ELSEVIER

European Journal of Pharmacology 444 (2002) 31–37

www.elsevier.com/locate/ejphar

Activation of IP and EP_3 receptors alters cAMP-dependent cell migration

Rüdiger Blindt [a,*], Anja-K. Bosserhoff [b], Jürgen vom Dahl [a], Peter Hanrath [a], Karsten Schrör [c], Thomas Hohlfeld [c], Jutta Meyer-Kirchrath [c]

[a] *Medical Clinic I, University Hospital RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany*
[b] *Institute of Pathology, University Regensburg, 93053 Regensburg, Germany*
[c] *Institute of Pharmacology and Clinical Pharmacology, Heinrich-Heine-University, Düsseldorf, Germany*

Received 7 January 2002; received in revised form 5 April 2002; accepted 12 April 2002

Abstract

Migration of vascular smooth cells from the media to the intima essentially contributes to neointima formation after percutaneous transluminal angioplasty and stent implantation. The stable prostacyclin mimetic iloprost has been shown to inhibit neointima formation in experimental restenosis, but it is currently unknown whether this may be caused by an antimigratory effect. Hence, the present study analyses (i) the influence of G_s-coupled prostacyclin (IP) receptors on cell migration and (ii) verifies whether EP_3 receptors with opposite (i.e., G_i) coupling may conversely stimulate cell migration. In a modified Boyden chamber model, it was shown that iloprost dose-dependently inhibits the migration of primary human arterial smooth muscle cells, which constitutively express the IP receptor. On the other hand, human arterial smooth muscle cell migration was stimulated by the EP_3 receptor agonist M&B 28.767. To independently study the effects of these receptors, IP or EP_3 receptors were stably overexpressed in chinese hamster ovary cells (CHO-IP and CHO-EP_3). Chemotaxis of CHO cells transfected with G_s-coupled IP receptors was concentration-dependently inhibited by iloprost (2–100 nM), while there was no effect of iloprost on mock-transfected CHO. By contrast, CHO-cells that overexpressed EP_3 receptors showed a significant, concentration dependent (1–100 nM) increase of cell migration in presence of the selective EP_3 agonist M&B 28.767. It is concluded that the prostacyclin mimetic iloprost inhibits vascular cell migration, which probably depends on a G_s-mediated increase of intracellular cAMP. EP_3 receptors conversely stimulate CHO migration.

Keywords: IP receptor; EP_3 receptor; cAMP; Cell migration

1. Introduction

The pathophysiology of vascular diseases, including atherosclerosis and restenosis after percutaneous transluminal angioplasty and stent implantation, involves a chronic inflammatory response to injury in the vessel wall and is linked to endothelial dysfunction resulting in a net loss of tissue protective endothelium-derived mediators (Schrör, 1997; Newby, 2000). This is considered to cause or contribute to smooth muscle dedifferentiation, migration and proliferation.

Vascular injury is associated with the induction of the inducible isoform of cyclooxygenase-2, which synthesizes prostaglandins from arachidonic acid, causing increased agonist concentrations at sites of atherosclerotic or restenotic vasular lesions (Schonbeck et al., 1999). While macrophages associated with atherosclerotic lesions mainly produce prostaglandin E_2, which is considered to mediate proinflammatory responses, endothelial and smooth muscle cells generate large amounts of prostacyclin (prostaglandin I_2) (Schonbeck et al., 1999). Prostaglandin I_2 increases intracellular cAMP via prostacyclin (IP) receptors coupled to G_s and adenylate cyclase. This is followed by vasodilation, inhibition of cholesterol accumulation and inhibition of vascular smooth muscle cell proliferation (Makary et al., 1992; Zucker et al., 1998). An anti-atherogenic action of prostaglandin I_2 has been suggested by the experimental delivery of the prostacyclin synthase gene in the balloon-injured rat artery model, where an enhanced vascular prostaglandin I_2 formation accelerated the recovery from endothelial damage and reduced neointima formation (Todaka et al., 1999). In pigs, stent-induced intimal hyperplasia was attenuated by stent coating which releases the prostaglandin I_2 mimetic iloprost (Alt et al., 2000).

* Corresponding author. Tel.: +49-241-8089300; fax: +49-241-8082414.
E-mail address: ruediger.blindt@post.rwth-aachen.de (R. Blindt).

0014-2999/02/$ - see front matter
PII: S0014-2999(02)01607-2

It is widely accepted that migration of vascular smooth cells from the medial to the intimal layer contributes to the development of atherosclerotic plaques and neointimal formation after angioplasty and stent implantation (Palmer et al., 1998; Le-Feuvre et al., 1998). Moreover, a growing body of evidence has emerged which implicates a crucial role of cAMP in the inhibition of vascular smooth muscle cell migration (Palmer et al., 1998). However, the effect of vasodilatory prostaglandins, including prostacyclin, has not been systematically analyzed in this context.

