Effect of cyclic AMP and prostaglandin E_2 on the induction of nitric oxide- and prostanoid-forming pathways in cultured rat mesangial cells

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Cyclic AMP (cAMP) represents an important cellular signalling molecule. We analysed the effect of dibutyryl cAMP (db-cAMP), a cell-permeable and stable derivative of cAMP, on the regulation and expression of cyclo-oxygenase 2, inducible NO synthase and argininosuccinate synthetase. We observed different transcriptional regulation of these enzymes depending on the db-cAMP concentration used. Low concentrations of db-cAMP in the range 10–50 μM elevated levels of cyclo-oxygenase 2 mRNA, protein and activity, but not the respective mRNA and protein concentrations of the inducible NO synthase or argininosuccinate synthetase. At higher concentrations a massive induction of the latter two enzymes was also apparent. Expression of prostacyclin synthase and argininosuccinate lyase, secondary enzymes of NO- and prostanoid-forming pathways, was not stimulated by db-cAMP. Prostaglandin E_2, known to be an intracellular physiological trigger of cAMP formation, stimulated only cyclo-oxygenase 2 expression and activity at a concentration of 10 μM, and not inducible NO synthase. The induction of the mRNA for the transcription factors JunB and p65, a component of the NFκB complex, by prostaglandin treatment of the cells might be a possible mechanistic explanation for this observation.

INTRODUCTION

Prostanoids and NO are important mediators in the control of physiological processes in the body, but are also connected with the pathogenesis of diverse diseases. The formation of these metabolites is regulated by the enzymes cyclo-oxygenase (COX) and NO synthase (NOS). Interestingly, for both enzymes at least two isoforms exist: a constitutive and an inducible form.

The constitutive cyclo-oxygenase COX-1 is present in low concentrations in almost all cells and tissues, and the prostanoids generated via this isoform are thought to be involved in physiological functions such as homoeostasis, gastroprotection and regulation of renal blood flow, salt and fluid transport. Growth factors, cytokines and endotoxins are reported to induce COX-2 [1–3]. This isoform is considered to be involved in the overproduction of prostanoids under pathological conditions, e.g. acute and chronic inflammatory disorders.

NO is a potent vasodilator derived from L-arginine. The responsible constitutive enzyme produces NO in nanomolar amounts. For different cell types [4], and also for glomerular mesangial cells [5], the presence of an inducible NOS (iNOS) has been described, which produces NO in micromolar amounts. Its overproduction in vascular smooth muscle for example is regarded as a main factor contributing to systemic hypotension during septic- and cytokine-induced circulatory shock. For a complete understanding of NO-synthetic capacity, it is necessary also to consider two cytosolic enzymes involved in the regeneration of arginine, argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) [6]. ASS catalyses the metabolism of citrulline, which is the catabolite of NOS action, and aspartate to argininosuccinate at the expense of ATP, and ASL converts this metabolite into arginine and fumarate. Recently it has been shown that ASL is constitutively expressed in vascular smooth-muscle cells, whereas ASS mRNA and activity are markedly induced in cells by treatment with endotoxin [7,8].

Both COX-2 and iNOS are considered to be encoded by inflammatory response genes, which are controlled by several response elements in their promoter regions. Interestingly, a cyclic AMP (cAMP)-response element (CRE) can be found only at the 5′-region upstream of the transcription-start site of the COX-2 gene [9]. However, the induction of iNOS via elevation of cAMP concentration has been reported for different types of smooth-muscle cells [10,11] including rat mesangial cells [12,13]. It was also realized that the cAMP stimulus represents only one component from a set of stimuli that control expression of iNOS. Lipopolysaccharide, interleukin 1 and tumour necrosis factor are others and it may require a certain balance between these mediators to synergistically reach a response under physiological conditions.

It was the aim of this study to define the physiological conditions under which cAMP mediates the expression of enzymes encoded by the early-response genes, such as iNOS, COX-2 and, as pointed out above, ASS. For the kidney, prostaglandin (PG) E_2 may be a physiological agonist of the mesangial cell and therefore its ability to affect the expression is observed as an appropriate physiological or pathophysiological stimulus for COX-2 but not for iNOS or ASS.

MATERIALS AND METHODS

Cell culture

Rat mesangial cells were cultures as previously described [14] and used between passage 5 and 10. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin 5 μg/ml transferrin. Cell growth was arrested by low serum conditions (0.5%) without penicillin and streptomycin for 24 h. Under low serum conditions cells were stimulated as indicated.

Abbreviations used: COX-2, cyclo-oxygenase 2; iNOS, inducible NO synthase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; db-cAMP, dibutyryl cyclic AMP; PG, prostaglandin; RT-PCR, reverse-transcription PCR; CRE, cAMP-response element.

