Prostaglandin E1 inhibits IL-6-induced MCP-1 expression by interfering specifically in IL-6-dependent ERK1/2, but not STAT3, activation

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INTRODUCTION

The immediate response of an organism towards inflammatory stimuli is the release of pro-inflammatory cytokines such as TNF (tumour necrosis factor) and IL (interleukin)-6. The activity of these cytokines is counteracted by anti-inflammatory cytokines such as IL-4 and IL-10. The members of the family of IL-6-type cytokines exert anti- as well as pro-inflammatory activities. Additionally, chemokines and PGs (prostaglandins) regulate inflammatory processes [1].

Further complexity results from the fact that the pro-inflammatory cytokine IL-1 induces other cytokines such as IL-6 or initiates the production of PGs by cyclo-oxygenase 2 induction, a key enzyme for the synthesis of PGs and thromboxanes. Furthermore, IL-1 blocks the functions of IL-6 by counteracting the synthesis of a set of acute-phase proteins in the liver [2]. Less is known about the regulation of IL-6 signal transduction by hormone/cytokine signalling through G-protein-coupled receptors. This is particularly surprising since many of these hormones (such as prostaglandins and chemokines) play an important role in inflammatory processes. In the present study, we have investigated the inhibitory activity of PGE₁ (prostaglandin E₁) on IL-6-induced MCP-1 expression and have elucidated the underlying molecular mechanism. Surprisingly, PGE₁ does not affect IL-6-induced STAT (signal transducer and activator of transcription) 3 activation, but does affect ERK (extracellular-signal-regulated kinase) 1/2 activation which is crucial for IL-6-dependent expression of MCP-1. In summary, we have discovered a specific cross-talk between the adenylate cyclase cascade and the IL-6-induced MAPK (mitogen-activated protein kinase) cascade and have investigated its impact on IL-6-dependent gene expression.

Key words: cytokine, inflammation, interleukin-6 (IL-6), Janus kinase (JAK), prostaglandin (PG), signal transducer and activator of transcription (STAT), signal transduction.

Abbreviations used: DMEM, Dulbecco’s modified Eagle’s medium; EP, prostaglandin E receptor; Epac, exchange protein directly activated by cAMP; ERK, extracellular-signal-regulated kinase; HRP, horseradish peroxidase; IBMX, 3-isobutyl-1-methylxanthine; IL, interleukin; IL-6R, IL-6 receptor; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; PI3K (phosphoinositide 3-kinase) cascades (reviewed in [4,5]). The mechanisms leading to STAT1 and STAT3 activation have been studied in detail: after activation of JAK1, 2 and Tyk2 (tyrosine kinase 2), the signal transducer gp130 of the IL-6R complex becomes phosphorylated on tyrosine motifs within the cytoplasmic region. The four most distal membrane phosphotyrosine modules exhibit recruitment sites for STAT3 and to a lesser extend STAT1 [6,7]. Subsequently, these transcription factors are also tyrosine phosphorylated, dimerize, translocate into the nucleus and bind to specific response elements within promoters of IL-6-inducible target genes.

Little is known about the initiation of IL-6-induced STAT-dependent signalling pathways. Activation of these pathways is crucial for the cell-type-specific activities of IL-6; it has been shown that IL-6-induced STAT3 activation elicits an anti-apoptotic signal, whereas activation of the MAPK cascade generates a pro-mitotic signal in cells responding with proliferation [8]. In other cells, STAT3 activation is crucial for IL-6-induced differentiation [9]. Finally, IL-6-induced neurite outgrowth depends on the activation of the MAPK cascade [10]. It has been suggested that binding of the adapter protein and protein-tyrosine phosphatase SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase 2) to the phosphorylated Tyr705 motif in gp130 is the initial step for activating the MAPK cascade in response to IL-6 [8,11]. Later, we identified this motif within gp130 to also be responsible for negative regulation of
the JAK/STAT pathway [12] by recruiting both the phosphatase SHP-2 [13] as well as the IL-6-induced feedback inhibitor SOCS (suppressor of cytokine signalling) 3 [14]. Obviously, Tyr705 does not simply mediate activation of the MAPK cascade but rather regulates the balance between activation of the JAK/STAT and MAPK cascades in response to IL-6 [15].

