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

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short title: Regulation of IL-6-induced MCP-1 expression by PGE1

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Synopsis

Interleukin-6 exerts pro- as well as anti-inflammatory activities. Beside many other activities, IL-6 is the major inducer of acute phase proteins in the liver, acts a differentiation factor for blood cells, as migration factor for T-cells and is a potent inducer of the chemokine monocyte chemoattractant protein-1 (MCP-1). Recent studies focussed on the negative regulation of interleukin-6 signal transduction through the IL-6-induced feedback inhibitors SOCS1 and SOCS3 or the protein tyrosine phosphatases SHP2 and TcPTP. Studies on the cross-talk between pro-inflammatory mediators (interleukin-1, tumour necrosis factor, lipopolysaccharide) and interleukin-6 elucidated further regulatory mechanisms. Less is known about the regulation of IL-6 signal transduction by hormones/cytokines signalling through G-protein-coupled receptors. This is particularly surprisingly since many of these hormones (such as prostaglandins and chemokines) play an important role in inflammatory processes. Here, we investigated the inhibitory activity of prostaglandin E1 on IL-6-induced MCP-1 expression and elucidated the underlying molecular mechanism. Surprisingly, PGE1 does not affect IL-6-induced STAT3- but ERK1/2-activation which is crucial of IL-6-dependent expression of MCP-1. In summary, a specific cross-talk between the adenylate cyclase cascade and the IL-6-induced MAPK cascade and its impact on IL-6-dependent gene expression was discovered.

Keywords: cytokines, signal transduction, Jak/STAT, inflammation

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The immediate response of the organism towards inflammatory stimuli is the release of pro-inflammatory cytokines such as TNF^1 and IL-1. 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 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 prostaglandins by cyclooxygenase 2 induction – a key enzyme for the synthesis of prostaglandins and thromboxans. Furthermore, IL-1 blocks IL-6 functions by counteracting the synthesis of a set of acute-phase proteins in the liver [2]. Less is known about the regulation of interleukin-6 signal transduction by prostaglandins or chemokines.

Interleukin-6 binds to a receptor complex composed of a ubiquitously expressed gp130 receptor chain and the more restrictively expressed IL-6 receptor α (IL-6R α). Cells which do not express IL-6R α respond to IL-6 in the presence of agonistically acting soluble IL-6R α at the site of inflammation [3]. Activation of the receptor complex by IL-6 leads to the initiation of the JAK/STAT-, MAPK-, and PI3K-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 the signal transducer gp130 of the IL-6-receptor complex becomes phosphorylated on tyrosine motifs within the cytoplasmic part. The four most membrane distal 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 to 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-independent 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-

¹ Abbreviations used: EP: PGE-receptor; Epac: exchange protein directly activated by cAMP; gp: glycoprotein; IBMX: 3-isobutyl-1-methylxanthine; IL: interleukin; JAK: Janus kinase;NHDF: normal human dermal fibroblasts; MAPK: mitogen activated protein kinase; MCP-1: monocyte chemoattractant protein-1; NF- κ B: nuclear factor κ B; PGE: prostaglandin E; PI3K: phosphatidyl-inositol 3 kinase; PKA: protein kinase A; PTX: pertussis toxin; Rap: Ras-proximate-1; SH2: Src-homology domain 2; SOCS: suppressor of cytokine signaling; STAT: signal transducer and activator of transcription; TcPTP: T-cell protein tyrosine phosphatase; TNF: tumor necrosis factor;

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 SHP2 to the phosphorylated tyrosine 759 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 be also responsible for negative regulation of the JAK/STAT pathway [12] by recruiting both, the phosphatase SHP2 [13] as well as the IL-6-induced feedback inhibitor SOCS3 [14]. Obviously, tyrosine 759 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 Janus kinases or to gp130, respectively, as already mentioned above [14, 16-20]. The phosphatase activity of SHP2 is responsible for tyrosine dephosphorylation of JAKs, gp130, and STATs in the cytoplasm [13], whereas TcPTP dephosphorylates STATfactors in the nucleus [21]. Furthermore, pro-inflammatory mediators such as LPS, TNF and IL-1 β counteract IL-6 signalling by inducing SOCS3 expression or by stabilizing SOCS3 mRNA [22, 23], by activating NF-KB 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 interleukin-6 signal transduction by mediators signalling through G-protein-coupled receptors such as chemokines and prostaglandins. The E-type prostaglandins 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 C β pathway through G α_q , whereas EP2 and EP4 activate the adenylate cyclase pathway through G α_s . Cells expressing EP3 activate G α_i and inhibit adenylate cyclase activity. Thus, the specific outcome of PGE-signalling is determined by the receptor type expressed at the cell surface.

