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Gene expression profile of the G_s-coupled prostacyclin receptor in human vascular smooth muscle cells

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Abstract

Migration and proliferation of medial smooth muscle cells (SMC) in the arterial intima contributes to the development of atherosclerotic plaques and restenotic processes after coronary angioplasty. Prostacyclin (PGI_2)-mediated stimulation of cyclic adenosine 3′5′-monophosphate (cAMP) signaling is believed to be important for maintaining SMC in a quiescent state. In order to identify new cellular targets of PGI_2/cAMP action, we have used microarray screening to examine changes in the transcriptional profile in human vascular SMC in response to exposure to the stable PGI_2 mimetic iloprost. We have identified 83 genes with significantly altered expression after iloprost (100 nM) exposure for 6 hr. Fifty-one genes were upregulated, among them stanniocalcin precursor (18.8 ± 2.7), zinc finger transcription factor (7.8 ± 2.0), hyaluronan synthase 2 (6.8 ± 1.8), cyclooxygenase 2 (4.7 ± 0.8), dual specific phosphatase (3.9 ± 0.5) and vascular endothelial growth factor (2.3 ± 0.4). Thirty-two genes were reduced, among them cystein-rich angiogenic protein (-14.9 ± 1.3), monocyte chemotactic protein 1 (-7.4 ± 1.1) and plasminogen activator inhibitor PAI-1 (-4.5 ± 0.5). By means of semi-quantitative RT-PCR, time-courses of gene expression were established. The present study identified genes not hitherto recognized to be targets of PGI_2 action, providing further insight into its cAMP-mediated effects on SMC growth, migration and matrix secretion.

Keywords: Atherosclerosis; cAMP; Gene expression; Prostacyclin; Vascular smooth muscle

1. Introduction

Atherosclerosis is a chronic inflammatory response to the injury in the arterial wall leading to endothelial dysfunction and, thereby, resulting in a net loss of tissue-protective endothelium-derived mediators [1]. This reduced vascular generation of vasoprotective mediators, among them nitric oxide (NO) and PGI_2, in conjunction with synergistic effects of various growth-regulatory factors, contributes to migration and activation of macrophages and also to the switch in SMC phenotype, which becomes proliferative, migratory and produces extracellular matrix proteins [1]. The complex of inflammatory and fibroproliferative processes results in intimal hyperplasia or the formation of fibrous plaques. Similar inflammatory and proliferative processes in the vascular wall are thought to underlie the intimal accumulation of SMC in the restenotic process in coronary arteries, occurring after balloon angioplasty which occurs in 30–50% of patients within 3–6 months [2].

PGI_2, the major arachidonic acid metabolite produced in vascular endothelium, is regarded an important endogenous atheroprotective factor in blood vessels [3]. PGI_2 exerts its multiple effects, such as vasodilation, inhibition of platelet aggregation and modulation of cholesterol turnover, by stimulation of cAMP formation subsequent

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Abbreviations: cAMP, cyclic adenosine 3′5′-monophosphate; COX-2, cyclooxygenase 2; Cyr61, cystein-rich angiogenic protein; Has2, hyaluronan synthase 2; hEZF, zinc finger transcription factor; hSMC, human vascular smooth muscle cell; IBMX, isobutylmethylxanthine; ICER, cAMP-responsive element modulator; IP-R, prostacyclin receptor; MCP-1, monocyte chemotactic protein; MKP-1, dual-specific phosphatase; PAI-1, plasminogen activator inhibitor; PGI_2, prostacyclin; STC, stanniocalcin precursor; VEGF, vascular endothelial growth factor.

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to its binding to a specific G_s-coupled IP-R [4]. Cyclic nucleotides like cAMP promote SMC quiescence *in vivo* and *in vitro*, acting as a control element to prevent SMC mitogenic response to growth factors in the vessel wall [5]. Consequently, PGI_2-mediated inhibition of SMC proliferation [6] and migration [7] have also been reported.

Reduced PGI_2 formation or action have been related to various cardiovascular disorders, including spontaneous angina or restenosis [8–10]. Transgenic mice lacking IP-R exhibit severe thrombotic events [11]. Therapeutic strategies, aimed to enhance local PGI_2 production by experimental delivery of the prostacyclin synthase gene, have beneficially influenced the recovery from endothelial damage and neointima formation in animal models [12,13] of restenosis. Moreover, in pigs, stent-induced intimal hyperplasia was attenuated by a stent coated with the stable IP receptor agonist iloprost [14].

While vasoprotective actions of PGI_2 and its mimetics are well documented, the molecular events underlying vessel protection are poorly understood. Possible alterations in gene expression during the transition of SMC from a quiescent to an activated state are largely unknown. In order to identify new targets of IP-R receptor-mediated vasoprotection, we performed microarray analysis to examine changes in the transcriptional profile of human SMC in response to exposure to the stable prostacyclin mimetic iloprost. This approach allows to study over 5800 functionally characterized genes at a time.