The requirement for a tight regulation of IL-6 signal transduction is obligatory. Several mechanisms counteracting IL-6-induced STAT activation and consequently STAT-dependent gene induction have been described. Most importantly, the IL-6-induced feedback inhibitors of the family of SOCS proteins, SOCS1 and SOCS3, inhibit STAT activation by binding to JAKs or to gp130 respectively, as already mentioned above [14,16–20]. The phosphatase activity of SHP-2 is responsible for tyrosine dephosphorylation of JAKs, gp130 and STATs in the cytoplasm [13], whereas TcPTP (T-cell protein tyrosine phosphatase) dephosphorylation of JAKs, gp130 and STATs in the cytoplasm [21]. Furthermore, pro-inflammatory mediators such as LPS (lipopolysaccharide), TNF and IL-1β counteract IL-6 signalling by inducing SOCS3 expression or by stabilizing SOCS3 mRNA [22,23], by activating NF-κB (nuclear factor κB), which competes with STAT3 for binding to promoters of IL-6-inducible genes [24,25] or by blocking STAT activation at the receptor, independent of SOCS induction [26,27].

Less is known about the regulation of IL-6 signal transduction by mediators signalling through G-protein-coupled receptors such as chemokines and PGs. The E-type PGs (PGEs) signal through the G-protein-coupled EP receptors which initiate different signalling pathways, dependent on the EP receptor subtype expressed by a specific cell type. Ligand-bound EP1 activates the phospholipase Cβ pathway through Gαs, whereas EP2 and EP4 activate the adenylate cyclase pathway through Gαs. Cells expressing EP3 activate Gαi and inhibit adenylate cyclase activity. Thus the specific outcome of PGE signalling is determined by the receptor type expressed at the cell surface.

PGE, exerts anti-inflammatory as well as vasodilatory activities. Synthetic analogues of PGE, ameliorate methotrexate-induced enterocolitis and counteract ROS (reactive oxygen species) production [28]. Recently, PGE, has been shown to protect against ischaemia/reperfusion-induced liver and lung damage [29,30]. In vivo studies demonstrated that PGE, in the blood efficiently decreases the levels of the CCL chemokines MCP-1 (monocyte chemotactrant protein 1; CCL2) and is therefore beneficial in peripheral arterial obstructive disease [31]. On the other hand IL-6, together with its soluble receptor, is a potent inducer of MCP-1 in fibroblasts [32]. Up to now, no results on a cross-talk between PGE, and IL-6 signal transduction and its impact on MCP-1 expression are available.

In the present study we describe a specific regulatory function of PGE, for IL-6 signal transduction. We show a series of initiators of cAMP signalling, including PGE, that specifically inhibit IL-6-dependent ERK (extracellular-signal-regulated kinase), but not STAT, activation through a PKA (protein kinase A)-dependent pathway which finally inhibits IL-6-induced MCP-1 expression. The present study shows a new and specific mechanism of negative regulation of IL-6-induced MAPK activity and its consequence on IL-6-induced MCP-1 gene expression.

