Inhibition by Prostaglandin E₂ of Anaphylatoxin C5a- but Not Zymosan-Induced Prostanoid Release from Rat Kupffer Cells

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SUMMARY: The proinflammatory anaphylatoxin C5a induces the release of prostanoids, ie, prostaglandins (PG) and thromboxane (TX), from the resident liver macrophages (Kupffer cells [KC]). Because KC themselves express prostanoid receptors, prostanoids—besides having paracrine functions—might regulate their own release in an autocrine loop. So far, such a possible feedback regulation has not been investigated systematically, probably because of methodological difficulties to measure newly synthesized prostanoids in the presence of added prostanoids. Here, after prelabeling of phospholipids with [¹⁴C]arachidonate, cellularly formed [¹⁴C]prostanoids were determined in the presence of added unlabelled prostanoids by thin layer chromatography. In cultured KC, recombinant rat C5a (rrC5a) rapidly increased PGD₂, PGE₂, and TXA₂ release, which was strongly reduced by PGE₂, but neither by PGD₂ nor by the TXA₂ analog U46619. The inhibitory effect of PGE₂ was mimicked by cAMP, indicating that the G_s-coupled PGE₂ receptors type 2 or 4 were involved. Zymosan also enhanced prostanoid release from KC, but with slightly slower kinetics; this action was neither inhibited by PGE₂ nor by cAMP. Also in perfused rat livers, rrC5a enhanced prostanoid release from KC as shown by prostanoid overflow and thereby indirectly increased glucose output from hepatocytes. Again, PGE₂, but not PGD₂, inhibited rrC5a-elicited prostanoid overflow. This resulted in a complete inhibition of rrC5a-induced, prostanoid-mediated glucose output. Thus, PGE₂ can inhibit specifically the C5a-induced prostanoid release from KC via a feedback mechanism and thereby limit prostanoid-mediated hepatocellular defense reactions, eg, glucose release. (*Lab Invest* 2002, 82:463–471).

Prostanoids, ie, prostaglandins (PG) and thromboxane (TX), regulate cellular functions locally within an organ. Their synthesis in and release from certain cell types is increased by a variety of stimuli. It can be induced either acutely or transiently within minutes via G-protein-coupled receptors or tyrosine kinases and constitutive enzymes or slowly with some delay within hours mainly via tyrosine-kinase-coupled receptors and de novo expressed enzymes (Murakami and Kudo, 2001). Prostanoids are known to act locally in a paracrine fashion on other cell types. In the perfused rat liver infusion of anaphylatoxins C5a and C3a (Püschel et al, 1996; Schieferdecker et al, 1999; Schieferdecker et al, 2001) or stimulation of hepatic nerves (Gardemann et al, 1992; Jungermann and Stümpel, 1999) in a first step rapidly elicited the release of PGF_{2α}, PGD₂, PGE₂, and TXA₂ from non-parenchymal cells such as Kupffer cells (KC) or hepatic stellate cells (HSC). In a second step, the prostaglandins then increased glucose output from hepatocytes to a maximum within 3 minutes (Iwai et al, 1988) by activating glycogen phosphorylase (Athari and Jungermann, 1989; Püschel et al, 1993). In con-

trast to recombinant rat C5a (rrC5a), which very rapidly stimulated prostanoid output from KC (Hespeling et al, 1995), lipopolysaccharide (LPS) only slowly induced PGD₂ and PGE₂ release from these cells with a delay of several hours reaching maximal values after 24 hours or thereafter (Peters et al, 1990). It is not known whether this slow prostaglandin release had any effect on prostanoid-mediated reactions of other liver cell types such as glucose release from hepatocytes (HC).

KC and HSC themselves express prostanoid receptors (Fennekohl et al, 1999). Thus, it seems possible that prostanoids in a feedback mode acutely regulate their own release from nonparenchymal cells and thus exert a fine control on prostanoid-modulated reactions of other liver cell types. So far, the putative feedback regulation of prostanoid synthesis by prostanoids has not been studied systematically neither in cell cultures nor in the intact organ. Only one study with cultured KC indicated that the slow and long-term LPS-elicited increase in PGD₂ and PGE₂ release after 24 hours was inhibited by PGE₂ (Peters et al, 1990). It was not investigated whether this feedback loop on slow increase in prostaglandin release could also be operative in the intact liver. The lack of more detailed information on short-term and long-term feedback inhibition of prostanoid synthesis by prostanoids is probably due to analytical difficulties. Prostanoids are normally quantified by ELISA, RIA, or HPLC. With

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immunoassays, and also with chromatographic techniques, it is impossible to measure the release of a given prostanoid when the same prostanoid is added to test its regulatory role. Very often it is equally impossible to determine the release of a given prostanoid when another prostanoid is applied in a relatively high concentration as a putative effector because of high immunological cross-reactivity or insufficient chromatographic separation.

