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Iloprost down-regulates the expression of the growth regulatory gene *Cyr61* in human vascular smooth muscle cells

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Abstract

Prostacyclin and its mimetics have repeatedly been shown to act antiatherogenic and to inhibit neointima formation in several animal models of vascular injury. Treatment of human vascular smooth muscle cells with the prostacyclin mimetic iloprost (100 nm) drastically reduces expression of *Cyr61*, encoding the growth-regulatory cystein-rich angiogenic protein, without affecting the degradation rate of *Cyr61* mRNA. Thrombin-induced *Cyr61* expression was inhibited completely in the presence of iloprost. It is concluded that vasoprotective actions of prostacyclin in vivo may in part be due to inhibition of expression of the growth regulatory gene *Cyr61* at sites of vascular lesions.

Keywords: CYR61; Iloprost; Prostacyclin; Smooth muscle; Atherosclerosis

1. Introduction

The accumulation of vascular smooth muscle cells in the arterial intima plays an important role in the development of atherosclerotic plaques and in the restenotic process occurring after balloon angioplasty (Ross, 1999). Accumulation is linked to endothelial dysfunction resulting in a net loss of tissue protective endothelium-derived mediators (Schrör, 1997) as well as up-regulation of various growth regulatory factors. This is considered as an important contribution to smooth muscle cell dedifferentiation, migration and proliferation.

Prostacyclin (PGI_2), the major arachidonic acid metabolite produced in vascular endothelium (Schönbeck et al., 1999), is regarded as an atheroprotective mediator in blood vessels (Schrör, 1997). Recently, it was shown that experimental delivery of the prostacyclin synthase gene in a balloon-injured rat artery model accelerated the recovery from endothelial damage and reduced neointima formation (Numaguchi et al., 1999), further substantiating the role of prostacyclin as a tissue-protective mediator. Prostacyclin exerts its multiple effects, such as vasodilation, inhibition of platelet aggregation and modulation of cholesterol turnover, mostly by stimulation of cyclic adenosine 3′,5′ monophosphate (cAMP) generation subsequent to its binding to a specific IP receptor (Narumiya et al., 1999). In addition to these well-known functions of prostacyclin, inhibition of human vascular smooth muscle cell migration and proliferation has also been reported (Zucker et al., 1998). At present, limited information is available regarding the genes, which are involved in these antimigratory and antimitogenic actions of prostacyclin and its mimetics.

Cell proliferation and migration are controlled by interactions between cells and the extracellular matrix (ECM). In this context, the CCN (*c*ystein rich angiogenic protein, *c*onnective tissue growth factor, *n*ephroblastoma overexpressed) family of secreted, extracellular matrix-associated heparin-binding proteins (Brigstock, 1999; Lau and Lam, 2000), which are likely to mediate cell-matrix interactions, has recently received broad attention. Among these, *Cyr61* (cystein-rich angiogenic protein gene) encodes an immediate early gene that is co-induced by serum, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF-β1) in fibroblasts (O'Brien et al., 1990). Although devoid of a RGD (arginine, glycine, aspartate) motif, CYR61 is a ligand of and binds directly to the integrins $\alpha_V\beta_3$, $\alpha_{IIb}\beta3$ (Kireeva et

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al., 1998) and $\alpha_v\beta_5$ (Grzeszkiewicz et al., 2001). Finally, purified CYR61 mediates cell adhesion, migration and augments growth factor-induced DNA synthesis (Kireeva et al., 1996; Grzeszkiewicz et al., 2002). Recently, expression of CYR61 in arteriosclerosis and its regulation by angiotensin II has been demonstrated (Hilfiker et al., 2002).

Here, we show for the first time that treatment of human vascular smooth muscle cells with the stable prostacyclin mimetic iloprost drastically reduces *Cyr61* mRNA expression. Furthermore, iloprost antagonized thrombin-induced *Cyr61* expression completely. These findings provide a putative new target of prostacyclin-mediated antimigratory and antiproliferative actions.

