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Biochemical Pharmacology 66 (2003) 643–652
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Inhibition of prostaglandin D_2 clearance in rat hepatocytes by the thromboxane receptor antagonists daltroban and ifetroban and the thromboxane synthase inhibitor furegrelate

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Received 24 February 2003; accepted 18 May 2003

Abstract

Prostanoids, i.e. prostaglandins and thromboxane, regulate liver-specific functions both in homeostasis and during defense reactions. For example, prostanoids are released from Kupffer cells, the resident liver macrophages, in response to the inflammatory mediator anaphylatoxin C5a, and mediate an enhanced glucose output from hepatocytes as energy supply. In perfused rat livers, the thromboxane receptor antagonist daltroban enhanced C5a-induced prostanoid overflow and reduced glucose output. It was the aim of this study to elucidate whether daltroban interfered with prostanoid release from Kupffer cells or prostanoid clearance by hepatocytes, and/or whether it directly influenced prostanoid-dependent glucose metabolism in these cells. In perfused rat livers, daltroban enhanced prostaglandin (PG)D_2 overflow not only after infusion of C5a (15-fold), but also after PGD_2 (10-fold). Neither daltroban nor another receptor antagonist, ifetroban, or the thromboxane synthase inhibitor furegrelate enhanced prostanoid release from Kupffer cells. In contrast, all inhibitors reduced clearance, i.e. uptake and degradation, of PGD_2 by hepatocytes: within 5 min uptake of 1 nmol/L PGD_2 was reduced from 43 ± 5 fmol (controls) to 22 ± 6 fmol (daltroban), 24 ± 6 fmol (ifetroban) and 21 ± 6 fmol (furegrelate). PGD_2 in the medium was reduced to $39 \pm 7\%$ in the controls, but remained at $93 \pm 9\%$, $93 \pm 11\%$ and $60 \pm 3\%$ in the presence of the inhibitors. PGD_2-dependent glucose output in the perfused liver or activation of glycogen phosphorylase in isolated hepatocytes remained unaffected by daltroban. These data clearly demonstrate that the thromboxane-inhibitors reduced PGD_2 clearance by hepatocytes, presumably by inhibition of prostanoid transport into the cells. In contrast, they did not interefere with PGD_2-dependent glucose metabolism, suggesting an independent mechanism for the inhibition of glucose output from the liver.

Keywords: Glucose metabolism; Kupffer cells; Liver perfusion; Prostaglandin transporter; Prostanoid uptake; Thromboxane synthase

1. Introduction

Prostanoids, i.e. prostaglandins (PG) and thromboxane (TX), are important mediators of cell-to-cell communication within the body. In the liver, prostanoids are exclusively released from nonparenchymal cells [1]; they control effector functions of those liver cell types expressing the respective prostanoid receptors. Prostanoids are generated by enzyme activation, and therefore can be released within seconds after stimulation. Accordingly, prostanoids are involved in the regulation of short-term reactions, such as an enhanced glucose output from hepatocytes under inflammatory conditions [1]. For anaphylatoxin C5a, an end product of the activated complement cascade [2], it has been shown that it rapidly enhanced glucose output and reduced flow in the perfused rat liver [3,4]. These effects are mediated solely by prostanoids, since the combined presence of the prostanoid synthesis inhibitor indomethacin and the TX-receptor antagonist daltroban inhibited not only C5a-induced overflow of prostanoids into the hepatic vein, but also the metabolic and hemodynamic C5a effects [4]. After stimulation with C5a, prostanoids were mainly

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Abbreviations: C5a, anaphylatoxin C5a; DMSO, dimethyl sulfoxide; GPH, glycogen phosphorylase; NA, noradrenaline; NCS, newborn calf serum; PGD_2, PGE_2, $PGF_{2\alpha}$, prostaglandin D_2, E_2, $F_{2\alpha}$; PGT, prostaglandin transporter; TXA_2 and TXB_2, thromboxane A_2 and B_2; rrC5a, recombinant rat C5a.

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doi:10.1016/S0006-2952(03)00398-8

released from Kupffer cells [5], but also from untransformed hepatic stellate cells [6]. Of the released prostanoids, only PGs but not TX *directly* induced glucose release from hepatocytes, which is in accord with the finding that hepatocytes expressed mRNA for PG-, but not TX-receptors [7]. In contrast to PGs, TX enhanced glucose output only *indirectly* as a consequence of hypoxia due to TX-dependent flow reduction [4].

Indomethacin, when given alone, only partially inhibited glucose release and flow reduction, even though by the used detection systems no prostanoids were detectable in the hepatic overflow (i.e. the perfusion medium collected behind the liver) [4]. This indicated, that also in the presence of 20 μmol/L indomethacin sufficient prostanoid levels were generated intrahepatically to allow residual metabolic and hemodynamic actions. The TX-receptor antagonist daltroban inhibited the metabolic and hemodynamic C5a actions to similar extents as indomethacin, but in contrast to indomethacin greatly enhanced PGD_2 and TXB_2 overflow 15- and 6-fold, respectively [4]. This could either be due to an enhanced release or due to an impaired elimination of prostanoids. In the liver, prostanoids are released exclusively by nonparenchymal cells [1]. They are then efficiently degraded by hepatocytes [8–10], so that during a single liver passage more than 90% of PGs infused in micromolar concentrations are eliminated [11]. Since prostanoids diffuse only poorly across biological membranes [12], a prerequisite for prostanoid degradation within hepatocytes is their carrier-mediated uptake into the cells. This uptake could be catalyzed by transport molecules such as the prostaglandin transporter (PGT), which is strongly expressed in rat liver [13]. Thus, the enhanced prostanoid overflow in the perfused liver in the presence of daltroban could be explained either by impairment of a thromboxane-dependent feedback mechansim for prostanoid release from nonparenchymal cells, such as Kupffer cells or by an inhibition of transport molecules mediating prostanoid uptake into hepatocytes as a prerequisite for their degradation.