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Assay of NO synthesis

NOS activity was measured as NO_2^- production in rat mesangial cells. At the end of the incubation, 200 μl of the culture medium was mixed with 0.1 vol. of 0.1 % naphthylethylenediamine dihydrochloride and 0.1 vol. of 1 % sulphanilamide in 1.2 M HCl. After 10 min of incubation at room temperature the A_{550} was measured NO_2^- concentration was determined using a calibration curve with $NaNO_2$ standards.

Measurement of COX-dependent arachidonic acid conversion

Cellular COX activity was determined by measuring the conversion of [^{14}C]arachidonic acid as reported by us recently [15]. After preincubation with different stimuli, cells were rinsed twice and stimulated with ^{14}C-labelled arachidonic acid (final concentrations 2 μM) for 15 min at 37 °C. The reaction was stopped by scraping off the cells with a 'rubber policeman' and the cell suspension transferred to four times its volume of ethyl acetate. Radioactive products were extracted at pH 3 into the organic solvent, gently evaporated under N_2 and subsequently separated by TLC (solvent: ethyl acetate/water/iso-octane/acetic acid, 90:100:50:20), by vol.). Prostanoid production was quantified by autoradiography, after visualization with a phosphoimaging system (Image Quant; Molecular Dynamics), and expressed as percentage of total radioactivity extracted. Thereafter the location of authentic prostanoids was visualized with iodine vapour, and the radioactive zones were identified. Prostacyclin synthesis was monitored by measuring the formation of its stable hydrolysis product 6-oxo-$PGF_{1\alpha}$.

Whole-cell reverse-transcription (RT)PCR analysis

After stimulation, 5×10^6 cells were washed in PBS, pH 7.4, resuspended in diethyl pyrocarbonate-treated water containing 10 units of RNasin and lysed for 30 min on ice as described recently by us [15]. Lysed cells were pelleted by centrifugation and one-fifth of the RNA-containing supernatant was used for target-specific RT with Superscript reverse transcriptase and specific primers to the different proteins to be analysed. The remaining cell pellet and supernatant were used for Western-blot analysis. The primers were deduced from the rat or mouse sequences and chosen to have a GC content of 50–60 % and to result in an amplification fragment of similar size. The wobble primer PCOXR1 [5′-A(G/C)AGCTCAGT-(G/T)GA(A/G)CG(C/T)CT-3′] complementary to the 3′-part of COX-2 was used for RT of the mRNA of this enzyme. Other primers were as follows: for iNOS, PNOSIRR1 (5′-AACGTT-TCTGGCTCTTGAGCTGGA-3′); for prostacylin synthase, PPCSBR1 (5′-CGGTAACGGACAGGCACATCGTGT--3′); for ASS, PASSRR1 (5′-AATGGGTTCATAGTCACCCT-3′); for ASL, PASLRR1 (5′-GACACTTCAAACACAGCCTC-3′); for β-actin, BAHR1 (5′-CTAGAAGCATTTGCGGTGGAC-3′). For target-specific RT of the PGE_2 receptor subtypes, the general primer PPRR0 [5′-CCA(A/G)GG(G/A)TC(C/T)A-(G/A)GAT(G/C)(T/G)GGTT-3′] was used. After cDNA synthesis, excess primers were removed and PCR amplification was performed using the cDNA template with the following nested primer pairs: for COX-2, PCOX2MR2 (5′-ATCTAGTCTGG-AGTGGGAGG-3′) and PCOX2F1 (5′-AATGAGTACCGCA-AACGTT-3′); for iNOS, PNOSIRR2 (5′-GCTTCTTCAAG-TGGTAGCCA-3′) and PNOSIRF1 (5′-AGTGTCAGTGGCT-TCCAGCTC-3′); for prostacyclin synthase, PPCSBR2 (5′-CTGCATCAGCCCAAAGCCATACCT-3′) and PPCSBF1 (5′-TGCTGAGTGAGAGCCTCAGGCTCA-3′); for ASS, PASSRR2 (5′-GTCTAAATGAGCGTGGTAAAGGAT-3′) and PASSRF1 (5′-CTGGAGCATGGATGAGAACCTTAT-3′); for ASL, PASLRR2 (5′-TCCTGTAAGTCCTTGTTGTA-GGTG-3′) and PASLRF1 (5′-CAGAACTGAACTTTGGAGC-CATTA-3′); for β-actin, BAHR1 and BAHF1 (5′-CATCACC-ATTGGCAATGAGCG-3′). For detection of the receptor subtype for PGE_2, the following primer pairs were used: for EP1 receptor subtype, PEP1F1 [5′-AGGCACTGCTTGC(C/T)GG-CCTGTT-3′] and PEP1R1 [5′-TGGCCCACCATCTCCAC(A/G)TCGT-3′]; for EP2 receptor subtype, PEP2F1 (5′-TCCGCA-TGCACCGCCAGTTCAT-3′) and PEP2R1 [5′-TC(G/T)GAT-GGCCTGCAAATCTGG-3′]; for EP3 receptor subtype, PEP3F1 (5′-ACTGGTATGCGAGCCACATGA-3′) and PEP3R1 [5′-CATAAGCTG(A/G)ATGGCCGTCTC-3′]. The reactions were cycled 32 times in a cycle profile of 30 s at 94 °C, 30 s at 56 °C and 30 s at 72 °C after a 5 min denaturing step at 95 °C. Amplification products were analysed by 2 % agarose-gel electrophoresis and ethidium bromide staining. No amplification products were found when RT was performed without a specific primer or PCR without a template. Fragments were identified by molecular mass, restriction enzyme analysis and sequencing. Samples were assayed at various dilutions to ensure proportionality in the yield of PCR products.