Prostaglandin PGE1 exerts anti-inflammatory as well as vasodilator activities. Synthetic analogues of PGE1 ameliorate methotrexate-induced enterocolitis and counteract ROS production [28]. Recently, PGE1 has been shown to protect against ischemia/reperfusion-induced liver and lung damage [29, 30]. In vivo studies demonstrated that PGE1 in the blood efficiently decreases the levels of the CCL chemokines MCP-1 (CCL2) and is therefore benefical 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 data on a cross-talk between PGE1 and IL-6 signal transduction and its impact on MCP-1 expression are available.

Here we describe a specific regulatory function of PGE1 for IL-6-signal transduction. We show a series of initiators of cAMP-signalling, including PGE1 to inhibit specifically IL-6-dependent ERK- but not STAT-activation through a PKA-dependent pathway which finally inhibits IL-6-induced MCP-1 expression. Our study elucidated 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 phosphorylated ERK1 and ERK2 ((p)ERK1 and (p)ERK2) as well as to activated STAT3 ((p)Tyr705-STAT3) and the PKA-substrate (PKAS) specific antibody (100G7) were obtained from Cell Signaling Technolgy (Frankfurt/Main, Germany). Antibodies to ERK1, ERK2 and SHP2 were purchased from Santa Cruz Biotechnology (Santa Crux, CA, USA). Pertussis toxin, forskolin, prostaglandin E1, the EP2-receptor agonist (R)-butaprost, the phosphodiesterase inhibitor IBMX, aprotinin, pepstatin, and gentamycin were from Sigma-Aldrich (Taufkirchen, Germany). Leupeptin was from MP Biomedical Inc. (Illkirch, France). Pefabloc was purchased from Roth (Karlsruhe, Germany). The PKA-inhibitor H89 was from Calbiochem (Bad Soden, Germany). The Src-inhibitor PP1 was from Biomol (Hamburg, Germany). The PKA and Epac agonists N-6Phe-cAMP and 8-pCTP2'-O-Me-cAMP were from Axxora (Grünberg, Germany). Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen-Gibco (Paislay, UK); fetal calf serum was from PAA (Pasching, Austria); Recombinant IL-6 and sIL-6R were prepared as described [33]. The specific activity of IL-6 was $2 \cdot 10^6$ B cell-stimulatory factor-2 units/mg of protein. Oligonucleotides were synthesized by Eurogenetec (Seraing, Belgium).

Western Blot

For the isolation of cellular proteins confluent cell cultures were lysed in 500 μ l lysis buffer (50 mM Tris/HCl, pH 7.5; 150 mM NaCl; 1 % NP-40; 1 mM NaF; 1 mM Na₃VO₄) supplemented with 10 μ g/ml of each aprotinin, pepstatin, and leupeptin as well as 1 mM pefabloc. Proteins were separated by SDS-PAGE and transferred to a poly(vinylidene difluoride) (PVDF) membrane (PALL, Dreieich, Germany). Antigens were detected by incubation with specific primary antibodies (1:1000) and horseradish-peroxidase (HRP)-coupled secondary antibodies (1:2000) (DAKO, Hamburg, Germany). The membranes were developed with an enhanced chemoluminescence kit GE-Healthcare (Munich, Germany).

Cell culture

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

Reverse transcription reaction (RT)

Total RNA isolation was done according to the manufacturer's instructions using RNAeasy kit provided by Qiagen (Hilden, Germany). The reverse transcription reaction was performed using the first strand cDNA synthesis kit (Roche, Ingelheim, Germany). 4µg of RNA was transcribed into cDNA and used for determining the prostaglandin receptor profile in NHDF cells according to manufacturer's instructions. 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 prostaglandin receptors EP1-EP4 was performed using the following program (35 cycles): 120s at 94°C for the first denaturation, 30 s at 94°C for denaturation, 30 s at 55°C for primer annealing, 40 s at 72°C for extension and 10 min at 72°C the final extension. The amplicons were analysed by agrose gel gelectrophoresis.