The present study examined the effect of the prostaglandin I_2 mimetic iloprost and the EP_3 selective prostaglandin E_2 mimetic M&B 28.767 on cell migration in primary human arterial smooth muscle cells, which constitutively express IP and EP_3 receptors. These receptors are of particular interest because both are expressed in vascular tissues. It was shown that iloprost in nanomolar concentrations inhibits dose-dependently human arterial smooth muscle cell migration and on the other hand, human arterial smooth muscle cell migration could be stimulated by the EP_3 receptor agonist M&B 28.767. To independently study the effects of these receptors, IP or EP_3 receptors were stably overexpressed in chinese hamster ovary cells (CHO). In this system, it could be shown that iloprost significantly inhibits migration of CHO cells expressing IP receptors via intracellular cAMP increase. Further, CHO cells expressing EP_3 receptors showed an increased migration in the presence of M&B 28.767 via intracellular cAMP decrease.

It is concluded that vascular prostaglandins of the E- and I-series constitute a system for dual (positive and negative) regulation of cell migration, which involves EP_3 and IP receptors.

2. Methods

2.1. Culture of human arterial smooth muscle cells

Human mammary arteries human arterial smooth muscle cells were obtained from patients during coronary artery bypass operation after obtaining written informed consent. Human arterial smooth muscle cells were isolated and cultured as described previously (Blindt et al., 2000). All cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM/Nut.Mix F-12 with Glutamax, Gibco-BRL) supplemented with penicillin (100 U/ml), streptomycine (10 μg/ml) (both Sigma-Aldrich) and 10% fetal calf serum (Gibco-BRL) and divided 1:2 at confluency. Cells were detached by incubation with 0.05% trypsin, 0.04% EDTA (Sigma-Aldrich) in phosphate buffered saline for 5 min at 37 °C. They were identified as vascular human arterial smooth muscle cells through their characteristic hill-and-valley growth pattern. Immunostaining confirmed the presence of smooth muscle actin as positive marker for smooth muscle cells, von Willebrand factor served as negative marker and confirmed the absence of endothelial cells. By this method, cultures of >95% human arterial smooth muscle cells purity were routinely obtained. The studies were performed with cells at passages 3–4. Cells were explanted from 20 different donors.

Shortly, human arterial smooth muscle cells with different migratory and invasive behavior were used. One phenotype resembling the non-proliferating, quiescent human arterial smooth muscle cells in the medial wall of undilated vessels was established. For this reason, a growth arrest was induced in human arterial smooth muscle cells. Quiescent human arterial smooth muscle cell were obtained by plating 2×10^6 cells/ml in a 75-cm^2 culture plastic flask. Cells were grown to confluency for at least 5 days prior to the assay with medium change containing DMEM (10% fetal calf serum) every third day.

On the other hand, human arterial smooth muscle cells, mimicking the proliferating, migrating and invasive phenotype during restenosis development, were gained by splitting subconfluent human arterial smooth muscle cells 2 days prior to the assay. For proliferation, human arterial smooth muscle cells 2×10^5 cells/ml were plated in a 75-cm^2 flask, maintained in DMEM (10% fetal calf serum) and split at subconfluency 1:2. Proliferating human arterial smooth muscle cells generated by this method, we were able to migrate in a Boyden chamber whereas the quiescent phenotype failed to migrate. All human arterial smooth muscle cells were tested in the Boyden chamber and only cells with a clear distinguishable migratory or non-migratory phenotype were evaluated in further assays.

2.2. Generation of CHO cells stably expressing human IP receptor or EP_3 receptor

The complete cDNA of the human IP receptor (hIP), cloned as a 1.4 kb fragment into pcDNAIamp (hIP11/6.pcDNAIamp) was a generous gift of Dr. M. Abramowitz (Merck Frosst). For stable transfection of CHO cells, the cDNA of the human IP receptor was subcloned as a 1.4 kb *Eco*RI-fragment into the expression plasmid pcDNA3 resulting in pcDNA-hIPR. Cloning of the complete cDNA of the porcine EP_3 receptor and subcloning into the expression plasmid pcDNA3 has been described previously (Meyer-Kirchrath et al., 1998).

CHO cells were cultured in Ham's F12 medium (Gibco BRL, Life Technologies) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cells were transfected by a modified polybrene method with linearized plasmid DNA as described elsewhere (Aubin et al., 1994). Clonal selection of stable integrants was performed in a medium containing 250 μg/ml G418. Expression of human IP receptor was verified reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated with TRIzol™ reagent. RNA was quantified by Gene Quant II (Amersham Pharmacia Biotech, Freiburg, Germany) and 500 ng RNA were used for RT-PCR with Ready To Go™ beads (Amersham Pharmacia Biotech) using

the gene specific primers for IP-receptor (sense: 5′AGCA GTAC-TGCCCCGGCAGCTGGTGCTTCC3′ and antisense: 5′GGTCCCCCATCTCACTGCTGCTGTCAGG3′) and EP_3 receptor (sense: 5′GGGCCAGTACACCATCCA GTG3′, antisense: 5′GATAAACCCAGGGATCCAAGAC TG3′). Receptor expression was verified by radioligand binding experiments as described elsewhere (Meyer-Kirchrath et al., 1998). Functional coupling was analyzed by measurement of receptor mediated cAMP formation (IP receptor) or inhibition of forskolin-stimulated cAMP-formation (EP_3 receptor) as described earlier (Meyer-Kirchrath et al., 1998).