Consistent with the atheroprotective PGI_2 actions, many iloprost-responsive genes are involved in growth regulation, cell/matrix interaction or immune response. Our data provide new insight into how smooth muscle cells respond to PGI_2 and the molecular targets involved in SMC phenotype determination (SMC quiescence). The identification of these genes might also be important for understanding the molecular events involved in the pathology of atherogenesis and restenosis.

2. Materials and methods

2.1. Cell culture

hSMC were isolated by the explant method from coronary arteries (for array experiments and RT-PCR) and mammary artery (RT-PCR) as described earlier [15]. hSMC were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal calf serum (FCS), 100 μg/mL streptomycin and 100 U/mL penicillin in a humidified atmosphere with 5% CO_2 at 37°. hSMC of passages 4–9 were used in the study. For acute experiments, cells were grown until subconfluence (70–80%) and then incubated in serum-free DMEM for 24 hr prior to stimulation with 100 nM iloprost for the indicated times. Iloprost was kindly provided by Schering AG.

2.2. cAMP measurements

hSMC were grown until subconfluency in 24-well plates. After preincubation in HBSS containing BSA (1 mg/mL), HEPES (10 mM, pH 7.3) and IBMX (1 mM) for 10 min at 37°, cells were stimulated with iloprost (0.1 μM) for 10 min. The reaction was stopped by aspiration and addition of ice-cold ethanol (96%). Dried samples were overlaid with 300 μL RIA buffer (NaCl 150 mM, Na_2HPO_4 8 mM, NaH_2PO_4, pH 7.4) and stored overnight at −80°. cAMP in the supernatant was determined by radioimmunoassay [16]. Protein determination was performed according to the method of Bradford [17]. The adenylyl cyclase activity was expressed as pmol cAMP/mg protein × 10 min.

2.3. RNA isolation

Total RNA from hSMC was prepared with Trizol-reagent (Gibco Life Technologies) following the manufacturer's manual. RNA was quantitated by spectrophotometric analysis and quality was proved by gel electrophoresis.

2.4. Affymetrix GeneChip probe array analysis

Iloprost stimulation experiments were performed with three independent primary cultures of coronary hSMC. Total RNA from untreated and iloprost-stimulated (100 nM, 6 hr) hSMC was isolated and cDNA synthesis of 16 μg total RNA was performed with Superscript™ Choice System for cDNA synthesis (Gibco Life Technologies) and T_7-$(dt)_{24}$ primer (MWG Biotech). Biotinylated target was prepared with BioArray™ HighYield™RNA Transcript Labeling Kit (Enzo Diagnostics Inc.). Five micrograms of labeled sample were hybridized for 16 hr at 45° to GeneChip Test-2 arrays (to assess sample quality), 15 μg cDNA were then hybridized to HU GeneFL-Arrays (Affymetrix). The arrays were washed and stained with streptavidin-phycoerythrin antibody. Data were collected by laser scanning (GeneArray scanner, Agilent Technologies) and expression levels were determined using Affymetrix GeneChip Analysis Suite 3.2 software. Before comparing two arrays to identify differentially expressed genes, global scaling was applied to correct for variations between the experimental and baseline array. When global scaling is applied, the intensity of all probe sets from the experimental array and the intensity of all probe sets from the baseline array are scaled to a user-defined target intensity. To identify differentially expressed transcripts, pairwise comparison analyses (iloprost-treated vs. untreated control cells) were carried out. Each probe set on the experiment array is then compared to its counterpart on the baseline array and the fold-change (increase or decrease) is calculated. Only genes with a fold-change $> \pm 2$ in all three experiments and $P < 0.05$ according to paired *t*-test on the three independent pairs

Table 1
Gene-specific oligonucleotides used for semi-quantitative RT-PCR

Gene	Sense primer	Antisense primer	Size (bp)	Annealing (°)
CYR61	GGCTGCGGCTGCTGTAAGGTCT	GTTCGGGGGATTTCTTGGTCT	739	60
MCP-1	CCAATTCTCAAACTGAAGCTC	AGGTGAATGAAGCACAGACCA	489	60
PAI-1	AAAGGTATGATCAGCAACTTG	CTGAAAGACTCGTGAAGTCAG	499	55
hEZF	CATCAGCGTCAGCAAAGGCAG	TCGCAGGTGTGCCTTGAGATG	514	60
VEGF	CTACTGCCATCCAATCGAGAC	TCACATCTGCAAGTACGTTCG	411	55
COX-2	AATGAGTACCGCAAACGCTTTATG	CATCTAGTCCGGAGCGGGAAGAAC	421	55
MKP-1	CATAGACTCCATCAAGAATGC	GAGTTCAGCAAATGTCTTGAC	559	55
Has2	GTCTCAAATTCATCTGATCTC	ACATTTCCTTAAGTAGTCTGG	419	51
STC-1	TGGTGATCAGTGCTTCTGCAAC	CTCAGTGATGGCTTCAGGGTTC	410	55
ICER	ATTATGGCTGTAACTGGA	TCATTAGCCTCAGCTCTC	213	55
GAPDH	TGATGACATCAAGAAGGTGGTGAA	TCCTTGGAGGCCATGTAGGCCAT	238	51, 55, 60

were considered to be significantly regulated by iloprost. Values of fold-change are given as mean ± SEM (N = 3).