It was shown in the present study that the enhancing effect of daltroban on C5a-dependent PGD_2 and TXB_2 overflow in the perfused liver was due to an impaired clearance rather than an enhanced prostanoid release: in isolated Kupffer cells daltroban did not enhance C5a-induced prostanoid release, and in the perfused liver it enhanced PGD_2 overflow not only after infusion of C5a, but also after PGD_2. Moreover, daltroban as well as another TX-receptor antagonist, ifetroban, and the TX synthase inhibitor furegrelate inhibited uptake as well as degradation of PGD_2 by isolated hepatocytes, supporting the hypothesis that the inhibitors reduced prostanoid clearance by hepatocytes *via* an inhibition of PG transport into the cells. In contrast, daltroban did not interfere with PGD_2-dependent activation of glycogen phosphorylase (GPH) and thus glucose output from hepatocytes.

2. Materials and methods

2.1. Chemicals

All materials were of analytical grade and from commercial sources. PGD_2 was obtained from Calbiochem-Novabiochem and [5,6,8,9,12,14,15 (*n*)-^{3}H]PGD_2 from Amersham Biosciences. Pronase was obtained from Merck, collagenase H and DNAse from Roche Diagnostics, nycodenz from Life Technologies, and percoll from Amersham Biosciences. RPMI 1640 was purchased from Biochrom, M199 from AppliChem, newborn calf serum (NCS) from PAA laboratories, and insulin and noradrenaline (NA) from Serva. Tissue culture dishes were obtained from Nunc, bisbenzimide, dexamethasone and indomethacin from Sigma-Aldrich. Radioimmunoassays for PGD_2, $PGF_{2\alpha}$ and TXB_2 (the stable derivative of the actually secreted, biologically active TXA_2, which decomposes with a half-life of only 37 s to TXB_2 [14]) were from Amersham Biosciences. Recombinant rat C5a (rrC5a) was prepared as described previously [15] and was kindly provided by Dr. Gerald Schlaf and Prof. Otto Götze, Abteilung für Immunologie, Georg-August-Universität, Göttingen, Germany.

TXA_2 receptor antagonists and synthase inhibitors were kindly provided as follows: daltroban (BM 13.505, [*p*-[2-(*p*-chlorobenzene sulfon amido)ethyl]phenyl] acetic acid): Roche, ifetroban (BMS-180291, [1*S*-(exo,exo)]-2-[[3-[4-[(pentylamino)carbonyl]-2-oxazolyl]-7-oxabicyclo-[2.2.1]hept-2-yl]methyl]-benzenepropanoic acid): Bristol Myers Squibb, furegrelate (U63.557, 5-(3-pyridinylmethyl) benzofurancarboxylic acid): Upjohn Laboratories.

2.2. Animals

Male Wistar rats (Winkelmann) were kept on a 12 hr day/night rhythm (light 7 a.m. to 7 p.m.) with free access to water and a standard rat diet (Ssniff) for at least 2 weeks before the experiments. Rats at a weight of 150–200 g were used for the perfusion experiments, which were started at about 9 a.m., i.e. after the feeding period at night to guarantee high glycogen levels in the liver. Rat weight was 350–450 g for the isolation of Kupffer cells, and 200–250 g for the isolation of hepatocytes. Treatment of the animals followed the German Law on the Protection of Animals and was performed with permission of the state animal welfare committee.

2.3. Liver perfusion

Rat livers were perfused *in situ via* the portal vein in a pressure-constant, nonrecirculating fashion with Krebs–Ringer bicarbonate buffer (pH 7.4) containing 5 mmol/L glucose, 2 mmol/L lactate and 0.2 mmol/L pyruvate at a rate of 4 mL × min^{-1} × g liver^{-1}. The buffer was equilibrated with 95% O_2 and 5% CO_2 at 37°. Livers were preperfused for 15 min with buffer and for additional

15 min with buffer containing 0.1% DMSO with either indomethacin, daltroban, ifetroban or furegrelate (20 μmol/L each, i.e. at concentrations, which in *in vitro* experiments have been shown to be effective but not toxic, cf. [4]), or the solvent DMSO alone as a control. rrC5a (final concentration 100 nmol/L) and PGD_2 (1 μmol/L) were infused at 1/15 of the total perfusion rate for 30 s and 1 min, respectively. The effluate was fractionated in 1 min intervals. Glucose was determined in these fractions using a commercial test kit (''glucose system'') from Merck. PGD_2 and TXB_2 were quantified by radioimmunoassays from Amersham Biosciences without further purification according to the instructions of the manufacturer.

2.4. Isolation and culture of Kupffer cells

Kupffer cells were isolated by combined pronase/collagenase perfusion and purified by nycodenz density gradient centrifugation and subsequent counterflow elutriation using a Beckman JE-6 elutriation rotor in a J-21 Beckman centrifuge [16,17]. 4×10^6 cells/dish were plated on 3.5 cm diameter tissue culture dishes in RPMI 1640 supplemented with 30% NCS and 1% penicillin/streptomycin. Cells were cultured for 72 hr before the experiments with medium changes every 24 hr.