EXPERIMENTAL

Materials

Antibodies to (p)ERK1 and (p)ERK2 (phosphorylated ERK1 and ERK2) as well as to activated STAT3 ([p]Tyr705–STAT3) and the PKA substrate-specific antibody (100G7) were obtained from Cell Signaling Technology. Antibodies to ERK1, ERK2 and SHP-2 were purchased from Santa Cruz Biotechnology. Pertussis toxin, forskolin, PGE1, the EP2 receptor agonist (R)-butaprost, the phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine), aprotinin, pepstatin and gentamycin were from Sigma–Aldrich. Leupeptin was from MP Biomedical. Pefabloc was purchased from Roth. The PKA inhibitor H89 was from Calbiochem. The Src inhibitor PP1 (protein phosphatase 1) was from Biomol. The PKA and Epac (exchange protein directly activated by cAMP) agonists N-6Phe-cAMP and 8-pCPT2-O-Me-cAMP were from Axonora. DMEM (Dulbecco’s modified Eagle’s medium) was from Invitrogen-Gibco and fetal calf serum was from PAA. Recombinant IL-6 and soluble IL-6R were prepared as described previously [33]. The specific activity of IL-6 was 2 × 106 B cell-stimulatory factor-2 units/mg of protein. Oligonucleotides were synthesized by Eurogentec.

Western blot analysis

For the isolation of cellular proteins, confluent cell cultures were lysed in 500 μl of lysis buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 % Nonidet P40, 1 mM NaF and 1 mM Na3VO4] supplemented with 10 μg/ml each of aprotinin, pepstatin and leupeptin as well as 1 mM pefabloc. Proteins were separated by SDS/PAGE and transferred on to a PVDF membrane (PALL). Antibodies were detected by incubation with specific primary antibodies (1:1000) and HRP (horseradish-peroxidase)-coupled secondary antibodies (1:2000; DAKO). The membranes were developed with an ECL (enhanced chemoluminescence) kit (GE Healthcare).

Cell culture

Primary NHDFs (normal human dermal fibroblasts) and MEFs (murine embryonic fibroblasts) were grown in DMEM supplemented with 10 % fetal calf serum and gentamycin (50 μg/l).

RT (reverse transcription)

Total RNA isolation was performed according to the manufacturer’s protocol using the RNAeasy kit provided by Qiagen. The RT reaction was performed using the first strand cDNA synthesis kit (Roche). RNA (4 μg) was transcribed into cDNA and used for determining the PG receptor profile in NHDFs according to manufacturer’s protocol. The first-strand cDNA synthesis (RT) was performed at 42 °C for 70 min (extension) followed by 5 min at 99 °C to inactivate reverse transcriptase. Subsequently, a PCR reaction with primers specific for the PG receptors EP1–EP4 was performed using the following programme (for 35 cycles): 120 s at 94 °C for the first denaturation, 30 s at 94 °C for further denaturation, 30 s at 55 °C for primer annealing, 40 s at 72 °C for extension and 10 min at 72 °C for the final extension. The amplicons were analysed by agarose gel electrophoresis. The primers used [34] were: EP1FW, 5′-TCTAATCCCTGT-CACCGGCCACTG-3′; EP1rev, 5′-GAAGTGCGCTAGGCGCCGCT-GTGGCCGGAGG-3′; EP2fw, 5′-TTCATCCGGACGGCGGCGACGGC-3′; EP2rev, 5′-GGTGCCCCGGTTTACTGCTAG-3′; EP3fw, 5′-GGAGACTGCAAGACACACCGGAG-3′; EP3rev, 5′-GATCTCCATGGTGATTTACTGCAACA-3′; EP4fw, 5′-CCTCTCCATGAGAAGACAGTGTC-3′; and EP4rev, 5′-AGG-ACTCAGAGGTCTT-3′.

Determination of MCP-1 expression

MCP-1 in the supernatant of NHDFs was determined with a quantitative sandwich ELISA according to the manufacturer’s
Protocol (R&D). Briefly, MCP-1 antibodies were immobilized in microtiter plates. MCP-1 standard solutions and the supernatant from stimulated NHDFs were applied to the pre-coated wells and incubated for 2 h at room temperature (27°C). Unbound proteins were washed away and HRP-conjugated MCP-1-antibody was added to each well and incubated for 1 h at room temperature. Following the washing steps, HRP substrate was added and further incubated at room temperature for 20 min. The reaction was stopped and the absorbance was measured in a microtiter plate reader (Molecular Dynamics) at 450 nm and 570 nm for λ-correction. MCP-1 concentrations in the analysed samples were re-calculated using the MCP-1 standard curve.