2.5. Semi-quantitative RT-PCR

RT-PCR was performed with a Qiagen® One-Step RT-PCR kit (Qiagen) using 250 ng RNA. Gene-specific primers were used according to Table 1. After reverse transcription for 30 min at 50° and an initial PCR activation step for 15 min at 95°, the following thermal profile was used: 1 min 94°, 1 min annealing, 1 min elongation at 72° (30 cycles) and a final elongation step at 72° for 10 min. Amplified fragments were analyzed by sequencing to prove specificity of selected primers.

3. Results

3.1. Microarray analysis

Functional coupling of the prostacyclin-receptor to the G_s/cAMP pathway was confirmed by stimulating hSMC with the stable prostacyclin mimetic iloprost. While in untreated hSMC basal cAMP levels accumulated to 86 ± 50 pmol/mg protein × 10 min, stimulation of hSMC with the stable prostacyclin mimetic iloprost (100 nM) resulted in significantly enhanced cAMP formation (2171 ± 45 pmol/mg protein × 10 min, N = 3), respectively.

The effect of iloprost on gene expression was then analyzed by microarray screening. Prior to comparison analysis, global scaling (scaling to all probe sets) was applied to correct for variations due to technical differences between the experimental and baseline array. Each probe set on the experiment array (iloprost-treated SMC) was then compared to its counterpart on the baseline array (untreated control SMC) and the fold-change (increase and decrease) calculated. By this procedure we identified 83 genes that responded to iloprost in all experiments. The expression of 51 genes was significantly increased, whereas the expression of 32 genes was significantly reduced by treatment with iloprost in three independent experiments using three different smooth muscle cell lines (Table 2).

3.2. Analysis of time-courses of iloprost-regulated genes

To further analyze iloprost-induced gene regulation, time-courses of 10 genes, chosen from different functional groups and expression levels, were established. Further criterion for selection of these genes was their known relevance to growth, cell cycle or matrix modification in the vasculature. Cell lines, different from those previously used for microarray analysis, were used and RNA samples from control and iloprost-treated cells (1–24 hr) were subjected to semi-quantitative RT-PCR analysis. The expression of all investigated genes was normalized to glycerol aldehyde phosphate dehydrogenase (GAPDH) expression, which was not influenced by iloprost. The fold-change in the experiments amounted to −1.05, 1.04 and 1.01, respectively, for AFFX-HUMGAPDH/M33197_5_at. Figure 1 shows representative semi-quantitative RT-PCR experiments and densitometric evaluation of three independent time-course experiments of the iloprost-induced genes ICER, STC-1, hEZF, Has2, COX-2, MKP-1, and VEGF. Under basal conditions, STC-1, Has2, ICER, and COX-2 were merely detectable, while MKP-1, hEZF and VEGF were easily detectable in untreated cells according to the fluorescence signal obtained in the microarray analysis. Expression of all genes was at least one magnitude below GAPDH expression. According to the microarray hybridization experiments, ICER was induced 6.1 ± 0.4-fold, STC-1 18.8 ± 2.7-fold, hEZF 7.8 ± 2.0-fold, Has2 6.8 ± 1.8-fold, COX-2 4.7 ± 0.8-fold, MKP-1 3.9 ± 0.5-fold and VEGF 2.3 ± 0.4-fold, respectively, by iloprost treatment. RT-PCR experiments confirmed the iloprost-induced expression of all six genes. Time-courses revealed that steady-state mRNA levels of all six induced genes were rapidly elevated above basal levels after 1 hr of iloprost treatment and reached maximal levels after about 3 hr of stimulation. Gene expression returned back to basal levels after prolonged (16–24 hr) iloprost treatment.

Figure 2 shows representative semi-quantitative RT-PCR experiments and densitometric evaluation of three independent time-course experiments of the downregulated genes Cyr61, MCP-1 and PAI-1. According to the