Primers used [34]: **EP1**fw: TCTAC CTCCC TCCAG CGGCC ACTG; **EP1**rev: GAAGTG GCTGA GGCCG CTGTG CCGGG A; **EP2**fw: TTCAT CCGGC ACGGG CGGAC CGC; **EP2**rev: GTCAG CCTGT TTACT GGCAT CTG; **EP3**fw: GAGCA CTGCA AGACA CACAC GGAG; **EP3**rev: GATCT CCCAT GGGTA TTACT GACAA; **EP4**fw: CCTCC TGAGA AAGAC AGTGT C; **EP4**rev: AGGAC TCAGA GAGTG TCTT.

Determination of MCP-1 expression

MCP-1 in the supernatant of NHDF was determined by with a quantitative sandwich enzyme immunoassay according to the supplier's manual (R&D, Wiesbaden-Nordenstadt, Germany). Briefly, MCP-1 antibodies were immobilised in microtiter plates. MCP-1 standard solutions and the supernatant from stimulated NHDF were applied to the pre-coated wells and incubated for 2h at RT. Unbound proteins were washed away and HRP-conjugated MCP-1-antibody was added to each well and incubated for 1h at RT. Following washing steps, HRP substrate was added and further incubated at RT for 20 min. The reaction was stopped and absorbance was measured in the microtiter plate reader (Molecular Dynamics, Krefeld, Germany) 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.

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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 act anti-inflammatory, 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 normal human dermal fibroblasts (NHDF) were preincubated with the phosphodiesterase inhibitor IBMX to block the breakdown of intracellular cAMP generated by PGE1. It is essential to add the phosphodiesterase inhibitors also to those samples without PGE1 to consider potential effects of basal cAMP in the absence of PGE1 (1st and 2nd bar). Whereas stimulation with IL-6/sIL-R results in a strong increase in MCP-1 gene expression (2nd bar) when compared to the non-stimulated control cells (1st bar), the IL-6-dependent MCP-1 expression was reduced in the presence of PGE1 (4th bar). These data further demonstrate the physiological impact of PGE1 on IL-6-induced gene expression.

MAPK activation but not STAT-activation by interleukin-6 is inhibited in the presence of forskolin

Prostaglandin E1 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 normal human dermal fibroblasts with interleukin-6 and the soluble IL-6-receptor for up to 60 minutes in the presences 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 (figure 2, 1st and 2nd panel). As a loading control we re-stained the blot for ERK1/2 and STAT3 (3rd and 4th panel). Figure 2 demonstrates that ERK1/2 phosphorylation is detectable 15 minutes after stimulation with IL-6/sIL-6R and fades out after one hour (1st panel, first 5 lanes). Remarkably, ERK-phosphorylation is severely impaired in cells treated with forskolin (1st panel, right half of the blot), whereas STAT3 phosphorylation (2nd panel) is hardly affected by treatment with forskolin. Inhibition of IL-6-dependent ERK-activation by forskolin treatment could be confirmed in murine embryonal fibroblast (MEF) (suppl. figure 1) suggesting that our observation is not only specific for human fibroblasts. These results indicate that stimuli inducing cAMPdependent 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_i -protein coupled receptors by freezing G_i 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 toxintreated NHDF cells (1st panel, right part), whereas STAT3 activation is hardly affected by the toxin (2nd panel). Again, inhibited ERK-activation but unchanged STAT3 activation in response to IL-6 could be confirmed in pertussis toxintreated MEFs (suppl. figure 2). These data suggest that activation of adenylate cyclase counteracts specifically 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

To test whether prostaglandin E1 influences IL-6-induced ERK-activation similarly, we monitored IL-6-dependent ERK-activation in whole cell lysates of NHDF in the presence or absence of prostaglandin E1 (PGE1). Figure 4 shows that PGE1 blocks ERK activation after IL-6 treatment (upper panel) but not IL-6-dependent STAT3 phosphorylation (2^{nd} panel).

To test whether inhibition of the MAPK-cascade has any relevance for IL-6induced MCP-1 expression, we compared MCP-1 induction by IL-6 in the absence or presence of the MEK-inhibitor UO126 (figure 5). Whereas stimulation with IL-6/sIL-R induces MCP-1 gene expression in the absence of UO126 (2nd bar), the presence of UO126 strongly reduces the potential of IL-6 to induce MCP-1 expression (4th bar). These data indicate a crucial role of the IL-6-intiated MAPK-cascade for the induction of MCP-1.