2.3. Migration assay

For chemotaxis experiments, Boyden-type blind well chambers were used (Costar, USA). Polycarbonate filters (13 mm diameter, 8 μm pore size; Nucleopore, USA) were coated with gelatin (5 mg/ml) as described previously (Blindt et al., 2000). Briefly, the lower compartment was filled with fibroblast-conditioned medium for CHO-cells or endothelial cell-conditioned medium for human arterial smooth muscle cells as chemoattractants and the gelatin-coated filter was placed above. Cells were harvested by trypsinization and resuspended in DMEM medium without fetal calf serum (2×10^5 cells/ml). A total of 800 μl of the cell suspension, 2×10^5 cells/ml, drug-treated or untreated, was added to the upper compartment and then the chambers were incubated at 37 °C in 5% carbon dioxide for 4 h. Cells on the upper side of the filter were removed mechanically. Cells which had migrated to the lower side were fixed on the filter with methanol, stained with hematoxylin–eosin and 15 random fields were counted at 200-fold magnification.

Iloprost and M&B 28.767 were added to the medium immediately before the experiments. For experiments with human arterial smooth muscle cell, the drugs were administered immediately before migration started or during a 24-h preincubation period. In an additional group, drugs were administered during preincubation and during the human arterial smooth muscle cell migration assay. In the combined preincubation and incubation assays, the medium was removed after the preincubation period and substituted by medium with the same concentration as used initially. Each sample was assayed in triplicate with the three different pools of human arterial smooth muscle cells from at least three to four donors, respectively.

2.4. cAMP measurement

CHO cells were grown to near-confluence and preincubated in Hank's balanced salt solution with 1 mg/ml BSA/10 mM HEPES and 1 mM isobutylmethylxanthine for 10 min at 37 °C. Forskolin, iloprost and M&B 28.767 were added as indicated. The reaction was stopped by ice-cold ethanol (75%) and the cells were overlaid with buffer (150 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.4) and cAMP determined by radioimmunoassay. Cell protein was determined by the Biorad method (Biorad, Munich, Germany).

2.5. Statistical analysis

All data are expressed as mean value±standard error of the means (S.E.M.). Differences between groups were analyzed by Student's *t*-test or one-factor analysis of variance, as required. Differences were considered significant if *P* values were <0.05.

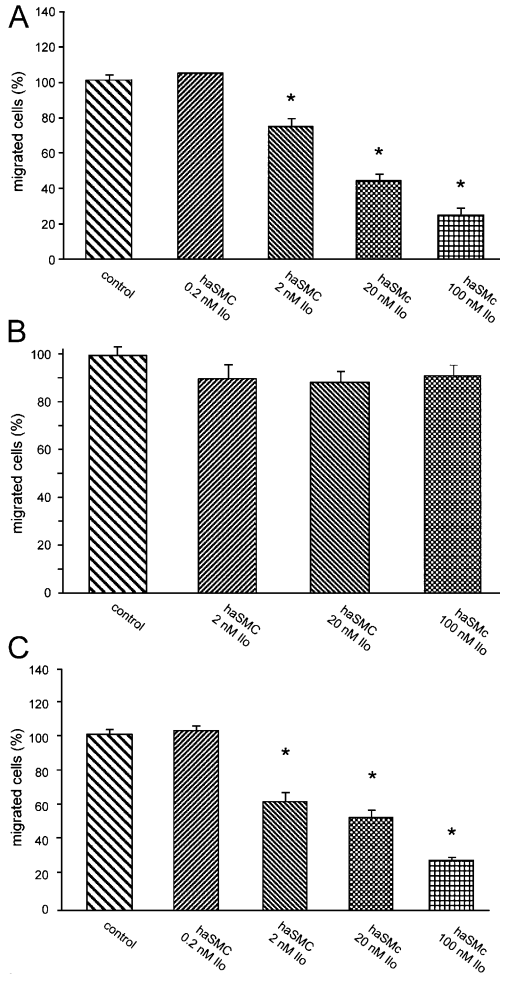

Fig. 1. (A) Effect of iloprost treatment on migration of human arterial smooth muscle cells (haSMC) during the assay. Migration of human arterial smooth muscle cells was dose-dependently inhibited (106% at 0.2 nM, 76% at 2 nM, 43% at 20 nM and 24% at 100 nM; **P*<0.05 for 2–100 nM; *n*=6). (B) Effect of iloprost pretreatment on migration of human arterial smooth muscle cells. Human arterial smooth muscle cells were preincubated for 24 h with iloprost before migration was determined. There was no treatment with iloprost during the migration assay. The experiments revealed no significant effect of pretreatment on human arterial smooth muscle cell migration (88% at 2 nM, 83% at 20 nM, 88% at 100 nM; not significant; *n*=6). (C) Effect of iloprost on human arterial smooth muscle cell migration after pretreatment for 24 h and treatment during the migration assay. The antimigratory effect of iloprost on human arterial smooth muscle cells was not influenced by iloprost pretreatment (104% at 0.2 nM, 64% at 2 nM, 53% at 20 nM, 29% at 100 nM; **P*<0.05 for 2–100 nM; *n*=6).