Table 2
Iloprost-regulated genes in hSMC

Identified gene	Accession no.	Fold-change
Hormones/growth factors		
Stanniocalcin precursor STC	U25997	18.8 ± 2.7
Activin β-A	X57579	3.4 ± 0.5
TGFβ	M60315	2.8 ± 0.3
VEGF	M27281	2.3 ± 0.4
Nerve growth factor β	X52599	−2.6 ± 0.2
Connective tissue growth factor (CTGF)	M92934	−5.5 ± 1.2
Cell cycle/apoptosis/proliferation		
CD24 signal transducer	L33903	3.8 ± 0.1
BCL3	U05681	2.8 ± 0.1
Growth arrest specific gene 1 (gas1)	L13698	2.5 ± 0.1
Growth arrest/damage induced protein (gadd45)	M60974	−3.9 ± 0.6
Immune/inflammation		
BSF2/IL6	Y00081	4.9 ± 0.9
Prointerleukin 1β	X04500	4.9 ± 1.1
Interleukin BSF-2	X04602	4.3 ± 1.3
TRAF-interacting protein	U59863	−2.7 ± 0.1
Pentraxin (PTX3)	X97748	−4.8 ± 0.9
Activation-induced C-type lectin (AICL)	X96719	−5.4 ± 1.4
MCP-1	M37719	−7.4 ± 1.1
Protein kinases		
Protein kinase A anchoring p rotein Ht31	AF387101	2.6 ± 0.2
DAP-kinase	X76104	−2.5 ± 0.2
Phosphatases		
MKP-1/CL100	X68277	3.9 ± 0.5
Protein phosphatase 2A	M65254	2.7 ± 0.3
Tyrosine phosphatase	X82676	−2.3 ± 0.1
GTPases/G-protein signaling		
Ras-related rho	M12174	6.5 ± 1.3
Rho E	S82240	4.8 ± 0.5
RP4	U27768	−4.6 ± 0.5
Phosphodiesterases		
Phosphodiesterase	L20971	3.2 ± 0.4
3,5-Cyclic nucleotide phosphodiesterase	U40372	−3.8 ± 0.6
Proteases/protease inhibitors		
Protease proMch 6	U60521	6.1 ± 0.6
Receptors		
Mitogen-induced orphan receptor (MINOR)	U12767	11.1 ± 3.5
Retinoic acid receptor	X06614	10.1 ± 0.5
Orphan G protein-coupled receptor (RDC1)	U67784	5.3 ± 1.3
Transferrin receptor	X01060	3.4 ± 0.5
PDGF receptor A	M21574	−2.2 ± 0.1
Glucocorticoid receptor β	HG4582	−2.8 ± 0.2
ARP-1	M64497	−2.9 ± 0.4
Progression associated protein	Y07909	−4.9 ± 0.8
Transcription factors/DNA-binding proteins		
HOX7	M76732	8.1 ± 1.9
Zinc finger transcription factor (hEZF)	U70663	7.8 ± 2.0
Jun-B	U20734	7.3 ± 0.8
cAMP-responsive element modulator	S68271	7.3 ± 0.6
AREB6	D15050	6.4 ±0.9
Helix loop helix basic phosphoprotein (G0S8)	L13391	5.7 ± 0.7
Jun-B	X51345	5.7 ± 1.0
MSX-2	D89377	5.4 ± 0.1
HOX7	M97676	3.8 ± 0.5
Id1	S78825	3.2 ± 0.6
MAD-3 (IkB-like)	M69043	2.5 ± 0.2
H4 histone	X60486	−3.5 ± 0.2
Osteoprotegerin (OPG)	U94332	−3.5 ± 0.8
CPBP	U44975	−3.6 ± 0.2
Msg1	U65093	−3.7 ± 0.7
Homeobox gene	M60721	−5.7 ± 1.0
Nucleases		
HEM45	U88964	3.3 ± 0.4
Transporter/channels		
Glucose transporter like protein (GLUT III)	M20681	5.8 ± 1.7
Na^+/K^+-transporting ATPase β3-subunit	U51478	3.0 ± 0.3
Cytoskeleton/motility		
Tubulin α-1	HG2259	21.6 ± 5.1
Tubulin β-3	U47634	5.3 ± 0.9
Extracellular matrix		
Has2	U54804	6.8 ± 1.8
TSG14	M31166	−3.6 ± 0.7
Cystein-rich angiogenic protein (CYR61)	U62015	−14.9 ± 1.3
Lipids and lipid turnover		
Adipogenesis inhibitor factor	X58377	5.0 ± 0.4
Cyclooxygenase 2 (hCOX-2)	U04636	4.7 ± 0.8
Hemostasis		
Thrombomodulin	J02973	6.2 ± 0.7
Plasminogen activator inhibitor 1 (PAI-1)	J03764	−4.5 ± 0.5
Metabolic enzymes		
Polyamine oxidase	U61836	6.8 ± 0.8
Cystathionine gamma lyase	S52028	5.2 ± 0.9
S-Adenosylmethionin synthase	X68836	4.7 ± 1.5
Methylene tetrahydrofolate dehyd.cyclohyd.	X16396	−2.1 ± 0.1
Ceramide glucosyltransferase	D50804	−2.9 ± 0.1
Antioxidants		
Heme oxygenase	X06985	3.9 ± 0.9
Other or unknown		
E16	M80244	9.5 ± 2.8
Cyclophilin (hCyP3)	M80254	3.3 ± 0.1
KIAA0071	D31888	3.2 ± 0.7
KIAA0025	D14695	3.2 ± 0.7
TSC-22	U35048	3.2 ± 0.6
AF1q	U16954	2.5 ± 0.2
Signal transducing adaptor	U43899	−2.2 ± 0.1
APC	M74088	−2.4 ± 0.1
KIAA0127	D50917	−2.5 ± 0.1
HMG isoform I-C	U28749	−2.9 ± 0.4
IFI16	M63838	−3.4 ± 0.4
MTG8a	D43638	−3.6 ± 0.3
Oncogene Tls/Chop	HG2724	−7.5 ± 1.5