RESULTS

PGE1 inhibits IL-6-induced MCP-1 chemokine gene expression

MCP-1 is induced during inflammation by a number of inflammatory agents, including IL-6. Since PGE1 is known to be anti-inflammatory, in the present study we asked whether IL-6-induced MCP-1 induction is influenced by PGE1. Figure 1). Prior to the stimulation with IL-6, PGE1 or IL-6 and PGE1, NHDFs were pre-incubated with the phosphodiesterase inhibitor IBMX to block the breakdown of intracellular cAMP generated by PGE1. It is essential to also add the phosphodiesterase inhibitors to those samples without PGE1 to consider potential effects of basal cAMP in the absence of PGE1 (Figure 1, 1st and 2nd bars). Although stimulation with IL-6/sIL-6R resulted in a strong increase in MCP-1 gene expression (Figure 1, 2nd bar) when compared with the non-stimulated control cells (Figure 1, 1st bar), the IL-6-dependent MCP-1 expression was reduced in the presence of PGE1 (Figure 1, 4th bar). These results further demonstrate the physiological impact of PGE1 on IL-6-induced gene expression.

IL-6-induced MAPK activation, but not STAT activation, is inhibited in the presence of forskolin

PGE1 signals through G-protein-coupled receptors. To check whether signalling through G-protein-coupled receptors affects IL-6-induced activation of the JAK/STAT or MAPK cascades we stimulated NHDFs with IL-6 and the sIL-6R for up to 60 min in the presence or absence of the adenylate cyclase activator forskolin. ERK1/2 phosphorylation as well as STAT3 phosphorylation in whole cell lysates was analysed by Western blotting (Figures 2A and 2B). As a loading control we re-stained the blot for ERK1/2 and STAT3 (Figures 2C and 2D). Figure 2 demonstrates that ERK1/2 phosphorylation is detectable 15 min after stimulation with IL-6/sIL-6R and fades out after 1 h (Figure 2A, first five lanes). Remarkably, ERK phosphorylation is severely impaired in cells treated with forskolin (Figure 2A, righthand side), whereas STAT3 phosphorylation (Figure 2B) is hardly affected by treatment with forskolin. Inhibition of IL-6-dependent ERK activation by forskolin treatment could be confirmed in MEFs (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/412/bj4120065add.htm) suggesting that our observation is not only specific for human fibroblasts. These results indicate that stimuli inducing cAMP-dependent signalling pathways specifically counteract IL-6-induced ERK activation but not STAT3 phosphorylation.

Pertussis toxin counteracts IL-6-induced ERK activation

Pertussis toxin inhibits signalling through G-protein-coupled receptors by halting Gs in its GDP-bound form. Consequently, the inhibition of target molecules such as the adenylate cyclase is impaired and the cellular cAMP concentration increases. Therefore we tested whether pertussis toxin leads to the same effect on IL-6-induced ERK activation as observed in response to forskolin. Figure 3 demonstrates reduced IL-6-dependent ERK activation in pertussis-toxin-treated NHDFs (Figure 3A, righthand side), whereas STAT3 activation is hardly affected by the toxin (Figure 3B). Again, inhibited ERK activation but unchanged STAT3 activation in response to IL-6 could be confirmed in pertussis-toxin-treated MEFs (see Supplementary Figure 2 at http://www.BiochemJ.org/bj/412/bj4120065add.htm). These results suggest that activation of adenylate cyclase specifically...
MAPK activation, but not STAT activation, induced by IL-6 is inhibited in the presence of pertussis toxin

Starved primary NHDFs were pretreated with pertussis toxin (PTX) (50 ng/ml) for 16 h or left untreated. Subsequently, the cells were stimulated with 800 units/ml IL-6 and 1 µg/ml sIL-6R for the times indicated. Whole cell lysates were prepared and ERK and STAT3 activation was monitored by Western blotting with antibodies specific for the activated forms of ERK [(p)ERK1 and (p)ERK2; (A)] or STAT3 [(p)Tyr705-STAT3; (B)]. ERK1/2 and STAT3 expression was additionally monitored to show equal loading of the lanes (C and D). IB, immunoblot.

