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Regulation of cyclooxygenase-2 expression by iloprost in human vascular smooth muscle cells Role of transcription factors CREB and ICER

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Abstract

Prostaglandin-endoperoxide synthase-2 (PGH-synthase) or cyclooxygenase-2 (COX-2) is inducible by a variety of stimuli, e.g. inflammatory mediators, growth factors and hormones and is believed to be responsible for the majority of inflammatory prostanoid production. Moreover, it has been demonstrated that COX-2 contributes substantially to prostacyclin-synthesis in patients with atherosclerosis. In this study, we demonstrate an up-regulation of COX-2 mRNA, protein and product formation by the prostacyclin-mimetic iloprost in human vascular smooth muscle cells (hSMC). COX-2 mRNA expression was induced transiently between 1 and 6 hr and returned to basal levels after 16 hr of iloprost stimulation. COX-2 protein was induced concomitantly between 3 and 6 hr of iloprost stimulation. This was accompanied by an increase in PGI_2 formation. Forskolin, a direct activator of adenylyl cyclase, and dibutyryl cAMP, a cell-permeable cAMP analogue-induced COX-2 mRNA, suggesting a cAMP-dependent COX-2 expression in hSMC. Iloprost-induced COX-2 protein expression and PGI_2 formation was synergistically elevated by co-stimulation with the phorbolester PMA (phorbol-12-myristate-13-acetate). It is concluded, that the observed up-regulation of COX-2 with subsequent release of newly synthesized PGI_2 and the synergistic effect of iloprost and phorbolester on PGI_2 formation provide a positive feedback of prostaglandins on their own synthesizing enzyme. This might be important for control of hSMC proliferation, migration and differentiation as well as inhibition of platelet aggregation.

Keywords: Iloprost; Prostacyclin; Cyclooxygenase-2; Vascular smooth muscle cell

1. Introduction

Prostaglandin endoperoxide synthase (PGH-synthase) or COX is the initial enzyme in the conversion of arachidonic acid to the different prostaglandins [1,2]. COX exists in two distinct isoforms, namely COX-1 and COX-2 [3,4] which are specifically regulated at the molecular level and seem to have different biological functions. While COX-1 is considered to be expressed constitutively and is generally believed to be responsible for physiological ''house keeping'' prostaglandin synthesis [5,6], COX-2 is inducible by a variety of stimuli, e.g. growth factors, inflammatory mediators, and hormones [6–10], and believed to be responsible for the majority of inflammatory prostanoid production [11–13].

Recent studies indicate that prostaglandins themselves could exert regulatory feedback actions on the expression of their own synthesizing enzyme COX-2 as demonstrated in macrophages, monocytes, keratinocytes and osteoblasts [14–17]. In vascular smooth muscle cells (SMC) it was demonstrated that exogenously applied prostaglandin E_2 (PGE_2) strongly inhibited COX-2 expression, whereas PGI_2 analogues had no effect on the expression of COX-2 [18,19].

PGI_2 is the main COX product in endothelial cells and SMC of large arteries [20], the primary sites of

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Abbreviations: hSMC, human vascular smooth muscle cells; cAMP, cyclic adenosine monophosphate; PGI_2, prostacyclin; PMA, phorbol-12-myristate-13-acetate; COX, cyclooxygenase; PKC, protein kinase C; PKA, protein kinase A; CRE, cAMP response element; CREB, CRE binding protein; CREM, CRE modulator; ICER, inducible cAMP early repressor.

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atherosclerotic lesions. PGI_2 acts *via* G-protein-coupled prostacyclin receptors (IP-R) and mediates several anti-atherosclerotic actions, e.g. inhibition of platelet aggregation, smooth muscle cell migration and proliferation [21], mostly by stimulation of cellular cAMP formation [22]. Proliferation of SMC is a critical event in the development of atherosclerosis and in restenosis following vessel injury, e.g. by coronary angioplasty (PTCA). Recently, Schönbeck *et al.* [23] reported enhanced expression of COX-2 in atherosclerotic blood vessels. The expression of COX-2 with subsequent production of atheroprotective PGI_2 might, therefore, be involved in the regulation of proliferative processes in the blood vessel.

In a recent study using microarray analysis to determine altered gene expression in human SMC (hSMC), we have found an up-regulation of COX-2 expression by the PGI_2 analogue iloprost. The present study investigates the regulation of COX-2 expression in hSMC by iloprost alone and under inflammatory and proatherogenic conditions, mimicked by phorbolester 12-myristate-13-acetate (PMA), a general PKC activator. We report that iloprost induces COX-2 expression and product formation *via* a cAMP-dependent mechanism and that this effect is synergistically enhanced by PMA.

2. Materials and methods

2.1. Materials

Cell culture reagents and TRIzol™ were purchased from Gibco Life Technologies. PMA, forskolin, dibutyryl cAMP (dbcAMP), bovine serum albumin (BSA) and isobutyl-methylxanthine (IBMX) were from Sigma. Iloprost was kindly provided by Schering. ^{3}H-6-keto-$PGF_{1\alpha}$ was from Amersham Pharmacia. Polyclonal COX-2 antibody was from Cayman Chemical Co and horseradish-peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology. Primers for RT-PCR were purchased from MWG-Biotech AG. Dextran 500 was derived from SERVA. Activated charcoal was from FLUKA-Chemie AG. The 6-keto-$PGF_{1\alpha}$-standard was derived from Biomol.

2.2. Cell culture

Human arterial smooth muscle cells (hSMC) were isolated from coronary artery or aorta, respectively, by the explant technique [24] and were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 15% fetal calf serum (FCS), 100 μg/mL streptomycin and 100 unit/mL penicillin in a humified atmosphere with 5% CO_2 at 37 °. In general, hSMC of the passages 4–9 were used in the different experiments. If not otherwise mentioned, cells were washed twice with phosphate-buffered saline and incubated in serum-free DMEM for 24 hr prior to acute experiments.

2.3. cAMP measurements

hSMC were grown until subconfluence 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) or forskolin (10 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 [25]. Protein determination was performed according to the method of Bradford [26]. The adenylyl cyclase activity was expressed as pmol cAMP mg^{-1} protein × 10 min.

2.4. Western blot analysis

SMC were stimulated with iloprost (0.1 μM) for the indicated times and then lysed in lysis buffer (sodium-phosphate, 62.5 mM pH 7.0, glycerol 10%, sodium dodecyl sulfate (SDS) 2% (w/v), bromphenol blue 0.01% (w/v)), sonicated for 3 s and heated for 5 min at 95°. Proteins were separated on a denaturing 10% SDS–polyacrylamide gel and then electrophoretically transferred to polyvinylidene difluoride membranes (PVDF, Immobilon™-P, Millipore). Membranes were blocked in TBSTM (Tris 25 mM, NaCl 0.5 mM, Tween-20 0.1 % (v/v), skim milk powder 5% (w/v)) for 1 hr at room temperature and then probed with anti COX-2 polyclonal antibody (1:1000) and anti-actin monoclonal antibody (1:5000) overnight at 4°. Membranes were washed three times with TBS containing 0.1% Tween-20 (v/v), and then incubated with peroxidase-conjugated secondary antibodies (1:3000 in TBSTM) for 1 hr at room temperature. Finally, the membranes were visualized with enhanced chemiluminescence detection reagent (ECL, Roche Diagnostics).