2.5. Determination of prostanoid release from cultured Kupffer cells

Seventy-two hours cultured Kupffer cells were washed three times with HEPES-buffered Hanks' balanced salt solution (HBSS, 137 mmol/L NaCl, 5.4 mmol/L KCl, 1.3 mmol/L $CaCl_2$, 0.8 mmol/L $MgCl_2$, 4.2 mmol/L $NaHCO_3$, 0.34 mmol/L Na_2HPO_4, 0.44 mmol/L KH_2PO_4, 20 mmol/L HEPES and 5 mmol/L glucose, pH 7.4) and preincubated at 37° for 5 min in HEPES-buffered HBSS and for another 5 min in HEPES-buffered HBSS containing 0.1% DMSO with either indomethacin, daltroban, ifetroban or furegrelate (20 μmol/L each, cf. [4]), or the solvent DMSO alone as a control. Cells were then stimulated with 100 nmol/L rrC5a. Immediately and at different time points, samples of the medium were taken from the supernatant and shock frozen in liquid nitrogen for the later determination of prostanoid concentrations. Prostanoids were determined by radioimmunoassays without further purification (see Section 2.3). Cell dishes were shock frozen in liquid nitrogen and Kupffer cells were collected using a rubber-policeman. DNA was determined in a fluorescence assay based on the intercalation of bis-benzimide into DNA [18].

2.6. Isolation and culture of hepatocytes

Hepatocytes were prepared according to Meredith by Ca^{2+}-free liver perfusion without the use of collagenase [19] as described previously [20]. For the experiments with cultured hepatocytes, cells were plated at 0.5×10^6 cells/dish on 3.5 cm diameter tissue culture dishes in M199 supplemented with 0.5 nmol/L insulin, 100 nmol/L dexamethasone, 1% penicillin/streptomycin, and additional 4% NCS during the first 4 hr. Medium was changed after 4 and 24 hr, and experiments were performed after 48 hr. Cell viability at the end of the experiments as judged by trypan blue exclusion was >98%.

2.7. Investigation of PGD_2 clearance by freshly isolated hepatocytes

Freshly isolated hepatocytes were resuspended at 100 mg/mL in Krebs–Ringer bicarbonate buffer, pH 7.4 supplemented with 20 mmol/L glucose, 2 mmol/L lactate, 0.2 mmol/L pyruvate and 1% BSA (preincubation medium) and incubated for 90 min at 37° under continuous gentle stirring and equilibration with 95% O_2/5% CO_2. After three washing steps for 2 min at 20 *g*, cells were resuspended at 50 mg/mL in the same medium without $NaHCO_3$ but with 20 mmol/L HEPES (incubation medium). 4 mL aliquots of this cell suspension were incubated for 10 min under continuous gentle shaking in a water bath at 37° in the presence of daltroban, ifetroban or furegrelate, each 20 μmol/L in 0.1% DMSO, or 0.1% DMSO as a control. PGD_2 was then given in a volume of 100 μL to a final concentration of 25 μmol/L. Immediately and at different time points as indicated, 150 μL aliquots of the cell suspension were taken, precipitated with 30 μL ice-cold 45% TCA (final TCA concentration 7.5%) and after centrifugation for 5 min at 2500 *g*, supernatants were frozen at −70° until determination of PGD_2 concentrations by radioimmunoassays (see Section 2.3).

2.8. Investigation of PGD_2 uptake in cultured hepatocytes

Hepatocytes cultured for 48 hr were washed three times with HEPES-buffered HBSS (see Section 2.5). After 10 min preincubation at 37° in a volume of 1 mL medium in the presence of daltroban, ifetroban or furegrelate (each 20 μmol/L in 0.1% DMSO) or 0.1% DMSO as a control, medium was replaced by medium with or without inhibitors and in addition with 1 nmol/L [^{3}H]PGD_2 or 1 nmol/L [^{3}H]PGD_2 plus an excess of 25 μmol/L unlabeled PGD_2 to calculate for unspecific binding. Immediately and after the indicated time points, cells were washed three times with HBSS and harvested in a volume of 500 μL H_2O. Incorporated radioactivity was determined by scintillation counting in hydroluma (Baker).

2.9. Determination of glycogen phosphorylase activity in freshly isolated hepatocytes

Freshly isolated hepatocytes were prepared as described in Section 2.7. Cells at 50 mg/mL were then incubated for

5 min at 37° in incubation medium. Then, 20 μmol/L daltroban in 0.1% DMSO (final concentrations) or 0.1% DMSO as a control were added and incubated for another 5 min. Cells were then stimulated with 1 μmol/L PGD_2 or 1 μmol/L noradrenaline. After 2 min of incubation, cells were frozen in liquid nitrogen and glycogen phosphorylase activity was determined using a standard assay [21].