The PGE1-receptor EP2 is expressed on NHDF cells and its activation counteracts IL-6-induced ERK activation and MCP-1 expression

We went on to elucidate the molecular mechanisms involved in PGE1-dependent inhibition of IL-6-induced MAPK activation. First, we clarified which EPreceptor is involved in PGE1-mediated repression of ERK activation in IL-6stimulated fibroblasts. We analysed the expression of EP1 to EP4 by an RT-PCR assay (figure 6A) and found EP1, EP2 and EP3 to be expressed in NHDF. Since EP2 and EP4 are known to activate adenylate cyclase we focussed on EP2 and used (R)-butaprost as a specific EP2 agonist. Similar to forskolin and PGE1, (R)butaprost inhibited IL-6-dependent ERK activation (figure 6B, upper panel) without affecting STAT3 phosphorylation (2nd panel). These data strongly suggest that PGE1 suppresses IL-6-dependent ERK activation through the EP2 receptor.

In line with these observations, we tested whether butatprost is potent to inhibit IL-6-dependent MCP1 expression. Figure 6C shows that MCP-1 protein expression induced by IL-6/sIL-6R is reduced in the presence of the EP2-agonist butaprost (compare 2^{nd} and 4^{th} bar). These data further indicate that IL-6-dependent MCP-1 expression can be inhibited by EP2 activation.

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 [35]. Thus, we tested whether the Src-kinase inhibitor PP1 restores IL-6-induced ERK activation in the presence of PGE1 (figure 7, upper panel). Surprisingly, the inhibitory potential of PGE1 was even more pronounced in the presence of PP1, clearly indicating that Src kinase activity is 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 directly activate protein kinase A (PKA) as well as the guanine-nucleotide exchange factor Epac. Our next aim was to elaborate which of these cAMP targets has the potential to inhibit IL-6-induced ERK-activation. We treated NHDF with either the specific agonist for PKA (N-6Phe-cAMP) or the specific agonist for Epac (8 CTP2-O-Me-cAMP) and monitored ERK-phosphorylation after stimulation with IL-6 (figure 8A). STAT3-phosphorylation was not affected by both agonists (2nd panel). In contrast, the PKA-agonist N-6Phe-cAMP inhibited IL-6-initiated ERK phosphorylation, whereas the Epacagonist 8 CTP2-OMe-cAMP did not affect ERK activation after IL-6 stimulation (upper panel). These observations suggest that not activation of Epac but activation of PKA by cAMP is crucial for the inhibition of IL-6-induced ERK activation by PGE1.

To further elaborate whether PGE1 acts inhibitory by activating PKA, we treated NHDF 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 8B shows a recovery of ERK phosphorylation when the cells were treated with PGE1 in the presences of the PKA-inhibitor H89 (upper panel). Obviously, H89 overrides basal inhibition of ERK activation in the absence of IL-6. STAT3 activation was unaltered by H89 treatment (2nd panel). These results further indicate that PGE1 inhibits ERK-phosphorylation in response to IL-6 via acting through PKA.

Previous studies on growth factor signalling indicated that PKA phosphorylates and thereby inhibits the MAPKKK c-Raf-1. Thus, we tested whether PGE1 induces phosphorylation of c-Raf-1 in NHDF (figure 8C). c-Raf-1 protein was precipitated from cellular extracts of NHDF treated with PGE for 5 minutes or of untreated NHDF. Phosphorylation of c-Raf-1 was monitored by Western blotting and subsequent staining with an antibody specific for phosphorylated PKA consensus sides. The right lane in the upper panel of figure 8C indicates that PGE1 induces phosphorylation of c-Raf-1 within a protein motif representing a substrate for PKA. In summary these data suggest that PGE1 counteracts IL-6dependent 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 the organism to cope with infections, sterile injuries and other traumata. The extent of an inflammation is controlled by a set of proand anti-inflammatory cytokines as well as chemokines and non-protein mediators such as prostaglandins, NO and reactive oxygen species. Whereas much information is available in 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 prostaglandins. Previous studies focussed on the induction of cytokine expression by cAMP [36-39] whereas the study presented here 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 that specifically IL-6-initiated ERK activation is counteracted by cAMP whereas STAT3 activation is not affected. Furthermore, we show that cAMP acts through protein kinase A and c-Raf phosphorylation to inhibit the MAPK cascade whereas activation of Epac by cAMP is not involved. This detail is important since very recently Sands and coworkers reported that cAMP is able to induce SOCS3 expression through an Epac-dependent pathway in vascular endothelial cells [40]. 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 likely, the induction of SOCS3 by Epac agonists and subsequent inhibition of STAT3 activation is specific for vascular endothelial cells.