3. Results

3.1. Iloprost inhibits and M&B 28.767 stimulates chemotaxis of human arterial smooth muscle cells

In order to evaluate the influence of iloprost and M&B 28.767 on migration of human arterial smooth muscle cells, the effects of both drugs were tested in a modified Boyden chamber assay.

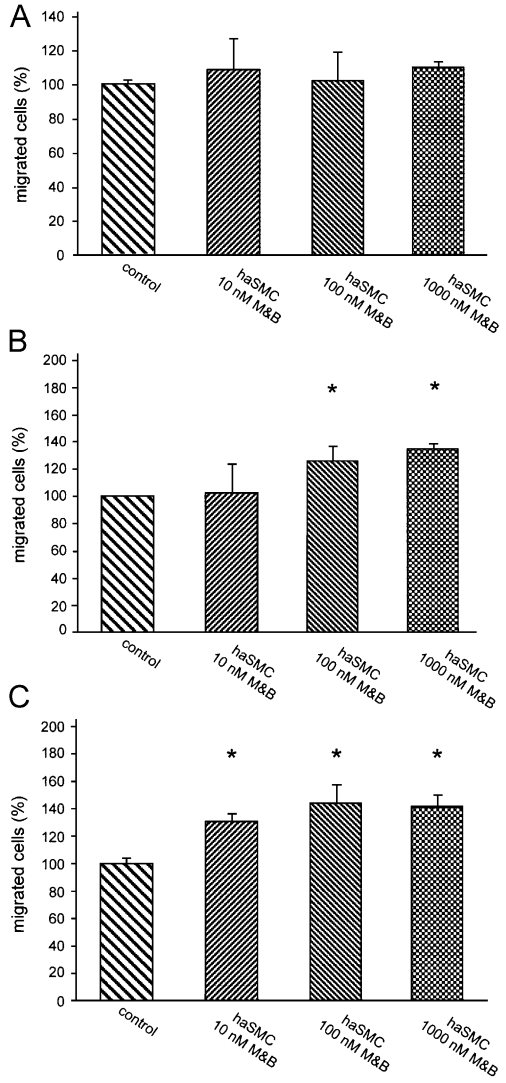

Fig. 2. (A) Effect of M&B 28.767 treatment on migration of human arterial smooth muscle cells (haSMC) during the assay. Migration of human arterial smooth muscle cells was not influenced by administration of M&B 28.767 during the assay (110% at 10 nM, 101% at 100 nM, 112% at 1000 nM; not significant; n=6). (B) Effect of M&B 28.767 pretreatment on migration of human arterial smooth muscle cells. Human arterial smooth muscle cells were preincubated for 24 h with M&B 28.767 before migration was determined. Migration of human arterial smooth muscle cells was dose-dependently inhibited (103% at 10 nM, 123% at 100 nM and 135% at 1000 nM; *P<0.05 for 100–1000 nM; n=6). (C) Effect of M&B 28.767 on human arterial smooth muscle cell migration after pretreatment for 24 h and treatment during the migration assay. The promigratory effect of M&B 28.767 on human arterial smooth muscle cell increased dose-dependently. (133% at 10 nM, 143% at 100 nM, 142% at 1000 nM; P<0.05 for 10–1000 nM, *P<0.05 for 10–1000 nM; n=6).

Table 1
cAMP formation in CHO cells expressing the expression plasmid pcDNA3 (mock control) and CHO cells overexpressing the human IP receptor (CHO-IP) treated with 1 μM forskolin or 100 nM iloprost, respectively

	cAMP (pmol/mg protein)	S.E.M.	P value
CHO mock basal	16	1	
CHO mock 1 μM forskolin	511	79	<0.05
CHO mock 100 nM iloprost	56	18	N.S.
CHO-IP basal	25	12	
CHO-IP 1 μM forskolin	508	134	<0.05
CHO-IP 100 nM iloprost	2463	299	<0.05

n=6. Statistical significance was always calculated compared to basal cAMP levels, N.S.=not significant.

If directly applied to migrating human arterial smooth muscle cells during the assay, iloprost concentration-dependently inhibited the migration of these cells as compared to control (Fig. 1A). If human arterial smooth muscle cells were pretreated with iloprost without treatment during the assay no inhibition of human arterial smooth muscle cell migration could be demonstrated (Fig. 1B). Combined administration of iloprost, e.g. pretreatment and treatment with the substance before and during the assay revealed no additional inhibition of cell migration compared to treatment during migration alone (Fig. 1C).