It was the aim of the present study to investigate the feedback regulation of prostanoid synthesis by prostanoids and the possible consequences for the regulation of prostanoid-dependent glucose output in liver using cell cultures and the perfused organ. C5a and zymosan were chosen to acutely stimulate prostanoid release from KC. As a prerequisite for the study, a radiochemical method was developed allowing the measurement of released labeled prostanoids in the presence of higher levels of added unlabeled prostanoids given as putative effectors: KC were prelabeled with [$1\text{-}^{14}\text{C}$]arachidonate and the released labeled prostanoids were separated by thin layer chromatography (TLC). It was found that C5a-, but not zymosan-induced prostanoid release from rat KC, was specifically inhibited by PGE_2 , most probably in a cAMP-mediated process via the G_s protein-coupled PGE_2 receptors subtype 2 and 4 (EP2R and EP4R). This inhibition, which could also be demonstrated in the perfused liver as an impaired prostanoid overflow, led to a complete inhibition of C5a-induced, prostanoid-mediated glucose output from HC.

Results

Differences in rrC5a- and Zymosan-Induced Prostanoid Release from Cultured Kupffer Cells

To investigate possible feedback effects of prostanoids on their own release, first rrC5a- and zymosan-induced prostanoid secretion from cultured KC was characterized: as measured with RIA or ELISA techniques, rrC5a rapidly induced the release of PGD_2 , PGE_2 , and TXA_2 (measured as its stable decomposition product TXB_2) from these cells (Fig. 1A). For PGD_2 and PGE_2 release, comparable results were obtained by RIA/ELISA (Fig. 1A) and, after [^{14}C]arachidonate prelabeling, by TLC separation of the [^{14}C]labeled prostanoids (Fig. 1B). In contrast, TXA_2 —for unknown reasons—was only barely [^{14}C]labeled, supporting the finding that the synthesis of prostaglandins and TXA_2 are differently regulated (Dieter et al, 1989a). TXA_2 could thus not be quantified by the radiochemical method.

The rrC5a-induced prostanoid secretion was maximal within the first 2 minutes (Fig. 1B). Zymosan at this time point did not significantly stimulate prostanoid release; it caused a weak PGD_2 and PGE_2 output after 5 minutes, which was then still clearly lower than after rrC5a (Fig. 1B). The release of PGD_2 , PGE_2 , and TXA_2 after zymosan was maximal after about 60 minutes; it then reached 10-fold higher levels than after rrC5a (Fig. 1A).

Inhibition by PGE_2 and cAMP of rrC5a-, but Not of Zymosan-Induced PGD_2 , PGE_2 , and TXA_2 Release from Cultured Kupffer Cells

PGE_2 at 10 μM almost totally inhibited rrC5a-induced PGD_2 and PGE_2 release from 72 hour-cultured KC to 17% and 29%, respectively (unstimulated controls: 14% and 25% of rrC5a-stimulated values, respectively, determination by the radiochemical method) (Fig. 2). PGE_2 at 1 μM strongly reduced TXA_2 release to 36% (unstimulated cells: 17% of the rrC5a-stimulated value, determination by ELISA) (Fig. 2); higher PGE_2 concentrations could not be tested because of the high crossreactivity of PGE_2 in the TXB_2 ELISA (see Introduction, Discussion, and Materials and Methods). PGD_2 and the TXA_2 analog U46619 at 10 μM , in contrast to PGE_2 , neither positively nor negatively influenced rrC5a-induced PGD_2 and PGE_2 release from the KC cultures (Fig. 2). The effect of PGD_2 or U46619 on TXA_2 release could not be measured for technical reasons (see above).

Not only in KC, but also in 48 hour-cultured HSC, PGE_2 at 1 μM clearly reduced C5a-induced PGD_2 output to $27 \pm 12\%$ ($n = 3$, $p < 0.05$), whereas the TXA_2 analog U46619 even at 10 μM had no influence ($86 \pm 7\%$; $n = 3$, not significant) (data not shown). These findings with HSC indicate, that the inhibitory effect of PGE_2 on prostanoid release was specific for the prostaglandin, ie, PGE_2 , but not for the cell type.