2. Materials and methods

2.1. Cell culture

Human arterial smooth muscle cells were isolated by the explant method. Cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 15% fetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin in a humified atmosphere with 5% CO_2 at 37 °C. In general, hSMC of the passages 4 to 9 were used in the different experiments. For acute experiments, cells were incubated in serum-free DMEM for 24 h and then treated with the different substances as indicated. Forskolin and dibutyryl cAMP were from Sigma (Deisenhofen, Germany); iloprost was kindly provided by Schering (Berlin, Germany) and α-thrombin was kindly provided by Dr. J. Stürzebecher (Zentrum für Vaskuläre Biologie und Medizin, Erfurt, Germany).

2.2. Microarray analysis and semiquantitative RT-PCR (reverse transcriptase polymerase chain reaction)

Total RNA from untreated and iloprost-stimulated (100 nM) human vascular smooth muscle cells was prepared with Trizol-reagent (Gibco Life Technologies, Karlsruhe, Germany) following the manufacturer's manual. Synthesis of cDNA, preparation of biotinylated target and hybridization to HUGeneFL-Arrays (Affymetrix, Santa Clara, USA) was performed according to the Affymetrix protocol. Data were collected by laser scanning and expression levels were determined using Affymetrix software. RT-PCR was performed with a Qiagen® One-Step RT-PCR kit (Qiagen, Hilden, Germany). A total of 250 ng RNA were amplified

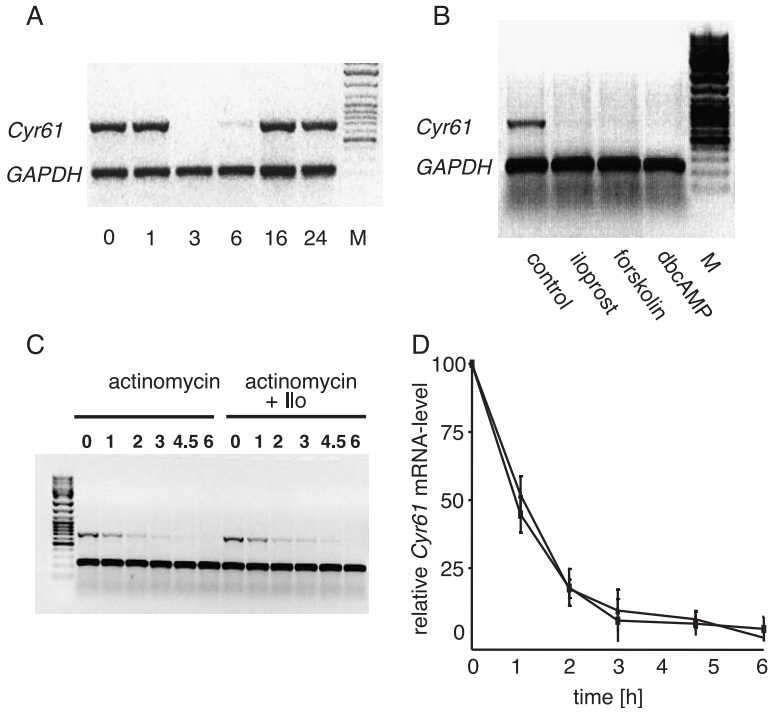

Fig. 1. Time-dependent reduction of *Cyr61* mRNA levels by iloprost. (A) Human vascular smooth muscle cells were treated with iloprost (100 nM) for varying time-periods as indicated. Total RNA was subjected to RT-PCR. The 739-bp *Cyr61* fragment and the 240-bp GAPDH fragment are indicated. M indicates the molecular weight marker. Results shown are representative for four independent experiments. (B) Human vascular smooth muscle cells were treated with forskolin (10 μM) or dibutyryl cyclic adenosine 3′,5′ monophosphate (1 mM) for 3 h. Total RNA was harvested and subjected to RT-PCR. Results shown are representative for three independent experiments. (C) Effect of iloprost on *Cyr61* mRNA stability. Human vascular smooth muscle cells were incubated with the transcription inhibitor actinomycin D (5 μg/ml), either alone or in combination with iloprost (100 nM), as indicated. Total RNA was isolated at the various time points and subjected to RT-PCR. Results shown are representative for three independent experiments. (D) Quantification of *Cyr61* band intensities of actinomycin (■) or actinomycin D plus iloprost (●)-treated cells. Band intensities were quantified using Quantity I software (BioRAD) and represent densitometric evaluation of three independent experiments.