3. Results

3.1. Enhancement of anaphylatoxin C5a-induced PGD_2 and TXB_2 overflow in the perfused rat liver, but not PGD_2 and TXB_2 release in isolated Kupffer cells by the TX-receptor antagonists daltroban and ifetroban and the TX synthase inhibitor furegrelate

As has been shown previously [4], the TX-receptor antagonists daltroban and ifetroban inhibit glucose output from the perfused liver to about 40% (Fig. 1). At the same time daltroban and ifetroban strongly enhanced C5a-dependent prostanoid overflow by 15- and 10-fold (PGD_2), and by 6- and 2-fold (TXB_2), respectively (Fig. 1). The enhanced prostanoid overflow in the presence of the inhibitors could not be explained by an enhanced prostanoid release from Kupffer cells, since both daltroban and ifetroban failed to influence C5a-dependent release of PGD_2, $PGF_{2\alpha}$ and TXB_2 from isolated Kupffer cells (Table 1). Thus, most likely the TX-receptor antagonists did not enhance prostanoid overflow in the perfused rat liver by impairing a possible negative feedback effect of TX on prostanoid release from Kupffer cells. This is in agreement with recent data, showing no feedback effect of the TXA_2 analog U46619 on C5a-dependent prostanoid release from Kupffer cells [22].

In the perfused liver the TX synthase inhibitor furegrelate strongly but not completely inhibited TXB_2 overflow and enhanced PGD_2 overflow by 9-fold (Fig. 1). In isolated Kupffer cells, furegrelate almost completely inhibited TXB_2 release, and slightly increased C5a-induced PGD_2 release (Table 1). The enhancement of PGD_2 release from Kupffer cells in the presence of furegrelate was far weaker

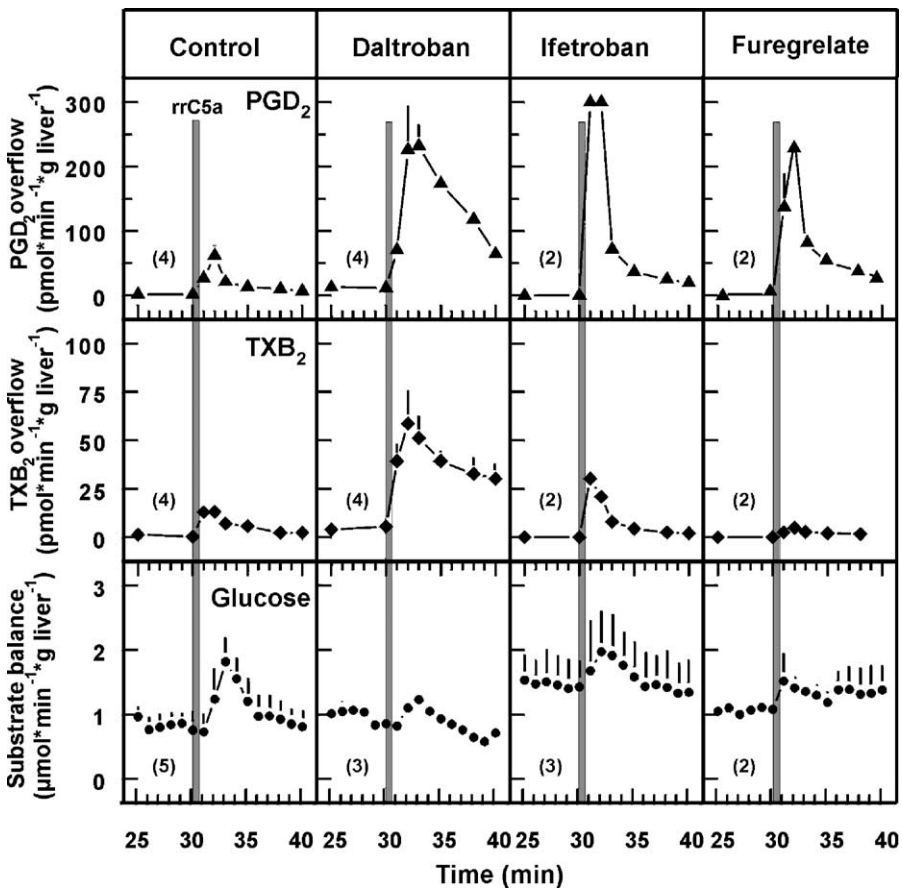

Fig. 1. Enhancement of anaphylatoxin C5a-induced prostanoid overflow in the perfused rat liver by the TX-receptor antagonists daltroban and ifetroban and the TX synthase inhibitor furegrelate. Rat livers were perfused *in situ via* the portal vein in a nonrecirculating perfusion system. After 15 min preperfusion with Krebs–Henseleit bicarbonate buffer and additional 15 min with the same buffer with or without daltroban, ifetroban or furegrelate (20 μmol/L each), rrC5a was infused for 30 s to a final concentration of 100 nmol/L. The perfusate was fractionated at 1 min intervals and prostanoid and glucose concentrations in the fractionated effluate were determined by radioimmunoassays and enzymatic tests, respectively. The labile TXA_2 was measured as the stable decomposition product TXB_2 (see Section 2). Values are means ± SEM of the number of experiments indicated in parentheses. Zero values of prostanoid overflow are given when prostanoids were below the detection limit of the assay (data correspond in part to Fig. 1 and Table 1 in [4]).