2.5. Determination of 6-keto-$PGF_{1\alpha}$

After stimulation with PMA (0.1 μM), iloprost (0.1 μM) or the combination of both, cells were washed twice with DMEM and incubated for another 30 min in 0.5 mL fresh DMEM. The supernatants were collected and the formation of 6-keto-$PGF_{1\alpha}$, the stable hydrolysis product of PGI_2, was determined by RIA as described previously by Schrör and Seidel [27]. Briefly, the 6-keto-$PGF_{1\alpha}$ content in the cell culture supernatants was determined as follows: the reaction mix containing 300 μL cell culture supernatant, 100 μL diluted specific 6-keto-$PGF_{1\alpha}$ antiserum (produced in rabbits) and 500 μL reaction buffer (200 mg gelatine plus 4000 pg ^{3}H-6-keto-$PGF_{1\alpha}$, dissolved in 100 mL RIA buffer) was incubated overnight at room temperature. Then, 500 μL dextran-activated charcoal-suspension was added and then mixed continuously for 5 min. Dextran-activated charcoal-suspension consists of

a mixture of equal volumes of dextran 500 solution (2.5 g/500 mL RIA buffer) and activated charcoal solution (25 g activated charcoal p.a. dissolved in 500 mL RIA buffer). After incubation with dextran-activated charcoal-suspension, samples were centrifuged at 11,600 *g* (3 min). Then, 1 mL of the supernatant was mixed with liquid scintillator (LUMASAFE-PLUS, PACKARD BioScience) and counted in a β-counter. The 6-keto-$PGF_{1\alpha}$-content of the samples was related to a 6-keto-$PGF_{1\alpha}$-standard curve (Biomol).

2.6. Semi-quantitative RT-PCR

Cells were treated with PMA (0.1 μM), iloprost (0.1 μM), forskolin (10 μM) or dbcAMP (1 mM) as indicated. Then total RNA was prepared with Trizol-reagent following the manufacturer's manual. If not otherwise mentioned, RT-PCR was performed with Ready-To-Go™ RT-PCR Beads (Amersham Pharmacia Biotech) using 0.5 μg total RNA. COX-2 mRNA was amplified using the gene-specific primers (15 pmol each), sense: AATGAGTACCGCAAACGCTTTATG; anti-sense: CATCTAGTCCGGAGCGGGAAGAAC, resulting in a 421 bp fragment. For semi-quantitative analysis GAPDH was co-amplified with the primers (15 pmol each), sense: TGATGACATCAAGAAGGTGGTGAA; antisense: TCCTTGGAGGCCATGTAGGCCAT; resulting in a 238 bp fragment. After reverse transcription for 30 min at 42° and a denaturation step for 5 min at 95°, the following thermal profile was used: 1 min 95°, 1 min 55°, 1 min 72° (30 cycles) and a final elongation step at 72° for 15 min.

RT-PCR for ICER was performed with Qiagen® one-step RT-PCR kit and the gene-specific primers, sense: ATTATGGCTGTAACTGGA; antisense: TCATTAGCCTCAGCTCTC; resulting in a 214 bp fragment. For semi-quantitative analysis GAPDH was co-amplified, as described above. A PCR amplification was subsequently performed with Ready-To-Go™ RT-PCR Beads (Amersham Pharmacia Biotech). A 5 μL aliquot of the cDNA generated in RT-PCR was subjected to 35 cycles of amplification with addition of fresh primers for ICER.

2.7. Statistics

Data are means ± SEM from N independent experiments. Statistical analysis was performed using ANOVA followed by Bonferroni's multiple comparisons test. $P < 0.05$ was considered significant.

3. Results

3.1. Time-dependent effects of iloprost and PMA on COX-2 mRNA expression in hSMC

Incubation of serum-deprived, unstimulated hSMC with the stable PGI_2 mimetic iloprost (0.1 μM) resulted in a time-dependent induction of COX-2 mRNA. As shown in Fig. 1A, iloprost induces up-regulation of COX-2 mRNA within 1–6 hr. The mRNA signal returned to basal levels after 16 hr of continuous iloprost stimulation. Comparable kinetics were observed when hSMC were treated with the phorbolester PMA (0.1 μM), as an PKC-activating inflammatory or proatherogenic stimulus (Fig. 1B).

3.2. Time-dependent effects of iloprost and PMA on COX-2 protein expression and prostacyclin synthesis in hSMC

Further experiments were focused on the functional consequences of iloprost- and PMA-induced expression

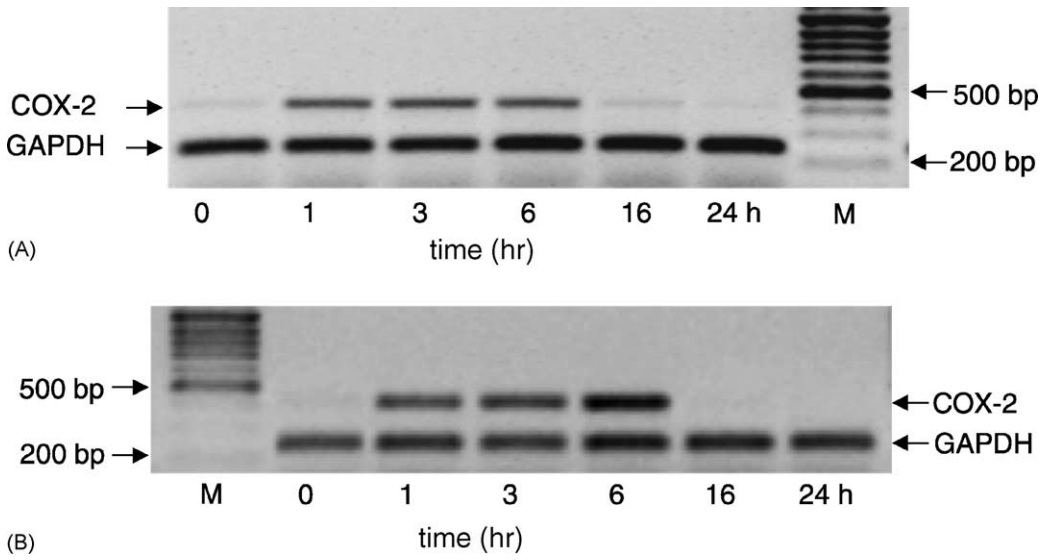

Fig. 1. RT-PCR analysis of time-dependent expression of COX-2 mRNA in hSMC. Panel (A) hSMC were incubated with iloprost (0.1 μM) for the times indicated. Shown is a representative experiment out of four with similar results. Panel (B) hSMC were incubated with PMA (0.1 μM) for the time indicated. Shown is a representative experiment out of three with similar results. $M = 100$ bp DNA-ladder.