Surprisingly, PGE_2 did not significantly influence PGD_2 , PGE_2 , and TXA_2 release from KC induced by zymosan at 0.5 mg/ml (Fig. 3). Also with a lower zymosan concentration of 0.1 mg/ml, which induced prostanoid synthesis submaximally to a similar extent as rrC5a, PGE_2 failed to reduce prostanoid release (data not shown). Thus, the inhibition of prostanoid synthesis by PGE_2 was also specific for the stimulus, ie, for C5a rather than for zymosan.

Because the EP2R and EP4R, which are expressed by KC (Fennekohl et al, 1999), are coupled to G_s -proteins enhancing intracellular cAMP levels, the effect of CPT-cAMP (a membrane-permeable and stable derivative of cAMP) on rrC5a- and zymosan-induced prostanoid release was investigated. CPT-cAMP (100 μM), like PGE_2 , reduced rrC5a-induced PGD_2 , PGE_2 , and TXA_2 release from KC to about 42%, 41%, and 44%, respectively (unstimulated controls: 14%, 25%, and 17% of the rrC5a-stimulated values, respectively, see Fig. 2) (Fig. 3). This result strongly suggests that the inhibition of prostanoid release by PGE_2 was regulated via the EP2R or EP4R. Zymosan-induced prostanoid release was neither influenced by CPT-cAMP (Fig. 3) nor by another cAMP-analog, dibutyryl-cAMP (db-cAMP) (not shown). This confirmed that the inhibitory effect of the cAMP-analogs was specific for the stimulus C5a.

Inhibition by PGE_2 of rrC5a-Induced Prostanoid Release in Isolated Perfused Rat Livers

It was then investigated whether PGE_2 would also inhibit C5a-induced prostanoid release from KC and HSC in the intact liver and not only from the isolated

Figure 1.

Kinetics of PGD₂, PGE₂, and TXA₂ release stimulated by rrC5a or zymosan in cultured Kupffer cells (KC). KC were cultured for 72 hours with medium changes every 24 hours. Newly formed prostanoids were determined by immunological (A) or radiochemical (B) techniques. If the latter method was to be applied, 0.5 μ Ci [¹⁴C]arachidonate were added for the last 20 hours before the experiment (B). KC were preincubated for 10 minutes in RPMI/1% penicillin-streptomycin (A) or in HEPES-buffered HBSS (B) and then stimulated with 1 μ g/ml rrC5a or 0.5 mg/ml zymosan. At the indicated time points, aliquots of the supernatants were shock-frozen, and prostanoid concentrations were determined later either immunologically by RIA (PGD₂) and ELISA (PGE₂ and TXB₂ as the stable decomposition product of the actually released TXA₂) (A) or radiochemically after ethylacetate extraction and separation by thin layer chromatography (TLC) (B). A, Shows increases in prostanoid release into the medium calculated as values of stimulated probes minus those of unstimulated controls. Note the different scales on the ordinates. Values are means $\pm$ SEM of three independent experiments; * $p < 0.05$, ** $p < 0.01$ = significant differences compared with zero time values (Student's *t* test for unpaired samples). At 0 minutes, absolute levels of released prostanoids were 0.23 ± 0.02 pmol PGD₂/ug DNA, 0.07 ± 0.01 pmol PGE₂/ug DNA, and 0.08 ± 0.01 pmol TXA₂/ug DNA. B, Shows the release of [¹⁴C]labeled PGD₂ and PGE₂ into the medium in pCi/10⁶ KC. Note the logarithmic scales on the ordinates. Values are means $\pm$ SEM of three independent experiments.

nonparenchymal cells in culture (Fig. 2 and data not shown). Prostanoids released in the intact organ have a dual fate: they will enter the HC via the prostaglandin

Figure 2.

Inhibition of rrC5a-induced PGD₂, PGE₂, and TXA₂ release by PGE₂ but not by PGD₂ or the TXA₂ analog U46619 in cultured KC. KC were cultured for 72 hours and prelabeled with [¹⁴C]arachidonate as described for Figure 1B. They were preincubated in HEPES-buffered HBSS for 10 minutes and then stimulated with 1 μ g/ml rrC5a with or without 10 μ M PGD₂, U46619, or PGE₂ (1 μ M for measurement of TXA₂ release to minimize cross-reactivity in the TXB₂ ELISA). After 30 minutes, the supernatants were shock-frozen and prostanoid release was determined either by the radiochemical method (PGD₂, PGE₂) or by ELISA (TXB₂). Values for rrC5a-stimulated KC were set equal to 100%. They are means $\pm$ SEM of three to five independent experiments (numbers in parentheses); * $p < 0.05$, ** $p < 0.01$ = significant differences compared with rrC5a-stimulated controls (Student's *t* test for unpaired samples). For better comparison, prostanoid release from unstimulated cells is indicated by a broken line. TXA₂ release in the presence of PGD₂ or U46619 could not be measured because of cross-reactivity of the TXB₂ ELISA with the respective prostaglandin.

transporter (Pucci et al, 1999) to be degraded there, and they will diffuse into the sinusoids to be removed from the organ with the blood stream. The resultant of prostanoid synthesis by the nonparenchymal cells and prostanoid uptake by the HC is prostanoid overflow into the hepatic vein.