Determination of intracellular cAMP concentrations

Confluent mesangial cells were incubated with PGE_2 for 20 h. The medium was then aspirated and 1 ml of ice-cold 5 % (w/v) trichloroacetic acid was added and left for 1 h. The trichloroacetic acid was then removed by extraction with 4×5 vol. of water-saturated diethyl ether, and cAMP was determined by radioimmunoassay (New England Nuclear).

Western-blot analysis

COX-2 and iNOS were analysed by the Western-blot technique as described by us [15]. After RNA isolation by cell lysis, the protein content of the remaining solution was determined and 200 μg was mixed with PAGE buffer (PBS containing 15 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin and 1 % Triton X-100). After separation by SDS/PAGE (10 % gel) the proteins were blotted on to a nitrocellulose membrane. The membrane was blocked with 5 % milk powder/0.1 % Tween 20 for 2 h. A polyclonal antibody raised against COX-2 (Cayman Chemicals) was applied at a dilution of 1:100 for 2 h. After washing, visualization was achieved using peroxidase-labelled goat anti-rabbit antibody and the enhanced chemiluminescence technique (ECL; Amersham). For detection iNOS the membrane was reprobed with a specific polyclonal antibody (kindly provided by Dr Lapetina, Burroughs Wellcome, RTP, NC, U.S.A.) at a dilution of 1:100 after removal of the antibodies for COX detection with 2 % SDS/100 mM 2-mercaptoethanol in 100 mM Tris/HCl (pH 6.7).

Measurement of the conversion of arachidonic acid from endogenous pools

After stimulation, the cells were washed and fresh medium was added. To trigger the release of arachidonic acid from endogenous pools, cells were stimulated with 1 μM ionophore A23187 for 10 min. Thereafter the supernatants were stored at −20 °C until determination of the 6-oxo-$PGF_{1\alpha}$ formed by GC–MS/MS as described previously [16]. Briefly, supernatant and deuterated reference substance were acidified to pH 3 with formic acid, and 1.5 M *O*-methylhydroxylamine hydrochloride was added to form the methoxime. After acidification to pH 2.5, the prostanoid derivatives were extracted twice with ethyl acetate/hexane (70:30,

v/v). After evaporation of the solvent, acetonitrile (80 μl), pentafluorobenzyl bromide (7 μl) and *NN*-di-isopropylethylamine (25 μl) were added and allowed to react at 40 °C for 25 min. The dry sample was purified by TLC (solvent: ethyl acetate/hexane, 90:10, v/v). The prostanoid-containing zone was eluted, the solvent evaporated and the prostanoids derivatized with bis(trimethylsilyl)trifluoroacetamide for 1 h at 40 °C. An aliquot of the solution was quantified by GC–MS/MS.

RESULTS

Recent studies have demonstrated that not only vascular smooth-muscle cells respond to artificially increased cAMP by enhanced NO production, but also mesangial cells [12,13,17]. Modulation of COX expression and activity or ASS expression in mesangial cells by cAMP is not known. We have used cultured rat mesangial cells to study the effect of cAMP on the COX-dependent pathway and compared it with the effect on the NO-synthetic pathway.

NO-forming activity was measured as NO_2^- production in culture supernatants. Whereas in untreated (control) cells negligible NO_2^- was detected, the cAMP-increasing agents db-cAMP, chlorophenylthio-cAMP (both 1 mM) and forskolin (20 μM) were potent stimuli of NO_2^- production (Table 1) in accordance with published data [12]. These agents also exerted a stimulatory effect on COX activity, increasing it 4–5-fold over basal levels (Table 1). The main arachidonic acid metabolite of cultured rat mesangial cells under these conditions is PGI_2 followed by PGE_2, as analysed by TLC (results not shown).

Table 1 Effect of different cAMP-increasing agents and PGE_2 on NO_2^- formation and on COX activity in rat mesangial cells

Results are given for NO_2^- production elicited by the indicated agents over 20 h, measured in cell supernatants. For assay of COX activity, cells were incubated with 2 μM arachidonic acid for 15 min after a 20 h incubation with the indicated agents. cp-cAMP, chlorophenylthio-cAMP. All data represent means ± S.D. of three to five values.