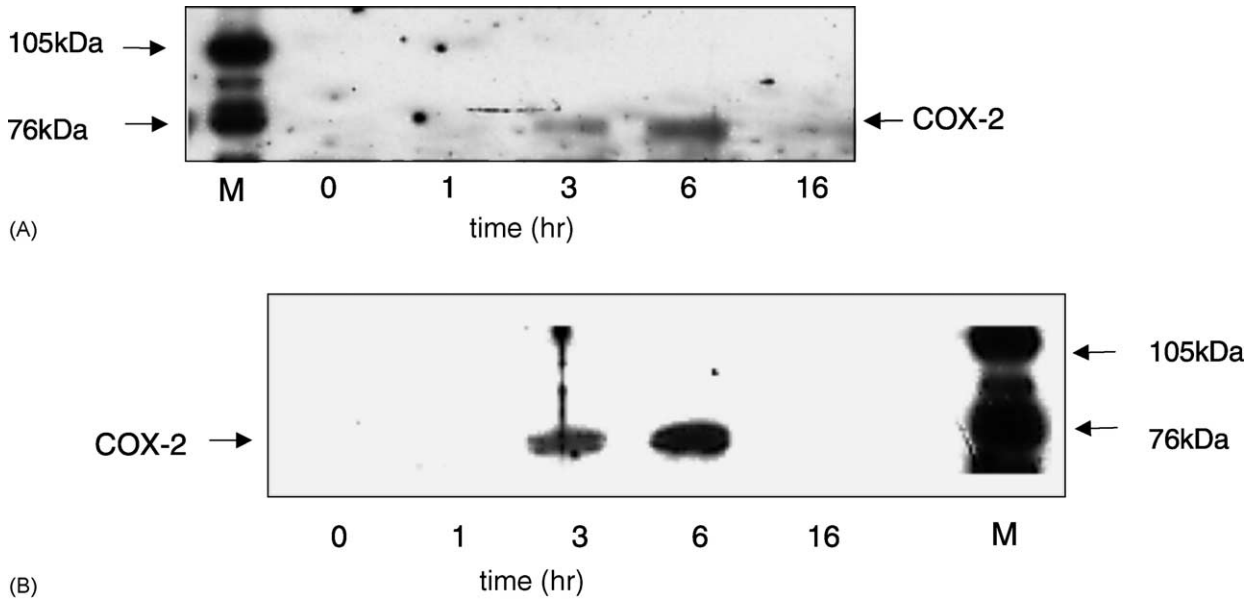

Fig. 2. Western blot analysis of time-dependent expression of COX-2 protein in human SMC. Panel (A) hSMC were incubated with iloprost (0.1 μM) for the times indicated. Shown is a representative experiment out of three with similar results. Panel (B) hSMC were incubated with PMA (0.1 μM) for the times indicated. Shown is a representative experiment out of three with similar results. *M*: molecular weight marker, 105 + 76 kDa markers are indicated.

of COX-2 mRNA. As shown in Fig. 2A, COX-2 protein was also transiently expressed upon iloprost stimulation (0.1 μM) as COX-2 mRNA. COX-2 protein could be detected within 3–6 hr and the expression returned to basal levels after 16 hr of iloprost treatment as examined by Western blotting. Similar kinetics were obtained when cells were stimulated with PMA. However, the response was somewhat stronger (Fig. 2B). As shown in Fig. 3A, iloprost-induced expression of COX-2 protein was accompanied by a slight and time-dependent stimulation of PGI_2 formation, determined as its stable metabolite 6-keto-$PGF_{1\alpha}$ in the cell culture supernatant. After 6 hr of iloprost stimulation, hSMC released 2.2 ± 0.5-fold more 6-keto-$PGF_{1\alpha}$ than untreated controls. Stimulation of cells with PMA (0.1 μM) also caused a significant elevation of 6-keto-$PGF_{1\alpha}$ formation after 4.5–8 hr of stimulation which returned near to basal levels after 16 hr. After 6 hr of PMA stimulation 6-keto-$PGF_{1\alpha}$ formation was elevated 4.5 ± 1.1-fold over basal values. These results are consistent with the up-regulation of COX-2 mRNA and protein expression shown in Figs. 1 and 2.

To examine a possible feedback action of iloprost on PMA-induced PGI_2 formation, hSMC were co-incubated with iloprost and PMA. As demonstrated in Fig. 3B, co-incubation of hSMC with iloprost and PMA resulted in a significantly increased 6-keto-$PGF_{1\alpha}$ formation between 4.5 and 8 hr of stimulation which was higher than PMA-induced 6-keto-$PGF_{1\alpha}$ formation alone. After 6 hr of stimulation with iloprost and PMA, release of 6-keto-$PGF_{1\alpha}$ was 7.5 ± 0.9-fold above basal levels. These data indicate an additive effect of PGI_2 on PMA-induced PGI_2 formation.

To test whether the additive effect of iloprost on PMA-induced PGI_2 synthesis was due to enhanced expression of COX-2, we examined the COX-2 protein expression by Western blot analysis. As demonstrated in Fig. 4, PMA-induced COX-2 protein expression was elevated by co-stimulation with iloprost, indicating that the additive effect of iloprost on PGI_2 synthesis can be attributed to enhanced protein expression and, therefore, exerts positive feedback actions.

3.3. Regulation of COX-2 expression by iloprost in hSMC is mediated by cAMP

In order to identify the mechanisms of iloprost action on COX-2 expression we measured initially cAMP levels in hSMC. In untreated hSMC basal cAMP formation accumulated to 86 ± 50 pmol/mg protein × 10 min. Stimulation of hSMC with iloprost in a concentration range between 1 and 100 nM resulted in a concentration-dependent increase in cAMP formation, accumulating to 351 ± 29 (1 nM), 1250 ± 130 (10 nM) and 2171 ± 45 (100 nM) pmol/mg protein × 10 min, respectively. Similar to cAMP levels, COX-2 mRNA was also concentration-dependently induced with a maximum at 10 nM iloprost, as examined by RT-PCR (Fig. 5A). These data strongly suggest a cAMP-depending mechanism in up-regulation of COX-2 expression. This was further confirmed by the finding that COX-2 mRNA was induced by forskolin (10 μM), a direct activator of adenylyl cyclase, or by the cell-permeable cAMP analogue dbcAMP (1 mM) as shown in Fig. 5B. As determined by RIA, forskolin (10 μM)-induced cAMP formation was 345 ± 62 pmol/mg protein × 10 min, while iloprost (100 nM) caused a strong induction of cAMP formation accumulating to 1814 ± 230 pmol/mg protein × 10 min vs. 67 ± 40 pmol/mg