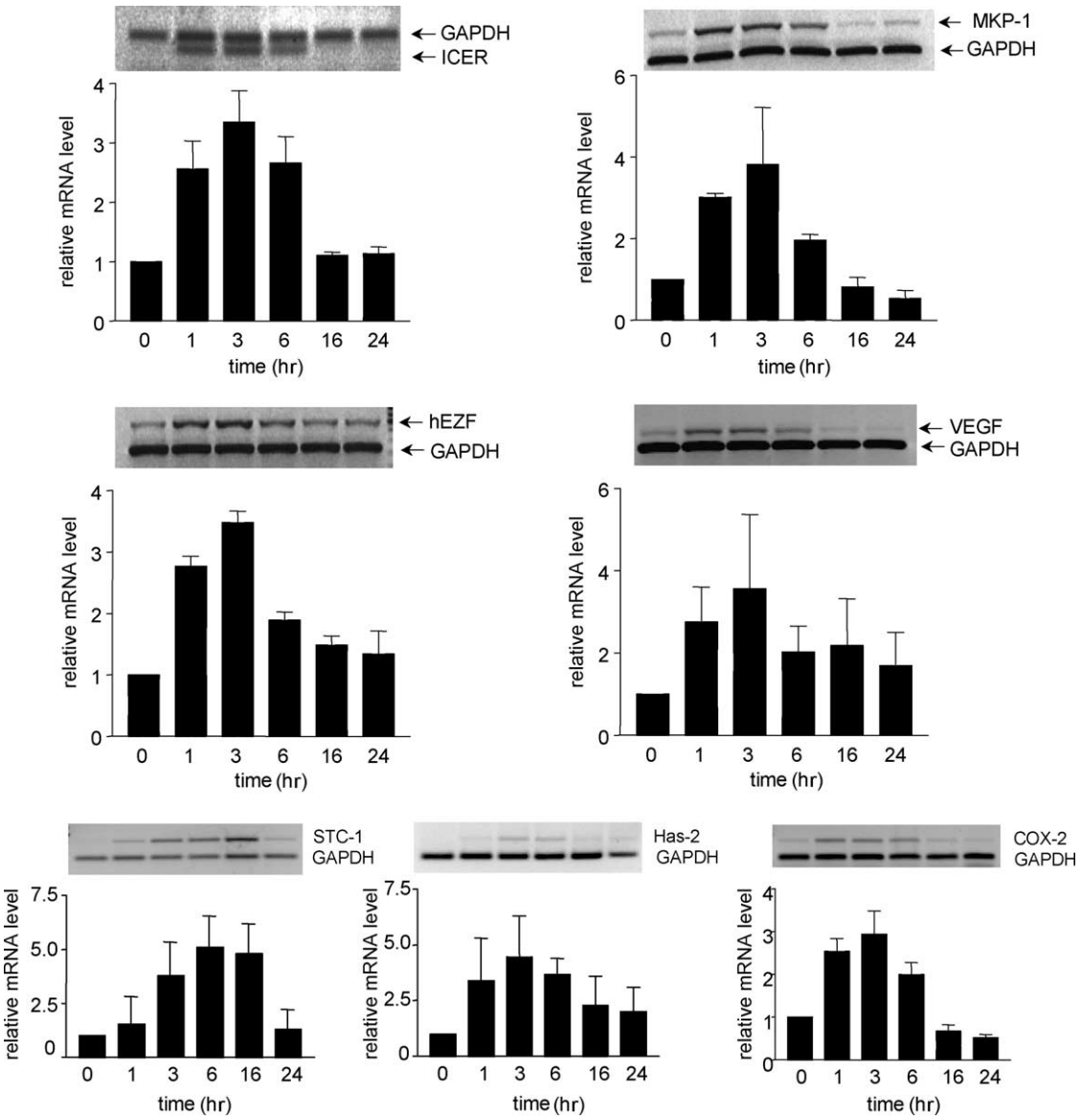

Fig. 1. Analysis of time-courses of iloprost-up-regulated genes. Given are representative semi-quantitative RT-PCR experiments and densitometric evaluation of three independent time-course experiments of the iloprost-induced genes ICER, MKP-1, hEZF, VEGF, STC-1, Has2, and COX-2. Expression ratios of the analyzed genes were normalized to GAPDH.

fluorescence signal obtained in the microarray analysis, MCP-1 and PAI-1 show strong basal expression, which was comparable to the GAPDH expression level, while Cyr61 basal expression was about one magnitude below GAPDH expression. Cyr61 mRNA levels were reduced -14.9 ± 1.3-fold, MCP-1 mRNA levels -7.4 ± 1.1-fold, and PAI-1 mRNA levels -4.5 ± 0.5-fold, respectively. Time-courses revealed a transient decline of all three mRNAs in iloprost-treated hSMC. However, while MCP-1 mRNA was already significantly reduced after 1 hr of iloprost treatment, PAI-1 and Cyr61 mRNA levels declined more slowly, with significant reduction after 3 hr of iloprost treatment.