	COX activity (% of 6-oxo-$PGF_{1\alpha}$/ 10 min per ml)	NO_2^- synthesis (μM)
Control	0.37 ± 0.19	< 0.1
1 mM db-cAMP	1.46 ± 0.05	9.96 ± 3.25
1 mM cp-cAMP	1.53 ± 0.08	10.41 ± 1.95
20 μM Forskolin	1.31 ± 0.12	7.66 ± 2.02
10 μM PGE_2	0.93 ± 0.18	< 0.1

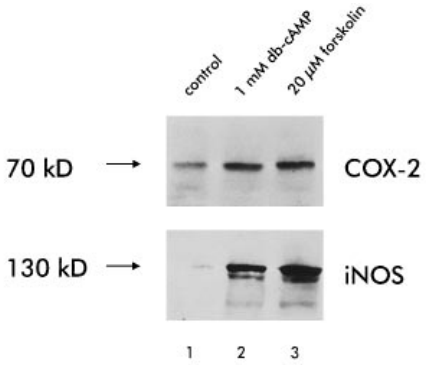

Figure 1 Western-blot analysis of COX-2 and iNOS in rat mesangial cells incubated with cAMP-increasing agents

Cells were incubated for 20 h in the presence of db-cAMP, forskolin or no additions and then COX-2 and iNOS protein concentrations were analysed by the Western-blot technique in whole-cell extracts as described in the Materials and methods section. Lane 1, control; lane 2, 1 mM db-cAMP; lane 3, 20 μM forskolin. A representative of three experiments is shown. Abbreviation: kD, kDa.

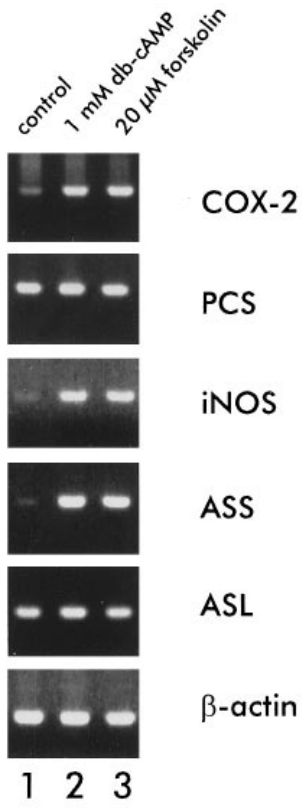

Figure 2 Detection of mRNA levels for different enzymes in rat mesangial cells treated with cAMP-augmenting agents

Mesangial cells were cultured for 20 h in the presence of db-cAMP, forskolin or no additions and RNA was prepared by cell lysis. One-fifth was used for target-specific RT-PCR, and the remaining suspension was used for parallel Western-blot analysis (Figure 1) Lane 1, control; lane 2, 1 mM db-cAMP; lane 3, 20 μM forskolin. PCS, prostacylcin synthase. A representative of three determinations is shown.

We next investigated the effect of db-cAMP and forskolin on protein concentration. In control cells basal amounts of COX-2 protein were detectable, but hardly any iNOS protein was observed. Both cAMP-increasing agents caused a marked increase in COX-2 protein but a dramatic severalfold higher induction of iNOS protein was observed, correlating with the measured activities (Figure 1). To determine whether this increase in enzyme protein was a result of enhanced transcription, we analysed mRNA levels of the enzymes involved by target-specific RT-PCR (Figure 2). db-cAMP (1 mM) and forskolin (20 μM) both caused a significant increase in the amount of COX-2 and iNOS mRNA. Furthermore ASS mRNA, an enzyme involved in L-arginine regeneration, was also found to be markedly increased. The specificity of this transcriptional regulation is seen in the unaffected mRNA levels of prostacyclin synthase, an important enzyme in PGH_2 conversion in rat mesangial cells, and ASL, considered to be constitutively expressed and involved in the arginine cycle. β-Actin mRNA, assessed as an internal control, remained unchanged during the different treatments and demonstrates equality of RNA preparation.