In the perfused rat liver, PGE₂ lowered rrC5a-elicited prostanoid overflow when applied 2 minutes before rrC5a addition: it reduced PGD₂ and TXB₂ (the stable decomposition product of the actually released TXA₂) overflow to 20% and to 45%—from 68 to 13

Figure 3.

Inhibition of rrC5a-, but not of zymosan-induced prostanoid release by PGE₂ or cyclic AMP, in cultured KC. KC were cultured for 72 hours and prelabeled with [¹⁴C]arachidonate as described for Figure 1B. They were preincubated in HEPES-buffered HBSS for 10 minutes and then stimulated with 1 μ M rrC5a or 0.5 mg/ml zymosan with or without 10 μ M PGE₂ (1 μ M for measurement of TXA₂ release to minimize cross-reactivity in the TXB₂ ELISA) or 100 μ M CPT-cAMP. After 30 minutes, the supernatants were shock-frozen and prostanoid release was determined later either by the radiochemical method (PGD₂, PGE₂) or by ELISA (TXB₂). Values for rrC5a- or zymosan-stimulated KC were set equal to 100%. They are means $\pm$ SEM of three to five independent experiments (numbers in parentheses); * $p < 0.05$, ** $p < 0.01$ = significant differences compared with rrC5a- or zymosan-stimulated controls (Student's *t* test for unpaired samples). For better comparison, prostanoid release from unstimulated cells is indicated by a broken line.

and from 24 to 11 pmol/g liver (AUC), respectively (Fig. 4, upper-middle versus left panels), in agreement with the inhibitory actions on prostanoid release from cultured nonparenchymal cells. PGD₂ did not lower but even increased rrC5a-stimulated prostanoid overflow: PGD₂ enhanced rrC5a-elicited TXB₂ overflow (PGD₂ overflow in the presence of exogenous PGD₂ cannot be measured) by almost fourfold—from 24 to 95 pmol/g liver (AUC) (Fig. 4, upper-right versus left panels). This unexpected increase in prostanoid overflow was probably due to a competitive inhibition by the high levels of exogenous PGD₂ of TXA₂/TXB₂ uptake into the HC via the prostaglandin transporter. Similarly, also high levels of exogenous PGE₂ would competitively inhibit PGD₂ and TXA₂/TXB₂ uptake into the hepatocytes via the prostaglandin transporter; thus, the inhibition by PGE₂ of rrC5a-dependent PGD₂ and TXB₂ overflow to only 20% and 45%, respectively

Figure 4.

Inhibition by PGE₂, but not by PGD₂, of rrC5a-induced prostanoid overflow as well as glucose release and flow reduction in isolated perfused rat livers. Isolated rat livers were perfused in a nonrecirculating manner with Krebs-Henseleit bicarbonate buffer equilibrated with O₂:CO₂ (19:1). After 30 minutes preperfusion, rrC5a was infused for 30 seconds to a final concentration of 1 μ M. When indicated, PGE₂ or PGD₂ were applied to final inflow concentrations of 10 μ M starting 2 minutes before rrC5a addition over a total period of 6 minutes. In the fractionated effluent, PGD₂ and TXB₂ were determined by ELISA and RIA, respectively, and glucose in a standard assay with glucose dehydrogenase. Flow rate was measured by quantitating the effluent. Filled circles show effects of rrC5a in the absence (left panels) or presence (middle and right panels) of PGE₂ or PGD₂, respectively; open circles show the actions of PGE₂ or PGD₂ alone. The alterations caused by rrC5a alone in the absence and those elicited by rrC5a in the additional presence of PGD₂ or PGE₂ are shown as hatched areas. Absolute values of the areas under the curves (AUC) (pmol/g, μ mol/g, or ml/g) are given numerically. Values are means $\pm$ SEM of three independent experiments. Cannot be measured: PGD₂ overflow in the presence of PGD₂ cannot be measured by ELISA techniques.

(Fig. 4, upper-middle versus left panels), probably reflects a much higher impairment of prostanoid release from the nonparenchymal cells.