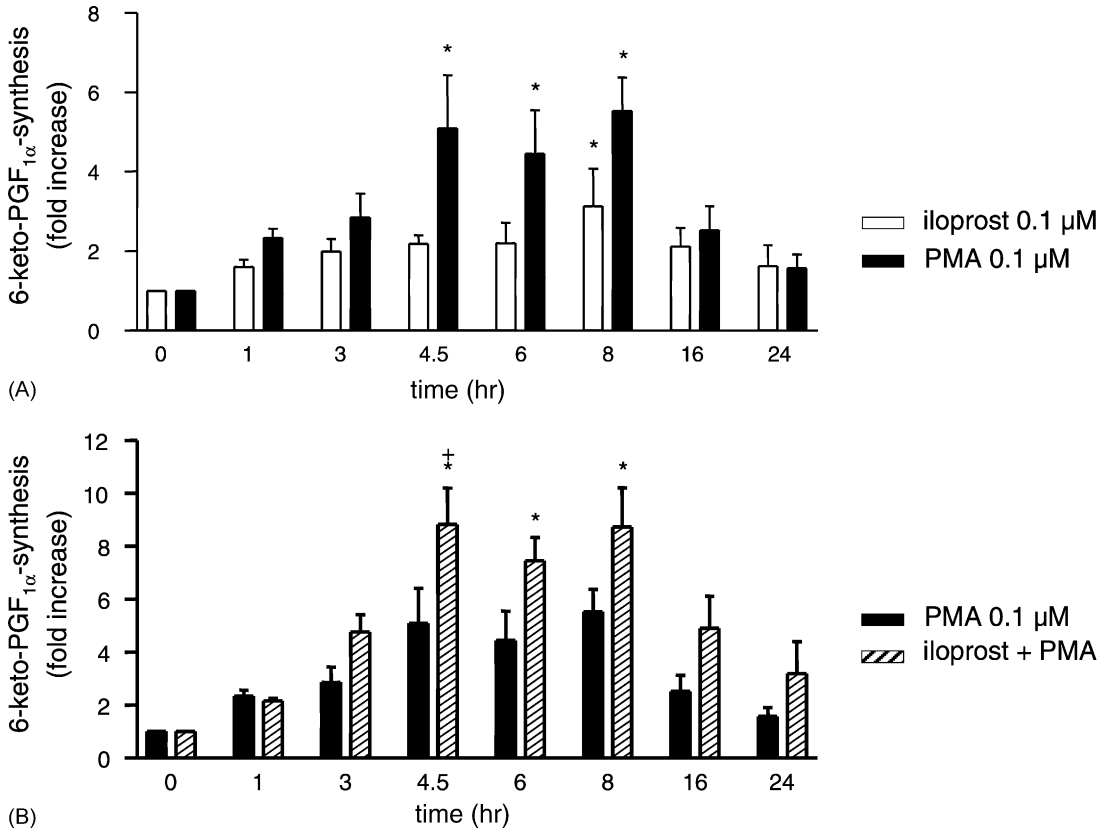

Fig. 3. Time-course of 6-keto-$PGF_{1\alpha}$ formation in hSMC. Panel (A) hSMC were treated with iloprost (0.1 μM, open bars) or with PMA (0.1 μM, black bars) for the times indicated. Values are means ± SEM of N = 5 (iloprost) or N = 3 (PMA) experiments. $^{*}P < 0.05$ significant vs. control. Panel (B) comparison of 6-keto-$PGF_{1\alpha}$ formation in hSMC treated with PMA (0.1 μM, black bars) or with iloprost (0.1 μM) plus PMA (0.1 μM; striped bars) for the times indicated. Values are means ± SEM of N = 3 experiments. $^{*}P < 0.05$ significant vs. control; $^{+}P < 0.05$ significant vs. PMA. The level of 6-keto-$PGF_{1\alpha}$ formation is expressed as fold increase above control.

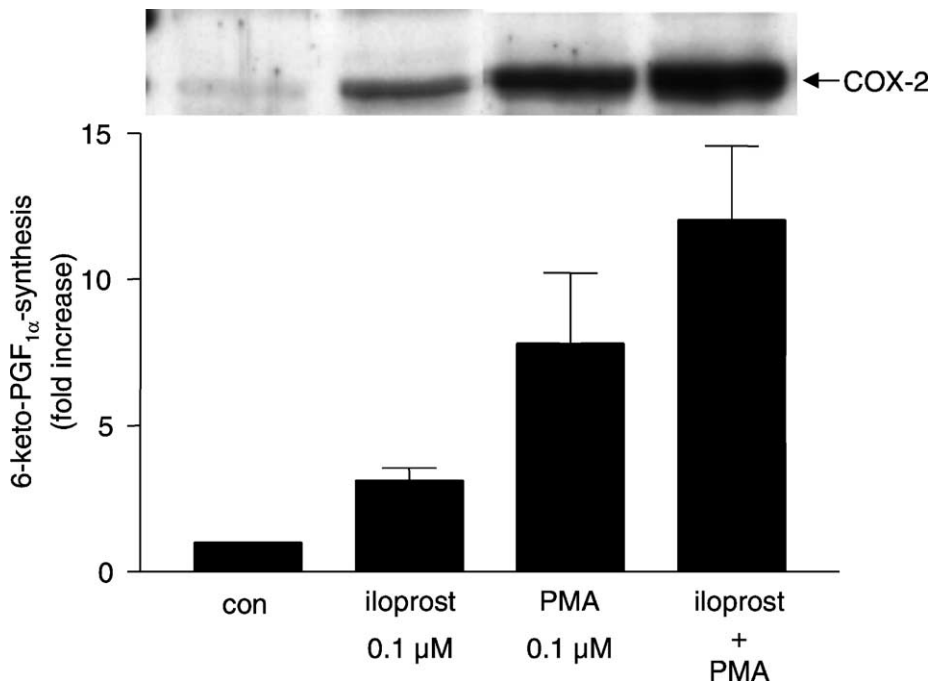

Fig. 4. Effect of iloprost on PMA-induced 6-keto-$PGF_{1\alpha}$ formation (lower panel) and COX-2 protein expression (upper panel). hSMC were treated with iloprost (0.1 μM), PMA (0.1 μM) or with iloprost plus PMA for 6 hr. Supernatants were collected and the stable PGI_2 hydrolysis product 6-keto-$PGF_{1\alpha}$ was determined by RIA. The level of 6-keto-$PGF_{1\alpha}$ formation is expressed as fold increase above control. Values are means ± SEM of N = 3 experiments. COX-2 protein expression was detected by Western blotting using anti-COX-2 antibody. Shown is a representative experiment out of three with similar results.