Table 2 Effect of PGE_2 on intracellular formation of cAMP in rat mesangial cells

Confluent mesangial cells were incubated with different PGE_2 concentrations for 20 min in medium containing the phosphodiester inhibitor 3-isobutyl-1-methylxanthine at a concentration of 500 μM. After extraction, cAMP concentration was determined by radioimmunoassay. All data represent means ± S.D. of five values.

	cAMP (pmol/mg of protein)
Control	18.4 ± 2.7
1 μM PGE_2	42.5 ± 9.2
10 μM PGE_2	112 ± 23

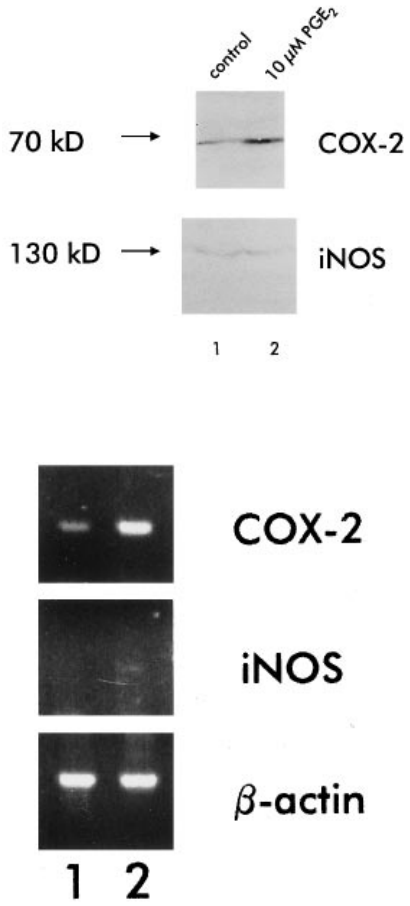

Figure 3 Western-blot and RT-PCR analysis of COX-2 and iNOS protein and mRNA in rat mesangial cells incubated with PGE_2

(Upper panel) Cells were incubated for 20 h in the presence of 10 μM PGE_2 or no addition and then the protein levels of COX-2 and iNOS were analysed by the Western-blot technique in whole-cell extracts. Lane 1, control cells; lane 2, 10 μM PGE_2. Abbreviation: kd, kDa. (Lower panel) For RT-PCR from the same cell preparation, RNA was prepared by cell lysis and subjected to target-specific RT-PCR with specific primers for the different mRNA species. Lane 1, control cells; lane 2, cells incubated in the presence of 10 μM PGE_2. Representatives of four experiments are shown.

Next we were interested in whether this regulation can be initiated by the physiological stimulus PGE_2, which is known to raise cAMP levels in different cell types, including mesangial cells. Under our conditions, 10 μM PGE_2 increased intracellular cAMP concentrations approx. 9-fold over controls (Table 2).

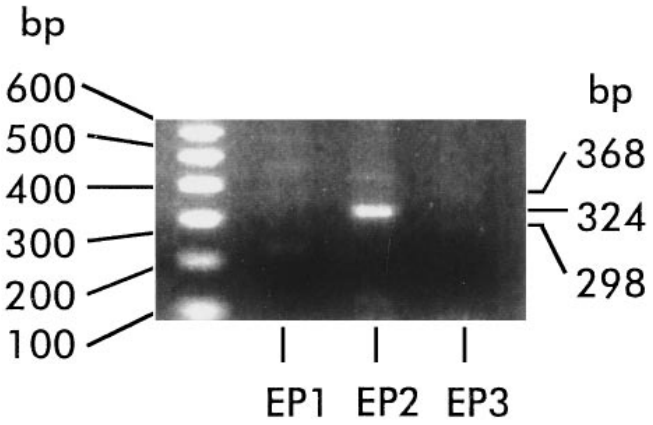

Figure 4 Determination of PGE_2 receptor subtype in cultured rat mesangial cells

Target-specific RT-PCR of rat mesangial cells was performed with specific primers to the different receptor sequences, and the amplification products were separated on a 2% agarose gel and stained by ethidium bromide. EP1, EP2, EP3, amplification with the primer pairs for EP1, EP2 and EP3 receptor subtype of the PGE_2 receptor with lengths of 298, 324 and 368 bp respectively. A representative of two experiments is shown.

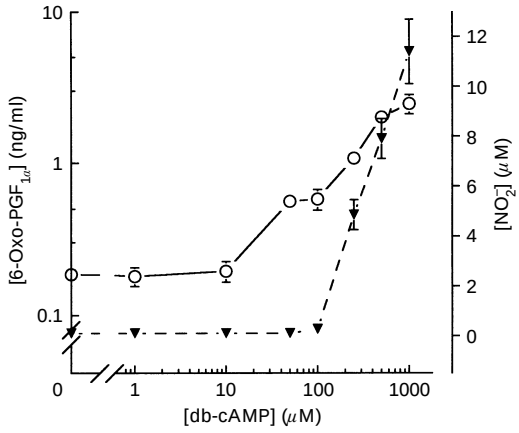

Figure 5 Concentration-dependent effect of db-cAMP on NO and on the biosynthesis of 6-oxo-$PGF_{1\alpha}$ from endogenous arachidonic acid pools in mesangial cells

Cells were incubated for 20 h with the indicated db-cAMP concentrations, and then NO_2^- (▼) was measured. The biosynthetic capacity to generate 6-oxo-$PGF_{1\alpha}$ from endogenous arachidonic acid (○) was determined in the supernatant by GC–MS/MS after stimulation of the cells with 1 μM A23187 for 10 min. The values represent means ± S.D. of four experiments.