In analogous assays analyzing the effect of the EP_3 receptor agonist M&B 28.767 on the migratory potential of human arterial smooth muscle cells, treatment with the substance during the assay caused no significant inhibition or stimulation of cell migration (Fig. 2A). In contrast, pretreatment with M&B 28.767 alone led to a concentration-dependant increase of human arterial smooth muscle cell migration with a maximal stimulation of 135% at 1000 nM (Fig. 2B). Combined pretreatment and treatment before and during the assay further increased the stimulating effect of M&B 28.767 with a maximum of 142% at 1000 nM significantly (Fig. 2C).

3.2. Iloprost inhibits chemotaxis of CHO cells overexpressing the human IP receptor via cAMP increase

To further analyze the mechanisms between IP receptor activation and cell migration CHO cells were stably transfected with the plasmid pcDNA3 carrying the human IP receptor (CHO-IP cells) and the migratory potential of these cells was further evaluated. The expression level of the receptor determined by radioligand binding was 2100 fmol/mg membrane protein. Functional coupling of the expressed receptor was monitored by analysis of the receptor-mediated stimulation of cAMP formation (Table 1). Mock transfected CHO cells carrying the pcDNA3 plasmid showed a basal level of 16±1 pmol/mg protein cAMP. Stimulation of adenylate cyclase with forskolin increased the basal level to 511±79 pmol/mg protein cAMP, stimulation with the

stable prostacyclin mimetic iloprost caused no significant increase of cellular cAMP levels (56±18 pmol/mg protein). The CHO-IP cells had a comparable basal cAMP level as mock transfected cells (25±12 pmol/mg protein), stimulation of cAMP formation by forskolin increased the cAMP level similar to mock tranfected cells (508±134 pmol/mg protein). By contrast to mock transfected cells, activation of the IP receptor by iloprost in the CHO-IP cells significantly increased cAMP formation above basal levels (2200±250 pmol/mg protein vs. 56±18 pmol/mg protein) which confirmed the iloprost driven cAMP dependent activation of the overexpressed IP receptor.

To analyze the effect of IP receptor stimulation on CHO cell migration, the appropriate stimulus for CHO cell migration was identified in separate experiments, which showed that fibroblast conditioned medium was the most efficient stimulus for reproducible migration of CHO cells (data not shown). Iloprost did not fibroblast conditioned medium stimulated migration in mock transfected CHO cells within a concentration range between 2 and 100 nM (Fig. 3). By contrast, iloprost significantly inhibited the migration of CHO cells which expressed functional IP receptors. This effect was concentration-dependent with a maximum of 44% cell migration at 100 nM iloprost in comparison to mock control.

3.3. M&B 28.767 stimulates chemotaxis of CHO cells overexpressing the EP_3 receptor via cAMP decrease

To further evaluate the effect of migration inhibition by activation of the EP_3 receptor CHO cells overexpressing prostaglandin EP_3 receptors were generated by stable transfection of cells with pCDNA3-EP_3. The level of receptor expression was 2200 fmol receptor/mg protein (radioligand binding). To demonstrate functional receptor coupling to an inhibitory G protein, receptor-mediated inhibition of forskolin stimulated cAMP formation was determined (Table 2). Mock transfected CHO cells carrying the pcDNA3 plasmid showed a basal cAMP level of 51±2 pmol/mg protein. The EP_3 receptor specific agonist M&B 28.767 did not alter cAMP formation in these cells. If CHO mock cells were significantly stimulated by forskolin, the cAMP level increased to 650±20 pmol/mg protein. Coincubation with M&B 28.767 had no influence on the forskolin triggered cAMP stimulation (630±9 pmol/mg protein). The basal level of cAMP production in CHO-EP_3 cells was comparable to mock control (41±10 pmol/mg), again M&B 28.767 did not alter cAMP formation. However, if cAMP formation in CHO-EP_3 cells was stimulated with forskolin (686±56 pmol/mg protein) and these cells were cocincubated with M&B 28.767, the EP_3 agonist significantly reduced forskolin stimulated cAMP formation in CHO-EP_3 cells from 686±56 to 346±18 pmol cAMP/mg protein at 100 nM M&B 28.767.

Table 2
cAMP formation in CHO cells expressing the expression plasmid pcDNA3 (mock control) and CHO cells overexpressing the EP_3 receptor (CHO-EP_3) treated with 1 μM forskolin, 100 nM M&B 28.767 (M&B), or forskolin (1 μM) plus M&B 28.767 100 nM), respectively

	cAMP (pmol/mg protein)	S.E.M.	*P* value
CHO mock basal	51	2	
CHO mock 100 nM M&B	51	1	N.S.
CHO mock 1 μM forskolin	650	20	<0.05
CHO mock 1 μM forskolin+ 100 nM M&B	630	9	<0.05
CHO-EP3 basal	41	10	
CHO-EP3 100 nM M&B	41	4	N.S.
CHO-EP3 1 μM forskolin	686	56	<0.05
CHO-EP3 1 μM forskolin+100 nM M&B	346	18	<0.05

n=6. Statistical significance was always calculated compared to basal cAMP levels, N.S.=not significant.