using following primers (MWG Biotech, Ebersbach, Germany): *CYR61* sense: GGCTGCGGCTGCTGTAAGGTCT, *CYR61* anti-sense: GTTCGGGGGATTTCTTGGTCT, GAPDH sense: TGATGACATCAAGAAGGTGGTGAA, GAPDH antisense: TCCTTGGAGGCCATGTAGGCCAT. After reverse transcription, the following thermal profile was used: 1 min 94 °C, 1 min 60 °C, 1 min 72 °C (30 cycles) and a final elongation step at 72 °C for 10 min.

3. Results

3.1. Effect of iloprost on Cyr61 mRNA expression in human vascular smooth muscle cells

To identify genes involved in the vasoprotective action mediated via prostacyclin, we have used microarray technology to examine changes in the transcriptional program in human vascular smooth muscle cells in response to exposure to the prostacyclin mimetic iloprost. Stimulation of quiescent human vascular smooth muscle cells with iloprost (100 nM, 6 h) led to a significant reduction in *Cyr61* mRNA levels (14.9 ± 1.3-fold reduction, $n=3$). To validate the data obtained in microarray analysis, RNA samples from control and iloprost-treated cells were subjected to RT-PCR analysis. *Cyr61* mRNA was down-regulated time-dependently by iloprost (100 nM) with the strongest reduction after 3–6 h ($n=4$). After continuous stimulation with iloprost (6–24 h), mRNA levels approached again control levels (Fig. 1A). Since prostacyclin and its mimetics exert their multiple effects mostly by the production of cAMP following binding to the specific IP receptor, we also tested the effect of the adenylyl cyclase activator forskolin (10 μM) and the cell-permeable cAMP analogue dibutyryl cyclic adenosine 3′,5′ monophosphate (1 mM) on *Cyr61* mRNA levels. Reduction of *Cyr61* mRNA was achieved with both substances (Fig. 1B), confirming cAMP dependency of *Cyr61* mRNA down-regulation.

3.2. Effect of iloprost on Cyr61 mRNA stability

To clarify the mechanisms of reducing the steady-state levels of *Cyr61* mRNA, we examined the effect of iloprost on the stability of the transcript. Therefore, human vascular smooth muscle cells were incubated with the transcription inhibitor actinomycin D (5 μg/ml), either alone or in combination with iloprost (100 nM). The disappearance of *Cyr61* mRNA was then monitored over a 6 h period. A similar decrease of *Cyr61* mRNA, in the absence or presence of iloprost, was observed (Fig. 1C,D). The observed short half-life of *Cyr61* mRNA is consistent with the generally observed rapid turnover of immediate early gene transcripts. The data suggest that transcriptional inhibition and not accelerated degradation of *Cyr61* mRNA are responsible for the reduced steady-state levels in iloprost-treated cells.