However, 10 μM PGE_2 had no effect on NO_2^- production in rat mesangial cells but COX activity appeared to be increased approx. 3-fold (Table 1). This differential activity was reflected in the protein and mRNA levels of COX-2 and iNOS respectively (Figure 3 upper and lower panels). Whereas the amount of iNOS protein is unchanged in PGE_2-treated cells, a significant increase in COX-2 protein was observed. As it is known that PGE_2 elicits different signalling pathways depending on the receptor to which it is coupled, we questioned with PGE_2 receptor subtype(s) is (are) present on rat mesangial cells. Using target-specific RT-PCR, we obtained an amplification product only with primers specific for the EP2 receptor subtype (Figure 4). This receptor, which is known to be coupled to cAMP increase appeared to be

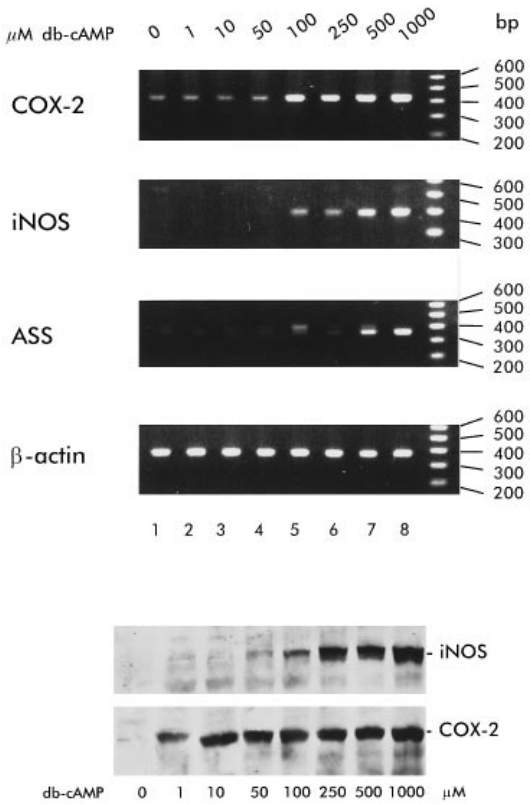

Figure 6 Concentration-dependent effect of db-cAMP on COX-2, iNOS and ASS mRNA expression and on COX-2 and iNOS protein

Four upper panels, rat mesangial cells were incubated for 20 h with the indicated db-cAMP concentrations. For RT-PCR, RNA was prepared by cell lysis and subjected to target-specific RT-PCR with specific primers for the different mRNA species. Two lower panels, from the same cell sample as described above COX-2 and iNOS protein levels were analysed by the Western-blot technique as described in Figure 1. Representatives of three independent experiments are shown.

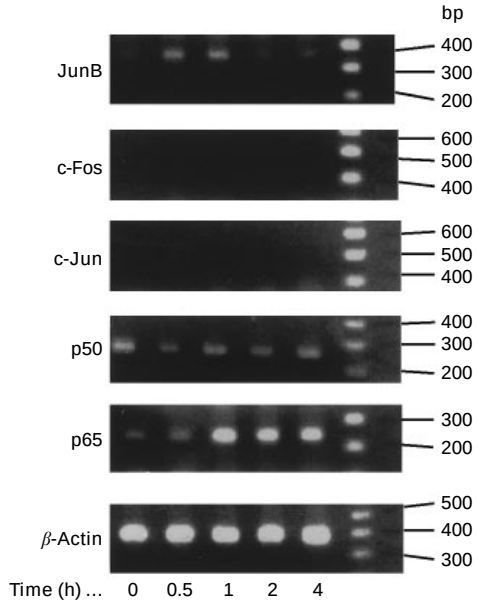

Figure 7 Detection of mRNA levels for different transcription factors in rat mesangial cells by RT-PCR

Mesangial cells were cultured for the indicated periods of time in the presence of 10 μM PGE_2. After cell lysis, target-specific RT-PCR for the indicated transcription factors was performed as described in the Materials and methods section. Visualization as achieved by ethidium bromide staining. A representative of three experiments is shown.

the only PGE_2 receptor type on rat mesangial cells. In RNA isolated from rat kidney all three subtypes were detectable (results not shown).