Figure 3

PGE1 blocks IL-6-induced ERK activation, which is crucial for IL-6-induced MCP-1 expression

To test whether PGE1 influences IL-6-induced ERK activation in a similar manner, we monitored IL-6-dependent ERK activation in whole cell lysates of NHDFs in the presence or absence of PGE1. Figure 4 shows that PGE1 blocked ERK activation after IL-6 treatment (Figure 4A) but not IL-6-dependent STAT3 phosphorylation (Figure 4B). To test whether inhibition of the MAPK cascade has any relevance for IL-6-induced MCP-1 expression, we compared MCP-1 induction by IL-6 in the absence or presence of the MEK inhibitor UO126 (Figure 5). Although stimulation with IL-6/sIL-R induces MCP-1 gene expression in the absence of UO126 (Figure 5, 2nd bar), the presence of UO126 strongly reduces the potential of IL-6 to induce MCP-1 expression (Figure 5, 4th bar). These results indicate a crucial role of the IL-6-initiated MAPK cascade for the induction of MCP-1.

The PGE1 receptor EP2 is expressed on NHDFs, and its activation counteracts IL-6-induced ERK phosphorylation but not activation of STAT3.

PGE1 blocks IL-6-induced ERK activation, which is crucial for IL-6-induced MCP-1 expression

In line with these observations, we tested whether butaprost could inhibit IL-6-dependent MCP-1 expression. Figure 6(C) shows that MCP-1 protein expression induced by IL-6/sIL-6R was reduced in the presence of the EP2 agonist butaprost (Figure 6C, compare the 2nd and 4th bars). These results further indicate that IL-6-dependent MCP-1 expression can be inhibited by EP2 activation.

Figure 6

IL-6-induced MCP-1 expression requires MAPK activation

Starved primary NHDFs were pre-incubated with the MEK inhibitor U0126 (20 µM; UO) for 30 min and subsequently stimulated with 800 units/ml IL-6 and 1 µg/ml sIL-6R for 1.5 h in the presence of UO126. IL-6/sIL-6R was eliminated by changing the medium. The time for stimulation was limited to 90 min to avoid long-term accumulation of the gene products and effects of autocrine IL-6 action. After incubation for a further 5 h in cytokine-free medium in the presence of IBMX (500 µM) and U0126 (20 µM) the MCP-1 concentration was determined as described in the legend for Figure 1. Basal MCP-1 levels were 65.8 ± 15.6 pg/ml. This amount of MCP-1 represents the MCP-1 which accumulates in non-stimulated cells within the last 5 h of the experiment. IBMX was added to allow a comparison of the results from this Figure with those from Figure 1 and to control PGE1-mediated inhibition of MCP-1 expression (results not shown).
Regulation of IL-6-induced MCP-1 expression by PGE₁

Figure 6  PGE₁ acts through the EP2 receptor

(A) Total RNA was isolated from starved primary NHDFs. RT–PCR analysis with specific primers for the EP1–4 receptors was performed and the PCR products separated on a 2 % agarose gel. The mobility of the bands corresponds to the predicted size (EP1, 210 bp; EP2, 510 bp; and EP3, 390 bp). (B) Starved NHDFs were pre-incubated with PGE₁ (7 µM for 5 min) or (R)-butaprost (10 µM for 10 min) or the solvent alone. Subsequently, cells were stimulated with 800 units/ml IL-6 and 1 µg/ml sIL-6R for the times indicated. Whole cell lysates were prepared and ERK and STAT activation was monitored by Western blotting with antibodies specific for the activated forms of ERK [(p)ERK1 and (p)ERK2; top panel] or STAT3 [(p)Tyr705-STAT3; 2nd panel]. ERK2 and STAT3 expression was additionally monitored to show equal loading of the lanes (two bottom panels). IB, immunoblot. (C) Starved NHDFs were pre-incubated with the phosphodiesterase inhibitor IBMX (500 µM) for 20 min. At 5 min prior to stimulation with 800 units/ml IL-6 and 1 µg/ml sIL-6R, cells were treated with (R)-butaprost (10 µM) or ethanol (carrier control). After 18 h incubation, the MCP-1 concentration in the medium was determined by an MCP-1 protein-specific ELISA. Results are presented with respect to IL-6/sIL-6R-treated cells. Basal MCP-1 levels were 0.92 ± 0.35 ng/ml. This amount of MCP-1 represents the MCP-1 which accumulates in non-stimulated cells from the beginning of the experiment.