Inhibition by PGE₂ of rrC5a-Induced Prostanoid-Mediated Glucose Output and Flow Reduction in Isolated Perfused Rat Livers

In the perfused rat liver, rrC5a previously had been shown to increase glucose output from HC and to reduce flow via prostanoids released from KC and HSC (Schieferdecker et al, 1999). Therefore, it was finally studied whether PGE₂ could act as a feedback inhibitor in these prostanoid-mediated hepatic reactions. In the perfused rat liver, exogenous PGE₂ alone

or PGD₂ alone, like rrC5a alone, increased glucose output and reduced flow (Fig. 4, lower panels). When rrC5a was applied 2 minutes after PGE₂, it did not cause an additional increase in glucose output and in flow reduction (Fig. 4, lower-middle versus left panels); apparently, PGE₂ inhibited the rrC5a-elicited, prostanoid-mediated metabolic and hemodynamic effects. In contrast, when rrC5a was applied 2 minutes after PGD₂, it caused an additional increase in glucose output and in flow reduction (Fig. 4, lower-right versus left panels); apparently, PGD₂ did not reduce the rrC5a-elicited, prostanoid-mediated metabolic and hemodynamic changes. Moreover, in the presence of PGD₂ the rrC5a-dependent increase in glucose output and reduction of flow were greater than in its absence, ie, 3.3 versus 2.0 μmol glucose/g liver and -1.7 versus -0.5 ml/g liver, respectively (Fig. 4, lower-right versus left panels). These changes were in line with the enhanced rrC5a-dependent prostanoid overflow in the presence of PGD₂ reflecting a higher availability of prostanoids in the liver (Fig. 4, upper-right panel).

Thus PGE₂ not only inhibited the rrC5a-elicited prostanoid release from nonparenchymal cells in culture (Fig. 2) and in the perfused liver (Fig. 4, upper panels), but it also impaired the rrC5a-dependent, prostanoid-mediated metabolic and hemodynamic reactions of the organ (Fig. 4, lower panels).

Discussion

In the present study it was shown that PGE₂, but not PGD₂ or TXA₂, acutely inhibited the rapid C5a-elicited prostanoid release from rat KC and HSC in culture (Fig. 2 and data not shown) and in the perfused rat liver (Fig. 4, upper panels), and that this inhibitory action of PGE₂ could be mimicked by cAMP (Fig. 3). Furthermore, it was found that PGE₂ or cAMP did not inhibit the rapid zymosan-stimulated prostanoid release (Fig. 3). Finally, it was demonstrated that in the perfused rat liver, PGE₂, but not PGD₂, inhibited the short-term, C5a-induced prostanoid-mediated increase in glucose output and reduction of flow (Fig. 4, lower panels).

Feedback Inhibition of Prostanoid Formation by PGE₂: Analytical Difficulties

So far, no information on a possible short-term feedback regulation of prostanoid formation by prostanoids has been available. This is probably due to analytical difficulties. The basal or stimulated release of a given prostanoid from a cell cannot be measured in the presence of the same prostanoid added as a putative effector using standard ELISA and RIA or HPLC and TLC methods. Very often, with the standard techniques it cannot be measured either in the presence of higher levels of another effector prostanoid because of cross-reactivities in the immunological tests or insufficient separation. Only a few combinations of cellularly-synthesized and of added prostanoids can be analyzed at the given concentrations using immunoassays.

To study a possible short-term feedback regulation of prostanoid synthesis and release by prostanoids, a radiochemical analytical procedure was developed. Cellular phospholipids as prostanoid precursors were prelabeled with [1-¹⁴C]arachidonate. The newly synthesized and released [¹⁴C]labeled prostanoids were separated by TLC. Quantification of PGD₂ and PGE₂ by this radiochemical technique yielded results comparable with those obtained with the immunoassays (Fig. 1, A and B). For unknown reasons, released TXA₂ was hardly labeled and could thus not be quantified by the radiochemical technique. However, the release of TXA₂ from the cultured cells resulting in accumulation of TXB₂, the stable TXA₂ decomposition product in the media could be measured with a TXB₂ ELISA in the presence of up to 1 μM PGE₂ because of a relatively low cross-reactivity of this immunoassay with PGE₂ (Figs. 2 and 3). The release of TXA₂ in the perfused liver resulting in TXB₂ overflow could also be measured even at the high PGE₂ and PGD₂ inflow concentrations of 10 μM , because more than 90% of a given prostanoid has been shown to be degraded during a single liver passage (Muschol et al, 1991; Tran-Thi et al, 1991); the same holds for PGD₂ overflow in the presence of the high PGE₂ inflow concentration of 10 μM (Fig. 4, upper panels).