To investigate further this divergence between the induction mechanism for the two enzymes by PGE_2, we looked at whether, for the regulation of expression of COX-2, iNOS and ASS, distinct different cAMP concentrations are necessary. First we studied the release of 6-oxo-$PGF_{1\alpha}$ from endogenous pools after ionophore stimulation of cell treated with various db-cAMP concentrations. In the range 20–100 μM, a plateau of prostaglandin formation was observed which was about 3 times control levels (Figure 5). With increasing db-cAMP concentrations a further massive release of 6-oxo-$PGF_{1\alpha}$ was observed. With regard to NO synthesis, a significant increase was observed only at db-cAMP concentrations of more than 100 μM (Figure 5). RT-PCR analysis of mesangial cells treated with increasing concentrations of db-cAMP revealed that iNOS gradually increases with db-cAMP concentrations, but only in the higher concentration range (Figure 6, upper panels). ASS mRNA was regulated in a similar way to iNOS (Figure 6, upper panel). For both enzymes the half-maximal effective dose was about 400 μM db-cAMP. However, for COX-2 a nearly complete induction response was observed already at a concentration of 100 μM db-cAMP which remained unchanged up to a concentration of 1 mM db-cAMP. The estimated half-maximal effective dose was approx. 50 μM. This was confirmed at the protein level, analysed in the same cell samples. At 10 μM db-cAMP an increase in COX-2 protein was already visible, and maximal effect was achieved with 50–100 μM db-cAMP (Figure 6, lower panels). iNOS protein was detectable only after incubation with db-cAMP concentrations above 100 μM.

Finally we questioned whether PGE_2 is able to trigger nuclear signalling pathways. Using the RT-PCR approach, we determined mRNA levels of different relevant transcription factors in mesangial cells treated with 10 μM PGE_2 at different time points. Whereas mRNA expression of the AP1 factors c-Fos and c-Jun was not detectable, JunB mRNA was transiently expressed from 30 min to 1 h (Figure 7). The p50 component of NFκB appeared to be constitutively expressed but the p65 component increased severalfold after 1 h. The expression of β-actin, assessed as a control, remained unchanged under these conditions.

DISCUSSION

The release of prostanoids and NO represents an important contribution of mesangial cells to the physiology and also the pathology of the kidney [18]. Under normal conditions, prostacyclin and NO perform important vasodilating functions. Of the prostanoids, PGI_2 is usually seen in a positive way because of its antiaggregation effects. However, excessive formation of NO may contribute to pathological alterations, resulting not only in an unwanted excessive vasodilatation but also in inhibition of aerobic energy metabolism [19], protein synthesis [20] and DNA replication [21].

Recent reports have demonstrated that the NO-synthesizing capacity of various cell types can be modulated by artificially

augmented cAMP concentrations by addition of stable cAMP derivatives or stimulation of adenylate cyclase by forskolin [11,12]. For mesangial cells a massive stimulation of NO synthesis was demonstrated when cAMP was added in combination with different cytokines [12]. Conflicting data have been reported, demonstrating an effect of cAMP-increasing agents on vascular smooth-muscle cells only in the presence of interleukin 1β [22]. Therefore it is still controversial whether the elevation of intracellular cAMP concentration alone is sufficient to regulate NOS activity and, more importantly, whether physiological stimuli known to raise intracellular cAMP levels are also able to stimulate NOS activity. Furthermore, it is not known whether the NO-regeneration cycle is also regulated and whether the prostanoid-synthetic pathway is influenced by cAMP in mesangial cells. Our data clearly point to a cAMP-dependent regulation of the important enzymes COX-2 and iNOS and for the first time demonstrate that the arginine-regeneration cycle is regulated in a similar way. It is generally recognized that the second messenger cAMP mediates its inductive action on various genes via protein kinase A-dependent phosphorylation of a nuclear phosphoprotein, termed CRE-binding protein, which binds as a transcriptional factor to a specific sequence-response element. This CRE is found near the transcription-initiation site not only of rat, but also of human and murine COX-2, genes [10]. For rat iNOS and ASS it remains to be clarified whether the sequences preceding the transcription-start site of these genes also contain the CRE. However, in our model different signal-transduction pathways appear to be triggered because mRNA and protein expression of iNOS and ASS were only stimulated in the presence of high cAMP concentrations, whereas COX-2 was regulated by low cAMP concentrations. Whereas COX-2 was enhanced by db-cAMP concentrations between 10 and 100 μM, reaching saturation, induction of iNOS activity required db-cAMP at a concentration of 100 μM or above and could not be saturated under non-damaging levels of this compound. ASS, an important enzyme in NO biosynthesis, behaved similarly. All data refer to increases in not only protein but also mRNA and hence reflect true transcriptional and possibly post-transcriptional processes.

These results throw new light on the regulation of different enzymes via cAMP. Furthermore, the human iNOS gene possesses no sequence resembling the CRE consensus sequence [23]. Therefore it remains speculative whether high cAMP concentrations are able to trigger pathways in a different way from classical cAMP signalling. The dramatic effect on these central enzymes of prostanoid and NO formation can be seen as response to a severe pathological situation. Recent evidence indicates that activation of iNOS in mesangial cells contributes to immune-mediated reoxygenation- and sepsis-induced renal injury [24–26]. High concentrations of NO may have toxic effects, and, at maximal iNOS stimulation, organ failure may occur. Even if this result can be accepted unambiguously only for the cells used, it clearly demonstrates that the conditions for induction of COX-2 and iNOS are not identical and this may be of broader interest for the pathophysiological process of inflammation in the kidney.