Table 1
Lack of influence of the TX-receptor antagonists daltroban and ifetroban on anaphylatoxin C5a-induced prostanoid release from isolated Kupffer cells

Stimulus (rrC5a+)	rrC5a-dependent prostanoid release (%)		
	PGD_2	$PGF_{2\alpha}$	TXB_2
None	100	100	100
Daltroban	99.4 ± 19.5	114.9 ± 30.3	87.7 ± 6.4
Ifetroban	132.2 ± 8.9	n.d.	103.3 ± 15.1
Furegrelate	161.0 ± 12.3*	n.d.	16.7 ± 4.3*
Indomethacin	3.1 ± 2.4**	14.4 ± 5.8**	8.9 ± 8.4**

Kupffer cells were isolated and cultured for 72 hr. After three washes with HEPES-buffered HBSS cells were preincubated for 10 min in the same buffer without or with daltroban, ifetroban, furegrelate or indomethacin (20 μmol/L each). Then, cells were stimulated with rrC5a at a final concentration of 100 nmol/L. Supernatants were removed after 5 min and increases in prostanoid concentrations (TXB_2 cf. Fig. 1) over basal levels at 0 min were determined by radioimmunoassays. Values of rrC5a-stimulated cells (control) were set equal to 100% and all other values are expressed as percent of rrC5a-stimulated PGD_2, $PGF_{2\alpha}$ or TXB_2 release. The values are means ± SEM of three to five experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$: significant differences compared with rrC5a-stimulated controls (Students' *t*-test for unpaired samples). n.d.: not determined (the values correspond in part to those of Figs. 2 and 3 in [4]).

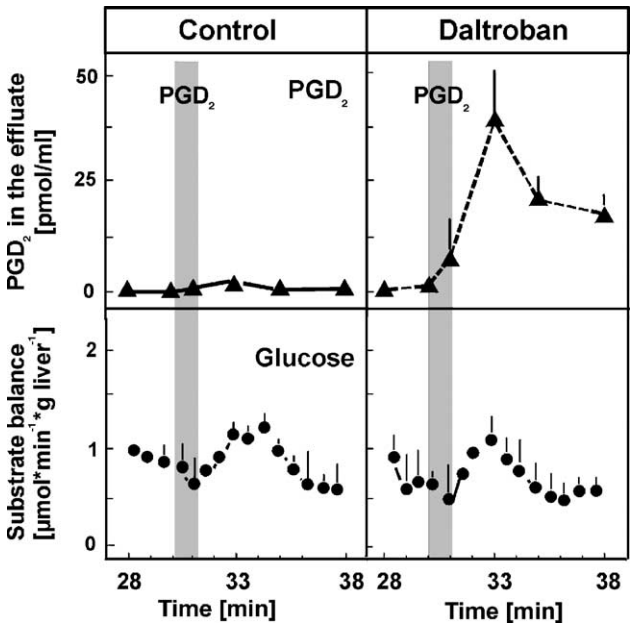

Fig. 2. Inhibition of PGD_2 elimination in the perfused rat liver by the TX-receptor antagonist daltroban. Rat livers were perfused as described in Fig. 1 and PGD_2 was infused for 1 min to a final concentration of 1 μmol/L in the absence or presence of daltroban (20 μmol/L). Glucose and PGD_2 concentrations were determined as in Fig. 1. Values are means ± SEM of four experiments.

than the increase in PGD_2 overflow in the perfused liver (less than 2-fold vs. 9-fold). Thus, the slightly enhanced PGD_2 release in the isolated cells was most likely a consequence of an enhanced substrate flow from PGH_2—the precursor of both TXA_2 and PGD_2—to PGD_2 synthase due to TX synthase blockade.

As expected, the prostanoid synthesis inhibitor indomethacin—used here as a control—almost completely inhibited C5a-dependent release of PGD_2, $PGF_{2\alpha}$ and TXB_2 from Kupffer cells (Table 1).

3.2. Enhancement of PGD_2 overflow after PGD_2 infusion in the perfused rat liver in the presence of the TX-receptor antagonist daltroban

The enhanced C5a-dependent prostanoid overflow in the perfused liver (Fig. 1) but unchanged prostanoid release from Kupffer cells (Table 1) in the presence of the TX-receptor antagonists indicated, that these substances interfered with prostanoid uptake and/or degradation in hepatocytes. If this would be the case, enhanced prostanoid levels should also be measurable after infusion of prostanoids. When 30 nmol PGD_2—corresponding to a concentration of 1 μmol/L PGD_2—were infused for 1 min into the liver *via* the portal vein (Fig. 2), PGD_2 overflow measured behind the liver in the hepatic vein was 1.0 ± 0.6 nmol PGD_2, i.e. 3% of the 30 nmol PGD_2 originally infused. This is in agreement with a previous study, demonstrating that more than 90% of prostanoids infused in micromolar concentrations were degraded during a single liver passage [11]. In contrast, daltroban enhanced PGD_2 overflow to 9.0 ± 1.8 nmol (Fig. 2), i.e. 30% of the infused PGD_2. Thus, daltroban increased PGD_2 overflow by 10-fold, clearly suggesting that daltroban interfered with hepatic PGD_2 uptake and/or degradation.

