesis occurs during atherogenesis, and in this study, we demonstrate that some have apoptogenic effects. It has been suggested that prostanoids, and in particular PGI2, secreted by endothelial cells can reduce VSMC proliferation. IP knockout mice are more susceptible to thrombotic stimuli than their wild-type littermates and deletion of the IP receptor results in an enhanced proliferative response of VSMCs to catheter-induced carotid vascular injury. In addition, several IP-receptor agonists have been shown to attenuate conditions such as restenosis, and COX-2 inhibitors can increase the risk of cardiovascular events.

The first report to show that intracellular PGI2 can promote apoptosis was published recently. Overexpression of COX-1 or COX-2 in bovine aortic endothelial cells, which express PGI2 synthase constitutively, was shown to increase apoptosis. In this study, we have demonstrated that PGI2 and iloprost when exogenously applied to VSMCs expressing IP receptors can also stimulate apoptosis. This was substantiated by showing an increase in the active form of caspase-3, the redistribution of membrane phosphatidylserine, and a change in the pattern and intensity of cytochrome c staining. The activation of caspase-3 by iloprost or PGI2 was dose-dependent, with EC50 values in the low nanomolar range. Although most studies using cell model systems have routinely used prostanoids at micromolar concentrations, the amounts found in vivo are reported to be in the low nanomolar range. Obviously local concentrations of PGI2 released at the sites of vascular disease may be much higher, but our study suggests that iloprost or PGI2 can regulate apoptosis at concentrations similar to those reported to have effects in vivo. In addition, it seems that the IP-receptor
agonists only need to remain in contact with the receptor for short lengths of time to induce signaling changes that occur many minutes later.

There was a significant drop in the number of viable VSMCs counted between those maintained under basal conditions and those treated with iloprost, consistent with apoptotic induction. In addition, the increase in cell number evoked by either bFGF or ET-1 was abolished by iloprost. However, confocal images and changes in caspase-3 activity showed that ET-1 or bFGF could prevent apoptosis induced by iloprost but at the apparent expense of a proliferative capacity. The survival of VSMCs thus appears to be regulated by a fine balance between proliferative and apoptotic signals. The point at which ET-1 and bFGF interfere with the apoptotic activity of iloprost must clearly be upstream of the impairment of the mitochondria and activation of caspase-3.

Considerable attention is currently being focused on the role played by the MAP kinase cascades in the control of cell survival and programmed death mechanisms. Iloprost did not change the activity status of the SAPK or the p38 family members and it thus seems unlikely that apoptotic induction is via the stimulation of these protein kinase cascades. An impairment of ERK signaling has been shown to induce cell death in a number of situations. For example, bFGF has been shown to prevent apoptosis induced in H-510 SCLC cells by the chemotherapeutic agent, etoposide. MEK was found to be crucial for this response as its inhibition abolished the prosurvival properties of bFGF. Administration of bFGF has also been shown to completely inhibit myocardial cell apoptosis induced by hydrogen peroxide or acidic medium. Again a MEK inhibitor blocked bFGF-induced activation of ERK1/2 and neutralized the apoptotic inhibitory effect of bFGF. These findings suggest that bFGF protects against apoptosis via the MEK/ERK pathway. In addition, ET-1 acts not only as a growth-promoting peptide but also as a potent survival factor against myocardial cell apoptosis.

Consistent with these findings, we have shown that bFGF or ET-1 application to VSMCs induced a sustained activation of MEK, ERK1/2 and isoforms of the PKC family with a concomitant proliferative activity. Iloprost administration prevented the sustained activation of both MEK and ERK but had no effect on the induced PKC activity. This would suggest that the mechanism through which iloprost is inhibiting proliferation must be upstream of MEK and cannot, for example, be via the induction of an ERK phosphatase. It has been suggested that the sustained activation of the ERK cascade is required for nuclear translocation and a subsequent increase in the proliferative activity of cells. The proliferation induced by bFGF and ET-1 was shown in this study to be blocked by UO126, which prevented sustained ERK activation. The MEK inhibitor could thus mimic the effects of iloprost at inducing both apoptosis of VSMCs and causing ERK to be dephosphorylated. However, the coinadministration of iloprost or UO126 with bFGF or ET-1 prevented ERK phosphorylation from dropping below basal levels, which would compromise cell viability.

In the present study, the decrease in cell numbers, the increase in caspase-3 activity, and the reduction in basal ERK activity induced by iloprost or UO126 in VSMCs were all prevented by ET-1 or bFGF. It would thus seem that ET-1 or bFGF can prevent apoptotic induction by preserving the basal levels of ERK and preventing them from falling to below a critical threshold. The time-lapse confocal series also showed a very small proportion of the cells to be entering the apoptotic program in the presence of iloprost and ET-1. This would suggest that the effects of iloprost and ET-1 are
essentially canceling one another out, rather than a situation where a proportion of cells are induced to die by iloprost and other cells are proliferating to maintain cell numbers.

The IP receptor signals primarily through activation of adenylyl cyclase,23 which is consistent with the increase in CREB phosphorylation observed on iloprost application. The only known intracellular effector to be regulated by cAMP is PKA, which has been shown to negatively regulate the Ras-ERK cascade by phosphorylating Raf and preventing its association with active Ras.24 The adenylyl cyclase inhibitor, SQ22536, promoted the basal phosphorylation of ERK, and counteracted the inhibition evoked by forskolin, suggesting that cAMP negatively regulates ERK activity in VSMCs. In addition, the forskolin-induced caspase-3 activity was also abolished by SQ22536, as well as by ET-1 or bFGF giving support to the hypothesis that the net amount of cAMP and its subsequent inhibition of ERK determines whether apoptosis or proliferation predominates. SQ22536 reversed the dephosphorylation of basal ERK and the activation of caspase-3 induced by iloprost or submaximal concentrations of U0126, placing cAMP production upstream to the effects on ERK and the subsequent changes in caspase-3 activity.

PGE2 can stimulate all EP receptor types, and in this study, it caused a reduction in the basal levels of ERK activity, presumably through the EP3 receptor. It would also appear that the inhibition of ERK activity via PGE2 can override the subsequent inhibition of ERK determined whether apoptosis or proliferation predominates. SQ22536 reversed the dephosphorylation of basal ERK and the activation of caspase-3 induced by iloprost or submaximal concentrations of U0126, placing cAMP production upstream to the effects on ERK and the subsequent changes in caspase-3 activity.

PGI2 agonists have found clinical use, along with prostaglandins, in the treatment of peripheral vascular disease, atherosclerosis, and restenosis. It is hoped that a better understanding of the mechanisms through which prostanooids can regulate VSMC growth will provide a better therapeutic strategy to be adopted in combating vascular disease.

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