Thus, the comparison of the gene expression levels of all 10 investigated genes showed a significant correlation between microarray data and RT-PCR. However, absolute quantitative values might vary due to technical differences in the detection mode.

4. Discussion

Understanding the mechanisms, underlying SMC quiescence, is of great importance because of the high incidence of SMC proliferation disorders such as atherosclerosis, restenosis and pulmonary hypertension. Despite this, little is known of the molecular events responsible for SMC quiescence or vice versa releasing SMC from quiescence. In the present study, we performed microarray analysis to examine changes in the transcriptional profile of human vascular smooth muscle cells in response to exposure to the vasoprotective prostacyclin mimetic iloprost, which is

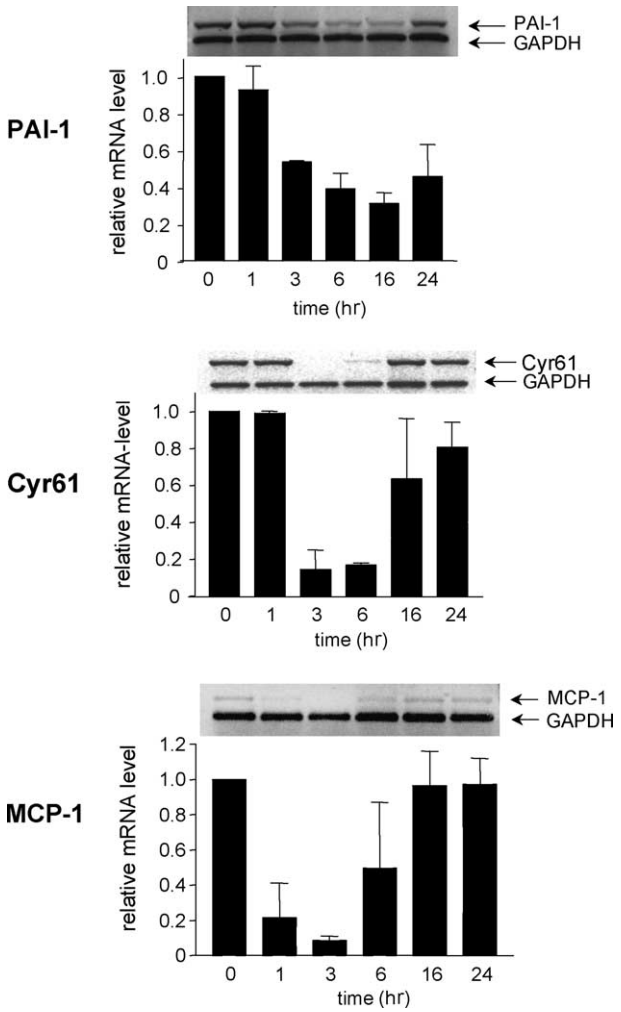

Fig. 2. Analysis of time-courses of genes down-regulated by iloprost. Given are representative semi-quantitative RT-PCR experiments and densitometric evaluation of three independent time-course experiments of the down-regulated genes PAI-1, Cyr61, and MCP-1. Expression ratios of the analyzed genes were normalized to GAPDH.

known to be coupled to the G_s/cAMP-pathway. We identified 51 genes that were upregulated significantly by iloprost in human vascular smooth muscle cells and 32 genes that were significantly down-regulated. Among these, we identified several genes related to proliferation and migration. Validation of array data was performed by means of RT-PCR for 10 selected genes.

Stimulation of the G_s-coupled PGI_2 receptor by iloprost induces cellular cAMP formation. Thus, signaling pathways, involved in transcriptional regulation of the identified genes, are related to the cAMP/PKA/CREB pathway [18]. This is consistent with the observed induction of the inducible cAMP early repressor (ICER) in iloprost-stimulated SMC. A cluster of four cAMP-responsive elements (CREs) in an intronic promoter inside the CREM gene directs cAMP inducibility due to binding of the transcription factors CREB and CREM [19]. Interestingly, it has been reported, that ICER blocks cells at the S and G2/M phases of the cell cycle [20], which results from transcriptional inhibition of various growth-related genes [21].

Microarray analysis revealed furthermore a significant induction of dual-specific MAP kinase phosphatase (MKP-1) mRNA in iloprost-stimulated cells. In general, MKPs have been shown to dephosphorylate and inactivate mitogen-activated protein kinases (MAPK) which are important regulators of cell growth, proliferation, and stress responsiveness [22]. Therefore, MKPs are regarded as critical counteracting factors that regulate the magnitude and duration of p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) activation, thereby opposing proliferative stimuli [23].

Moreover, the expression of the zinc finger protein hEZF mRNA was strongly upregulated in iloprost-treated SMC. Although relative little information is available concerning hEZF [24], it is regarded as the human homologue of the mouse gut-enriched Krüppel-like factor (GKLF), which plays a role in regulating growth arrest by repressing cyclin D1 expression [25]. The growth arresting nature of EZF/GKLF was demonstrated by its ability to inhibit DNA synthesis in fibroblasts, over-expressing this gene [26]. Increased hEZF expression after iloprost treatment suggests its involvement in IP-R-mediated growth inhibition. However, it should be mentioned, that the GKLF family of transcription factors is complex and involves numerous members with opposing actions on cellular proliferation. Interestingly, the growth arrest specific 1 gene (gas1) [27] was also found to be up-regulated in iloprost-treated SMC. Gas1 is expressed in fibroblasts in response to stimuli driving cells into Go phase and has been demonstrated to inhibit cell proliferation when overexpressed in fibroblasts [28].