3.3. Inhibition of PGD_2 clearance by isolated hepatocytes by the TX-receptor antagonists daltroban and ifetroban and the TX synthase inhibitor furegrelate

Since prostanoids are known to be degraded by hepatocytes [8–10], in a next step a potential inhibition of prostanoid degradation by the TX-receptor antagonists was investigated on the cellular level. PGD_2 given at a concentration of 25 μmol/L to freshly isolated hepatocytes was rapidly cleared from the medium to 39 ± 7% within the first 5 min (Fig. 3). A similar clearance rate had previously been reported for the clearance of $PGF_{2\alpha}$ in hepatocytes [23]. Daltroban as well as ifetroban almost completely inhibited the short-term prostanoid clearance: after 5 min the media still contained 93 ± 9% and 93 ± 11%, respectively, of the administered PGD_2. After 10 min, controls contained only 31 ± 4% of initial PGD_2 compared to 85 ± 12% in the presence of daltroban and 73 ± 20%, in the presence of ifetroban. After 30 min, of the given PGD_2 60 ± 13% was still detectable in the presence of daltroban, while in the presence of ifetroban PGD_2 levels no longer differed significantly from those of the controls (26 ± 10% and 18 ± 3%, respectively). Thus, the TX-receptor antagonists daltroban and ifetroban inhibited PGD_2 clearance not only in the perfused liver but also in isolated hepatocytes. Also in the presence of the TX

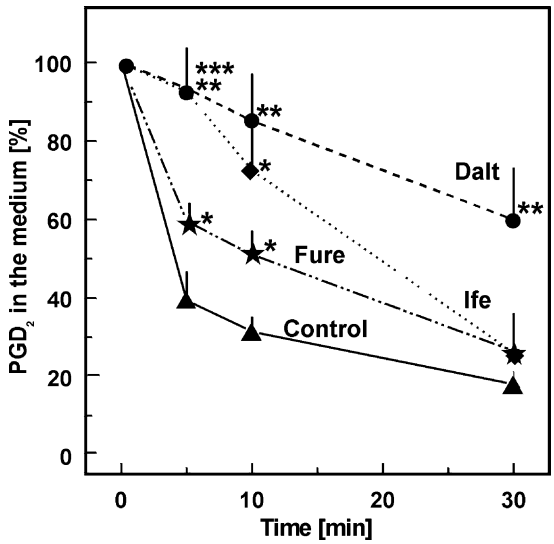

Fig. 3. Inhibiton of PGD_2 clearance by isolated hepatocytes by the TX-receptor antagonists daltroban and ifetroban and the TX synthesis inhibitor furegrelate. Freshly isolated hepatocytes were preincubated for 90 min in preincubation medium (see Section 2). After 10 min incubation in incubation medium without (control) or with daltroban (Dalt), ifetroban (Ife), or furegrelate (Fure) (20 μmol/L each), PGD_2 was given to a final concentration of 25 μmol/L. At the given time points aliquots of the cell suspensions were taken, precipitated with ice-cold TCA, and PGD_2 concentrations in the supernatants were determined as described in Fig. 1. Values were calculated as percentages of the given PGD_2 concentrations measured at 0 min and are means ± SEM of seven (controls and daltroban) or three experiments (ifetroban, furegrelate). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$: significant differences compared to control values after 5, 10 and 30 min, respectively (Student's t test for unpaired samples).

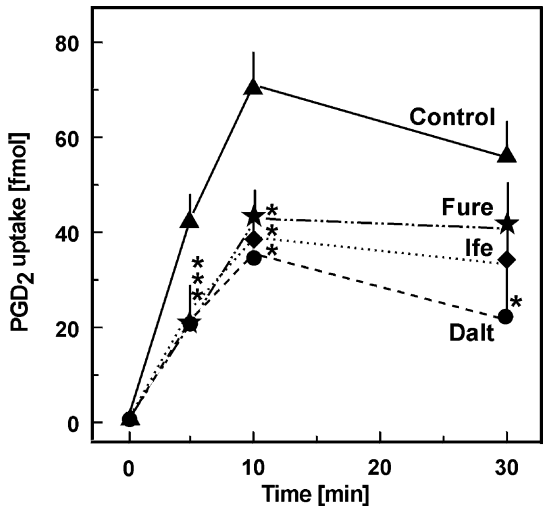

Fig. 4. Inhibiton of PGD_2 uptake in isolated hepatocytes by the TX-receptor antagonists daltroban and ifetroban and the TX synthase inhibitor furegrelate. Hepatocytes were isolated and cultured for 48 hr. After three washes with HEPES-buffered HBSS, cells were preincubated for 10 min in the same medium without or with daltroban (Dalt), ifetroban (Ife), or furegrelate (Fure) (20 μmol/L each). Then, the medium was replaced by the same medium with or without inhibitors and 1 nmol/L (600 fmol) [^{3}H]PGD_2. At the given time points, aliquots of the cell suspensions were taken and incorporated radioactivity was determined by scintillation counting. All values were corrected for unspecific uptake by measuring PGD_2 uptake in the presence of an excess (25 μmol/L) of unlabeled PGD_2. Values are means ± SEM of three experiments. $^{*}P < 0.05$, significant differences compared with control values at 5, 10 and 30 min (Students' t-test for unpaired samples).

synthase inhibitor furegrelate, PGD_2 clearance was reduced (Fig. 3): PGD_2 levels after 5 and 10 min were clearly higher than in the controls (60 ± 3% and 51 ± 6% of the infused PGD_2), but reached control values after 30 min (27 ± 2%).

3.4. Inhibition of PGD_2 uptake in isolated hepatocytes by the TX-receptor antagonists daltroban and ifetroban and the TX synthase inhibitor furegrelate

A prerequisite for prostanoid degradation by hepatocytes is their uptake into the cells. Thus, a reduced prostanoid transport in the presence of the TX-receptor inhibitors would inhibit prostanoid degradation indirectly by reducing prostanoid uptake into the hepatocytes.