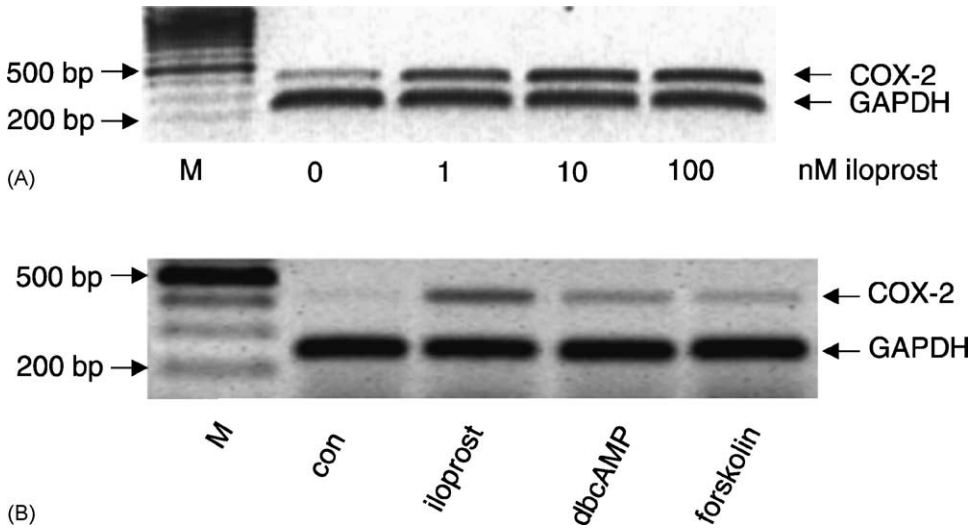

Fig. 5. cAMP-dependent induction of COX-2 mRNA expression in hSMC. Panel (A) RT-PCR analysis of concentration-dependent induction of COX-2 mRNA expression after stimulation with iloprost for 3 hr. Shown is a representative experiment out of three with similar results. Panel (B) influence of iloprost (0.1 μM), forskolin (10 μM) and dbcAMP (1 mM) on COX-2 mRNA expression in hSMC. Cells were treated for 3 hr with the substances as indicated. Thereafter, total RNA was isolated and COX-2 mRNA expression was analyzed by semi-quantitative RT-PCR. Shown is a representative experiment out of three with similar results. $M = 100$ bp DNA-ladder.

protein × 10 min in control cells. The lesser extent of forskolin-induced cAMP formation could explain why forskolin was less potent than iloprost in induction of COX-2 mRNA and suggests that the intensity of COX-2 mRNA expression indeed correlates with cAMP formation.

Besides its coupling to G_s, IP-R may also couple to G_q protein-mediated stimulation of phospholipase C/PKC pathway [28]. To investigate a possible involvement of G_q-coupling in iloprost-mediated COX-2 induction, hSMC were pretreated for 24 hr with PMA (0.1 μM). After that

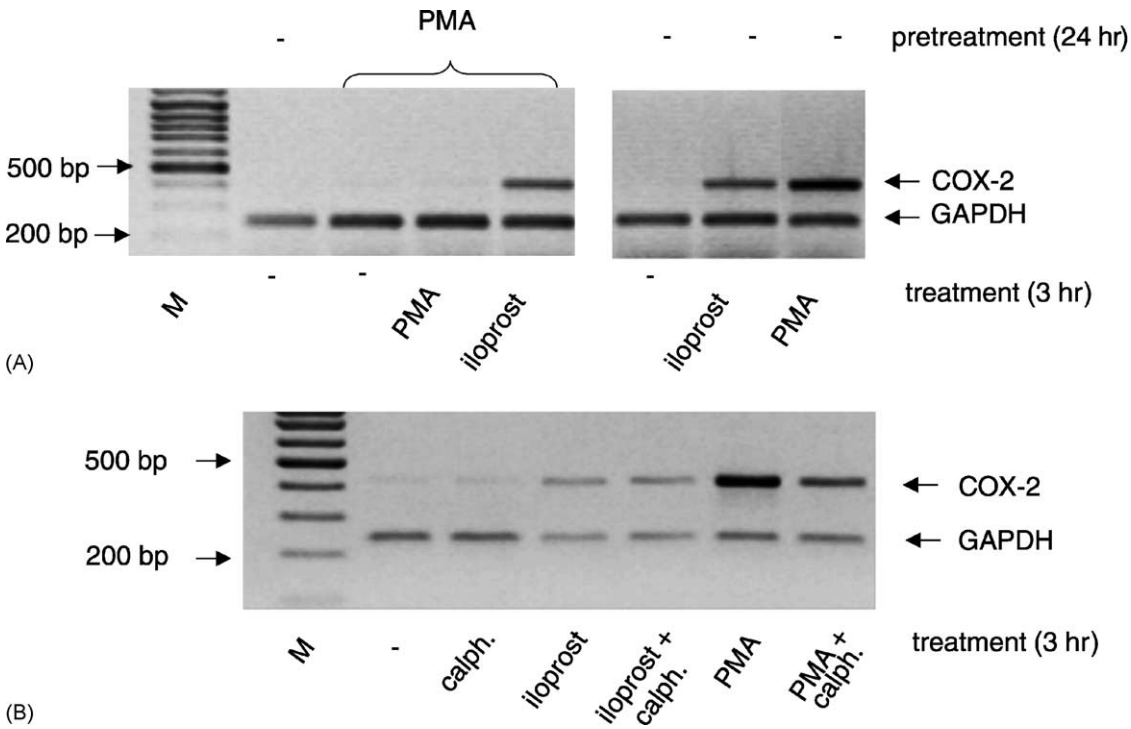

Fig. 6. (A) hSMC were treated for 24 hr with PMA (0.1 μM) to reach PKC-desensitization. After that, cells were restimulated with iloprost (0.1 μM) or PMA (0.1 μM) for 3 hr (left panel). For comparison nonpretreated cells were also stimulated with iloprost (0.1 μM) or PMA (0.1 μM) for 3 hr (right panel) and COX-2 mRNA expression was determined by RT-PCR. Shown is a representative experiment out of three with similar results. $M = 100$ bp DNA-ladder. (B) hSMC were either untreated or treated with the PKC-inhibitor calphostin (1 μM) in the indicated lanes 30 min prior to stimulation with either iloprost (0.1 μM) or PMA (0.1 μM) for 3 hr. COX-2 mRNA expression was determined by RT-PCR. Shown is a representative experiment out of two with similar results. $M = 100$ bp DNA-ladder.