Src does not contribute to PGE₁-mediated inhibition of ERK activation

Previous reports have shown that Src is required for cAMP-mediated inhibition of growth-factor-induced ERK phosphorylation by activating Rap1 (Ras-proximate 1) [35]. Thus we tested whether the Src-kinase inhibitor PP1 restored IL-6-induced ERK activation in the presence of PGE₁ (Figure 7A). Surprisingly, the inhibitory potential of PGE₁ was even more pronounced in the presence of PP1, clearly indicating that Src kinase activity was not crucial for the inhibitory activity of PGE₁ on IL-6 signalling.

PGE₁ acts through cAMP-activated PKA and not through cAMP-activated Epac

cAMP is known to activate directly PKA as well as the guanine-nucleotide-exchange factor Epac. Our next aim was to investigate which of these cAMP targets has the potential to inhibit IL-6-induced ERK activation. We treated NHDFs with either the specific agonist for PKA (N-6Phe-cAMP) or the specific agonist for Epac (8-pCTP2′-O-Me-cAMP) and monitored ERK phosphorylation after stimulation with IL-6 (Figure 8A). STAT3 phosphorylation was not affected by either agonist (Figure 8A, 2nd panel). In contrast, the PKA agonist N-6Phe-cAMP inhibited IL-6-initiated ERK phosphorylation, whereas the Epac agonist 8-pCTP2′-O-Me-cAMP did not affect ERK activation after IL-6 stimulation (Figure 8A, top panel). These observations suggest

Figure 7  Src does not contribute to PGE₁-dependent inhibition of IL-6-induced ERK activation

Starved primary NHDFs were pre-incubated with the Src inhibitor PP1 (10 µM) or DMSO for 40 min. Five min prior to stimulation with 800 units/ml IL-6 and 1 µg/ml sIL-6R, cells were treated with PGE₁ (1 µM) or ethanol (carrier control). After the times indicated in the Figure, whole cell lysates were prepared and ERK and STAT activation was monitored by Western blotting with antibodies specific for the activated forms of ERK [(p)ERK1 and (p)ERK2; (A)] or STAT3 [(p)Tyr705-STAT3; (B)]. ERK1/2 and STAT3 expression was additionally monitored to show equal loading of the lanes (C and D). IB, immunoblot.
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Figure 8  PKA, but not Epac, agonists mimic cAMP effects on IL-6-induced ERK activation