Feedback Inhibition of Prostanoid Formation by PGE₂: Specificity for the Prostanoid but Not for the Cell Type

RrC5a stimulated the release of PGD₂, PGE₂, and TXA₂ from cultured KC rapidly within the first 2 minutes (Fig. 1), in line with previous studies, which in addition showed an increase in PGF_{2 α} release (Hespeeling et al, 1995). Only PGE₂, but neither PGD₂ nor the TXA₂ analog U46619, acutely inhibited the C5a-induced PGD₂ and PGE₂ release from KC (Fig. 2), analogous to the inhibition by PGE₂, but not PGD₂, of the slow and delayed prostanoid formation induced by LPS (Peters et al, 1990). CPT-cAMP had a similar effect on C5a-induced prostanoid release as PGE₂ (Fig. 3). This strongly suggests that the short-term feedback inhibition of prostanoid release by PGE₂ was mediated by G_s-coupled, cAMP-enhancing EP2R or EP4R present in KC (Fennekohl et al, 1999). More direct evidence for the role of these PGE₂ receptor subtypes in the inhibition of C5a-induced prostanoid release from KC (Figs. 2 and 3) and glucose output from HC in the perfused liver (Fig. 4, lower panels) would come from experiments investigating the effects of C5a on prostanoid release in cultured KC and on glucose output in the perfused liver in the presence of specific EP2R or EP4R antagonists; these, however, are not available so far. A mediation by cAMP of the effect of PGE₂ had also been proposed but not experimentally shown for LPS-induced prostanoid formation (Peters et al, 1990).

PGD₂, in contrast to PGE₂, did not inhibit the C5a-induced prostanoid release from KC (Fig. 2). This finding is in line with the very low expression of G_s-coupled, cAMP-elevating PGD₂ receptors (DPR) in KC (Fennekohl et al, 1999). The TXA₂ analog U46619

did not impair the C5a-elicited prostanoid formation in KC neither (Fig. 2), in line with the observation that TXA₂ acts via G_o-coupled TXA₂ receptors (TPR) (Fenekohl et al, 1999), enhancing inositol-1,4,5-trisphosphate rather than cAMP.

The short-term inhibitory effect of PGE₂ on C5a-induced prostanoid release was not cell specific: also in HSC only PGE₂, but not the TXA₂ analog U46619, reduced C5a-induced PGD₂ release to similar levels as in KC. Information on the cell specificity of the long-term inhibition by PGE₂ of LPS-induced prostanoid formation is not available.

Feedback Inhibition of Prostanoid Formation by PGE₂: Specificity for the Stimulus

Only the C5a-, but not the zymosan-induced prostanoid release, was inhibited by PGE₂ and CPT-cAMP (Fig. 3). This was not due to the higher amounts of prostanoids released after zymosan stimulation (Fig. 1), because PGE₂ also failed to inhibit prostanoid release stimulated by zymosan concentrations, which enhanced prostanoid output to comparable levels as C5a (see Results, data not shown). In contrast to the present data, Dieter et al, 1989b showed a partial inhibition of zymosan-induced prostanoid release from KC by db-cAMP. These differences cannot be ascribed to differences in the experimental procedure, because neither preincubation with CPT-cAMP nor the use of db-cAMP—a membrane permeable, but with respect to CPT-cAMP, less stable derivative of cAMP—reduced zymosan-dependent PGD₂ release from KC (see Results, data not shown).

The differences in the kinetics (Fig. 1B) and in the inhibition by PGE₂ or cAMP (Fig. 3) between C5a- and zymosan-induced prostanoid release implicate different intracellular signaling cascades and/or activation of different (iso-)enzymes used by the two stimuli. PGE₂ via cAMP could thus impair the signal transduction from the C5a receptor to the first major rate limiting enzyme of prostanoid synthesis, phospholipase A₂ (Decker, 1990), or it could inhibit an isoen-

zyme of phospholipase A₂ specifically activated by the C5a—but not by the zymosan-stimulated signaling chain (Fig. 5).

Feedback Inhibition of Prostanoid Formation by PGE₂: Functional Significance in Liver

In the perfused rat liver, PGE₂ at an inflow concentration of 10 μM strongly reduced PGD₂ and TXB₂ overflow into the hepatic vein (Fig. 4, upper-middle and left panels). This reduction of overflow was most probably due to a PGE₂-dependent inhibition of PGD₂ and TXA₂ release mainly from KC (Figs. 2 and 3) and also from HSC (not shown) in line with the respective findings with the cultured nonparenchymal cells. A possible PGE₂-dependent increase in PGD₂ and TXA₂/TXB₂ uptake into the HC via the prostaglandin transporter contributing to the lowering of prostanoid overflow is highly unlikely.