Cell proliferation and migration are controlled by interactions between cells and the extracellular matrix (ECM). Therefore, the composition of the ECM is regarded as an important atherogenic factor. Interestingly, several genes, involved in cell/matrix interaction or matrix synthesis, were found to be regulated by iloprost. Microarray analysis revealed that the mRNA of Cyr61, a member of the CCN (Cyr61, CTGF, NOV) family of secreted, extracellular matrix-associated, heparin-binding proteins [29], was down-regulated markedly by iloprost. It has recently been shown, that purified Cyr61 mediates cell adhesion, migration and augments growth factor-induced DNA synthesis in fibroblasts [30]. Moreover, expression of Cyr61 in atherosclerotic lesions has been demonstrated recently [31]. Likewise, in atherosclerosis, high levels of CTGF expression, another closely related member of the CCN protein family, were assumed to be involved in extracellular matrix accumulation and progression of atherosclerotic lesions [32]. Iloprost-mediated down-regulation of Cyr61 and CTGF, as assessed by microarray analysis, might, therefore, contribute to antiproliferative and antimigratory PGI_2 actions. The observed marked induction of Has2 mRNA expression in hSMC after iloprost treatment, is more

difficult to interpret. Hyaluronan, the principal ligand for CD44, reveals two opposing effects depending on its molecular weight. While low-molecular weight pro-inflammatory forms of hyaluronan stimulate proliferation of cultured primary aortic smooth muscle cells, high-molecular weight forms of hyaluronan inhibit smooth muscle cell proliferation [33]. It is, therefore, difficult to determine the physiological consequence of iloprost-mediated Has2 induction in SMC.

Interestingly, we also observed iloprost-mediated induction of stanniocalcin 1 (STC-1) expression (18.8 ± 2.7-fold). Recently, Zhang *et al.* [34] demonstrated, that STC-1 increases resistance to ischaemic challenge and to elevated intracellular free calcium in neurons. These findings suggest a possible cell-protective action in SMC, which might be relevant under ischaemic oxidative stress situations. Moreover, according to recent findings of Filvaroff *et al.* [35], STC-1 seems also to be involved in endothelial activation and angiogenesis.

Decreased fibrinolytic capacity has been suggested to accelerate the process of arterial atherogenesis by facilitating thrombosis and fibrin deposition within the developing atherosclerotic lesion [36]. Interestingly, iloprost stimulation of vascular SMC results in decreased expression of type 1 PAI-1 mRNA, as demonstrated by both, microarray and RT-PCR analysis. PAI-1 is the primary inhibitor of tissue-type plasminogen activator and thereby prevents plasmin generation and fibrinolysis. Increased expression of PAI-1 mRNA in severely atherosclerotic human arteries suggests a role for PAI-1 in the progression of human atherosclerotic disease [37]. Recently, it has been demonstrated that PAI-1 enhances neointima formation after vascular injury in different animal models of restenosis [38]. Decreased PAI-1 expression after iloprost treatment, therefore, correlates with enhanced fibrinolytic capacity and might be regarded as a vasoprotective mechanism. Moreover, thrombomodulin is up-regulated in iloprost-stimulated hSMC, according to the microarray data. This glycoprotein can bind to thrombin and activate protein C, thus exerting anticoagulant activity and mitigating the effects of inflammatory cytokines. Thrombomodulin, therefore, exerts a protective function on endothelial cells [39] and down-regulation of its expression has been reported in coronary atherosclerosis [40].

A growing body of experimental evidence suggests a pivotal role of chemokines in the pathogenesis of vascular disease. Monocytes are attracted to sites of vascular endothelial cell injury by a chemokine known as monocyte chemotactic protein-1 (MCP-1). This migration of monocytes into the arterial wall is regarded as the initiating event in the development of atheromas [41]. MCP-1 is selectively secreted by injured vascular endothelial and smooth muscle cells [42]. According to our microarray and RT-PCR data, MCP-1 mRNA expression was significantly reduced in iloprost-treated cells, suggesting another aspect of iloprost-mediated vasoprotection by reducing the attraction of monocytes.

Several other genes involved in vascular protection, among them VEGF or heme oxygenase-1 (HO-1), were found to be upregulated by iloprost. It has been reported that the growth- and survival-promoting functions of VEGF support endothelial regeneration, whereas the VEGF-induced endothelial production of NO and PGI_2 inhibits vascular smooth muscle cell proliferation and thus may be useful in atherosclerotic/restenotic vessels [43]. Likewise, systemic pharmacological induction or adenoviral delivery of heme oxygenase-1, the inducible isoform of the initial and rate-limiting enzyme for heme catabolism, attenuates neointima formation after experimental vascular injury [44]. Therefore, HO-1 is regarded an important vasoprotective mediator *in vivo* that is capable of attenuating the pathophysiological response to endovascular injury.