(A) Starved primary NHDFs were pretreated with the PKA agonist N-6Phe-cAMP (300 µM) or the Epac agonist 8-pCPT2'-O-Me-cAMP (100 µM) for 15 min or left untreated as indicated. Subsequently, the cells were stimulated with 800 units/ml IL-6 and 1 µg/ml sIL-6R for the times indicated. Whole cell lysates were prepared and ERK and STAT3 activation was monitored by Western blotting with antibodies specific for the activated forms of ERK (pERK1 and pERK2; top panel) or STAT3 (pTyr705-STAT3; 2nd panel). ERK2 and STAT3 expression was additionally monitored to show equal loading of the lanes (two bottom panels). (B) Starved NHDFs were pre-incubated with the PKA inhibitor H89 (10 µM) or DMSO for 20 min. At 5 min prior to stimulation with 800 units/ml IL-6 and 1 µg/ml sIL-6R cells were treated with PGE1 (1 µM) or ethanol (carrier control) for the times indicated in the Figure. Whole cell lysates were prepared and ERK and STAT3 activation was monitored by Western blotting with antibodies specific for the activated forms of ERK (pERK1 and pERK2; top panel) or STAT3 (pTyr705-STAT3; 2nd panel). ERK2 and STAT3 expression was additionally monitored to show equal loading of the lanes (two bottom panels). (C) Starved NHDFs were stimulated with PGE1 (1 µM) for 5 min. Cell lysates were prepared and Raf-1 kinase was precipitated with antibodies against c-Raf-1. PKA-dependent phosphorylation of c-Raf was monitored with an antibody recognizing the PKA-substrate sequence RRX(p)S/(p)T by Western blotting (top panel). Precipitation of c-Raf was controlled by re-staining the blot with an antibody against c-Raf-1 (bottom panel). IB, immunoblot; IP, immunoprecipitate.

that not the activation of Epac but the activation of PKA by cAMP is crucial for the inhibition of IL-6-induced ERK activation by PGE1.

To elaborate further whether PGE1 acts as an inhibitor by activating PKA, we treated NHDFs with the PKA inhibitor H89 and monitored whether blocking PKA by H89 renders these cells resistant to PGE1-mediated inhibition of IL-6-dependent ERK activation. Figure 8(B) shows the recovery of ERK phosphorylation when the cells were treated with PGE1 in the presence of the PKA inhibitor H89 (Figure 8B, top panel). Obviously, H89 overrides basal inhibition of ERK activation in the absence of IL-6. STAT3 activation was unaltered by H89 treatment (Figure 8B, 2nd panel). These results further indicate that PGE1 inhibits ERK phosphorylation in response to IL-6 by acting through PKA.

Previous studies on growth-factor signalling indicated that PKA phosphorylates and thereby inhibits the MAPKKK (MAPK kinase kinase) c-Raf-1. Thus we tested whether PGE1 induces phosphorylation of c-Raf-1 in NHDFs (Figure 8C). c-Raf-1 protein was precipitated from cellular extracts of NHDFs treated with PGE1 for 5 min or from untreated NHDFs. Phosphorylation of c-Raf-1 was monitored by Western blotting and subsequent staining with an antibody specific for phosphorylated PKA consensus sides. The right-hand lane in the top panel of Figure 8(C) indicates that PGE1 induced phosphorylation of c-Raf-1 within a protein motif representing a substrate for PKA. In summary these results suggest that PGE1 counteracts IL-6-dependent ERK activation by activating PKA which leads to c-Raf-1 phosphorylation to block the initiation of the MAPK cascade.

DISCUSSION

Inflammation is a response of an organism to cope with infections, sterile injuries and other trauma. The extent of inflammation is controlled by a set of pro- and anti-inflammatory cytokines as well as chemokines and non-protein mediators such as PGs, NO and ROS. Although much information is available with respect to the signal transduction of the individual mediators, only recently attention has been put on the mutual regulation of the signalling pathways. Regulating the cellular cAMP concentration is a crucial event for the signal transduction of chemokines and PGs. Previous studies have focused on the induction of cytokine expression...
by cAMP [36–39], whereas the present study investigates the influence of cAMP signalling on IL-6 signal transduction and IL-6-induced MCP-1 gene induction.

We analysed the influence of cellular cAMP on IL-6 signal transduction in fibroblasts and demonstrate specifically that IL-6-initiated ERK activation is counteracted by cAMP whereas STAT3 activation is not affected. Furthermore, we show that cAMP acts through PKA and c-Raf phosphorylation to inhibit the MAPK cascade, whereas activation of Epac by cAMP is not involved. This detail is important, since recently Sands and co-workers [40] reported that cAMP is able to induce SOCS3 expression through an Epac-dependent pathway in vascular endothelial cells. Obviously our observations reflect a different mechanism, since we did not detect reduced STAT3 activation in response to Epac agonists but specifically a reduction of IL-6-dependent ERK activation by PKA agonists (Figure 8A). Very probably, the induction of SOCS3 by Epac agonists and subsequent inhibition of STAT3 activation is specific for vascular endothelial cells.