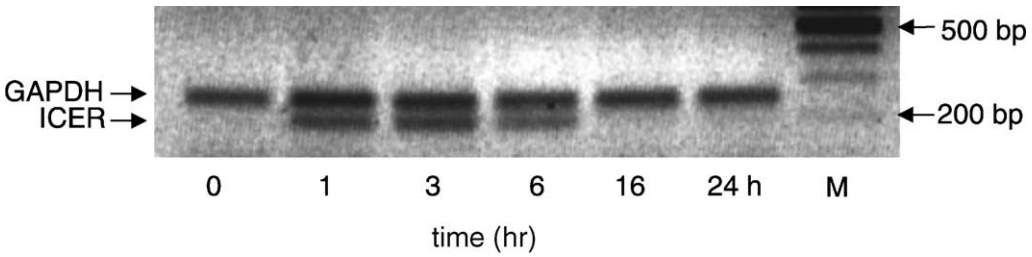

Fig. 7. RT-PCR analysis of time-dependent expression of ICER mRNA in hSMC in response to iloprost (0.1 μM) for the times indicated. Shown is a representative experiment out of three with similar results. $M = 100$ bp DNA-ladder.

time COX-2 mRNA levels returned to control levels and reapplication of PMA had no effect on COX-2 expression, indicating complete desensitization of PKC (Fig. 6A). When PMA-pretreated cells were stimulated with iloprost (0.1 μM), a comparable induction of COX-2 mRNA occurred as in control cells. These data suggest that induction of COX-2 mRNA upon iloprost stimulation is substantially not influenced by the G_q/PKC pathway. These findings were further substantiated by the observation that the PKC-inhibitor calphostin C (1 μM) had no effect on iloprost-induced COX-2 induction. As expected, PMA-induced COX-2 expression was markedly reduced by calphostin C (Fig. 6B), reflecting its efficient PKC-inhibition.

3.4. Iloprost induces the expression of the transcriptional repressor ICER

The promotor of the COX-2 gene contains a consensus-site for a cAMP responsive element (CRE) [29], which could control transcriptional activation in hSMC upon iloprost treatment. To test this hypothesis, we investigated the expression of the transcriptional repressor ICER. cAMP-regulated expression of ICER is directed by a cluster of four cAMP responsive elements (CREs) in an intronic promoter inside the CREM gene due to binding of the transcription factors CREB and CREM. ICER, in turn, strongly binds to CREs as a homodimer or heterodimer with CREB or CREM and thereby represses the activity of its own promoter, thus constituting a negative autoregulatory loop [30]. Accordingly, ICER is known to turn off the expression of cAMP responsive genes in general.

As shown in Fig. 7, iloprost (0.1 μM)-induced transient ICER mRNA expression, which was similar to the COX-2 mRNA kinetics shown in Fig. 1. ICER mRNA is induced over a period of 1–6 hr and declines after 16 hr of iloprost treatment.

4. Discussion

The present study demonstrates an up-regulation of COX-2 mRNA and protein expression by the PGI_2 mimetic iloprost in hSMC. Several previous studies have demonstrated that prostaglandins could exert regulatory feedback actions *via* the expression of their own synthesizing enzymes, however, the results are nonequivalent. In RAW 264.7 macrophages and in human monocytes, PGE_2 has no direct effect on COX-2 expression but potentiates LPS-induced COX-2 expression in a cAMP-dependent manner [14,15]. A direct effect of PGE_2 and cAMP on COX-2 expression was seen in rat mesangial cells [31] and murine keratinocytes [16]. These conflicting data suggest that prostaglandins regulate COX-2 expression in a cell-specific manner. Interestingly, in porcine and hSMC, PGE_2 and other cAMP-elevating substances caused a decrease in COX-2 expression, whereas PGI_2 analogues had no effect [18,19]. These findings are in contrast to our results, demonstrating up-regulation of COX-2 by iloprost and other cAMP-elevating substances. This discrepancy might be explained by variations in the prostaglandin receptor population expressed on the SMC of different origins. Moreover, the above mentioned studies used PGE_2, whose cAMP stimulation was considered to be mediated mainly *via* the EP_2 receptor, and the failure of PGI_2 analogues to modulate COX-2 expression indicates low expression of IP-receptors. In contrast to that, our results indicate strong expression of IP-receptors and only little presence of the G_s-coupled EP_2- and EP_4-receptors as determined by RIA with the specific agonists butaprost (EP_2) or Ono AE-1329 (EP_4), respectively (data not shown). The opposite actions of cAMP in regulating COX-2 expression may be explained by different effector systems coupled to PKA [32].

Our results on iloprost-mediated up-regulation of COX-2 expression point toward a crucial role for cAMP, the second messenger generated upon activation of the G_s coupled IP-R, without participation of the G_q/PKC signal transduction pathway. According to these findings, COX-2 mRNA was up-regulated by the cell-permeable cAMP analogue dbcAMP or the adenylyl cyclase activator forskolin, respectively. These findings are in agreement with Samokovlisky *et al*. [33] who demonstrated up-regulation of COX-2 by cAMP in bovine aortic endothelial cells.

The elevation of COX-2 mRNA level upon iloprost treatment might be attributed to two mechanisms: a stabilization of COX-2 mRNA or an enhanced transcription of the COX-2 gene. In recent studies, we have observed that up-regulation of COX-2 mRNA was completely abolished when the transcription inhibitor actinomycin D was added