It has been known previously and also confirmed in our study (100-fold, data not shown) that the intracellular concentration of cAMP in fibroblasts increases drastically in response to PGE1 [41-43]. Accordingly, we observed a strong reduction of IL-6-dependent ERK activation in response to PGE1 in primary human dermal fibroblasts. 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 described that PGE2 augments IL-10-mediated STAT3 and STAT1 activation in THP-1 cells. In contrast, PGE2 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 our data, cAMP alone did not affect STAT activation after IL-6. In addition to these observations, we show here for the first time that PGE1 suppresses IL-6-induced ERK activation and MCP-1 gene induction.

We focussed on the mechanism how PGE1 affects IL-6-induced ERK activation. The fact that STAT3 activation by IL-6 is not affected by PGE1 argues to look for targets downstream of the activated receptor and JAK-kinases. Although de Silva and colleagues demonstrated PGE2-induced down regulation of IL-6R α expression in NFS-60 cells [45], 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 soluble IL-6R α . Furthermore, the cell surface expression of gp130 was controlled by FACS analyses (data not shown).

From previous studies by Schmitt and Stork we know that cAMP also antagonizes ERK activation by growth factors. In this context PKA, activated through elevated intracellular cAMP concentration, 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 this manuscript Stork and colleges further elucidated the mechanism how cAMP activates the MAPK cascade through B-Raf [49]. The authors demonstrate that activation of B-Raf through 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 growth factor induced MAPK cascade, dependent on the presence or absence of B-Raf.

In our study, we got no evidence for a contribution of Epac (figure 8A) or Src (figure 7) to the inhibition of IL-6-dependent ERK activation. Src rather suppresses the inhibitory activity of PGE1 than mediating its inhibitory function on IL-6-dependent ERK activation (figure 7). Instead, we demonstrate a crucial role of PKA (figures 8A and B) 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 adenylate cyclase, protein kinase A 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 prostaglandin E1 and interleukin-6.

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Figure legends

Figure 1: PGE1-inhibits IL-6-induced MCP-1 gene expression

Starved primary normal human dermal fibroblasts (NHDF) were preincubated with the phosphodiesterase inhibitor IBMX (500 μ M) for 20 min. 5 minutes prior to stimulation with 800U/ml IL-6 : 1 μ g/ml sIL-6R cells were treated with PGE1 (1 μ M) or EtOH. After 18 hours incubation, MCP-1 concentration in the medium was determined by an MCP-1 protein-specific ELISA. Results are presented in respect to IL-6/sIL-6R-treated cells. Basal MCP-1 levels were 0.81 \pm 0.32 ng/ml. This amount of MCP-1 represents the MCP-1 which accumulates in non-stimulated cells from the beginning of the experiment.

Figure 2: Forskolin counteracts IL-6-induced ERK but not STAT3 activation

Starved primary normal human dermal fibroblasts (NHDF) were pretreated with forskolin (10 μ M) for 15 min or with the solvent alone as indicated. Subsequently, cells were stimulated with 800U/ml IL-6 : 1 μ g/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 or ERK ((p)ERK1 and (p)ERK2; upper panel) or STAT3 ((p)Tyr705-STAT3; 2nd panel). ERK1/2 and STAT3 expression was additionally monitored to show equal loading of the lanes (two lowest panels).

Figure 3: MAPK activation but not STAT activation by IL-6 is inhibited in the presence of pertussis toxin

Starved primary normal human dermal fibroblasts (NHDF) were pretreated with pertussis toxin (PTX) (50ng/ml) for 16 hrs or left untreated. Subsequently, the cells were stimulated with 800U/ml IL-6 : 1μ g/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; upper panel) or STAT3 ((p)Tyr705-STAT3; 2nd panel). ERK1/2 and STAT3 expression was additionally monitored to show equal loading of the lanes (two lowest panels).