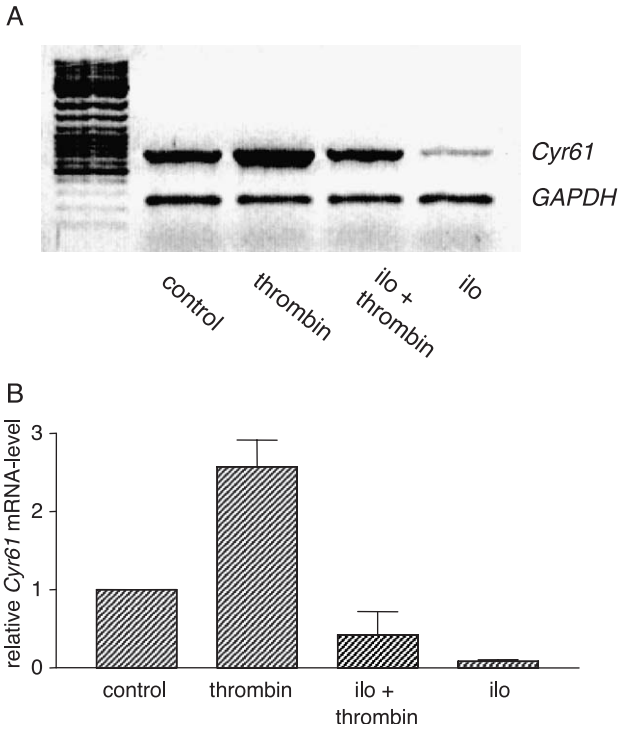

Fig. 2. Effect of iloprost on thrombin-induced *Cyr61* mRNA expression. (A) Quiescent human vascular smooth muscle cells were treated with iloprost (100 nM), thrombin (2 U/ml) or a combination of both for 3 h. Total RNA was harvested and subjected to RT-PCR. The picture shows one representative experiment out of three independent experiments with similar results. (B) Densitometric quantification of *Cyr61* band intensities (mean ± S.E.M. of $n=3$ independent experiments).

3.3. Iloprost antagonizes Cyr61 mRNA induction by thrombin

Thrombin generated at sites of vascular injury not only participates in the coagulation cascade but can also signal cellular events related to mitogenesis and migration. We therefore tested the effect of thrombin for its ability to induce the expression of *Cyr61* in human vascular smooth muscle cells. RT-PCR of cells treated with thrombin (2 U/ml) for 3 h revealed enhanced *Cyr61* mRNA levels as compared to control cells (2.6 ± 0.3 fold, $n=3$) (Fig. 2A,B). The thrombin-induced *Cyr61* mRNA expression, like basal *Cyr61* mRNA expression, was markedly decreased by iloprost treatment.

4. Discussion

To date, the factors that mediate human vascular smooth muscle cell dedifferentiation, migration and proliferation in relation to atherogenesis and restenosis are poorly understood. It is assumed that these factors are under control of tissue protective mediators, among them prostacyclin. In order to obtain further insights into the mechanisms of vasoprotective prostacyclin actions, we have analyzed the

gene expression profile of human vascular smooth muscle cells treated with the stable prostacyclin mimetic iloprost and found a significant down-regulation of the extracellular matrix-associated, growth-regulatory protein CYR61 (cysteine-rich angiogenic protein) in comparison to untreated control cells. Since we have used different cultured cell lines, cell line-specific variations in basal Cyr61 mRNA levels as well as iloprost-sensitivity and time kinetics were observed. Nevertheless, in all experiments, *Cyr61* mRNA was down-regulated time-dependently by iloprost with the strongest reduction after 3-6 h.

CYR61 was shown to promote the attachment and spreading of endothelial cells or to enhance the effects of growth factors on the rate of DNA synthesis of fibroblasts and vascular endothelial cells (Kireeva et al., 1998). Moreover, it promotes cell migration in both, fibroblasts and endothelial cells. The recent observation, that *Cyr61* is highly expressed in the neointima of balloon-injured arteries, provides the first link of CYR61 to the pathogenesis of vascular restenosis (Wu et al., 2000). Moreover, expression of CYR61 in atherosclerotic lesions has been demonstrated recently (Hilfiker et al., 2002). Likewise, in atherosclerosis, high levels of connective tissue growth factor (CTGF) expression, another closely related member of the CCN protein family, are discussed to be involved in extracellular matrix accumulation and thus progression of atherosclerotic lesions (Oemar et al., 1997). Interestingly, CTGF has also been reported to be down-regulated by iloprost (Stratton et al., 2001).

In this study, we have also shown that thrombin significantly up-regulates *Cyr61* mRNA levels in human vascular smooth muscle cells. Besides its function in the coagulation cascade, thrombin is known as a potent mitogen and promigratory stimulus in vascular smooth muscle cells (McNamara et al., 1993; Bretschneider et al., 2001). It might be speculated that thrombin could regulate cellular functions like migration and proliferation at least partly by enhancing CYR61 secretion.

Our observation that the stable prostacyclin mimetic iloprost down-regulates *Cyr61* expression in human vascular smooth muscle cells and antagonizes thrombin-induced up-regulation in human vascular smooth muscle cells suggests that *Cyr61* may be an important down-stream target for antiatherogenic prostacyclin effects, such as inhibition of cell proliferation or migration.

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