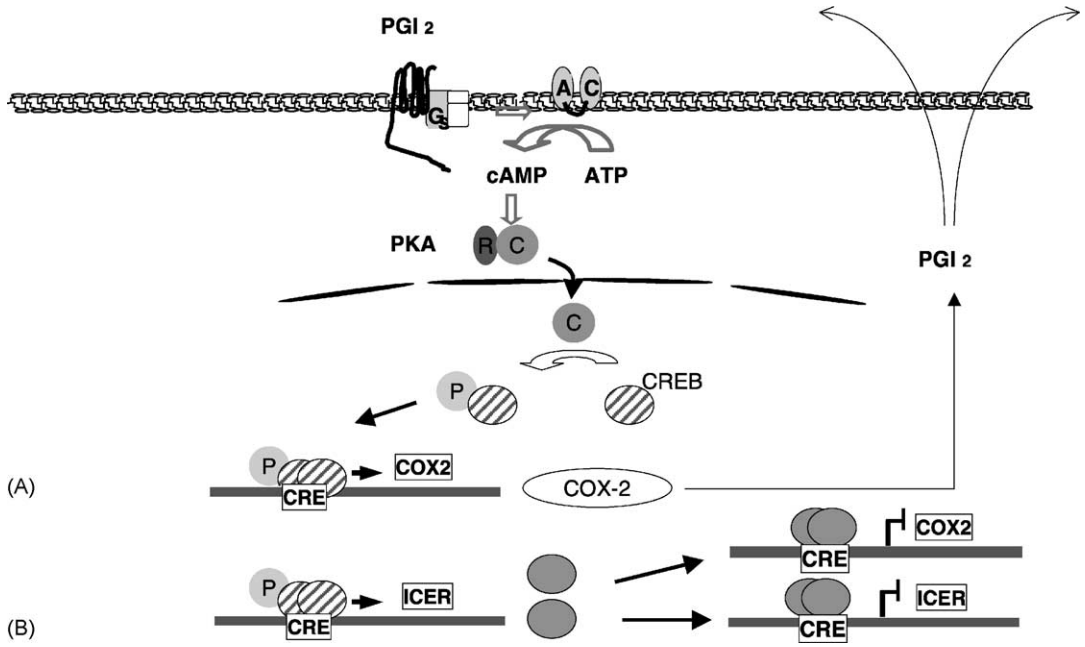

Fig. 8. Schematic representation of the iloprost-induced expression of COX-2. Prostacyclin (PGI_2) binding to the G_s-coupled IP-R activates the membrane-associated adenylyl cyclase (AC). This converts ATP to cAMP which causes dissociation of the inactive tetrameric PKA complex into the active catalytic subunits (C) and the regulatory subunits (R). The catalytic subunits migrate into the nucleus where they phosphorylate (P) and thereby activate transcriptional activators like CREB. (A) Phosphorylated CREB then interacts as a dimer with the cAMP response element (CRE) in the promotor of the COX-2 gene and activates transcription. Synthesis of COX-2 protein is accompanied by synthesis of PGI_2 which in turn could activate IP-R. (B) Phosphorylated CREB interacts with CRE in the promotor of the CREM-gene and activates the transcription of ICER. ICER then binds to the CRE regions of cAMP inducible genes, and thereby interrupts their transcription, e.g. COX-2. Furthermore, the expression of ICER itself is repressed by a negative autoregulatory feedback.

to hSMC, indicating that iloprost-mediated up-regulation depends on transcriptional activation of the COX-2 gene. According to these findings, iloprost-induced up-regulation of COX-2 might be mediated by a cAMP/PKA-dependent phosphorylation of the transcription factor CREB which subsequently binds to the CRE region of the human COX-2 promotor [29] resulting in enhanced transcription (Fig. 8). This hypothesis is supported by the observation that iloprost induces ICER mRNA expression. ICER is strongly induced by cAMP/CREB by an internal promotor of the CREM gene, containing four CRE binding sites [30,34]. ICER is known to block the transcription of cAMP-inducible genes by binding as homodimers or heterodimers with CREB or CREM to the CREs in the promotor of the respective gene (reviewed in [35]). The induction of ICER upon iloprost treatment, therefore, represents a regulatory mechanism to turn-off the expression of cAMP-inducible genes. It might be speculated, that the induction of ICER is involved in the down-regulation of COX-2 expression following persistent (16–24 hr) iloprost stimulation (Fig. 8), in addition to instability of COX-2 mRNA and protein, common regulatory mechanisms of immediate early gene expression.

The duration of the IP-R-mediated signaling is critically dependent on the number of functionally active receptors. Thus, the agonist-induced down-regulation of IP-R [36,37] might be an alternative mechanism in the regulation of iloprost-mediated COX-2 expression. However, in recent studies we demonstrate that forskolin, a direct activator of the adenylyl cyclase, induces as well transient expression of COX-2 mRNA as iloprost, indicating a receptor-independent mechanism to turn-off COX-2 expression.

In this study, we demonstrate that time-dependent up-regulation of COX-2 mRNA and protein expression by iloprost was accompanied by a slight increase in PGI_2 synthesis, whereas PMA induced significant production of PGI_2 after 4.5–8 hr of stimulation. Furthermore, when hSMC were co-incubated with iloprost and PMA to mimick an inflammatory state, a synergistic effect in PGI_2 synthesis could be demonstrated, which seems to depend on increased COX-2 protein expression, as demonstrated in Western blot studies. Similar results were obtained when hSMC were co-incubated with iloprost and platelet-derived growth factor (PDGF-BB, data not shown). The synergism of iloprost- and PMA-actions suggests that the PKA and PKC signaling pathways converge at the transcriptional level, as described in other systems [38], leading to increased COX-2 expression in hSMC. Therefore, the synergistic effect of iloprost and PKC activators in COX-2 induction in hSMC might reflect situations of inflammatory states in vascular injury and atherosclerotic lesion where several mediators of COX-2 induction, including proinflammatory cytokines, tumor necrosis factor and growth factors act on SMC. This could finally contribute to increased COX-2 expression and prostaglandin formation [23,39].

Recently Caughey *et al.* [40] demonstrated that platelet-derived thromboxane A_2 (TXA_2), an activator of the G_q/PKC-signal transduction pathway, up-regulates endothelial COX-2 expression and PGI_2 synthesis. Thus, endothelial-derived PGI_2 might contribute to enhanced COX-2 expression and PGI_2 formation in hSMC.

In conclusion, the augmentation of COX-2 induction by a prostaglandin-dependent mechanism provides a positive feedback loop in prostaglandin synthesis by hSMC. The subsequent increased release of PGI_2 upon induction of COX-2 might contribute to its anti-atherosclerotic actions, i.e. inhibition of monocyte/macrophage adhesion to the endothelium, inhibition of smooth muscle cell proliferation and migration as well as inhibition of platelet aggregation and secretion.