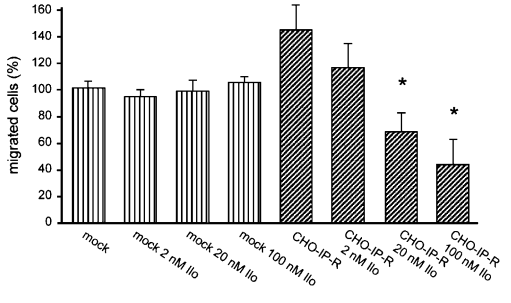

Fig. 3. Effect of iloprost on migration of mock transfected CHO cells and CHO cells expressing the human IP receptor. Only migration of IP receptor-transfected CHO cells was significantly inhibited by iloprost in comparison to mock control (117% at 2 nM, 69% at 20 nM and 44% at 100 nM; **P*<0.05 for 20–100 nM; *n*=6).

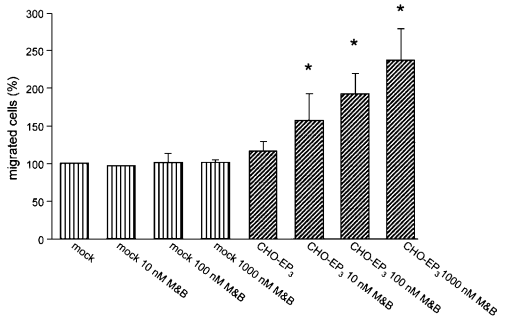

Fig. 4. Effect of M&B 28.767 on migration of mock transfected CHO cells or CHO cells expressing the EP_3 receptor. Migration of CHO cells expressing the EP_3 receptor was dose-dependently stimulated (158% at 10 nM, 192% at 1000 nM and 240% at 1000 nM; **P*<0.05 for 10–1000 nM; *n*=6).

Again, the effect of the EP_3-agonist M&B 28.767 on the migratory behavior of CHO-EP_3 cells was tested in the Boyden chamber model. M&B 28.767 caused a concentration-dependent stimulation of CHO-EP_3 cells with a maximum of 240% at 1000 nM M&B while the substance had no effect on the migratory potential of mock transfected CHO cells (Fig. 4). Since the prostacyclin mimetic iloprost has been reported to show high affinity binding to prostaglandin EP_3 receptors (Kiriyama et al., 1997), we also tested the effect of iloprost on chemotaxis of CHO cells expressing EP_3 receptors. These experiments did not show an effect of iloprost (1–100 nM) on migration of these cells (data not shown).

4. Discussion

The present study investigates the effects of prostaglandin IP and EP_3 receptor agonists, i.e., iloprost and M&B 28.767, on cell migration. The most important finding is that iloprost, by stimulating G_s coupled IP receptors with a consequent increase in cellular cAMP, inhibits chemotaxis of human arterial vascular smooth muscle cells and CHO cells overexpressing the human IP receptor. These results could be substantiated by demonstrating that stimulation of the EP_3 receptor increased the migratory potential of human arterial smooth muscle cells and CHO cells overexpressing this receptor which was accompanied by a decrease in cellular cAMP. An influence of iloprost on the process of cell migration via activation of the EP_3 receptor could be excluded.

Vascular cells like human arterial smooth muscle cell are likely to coexpress different types of prostaglandin receptors. Therefore, this study not only analyses the prostaglandin effects on cell migration in primary human arterial smooth muscle cell but also uses CHO cells which were stably transfected with the G_s-coupled prostacyclin receptor (CHO-IP receptor) as a model system. CHO cells expressing adequate receptors have been successfully used to analyze the chemotactic response to different stimuli, including *N*-formyl peptide (Miettinen et al., 1998) and platelet activating receptor (Boccellino et al., 2000).

It is unknown, however, whether IP receptor agonists influence migration of vascular cells, and if so, which signaling events may be involved. Remarkably, Ai et al. (2001) showed that Rho–Rho kinase is involved in smooth muscle cell migration and Shibata et al. (2001) reported that Rho-kinase blockade reduces neointima formation after vascular injury. In turn, prostacyclin has been shown to transiently reduce Rho in osteoclasts (Adebanjo et al., 1994). In the migration experiments with primary human arterial smooth muscle cell, it was found that migration was inhibited by iloprost at a concentration in the nanomolar range, which is compatible with an IP receptor mediated effect. This effect could also be demonstrated in the CHO-IP model. In agreement with the inhibitory effect on human arterial smooth muscle cell migration reported here, Alt et al. (2000) recently reported (3) a significant reduction of restenosis in pigs and sheep after implantation of coated stents that release iloprost and hirudin.

The molecular mechanisms distal to the IP receptor-mediated cAMP response, which finally lead to the observed inhibition of cell migration, are currently hypothetical. Nevertheless, it is well established that the small GTPase Rho is, among a variety of physiological functions, involved in cell adhesion and migration (Nobes and Hall, 1999). O'Connor et al. (2000) recently highlighted the importance of cAMP metabolism in the activation and localization of RhoA by their findings that cAMP inhibited RhoA activation and translocation to membrane ruffles. Hence, IP receptors may cause RhoA inactivation by stimulation of adenylate cyclase which could cause inhibition of cell migration.