Figure 4: PGE1 counteracts IL-6-induced ERK activation

Starved primary normal human dermal fibroblasts (NHDF) were pretreated with prostaglandin E1 (PGE1) (5 μ M) for 5 min or left untreated as indicated. Subsequently, cells were stimulated with 800U/ml IL-6 : 1 μ g/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 or ERK ((p)ERK1 and (p)ERK2; upper panel) or STAT3 ((p)Tyr705-STAT3; 2nd panel). ERK1/2 and STAT3 expression was additionally monitored to show equal loading of the lanes (two lowest panels).

Figure 5: IL-6-induced MCP-1 expression requires MAPK-activation

Starved primary normal human dermal fibroblasts (NHDF) were preincubated with the MEK-inhibitor UO126 (20 μ M) for 30 min and subsequently stimulated with 800U/ml IL-6 : 1 μ g/ml sIL-6R for 1.5 hrs in the presence of UO. IL-6 : sIL-6R was eliminated by changing the medium. Time for stimulation was limited to 90 min to avoid long term accumulation of the gene products and effects of autokrine IL-6 action. After incubation for further 5 hours in cytokine-free medium in the presence of IBMX (500 μ M) and UO126 (20 μ M) 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 hours 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 (not shown).

Figure 6: PGE1 acts through the EP2 receptor

(A) Total RNA was isolated from starved primary normal human dermal fibroblasts (NHDF). RT-PCR analysis with specific primers for the EP1-4 receptors was performed and the PCR products separated in 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 NHDF were preincubated with PGE1 (5µM, 5 min) or (R)-butaprost (10 µM, 10 min) or the solvent alone. Subsequently, cells were stimulated with 800U/ml IL-6 : 1µg/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; upper panel) or STAT3 ((p)Tyr705-STAT3; 2nd panel). ERK2 and STAT3 expression was additionally monitored to show equal loading of the lanes (two lowest panels). (C) Starved NHDF were preincubated with the phosphodiesterase inhibitor IBMX (500 µM) for 20 min. 5 minutes prior to stimulation with 800U/ml IL-6 : 1µg/ml sIL-6R cells were treated with (R)butaprost (10 µM) or EtOH. After 18 hours incubation, MCP-1 concentration in the medium was determined by an MCP-1 protein-specific ELISA. Results are presented in respect to IL-6/sIL-6R-treated cells. Basal MCP-1 levels were 0.92 \pm 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.

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

Starved primary normal human dermal fibroblasts (NHDF) were preincubated with the Src-inhibitor PP1 (10 μ M) or DMSO for 40 min. 5 minutes prior to stimulation with 800U/ml IL-6 : 1 μ g/ml sIL-6R cells were treated with PGE1 (1 μ M) or EtOH. 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 or ERK ((p)ERK1 and (p)ERK2;

upper panel) or STAT3 ((p)Tyr705-STAT3; 2nd panel). ERK1/2 and STAT3 expression was additionally monitored to show equal loading of the lanes (two lowest panels).

Figure 8: PKA- but not Epac-agonists mimic cAMP effects on IL-6-induced ERK activation

(A) Starved primary normal human dermal fibroblasts (NHDF) were pretreated with the PKA agonist N6-Phenyl-cAMP (300 µM) or the Epac agonist 8-pCPT-2'-O-Me-cAMP (100 µM) for 15 min or left untreated as indicated. Subsequently, the cells were stimulated with 800U/ml IL-6 : 1µg/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 or ERK ((p)ERK1 and (p)ERK2; upper panel) or STAT3 ((p)Tyr705-STAT3; 2nd panel). ERK2 and STAT3 expression was additionally monitored to show equal loading of the lanes (two lowest panels). (B) Starved NHDF were preincubated with the PKA-inhibitor H89 (10 µM) or DMSO for 20 min. 5 minutes prior to stimulation with 800U/ml IL-6 : 1µg/ml sIL-6R cells were treated with PGE1 (1 μ M) or EtOH 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 ((p)ERK1 and (p)ERK2; upper panel) or STAT3 ((p)Tyr705-STAT3; 2nd panel). ERK2 and STAT3 expression was additionally monitored to show equal loading of the lanes (two lowest panels). (C) Starved NHDF 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 (upper panel). Precipitation of c-Raf was controlled by restaining the blot with an antibody against c-Raf-1 (lower panel).











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