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References

[1] Wu KK. Inducible cyclooxygenase an nitric oxide synthase. Adv Pharmacol 1995;33:179–207.
[2] Smith WL, DeWitt DL. Prostaglandin endoperoxide H synthases-1 and -2. Adv Immunol 1996;62:167–215.
[3] Otto JC, Smith WL. Prostaglandin endoperoxide synthase-1 and -2. J Lipid Mediat Cell Signal 1995;12:139–56.
[4] Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. Ann Rev Pharmacol Toxicol 1998;38:97–120.
[5] Vane JR, Botting RM. New insights into the mode of action of anti-inflammatory drugs. Inflamm Res 1995;44:1–10.
[6] DeWitt DL, Meade EA. Serum and glucocorticoid regulation of gene transcription and expression of the prostaglandin H synthase-1 and prostaglandin H synthase-2 isozymes. Arch Biochem Biophys 1993;306:94–104.
[7] Ristimaki A, Garfinkel S, Wessendorf J, Maciag T, Hla T. Induction of cyclooxygenase-2 by interleukin-1-alpha. Evidence for post-transcriptional regulation. J Biol Chem 1994;269:11769–75.
[8] Xie W, Chipman JG, Robertson DL, Erikson RL, Simmons DL. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc Natl Acad Sci USA 1991;88:2692–6.
[9] Lee SH, Soyoola E, Chanmugam P, Hart S, Sun W, Zhong H, Liou S, Simmons D, Hwang D. Selective expression of mitogen-inducible COX in macrophages stimulated with LPS. J Biol Chem 1992;267: 25934–8.
[10] Schrör K, Zhu Y, Saunders MA, Wu-Guo D, Xu XM, Meyer-Kirchrath J, Wu KK. Obligatory role of cyclic adenosine monophosphate response element in cyclooxygenase-2 promoter induction and feedback regulation by inflammatory mediators. Circulation 2002;105:2760–5.
[11] Xie W, Robertson DL, Simmons DL. Mitogen inducible prostaglandin G/H synthase: a new target for nonsteroidal anti-inflammatory drugs. Drug Dev Res 1992;25:249–65.
[12] Sano H, Hla T, Maier JA, Crofford LJ, Case JP, Maciag T, Wilder RL. In vivo cyclooxygenase expression in synovial tissues of patients with rheumatoid arthritis and osteoarthritis and rats with adjuvant and streptococcal cell wall arthritis. J Clin Invest 1992;89:97–108.
[13] Seibert K, Zhang Y, Leahy K, Hauser S, Masferrer J, Perkins W, Lee L, Isakson P. Pharmacological and biochemical demonstration of the role of cyclooxygenase-2 in inflammation and pain. Proc Natl Acad Sci USA 1994;91:12013–7.
[14] Hinz B, Brune K, Pahl A. Prostaglandin E_2 up-regulates cyclooxygenase-2 expression in lipopolysaccharide-stimulated RAW 264.7 macrophages. Biochem Biophys Res Commun 2000;272:744–8.
[15] Hinz B, Brune K, Pahl A. Cyclooxygenase-2 expression in lipopolysaccharide-stimulated human monocytes is modulated by cyclic AMP, prostaglandin E_2, and nonsteroidal anti-inflammatory drugs. Biochem Biophys Res Commun 2000;278:790–6.
[16] Maldve RE, Kim Y, Muga SJ, Fischer SM. Prostaglandin E_2 regulation of cyclooxygenase expression in keratinocytes is mediated via cyclic nucleotide-linked prostaglandin receptors. J Lipid Res 2000;41: 873–81.
[17] Pilbeam CC, Raisz LG, Voznesensky O, Alander CB, Delman BN, Kawaguchi H. Autoregulation of inducible prostaglandin G/H synthase in osteoblastic cells by prostaglandins. J Bone Miner Res 1995;10:406–14.
[18] Bishop-Bailey D, Pepper JR, Larkin SW, Mitchell JA. Differential induction of cyclooxygenase-2 in human arterial and venous smooth muscle. Arterioscler Thromb Vasc Biol 1998;18:1655–61.
[19] Karim S, Berrou E, Levy-Toledano S, Bryckaert M, MacLouf J. Regulatory role of prostaglandin E_2 in induction of cyclo-oxygenase-2 by a thromboxane A_2 analogue (U46619) and basic fibroblast growth factor in porcine aortic smooth muscle cells. Biochem J 1997;326:593–9.
[20] Smith WL. Prostaglandin biosynthesis and its compartmentation in vascular smooth muscle and endothelial cells. Ann Rev Physiol 1986; 48:251–62.
[21] Schrör K, Weber A-A. Roles of vasodilatory prostaglandins in mitogenesis of vascular smooth muscle cells. Agents Actions Suppl 1997;67:62–90.
[22] Schrör K. Prostacyclin (PGI_2) and atherosclerosis. In: Rubanyi GM, Dzau VJ, editors. The endothelium in clinical practice: source and target of novel therapies. New York: Marcel Dekker; 1997. p. 1–44.
[23] Schönbeck U, Sukhova GK, Graber P, Coulter S, Libby P. Augmented expression of cyclooxygenase-2 in human atherosclerotic lesions. Am J Pathol 1999;155:1281–91.
[24] Fallier-Becker P, Rupp J, Fingerle J, Betz E. Smooth muscle cells from rabbit aorta. In: Piper HM, editor. Cell culture techniques in heart and vessel research. New York: Springer-Verlag; 1990. p. 247–70.
[25] Steiner AL, Parker CW, Kipnis DM. Radioimmunoassay for cyclic nucleotides. J Biol Chem 1972;247:1106–13.
[26] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[27] Schrör K, Seidel H. Blood-vessel wall arachidonate metabolism and its pharmacological modification in a new in vitro assay system. Naunyn Schmiedeberg's Arch Pharmacol 1988;337:177–82.
[28] Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. Physiol Rev 1999;79:1193–226.
[29] Kosaka T, Miyata A, Ihara H, Hara S, Sugimoto T, Takeda O, Takahashi E, Tanabe T. Characterization of the human gene (PTGS2) encoding prostaglandin-endoperoxide synthase-2. Eur J Biochem 1994;221:889–97.
[30] Molina CA, Foulkes NS, Lalli E, Sassone-Corsi P. Inducibility and negative autoregulation of CREM: an alternative promotor directs the expression of ICER, an early response repressor. Cell 1993;75: 875–86.
[31] Nüsing RM, Klein T, Pfeilschifter J, Ullrich V. Effect of cyclic cAMP and prostaglandin E_2 on the induction of nitric oxide- and prostanoid-forming pathways in cultured rat mesangial cells. Biochem J 1996; 313:617–23.

[32] Lalli E, Sassone-Corsi P. Signal transduction and gene regulation: the nuclear response to cAMP. J Biol Chem 1994;269:17359–62.
[33] Samokovlisky A, Rimon G, Danon A. Differential regulation of cyclooxygenase isozymes by cAMP-elevating agents. Eur J Pharmacol 1999;378:203–11.
[34] Servillo G, Penna L, Foulkes NS, Magni MV, Della-Fazia MA, Sassone-Corsi P. Cyclic AMP signalling pathway and cellular proliferation: induction of CREM during liver regeneration. Oncogene 1997;14:1601–6.
[35] Sassone-Corsi P. Coupling gene expression to cAMP signalling: role of CREB and CREM. Int J Biochem Cell Biol 1998;30:27–38.
[36] Nilius SM, Hasse A, Kuger P, Schrör K, Meyer-Kirchrath J. Agonist-induced long-term desensitization of the human prostacyclin receptor. FEBS Lett 2000;484:211–6.
[37] Smyth EM, Austin SC, Reilly MP, FitzGerald GA. Internalization and sequestration of the human prostacyclin receptor. J Biol Chem 2000;275:32037–45.
[38] Hoeffler JP, Deutsch PJ, Lin J, Habener JF. Distinct adenosine 3′,5′-monophosphate and phorbol ester-responsive signal transduction pathways converge at the level of transcriptional activation by the interactions of DNA-binding proteins. Mol Endocrinol 1989;3:868–80.
[39] FitzGerald GA, Smith B, Pedersen AK, Brash AR. Increased prostacyclin biosynthesis in patients with severe atherosclerosis and platelet activation. N Engl J Med 1984;310:1065–8.
[40] Caughey GE, Cleland LG, Gamble JR, James MJ. Up-regulation of endothelial cyclooxygenase-2 and prostanoid synthesis by platelets. Role of thromboxane A_2. J Biol Chem 2001;276:37839–45.