In contrast to iloprost, the specific agonist M&B 28.767 concentration-dependently stimulated cell migration in the human arterial smooth muscle CHO cells expressing G_i coupled EP_3 receptors. Interestingly, the effect of M&B 28.767 on cell migration is different in human arterial smooth muscle cell and CHO-EP_3 cells. In CHO cells with EP_3 receptor treatment with M&B 28.767 causes a dose dependant increase in cell migration. Stimulation of human arterial smooth muscle cell depends of pretreatment of the cells, an effect that could be explained by an activation of the EP_3 receptor.

In this context, it is of importance that the EP_3 subtype activates Rho (Hasegawa et al., 1997) and mediates the prostaglandin E_2-induced augmentation of matrix metalloproteinase 9 expression in cultured T-cells (Zeng et al., 1996), an enzyme involved in the degradation of basal laminae and considered crucial in cell migration and evolution of vulnerable plaques. These findings are in agreement with the pro-migratory action of the EP_3-selective agonist M&B 28.767 observed with CHO-EP_3 cells.

While the present study shows that iloprost inhibits CHO and smooth muscle cell migration, exogenous supplementation of prostaglandin I_2 has been of limited success for the prevention of clinical arterial restenosis (Knudtson et al., 1990; Gershlick et al., 1994). This might be caused by insufficient local prostaglandin I_2 concentrations or by desensitization of the IP receptor. In fact, several studies have shown that responses to prostaglandin I_2 or prostaglandin I_2 mimetics are attenuated or even lost when continuously exposed to elevated agonist concentrations (Jaschonek et al., 1988; Nilius et al., 2000). Nevertheless, iloprost inhibited human arterial smooth muscle cell migration by a similar extent independent whether or not iloprost was administered over 24 h before human arterial smooth muscle cell migration was measured. Apparently, if any desensitization occurs, sufficient receptor reserve appears to exist allowing a prolonged antimigratory action on human arterial smooth muscle cells.

In conclusion, inhibition of smooth muscle cell migration by IP receptor agonists, such as iloprost, seems to be a therapeutic strategy to control migration of vascular cells after vascular injury with consequent reduction of neointima formation. This concept is also of physiological importance, because prostaglandin I_2 formation is enhanced at sites of vascular injury and local inflammation as a consequence of an induction of the inducible isoform of cyclooxygenase-2, which provides increased agonist (prostacyclin) concentrations at sites of atherosclerotic or restenotic vasular lesions (Schonbeck et al., 1999; Belton et al., 2000). In the future, restenosis therapy with IP receptor agonists may become particularly effective by the administration of selective IP receptor agonists with local drug delivery strategies, supplemented by local overexpression of IP receptors in vascular cells by gene transfer techniques.

Acknowledgements

The authors wish to thank Petra Kuger, Irmhild Rüther and Nicole Krott for their excellent technical assistance.

References

Adebanjo, O.A., Pazianas, M., Zaidi, A., Shankar, V.S., Bascal, Z.A., Dacke, C.G., Huang, C.L., Zaidi, M., 1994. Quantitative studies on the effect of prostacyclin on freshly isolated rat osteoclasts in culture. J. Endocrinol. 143, 375–381.

Ai, S., Kuzuya, M., Koike, T., Asai, T., Kanda, S., Maeda, K., Shibata, T., Iguchi, A., 2001. Rho–Rho kinase is involved in smooth muscle cell migration through myosin light chain phosphorylation-dependent and independent pathways. Atherosclerosis 155, 321–327.

Alt, E., Haehnel, I., Beilharz, C., Prietzel, K., Preter, D., Stemberger, A., Fliedner, T., Erhardt, W., Schömig, A., 2000. Inhibition of neointima formation after experimental coronary artery stenting. Circulation 101, 1453–1458.

Aubin, R.A., Weinfeld, M., Mirzayans, R., Paterson, M.C., 1994. Polybrene/DMSO-assisted gene transfer. Generating stable transfectants with nanogram amounts of DNA. Mol. Biotechnol. 1, 29–48.

Belton, O., Byrne, D., Kearney, D., Leahy, A., Fitz-Gerald, D.J., 2000. Cyclooxygenase-1 and -2 dependent prostacyclin formation in patients with atherosclerosis. Circulation 102, 840–845.

Blindt, R., Bosserhoff, A.K., Zeiffer, U., Krott, N., Hanrath, P., vom Dahl, J., 2000. Abciximab inhibits the migration and invasion potential of human coronary artery smooth muscle cells. J. Mol. Cell. Cardiol. 2195–2206.

Boccellino, M., Biancone, L., Cantaluppi, V., Ye, R.D., Camussi, G., 2000. Effect of platelet-activating factor receptor expression on CHO cell motility. J. Cell. Physiol. 183, 254–264.