PGE_2 is a physiological autacoid in the kidney and is known to function via different receptors coupled to different signalling pathways. Three subtypes of PGE receptors have been described pharmacologically: EP1, EP2 and EP3. These subtypes are believed to utilize the Ca^{2+}/inositol phospholipid pathway, stimulation of adenylate cyclase and inhibition of adenylate cyclase respectively. We were able to demonstrate the presence of the mRNA for the EP2 receptor, indicating that this receptor subtype is probably the main PGE receptor on rat mesangial cells. EP2 receptor is known to increase cAMP concentration. Our observation is consistent with *in situ* hybridization studies demonstrating the dominance of EP2 receptors in glomeruli [27].

In our study PGE_2 promoted a selective increase in COX-2 mRNA, protein and product which can be mimicked by low db-cAMP concentrations (10–100 μM). At higher concentrations (200–1000 μM), the further dramatic increase in prostacyclin release observed may be the result of induction of phospholipases. In line with this result is our previous observation that 1 mM cAMP stimulates phospholipase A_2 secretion [28]. This is the first hint that specific stimulatory concentrations of cAMP trigger specific signalling pathways. However, one has to question how physiological studies are that use only high cAMP agonist concentrations to investigate cAMP-dependent signalling.

The function of this regulatory mechanism by PGE_2 in renal physiology can only be speculated on. PGE_2 is known to be an important modulator of kidney function, and inhibits net Cl^- reabsorption in the cells of the thick ascending limb of Henle [29]. Rich sources of this prostaglandin are medullary collecting ducts, interstitial cells and epithelial cells of the more distal nephron structures, cellular compartments that host primarily COX-1. Normally, decreased Cl^- reabsorption supervised by the macula densa increases renin secretion. Furthermore, in our study the main arachidonic acid metabolite of rat mesangial cells after induction of COX-2 was PGI_2 followed by PGE_2, the concentration of which was approx. 2–3 times less. Therefore it is possible that the COX-2 coupled increased PGI_2 formation in mesangial cells directly stimulates renin secretion in juxtaglomerular cells as a kind of feedback to control salt reabsorption. An interesting question remains of how other cells of the kidney that are able to express COX are affected by high PGE_2 concentrations. Recent studies have noted that the kidney has low but measurable levels of COX-2 mRNA [30,31]. The fact that COX-2 mRNA has the AUUUA motif in its 3′-untranslated region [1], which is considered an mRNA-instability determinant, indicates the necessity of continuous stimulation for its expression. This is supported by our observation of COX-2-positive glomerular cells in normal rat kidney and the expression of COX-2 mRNA but not iNOS mRNA in rat kidney (M. Kömhoff, and R. M. Nüsing, unpublished work).

A possible explanation for the inductive action of PGE_2 may be modulation of the expression of different transcription factors. PGE_2 appeared to be able to induce the expression of JunB and p65 mRNA but not c-Fos or c-Jun mRNA. However, this is contrary to the recent observation by Simonson et al. [32] that PGE_2 rapidly induces c-Fos and c-Jun mRNA levels in glomerular mesangial cells. This effect was independent of cAMP, and activation of the serum response element located in the c-*fos* gene by PGE_2 was demonstrated by reporter gene assay. Although we have no conclusive explanation for this difference, variations in culture conditions which may influence the behaviour of the cells cannot be excluded. In our study early-passage (5–10) mesangial cells were used; because these cells rapidly change their behaviour with long passanging, another study used late-passage (15–30) cells with high serum concentrations. We suggest that the rapid but transient expression of Jun B mRNA contributes to the induced expression of COX-2. We recently reported on the participation of JunB mRNA expression in COX induction in a macrophage cell line [33]. Like the Jun–Fos heterodimers which mainly form the AP-1 factor JunB homodimers also possess *trans*-activity [34] and are thought to serve as tissue-specific activators of target genes regulated by AP-1.

The existence of the transcription-regulatory element for NFκB on the COX gene is known and therefore the induced expression of a component of this complex transcription factor may promote

its activity next to the known activation mechanism by phosphorylation. NFκB is a heterodimeric protein composed of 50 kDa (p50) and 65 kDa (p65) subunits [35]. The complex contains a strong transcription-activation domain and an intrinsic nuclear localization signal. This is recognized and masked by IκB, a cytoplasmic inhibitor of NFκB, which binds to the p65 subunit. Although the direct link between induced p65 expression and induced COX-2 expression remains to be verified, we suggest that overexpressed p65 may function as a scavenger of IκB resulting in free and active NFκB protein. Further studies are necessary to prove this hypothesis.

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