It has been shown previously and also confirmed in the present study (results not shown) that the intracellular concentration of cAMP in fibroblasts increases drastically (100-fold) in response to PGE1 [41–43]. Accordingly, we observed a strong reduction of IL-6-dependent ERK activation in response to PGE1 in primary NHDFs. This observation indicates that not only pharmaceuticals which increase the cAMP concentration in the cell but also natural inflammatory regulators are potent regulators of IL-6 signal transduction. Recently, Cheon and co-workers [44] described that PGE2 augments IL-10-mediated STAT3 and STAT1 activation in THP-1 cells. In contrast, PGE1 suppresses IL-6-induced STAT3 and STAT1 phosphorylation through mechanisms requiring de novo protein synthesis, probably SOCS3 expression [44]. Corroborating this hypothesis, and in line with the results of the present study, cAMP alone did not affect STAT activation after IL-6 treatment. In addition to these observations, we show in the present study for the first time that PGE1 suppresses IL-6-induced ERK activation and MCP-1 gene induction.

We focused on the mechanism of how PGE1 affects IL-6-induced ERK activation. The fact that STAT3 activation by IL-6 is not affected by PGE1 argues the case to look for targets downstream of the activated receptor and JAKs. Although de Silva and co-workers [45] demonstrated PGE2-mediated downregulation of IL-6Rα expression in NFS-60 cells, we could exclude an effect of PGE1 on receptor expression because of ongoing STAT activation in the presence of PGE1 and the stimulation with the agonistically acting sIL-6Rα. Furthermore, the cell-surface expression of gp130 was controlled by FACS analyses (results not shown).

From previous studies by Schmitt and Stork [46–48] we know that cAMP also antagonizes ERK activation by growth factors. In this context PKA, activated through elevated intracellular cAMP concentrations, phosphorylates and activates Src kinase, which in turn leads to Rap1 activation [46]. Rap1 counteracts Ras function in cells such as fibroblasts, which do not express B-Raf but activates ERK in cells expressing B-Raf [47,48]. In the course of preparing the present manuscript Stork and co-workers [49] further elucidated the mechanism of how cAMP activates the MAPK cascade through B-Raf. The authors demonstrate that activation of B-Raf by PKA but not through the cAMP-dependent guanine-nucleotide-exchange factor Epac is crucial for the initiation of the MAPK cascade in B-Raf-expressing cells. In summary, in both cases PKA accounts for the induction, as well as for the repression, of the growth-factor-induced MAPK cascade, dependent on the presence or absence of B-Raf.

In the present study, we found no evidence for a contribution of Epac (Figure 8A) or Src (Figure 7) to the inhibition of IL-6-dependent ERK activation. Src suppresses the inhibitory activity of PGE1 rather than mediating its inhibitory function on IL-6-dependent ERK activation (Figure 7). Instead, we demonstrated a crucial role of PKA (Figures 8A and 8B) for PGE1-mediated inhibition of ERK activation by IL-6. PKA is also known to inhibit c-Raf-1 by phosphorylation [48]. Indeed, we could demonstrate PGE1-dependent phosphorylation of PKA-target sites within c-Raf-1 (Figure 8C) and a repression of IL-6-induced MCP-1 expression by PGE1, (Figure 1) which is in line with a crucial role of the IL-6-initiated MAPK cascade for MCP-1 expression as shown in Figure 5.

In summary our results show adenylyl cyclase, PKA and c-Raf-1 to be involved in the inhibition of the MAPK cascade by PGE1, and its consequence on IL-6-induced MCP-1 gene expression. Understanding this cross-talk will help to critically judge the outcome of pharmaceutical approaches targeting PGE1 and IL-6.

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