Gershlick, A.H., Spriggins, D., Davies, S.W., Syndercombe, C.Y., Timmins, J., Timmis, A.D., Rothman, M.T., Layton, C., Balcon, R., 1994. Failure of epoprostenol (prostacyclin, PGI2) to inhibit platelet aggregation and to prevent restenosis after coronary angioplasty: results of a randomised placebo controlled trial. Br. Heart J. 71, 7–15.

Hasegawa, H., Negishi, M., Katoh, H., Ichikawa, A., 1997. Two isoforms of prostaglandin EP3 receptor exhibiting constitutive activity and agonist-dependent activity in rho-mediated stress fiber formation. Biochem. Biophys. Res. Commun. 234, 631–636.

Jaschonek, K., Faul, C., Schmidt, H., Renn, W., 1988. Desensitization of platelets to iloprost. Loss of specific binding sites and heterologous desensitization of adenylate cyclase. Eur. J. Pharmacol. 147, 187–196.

Kiriyama, M., Ushikubi, F., Kobayashi, T., Hirata, M., Sugimoto, Y., Narumiya, S., 1997. Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. Br. J. Pharmacol. 122, 217–224.

Knudtson, M.L., Flintoft, V.F., Roth, D.L., Hansen, J.L., Duff, H.J., 1990. Effect of short-term prostacyclin administration on restenosis after percutaneous transluminal coronary angioplasty. J. Am. Coll. Cardiol. 15, 691–697.

Le-Feuvre, C., Tahlil, O., Paterlini, P., Maillard, L., Brousse, N., Lacour, B., Guettier, C., Vacheron, A., Feldman, L., Steg, P.G., 1998. Arterial response to mild balloon injury in the normal rabbit: evidence for low proliferation rate in the adventitia. Coron. Artery Dis. 9, 805–814.

Makary, A., Kolthay, E., Pataki, M., Peto, J., Kovacs, G., Lusztig, G., 1992. Inhibition of ACAT activity after 7-oxo-PGI2 treatment in cholesterol-fed rabbits. Exp. Toxicol. Pathol. 44, 105–107.

Meyer-Kirchrath, J., Kuger, P., Hohlfeld, T., Schrör, K., 1998. Analysis of a porcine EP3-receptor: cloning, expression and signal transduction. Naunyn-Schmiedeberg's Arch. 160–167.

Miettinen, H.M., Gripentrog, J.M., Jesaitis, A.J., 1998. Chemotaxis of chinese hamster ovary cells expressing the human neutrophil formyl peptide receptor: role of signal transduction molecules and alpha 5beta1 integrin. J. Cell Sci. 111, 1921–1928.

Newby, A.C., 2000. An overview of the vascular response to injury: a tribute to the late Russell Ross. Toxicol. Lett. 112–113, 519–529.

Nilius, S.M., Hasse, A., Kuger, P., Schror, K., Meyer-Kirchrath, J., 2000. Agonist-induced long-term desensitization of the human prostacyclin receptor. FEBS Lett. 484, 211–216.

Nobes, C.D., Hall, A., 1999. Rho GTPases control polarity, protrusion, and adhesion during cell movement. J. Cell Biol. 144, 1235–1244.

O'Connor, K.L., Nguyen, B.K., Mercurio, A.M., 2000. RhoA function in lamellae formation and migration is regulated by the alpha6beta4 integrin and cAMP metabolism. J. Cell Biol. 148, 253–258.

Palmer, D., Tsoi, K., Maurice, D.H., 1998. Synergistic inhibition of vascular smooth muscle cell migration by phosphodiesterase 3 and phosphodiesterase 4 inhibitors. Circ. Res. 82, 852–861.

Schonbeck, U., Sukhova, G.K., Graber, P., Coulter, S., Libby, P., 1999. Augmented expression of cyclooxygenase-2 in human atherosclerotic lesions. Am. J. Pathol. 155, 1281–1291.

Schrör, K., 1997. The Endothelium in Clinical Practice. Marcel Dekker, New York.

Shibata, R., Kai, H., Seki, Y., Kato, S., Morimatsu, M., Kaibuchi, K., Imaizumi, T., 2001. Role of rho-associated kinase in neointima formation after vascular injury. Circulation 103, 284–289.

Todaka, T., Yokoyama, C., Yanamoto, H., Hashimoto, N., Nagata, I., Tsukahara, T., Hara, S., Hatae, T., Morishita, R., Aoki, M., Ogihara, T., Kaneda, Y., Tanabe, T., 1999. Gene transfer of human prostacyclin synthase prevents neointimal formation after carotid balloon injury in rats. Stroke 30, 419–426.

Zeng, L., An, S., Goetzl, E.J., 1996. Regulation of expression of matrix metalloproteinase-9 in early human T cells of the HSB.2 cultured line by the EP3 subtype of prostaglandin E2 receptor. J. Biol. Chem. 271, 27744–27750.

Zucker, T.P., Bönisch, D., Hasse, A., Grosser, T., Weber, A.A., Schrör, K., 1998. Tolerance development to antimitogenic actions of prostacyclin but not of prostaglandin E1 in coronary artery smooth muscle cells. Eur. J. Pharmacol. 345, 213–220.