The circulating concentrations of prostanoids are in the nanomolar range (Wernze et al, 1986). In the liver, locally in the space of Disse, the concentrations of prostanoids released from nonparenchymal cells were calculated to reach the 1 to 10 micromolar range within 1 minute after stimulation (Neuschäfer-Rube et al, 1993). The short-term increase in glucose output and decrease in flow by exogenous prostaglandins in the perfused rat liver were found to be half-maximal at 3 to 8 micromolar levels of prostaglandins (Iwai et al, 1988). In the present study, the PGE₂-elicited inhibition of short-term prostanoid synthesis and release from nonparenchymal cells in isolated cells (Figs. 2 and 3) and in the perfused rat liver (Fig. 4) was shown to be effective in the same concentration range. Therefore, the acute feedback inhibition of prostanoid synthesis by PGE₂ should be a functionally relevant process.

Direct evidence for such a feedback loop being operative in the whole organ would be given by the use of specific blockers of cAMP-enhancing EP2R and EP4R; such blockers, however, are not available so far. Therefore, it was shown that exogenous PGE₂

Figure 5.

Feedback inhibition by PGE₂ of C5a-induced prostanoid release from KC limiting C5a-induced prostanoid-mediated glucose output and flow reduction in the liver (in part hypothesis). C5a increases the output of PGD₂, PGE₂, PGF_{2α}, and TXA₂ from KC. PGD₂, PGE₂, PGF_{2α}, and TXA₂ have paracrine effects on hepatocytes (HC) and sinusoidal endothelial cells (SEC) or hepatic stellate cells (HSC), respectively, such as an increase in glucose output and a decrease in flow. PGE₂, in addition to its paracrine functions, reduces C5a-induced prostanoid release from KC in an autocrine manner. This negative feedback loop is mediated by the G_s protein-coupled PGE₂ receptors subtype 2 or 4 (EP2R or EP4R) via an increase of cAMP. The target of cAMP action could be the signaling chain from the C5a receptor (C5aR) to phospholipase A₂ (PLA₂) or a PLA₂ isoenzyme specifically activated by C5a.

prevented the C5a-elicited prostanoid-mediated increase in glucose output and decrease in flow (Fig. 4, lower-middle and right panels).

In summary, these data indicate that PGE₂ impaired the C5a-elicited release of PGE₂, PGD₂, and TXA₂ from KC and HSC, and thus inhibited the C5a-induced, prostanoid-mediated glucose output from HC and flow reduction in perfused rat livers. Thus, PGE₂ could, via a negative feedback loop, prevent an overshoot of prostanoid-dependent defense reactions of the liver, such as the C5a-induced, prostanoid-dependent glucose release (Fig. 5).

Materials and Methods

Animals

Male Wistar-Unilever rats (Winkelmann, Borcheln, Germany), weighing 150 to 200 g for perfusion experiments, 300 to 350 g for isolation of KC, and 400 to 500 g for isolation of HSC were kept on a 12 hour day/night rhythm with free access to water and a standard rat diet (Ssniff, Soest, Germany) for at least 2 weeks before the experiment. Treatment of the animals followed the German Law on the Protection of Animals.

Materials

Pronase was obtained from Merck (Darmstadt, Germany), collagenase H and DNase from Roche (Mannheim, Germany), and Nycodenz from Life Technologies (Eggenstein, Germany). Zymosan A from *Saccharomyces cerevisiae* and the TXA₂ analog U46619 were delivered by Sigma-Aldrich (Deisenhofen, Germany), PGD₂ and PGE₂ by Serva (Heidelberg, Germany), and chlorophenylthio-cAMP (CPT-cAMP) and dibutyl-cAMP (db-cAMP) by Roche. [³H]PGD₂-RIA and [1-¹⁴C]arachidonate (activity 50–62 mCi/mmol) were purchased from Amersham (Braunschweig, Germany). ELISAs for PGE₂ and TXB₂ were from R&D Systems (Wiesbaden-Nordenstadt, Germany), and silica gel plates (F1500, acid resistant) were from Schleicher & Schüll (Dassel, Germany). All other chemicals were of analytical grade and from commercial sources. Daltroban (BM 13.505: 4-[2-(4-chlorobenzenesulphonylamino)-ethyl] benzene-acetic acid) was kindly provided by Roche. RrC5a was prepared as previously described (Rothermel et al, 1997).