Finally, we found up-regulation of the inducible cyclooxygenase (COX) isoform COX-2 mRNA in iloprost-stimulated vs. untreated control cells. COX catalyzes the initial step in the conversion of arachidonic acid to prostaglandins and thromboxane [45]. Autoregulation of COX-2 expression by prostaglandins was suggested earlier by findings of Oshima *et al.* [46], who investigated the cAMP-dependent induction of COX-2 in mouse osteoblastic cells. Iloprost-induced COX-2 expression, therefore, represents a positive auto-regulatory mechanism further enhancing PGI_2 formation in hSMC.

To date, limited information is available on the molecular mechanisms that control integrity of the blood vessel, and more specifically, the smooth muscle cell phenotype. This study is the first report on hSMC gene expression in response to stimulation with a vasoprotective PGI_2 mimetic. Besides genes which are already known to be regulated by cAMP, like ICER, VEGF, PDGF receptor or CTGF [5,47], a large number of additional genes was identified, which were hitherto unknown to be regulated by PGI_2 or cAMP, respectively, in human vascular SMC.

Several of the iloprost-induced genes like COX-2, VEGF or Has2 are also known to be induced by different growth factors. However, assuming, that IP-receptor-induced genes reflect mostly those, which are controlled by cAMP and thus the PKA/CREB signaling pathway, this seems not to be a contradiction. It is well known, that the transcription factor CREB binds the cyclic AMP response element (CRE) and activates transcription in response to a variety of extracellular signals including neurotransmitters, hormones, membrane depolarization and growth factors. Not only protein kinase A but also the calmodulin-dependent protein kinases CaMKII, p44/42 MAP Kinase, p90RSK, p38 MAP Kinase and MSK1 stimulate CREB phosphorylation at Ser133, a key regulatory site controlling transcriptional activity [48].

The consequences for the smooth muscle cell phenotype are obvious for those genes, directly affecting growth control, inflammation, extracellular matrix generation, matrix composition or hemostasis. The results of this study,

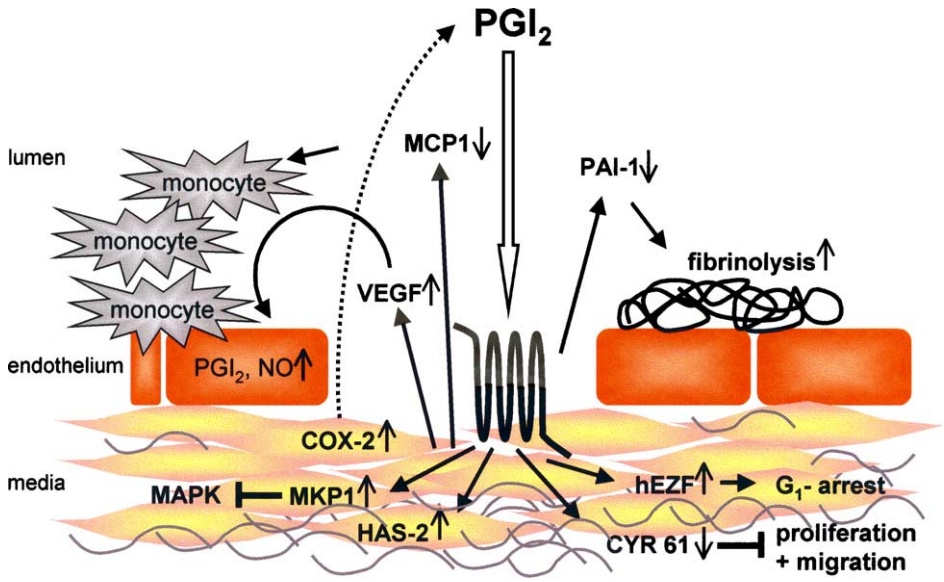

Fig. 3. Summary of atheroprotective PGI_2 actions in the blood vessel. Depicted is the effect of IP-receptor stimulation on hSMC gene expression. Receptor stimulation results in decreased expression of monocyte chemotactic protein (MCP-1), plasminogen activator inhibitor 1 (PAI-1) and cystein-rich angiogenic protein (Cyr61). On the other hand, expression of vascular endothelial growth factor (VEGF), zinc finger transcription factor (hEZF), stanniocalcin (STC-1), hyaluronan synthase 2 (Has2), cyclooxygenase 2 (COX-2) and dual-specific phosphatase (MKP-1) is elevated. Altogether, these effects support SMC quiescence and endothelial regeneration.

as far as confirmed by RT-PCR analysis, are summarized in Fig. 3. In case of many other genes, the *in vivo* relevance has to be established in further experiments. However, the initial identification of these genes might be important not only for a more detailed understanding of how smooth muscle phenotype is modulated by endogenous PGI_2 in the blood vessel, but also for understanding and treating pathological conditions that are influenced by prostaglandins.

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