After addition of 1 nmol/L [^{3}H]PGD_2 to cultured hepatocytes without inhibitors, 43 ± 5 fmol and 71 ± 7 fmol had been taken up after 5 and 10 min, respectively (Fig. 4). After 30 min, a slight decrease to 56 ± 8 fmol was observed, which was not significant as compared with the 10 min value. In the presence of daltroban, ifetroban and furegrelate, this PGD_2 uptake was strongly inhibited and only reached 22 ± 6 fmol, 24 ± 6 fmol and 21 ± 6 fmol, respectively, after 5 min, and 36 ± 9 fmol, 39 ± 6 fmol and 42 ± 6 fmol, respectively, after 10 min. Taken together, these data clearly demonstrate, that daltroban and ifetroban as well as furegrelate indeed inhibited PGD_2 uptake.

3.5. Lack of influence of the TX-receptor antagonist daltroban on PGD_2-induced glucose output in the perfused liver and activation of glycogen phosphorylase in isolated hepatocytes

In the perfused liver daltroban, ifetroban and furegrelate on the one hand enhanced C5a-dependent prostanoid overflow, but on the other hand inhibited C5a-induced glucose output (Fig. 1). Thus, possibly these compounds not only inhibited prostanoid clearance by hepatocytes, but also interfered with prostanoid-induced activation of glycogen phosphorylase in these cells thereby reducing C5a-induced glucose output. Of those prostanoids released after C5a from nonparenchymal cells [5,6], only PGs but not TX *directly* enhanced glycogen phosphorylase activity in hepatocytes [23,24]. Therefore, it was investigated, whether daltroban influenced glucose output and activation of glycogen phosphorylase induced by PGD_2, the PG released in highest amounts from Kupffer cells [5] and hepatic stellate cells [6] after stimulation with C5a. In contrast to the *indirect* C5a-induced glucose output mediated by prostanoids, the direct PGD_2-dependent glucose output was not significantly influenced by daltroban (5.8 ± 1.0 μmol × g liver^{-1} with daltroban, 7.4 ± 1.0 μmol × g liver^{-1} without daltroban)

Table 2
Lack of influence of the TX-receptor antagonist daltroban on PGD_2-induced activation of glycogen phosphorylase in isolated hepatocytes

Stimulus	Glycogen phosphorylase activity (%)	
	Without inhibitor	Daltroban
None	100	100
PGD_2	155.5 ± 26.4*	161.2 ± 32.5* n.s.
Noradrenaline	352.2 ± 109.8*	331.3 ± 97.8* n.s.

Hepatocytes were isolated and cultured for 48 hr. After three washes in HEPES-buffered saline, cells were incubated in the same buffer with 0.1% DMSO in the absence or presence of 20 μM daltroban. After 10 min, cells were stimulated with 1 μmol/L PGD_2 or 1 μmol/L noradrenaline. Supernatants were removed after 2 min and the increases in glycogen phosphorylase activity compared to controls were determined in cell homogenates as mU × μg DNA^{-1}. Values were calculated as percentages of glycogen phosphorylase activity in unstimulated cells (basal values: 2.67 ± 0.96 mU × μg DNA^{-1} without inhibitor, 2.70 ± 0.92 mU × μg DNA^{-1} with daltroban) and are means ± SEM of three experiments. *$P \leq 0.05$, significant differences compared with controls (stimulus: none), n.s.: not significant compared to values without inhibitor (Students' *t*-test for unpaired samples).

(Fig. 2). Moreover, daltroban neither increased nor decreased the enhancing effect of 1 μmol/L PGD_2 on glycogen phosphorylase activity in these cells. Daltroban also did not have any effect on NA-induced activation of glycogen phosphorylase, which was used as a control (Table 2).

Thus, the inhibiting effect of daltroban on C5a-induced prostanoid-dependent glucose output was not mechanistically linked to the reduced prostanoid clearance by hepatocytes, but was mediated by an independent pathway.

4. Discussion

In the present study it was shown, that in the perfused rat liver the TX-receptor antagonist daltroban not only enhanced the C5a-induced prostanoid overflow into the hepatic vein (Fig. 1) but also PGD_2 overflow after infusion of PGD_2 (Fig. 2). It was thus suggested, that daltroban might interfere with the elimination of prostanoids by hepatocytes. In accord with this hypothesis, neither daltroban nor another TX-receptor antagonist ifetroban interfered with prostanoid release from Kupffer cells (Table 2), but both substances efficiently inhibited PGD_2 uptake (Fig. 4) and clearance (Fig. 3) by isolated hepatocytes.

Surprisingly, even though daltroban enhanced prostanoid overflow, it inhibited C5a-dependent glucose output in the perfused liver ([4] and Fig. 1). However, daltroban did neither interfere with PGD_2-induced glucose release in the perfused liver (Fig. 2) nor with PGD_2-dependent activation of glycogen phosphorylase in isolated hepatocytes (Table 2). Thus, the enhancing effect of daltroban on C5a-dependent prostanoid overflow and the inhibiting effect on C5a-induced glucose output most probably are mediated by independent mechanisms.