Isolation and Culture of Kupffer Cells

KC were isolated by combined pronase/collagenase perfusion and purified by density gradient centrifugation through 16.7% Nycodenz and subsequent counterflow elutriation using a Beckmann J-21 centrifuge with a Beckmann JE-6 elutriation rotor (Beckmann, München, Germany) (Brouwer et al, 1984). 4*10⁶ KC were plated on 35 mm culture dishes (Nunc, Wiesbaden, Germany) in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 30% newborn calf serum (PAA Laboratories, Cölbe, Germany) and 1% penicillin-streptomycin (Serva). KC were cultured for

72 hours, with medium changes every 24 hours, and were then > 99% pure as determined by phagocytosis of fluorescent latex beads (fluoresbrite, diameter 1 µm; Polysciences, Eppelheim, Germany) (Shaw et al, 1984) and/or intracellular staining of added diamino-benzidine (Sigma-Aldrich) by endogenous peroxidases (Widman et al, 1972).

Isolation and Culture of Hepatic Stellate Cells

HSC were obtained by Ca²⁺-free, EGTA-supplemented perfusion of the liver, followed by combined pronase/collagenase-digestion (Schieferdecker et al, 1997). HSC were concentrated at the interphase of an 8.1% Nycodenz density gradient. 0.4*10⁶ HSC were plated on 24 well culture dishes in RPMI 1640 supplemented with 30% newborn calf serum and 1% penicillin-streptomycin. HSC were cultured for 48 hours, with one medium change after 24 hours, and were then > 95% pure as determined by vitamin A autofluorescence (Suematsu et al, 1993).

Cell Culture Experiments

For experiments up to 1 hour, KC or HSC were washed with and incubated in HBSS supplemented with 20 mM HEPES, pH 7.4; for longer experiments cells were kept in RPMI 1640 with 1% penicillin-streptomycin, pH 7.4. Cells were preincubated for 10 minutes at 37° C and then stimulated with rrC5a or zymosan at final concentrations of 1 µg/ml (100 nM) and 0.5 mg/ml, respectively. When indicated, CPT-cAMP (100 µM), db-cAMP (100 µM), or PGE₂, PGD₂, as well as the TXA₂ analog U46619 (10 µM each for the detection of synthesized prostanoids by the radiochemical method, and 1 µM each for the detection by RIA or ELISA), were added simultaneously with rrC5a or zymosan. Aliquots of the cell culture supernatants were shock-frozen in liquid nitrogen and stored at -20° C until determination of prostanoid concentrations.

Perfusion of Isolated Rat Liver

Livers were perfused in a nonrecirculating manner via the portal vein with a flow rate of about 4 ml/minutes/g liver with an erythrocyte-free Krebs-Henseleit bicarbonate buffer containing 5 mM glucose, 2 mM lactate, and 0.2 mM pyruvate, equilibrated with O₂:CO₂ (19:1) at 37° C. After 30 minutes of preperfusion, livers were stimulated for 30 seconds with 1 µg/ml rrC5a. In case of prostaglandin coinfusion, 10 µM PGE₂ or PGD₂ were given over 6 minutes, starting 2 minutes before rrC5a infusion. Every minute, flow rates were determined by quantitating the effluent, and aliquots of the perfusate were taken for quantitation of glucose and prostanoid concentrations.

Biochemical Determinations

Glucose concentrations were measured with a commercial enzyme test kit using glucose dehydrogenase (granustest; Merck) (Banauch et al, 1975). DNA content

of cultured cells was determined by intercalation of bis-benzimide (Sigma-Aldrich), followed by fluorometric quantitation (Labarca and Paigen, 1980). Prostanoid concentrations in the culture media or perfusion effluats were quantified without further purification with commercial RIAs (PGD₂) or ELISAs (PGE₂ or TXA₂, which was determined as its stable decomposition product TXB₂). In the experiments for the investigation of feedback effects of added prostanoids, RIAs or ELISAs could not generally be applied because of high cross-reactivities of the immunoassays with the added prostanoids. In these cases, prostanoids were determined by the radiochemical method. Cellular phospholipids as prostanoid precursors were prelabeled by addition of 0.5 μ Ci [1-¹⁴C]arachidonate to KC cultures for 20 hours before the experiment. At the end of the experiment the newly formed [¹⁴C]labeled prostanoids were extracted from the cell culture supernatant with ethylacetate. They were then separated by thin layer chromatography (TLC) on silica plates with the organic phase of ethylacetate/water/isooctane/acetate (110:100:50:20, v/v) (Hamberg and Samuelsson, 1966). Radioactivity of the newly synthesized [¹⁴C]labeled prostanoids was determined with a phosphorimager (Storm 860; Molecular Dynamics, Krefeld, Germany) and quantified with Image Quant (Molecular Dynamics) using diluted standards of [¹⁴C]arachidonate.

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