4.1. TX as a mediator of C5a-dependent flow reduction and glucose output in the perfused liver

Prostanoids regulate effector functions in different liver cell types both in homeostasis and during defense reactions [1]. In response to different inflammatory mediators, such as lipopolysaccharides, zymosan or factors of the activated complement system as C5a, TX as well as the different PGs are released from nonparenchymal cells [1]. Prostanoids then act locally on those cells, which express the respective receptors. In the liver, TX-receptor mRNA has been shown to be expressed solely in nonparenchymal cells, i.e. in sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells in decreasing amounts, but not in hepatocytes [7]. In agreement with this expression pattern, TX has been shown to be involved in the regulation of hepatic flow presumably by action on sinusoidal endothelial cells [3,24–26], but not in the direct regulation of glucose output from hepatocytes [23,24].

Since hepatocytes do not express TX-receptors, neither the inhibiting effect of daltroban on C5a-dependent glucose output (Fig. 1) nor the enhancing effect of daltroban on C5a-induced prostanoid overflow (Fig. 1) could be explained by the inhibition of a direct effect of TX *via* TX-receptors on hepatocytes.

The following two hypotheses could explain the observed inhibiting effects of the TX-receptor antagonists and the TX synthase inhibitor on glucose output on the one hand and their enhancing effects on prostanoid overflow on the other hand: daltroban, ifetroban and furegralate could (i) inhibit C5a-induced glucose output *via* prevention of TX-dependent flow reduction or (ii) enhance C5a-induced prostanoid overflow *via* inhibition of prostanoid elimination by hepatocytes.

4.2. Inhibition by daltroban, ifetroban and furegrelate of C5a-induced glucose output via prevention of TX-dependent flow reduction

Daltroban, ifetroban and furegrelate had been shown previously to reduce glucose output *via* inhibition of C5a-induced flow reduction [4] and the thus resulting hypoxia [24,25]. The inhibitors reduced TX-mediated flow reduction and thereby, indirectly, hypoxia-dependent enhancement of glucose output. In the present study it was shown that there was no additional direct effect of daltroban on prostaglandin-dependent glucose output: daltroban did neither interfere with PGD_2-dependent glucose output in the perfused liver (Fig. 2) nor with PGD_2-induced activation of glycogen phosphorylase in isolated hepatocytes (Table 2). Thus, daltroban reduced C5a-dependent glucose output in the perfused rat liver only indirectly, e.g. *via* inhibition of TX-dependent flow reduction [4].

4.3. Enhancement by daltroban, ifetroban and furegrelate of C5a-induced prostanoid overflow via inhibition of prostanoid elimination by hepatocytes

Three hypotheses were developed to explain the observed increase in prostanoid overflow in the perfused liver in the presence of the TX-receptor antagonists and the TX synthase inhibitor:

(i) First it was suggested that daltroban, ifetroban and furegrelate influenced prostanoid overflow by action on Kupffer cells [4], the cell type releasing the by far highest prostanoid levels after stimulation with C5a [6]. However, it was shown that neither daltroban nor ifetroban enhanced C5a-induced prostanoid release from isolated Kupffer cells ([4] and Table 1). Also the slight increase in PGD_2 release in the presence of furegrelate most likely resulted from a redistribution of substrate flow from the precursor PGH_2 to PGs due to the blockade of TX synthase (see Section 3). Thus, the strongly enhanced prostanoid overflow in the perfused liver at least in the presence of daltroban and ifetroban and most probably also of furegrelate was not due to a direct effect of these compounds on prostanoid synthesis in Kupffer cells.

(ii) The second possibility taken into consideration was, that TX might act by reducing the size of fenestrae in the sinusoidal endothelial cells, which are known to regulate substrate exchange between blood sinusoids and the Space of Disse. A regulation of fenestrae size has previously been demonstrated for substances like nicotin [27], alcohol [28–30], serotonin [30,31] and during lipopolysaccharide-induced inflammation [32]. A TX-dependent reduction of fenestrae size would cause a retainment of directionally released PGs within the liver tissue, thereby on the one hand enhancing their efficacy, e.g. on glucose output, on the other hand facilitating their degradation by hepatocytes. Then, in the presence of daltroban, ifetroban and—perhaps—furegrelate, prostanoids would be flushed into the hepatic vein, which would explain both the reduced metabolic C5a effect and the enhanced C5a-dependent prostanoid overflow. Preliminary data showing an effect of the TXA_2-analog U46619 on fenestrae support this hypothesis [33], but have to be strengthened by the investigation of the time- and dose-dependency of possible effects of TX on fenestrae size in isolated sinusoidal endothelial cells and in the perfused rat liver.

(iii) A third explanation for the enhanced C5a-dependent prostanoid overflow in the presence of the TX-receptor antagonists and TX synthase inhibitor is an inhibition of prostanoid elimination by hepatocytes in a TX-receptor independent manner. Indeed, clear experimental evidence for this is given in the present study: daltroban, ifetroban and furegrelate inhibited uptake (Fig. 4) as well as clearance (Fig. 3) of PGD_2 by hepatocytes. Since hepatocytes do not express TX-receptors (see above), these inhibitory effects could not be explained by inhibition of a TX-receptor-dependent action on hepatocytes, as was described recently for $PGF_{2\alpha}$ receptor-dependent regulation of PG transport [34]. Thus, these substances clearly interfere with molecules different from the TX-receptor, which are involved in the transport of PGs into the cells.

4.4. Possible targets for the inhibiting effects of daltroban, ifetroban and furegrelate on prostanoid uptake into hepatocytes

In the liver, the necessary tight regulation of prostanoid availability is achieved by immediate degradation of prostanoids in hepatocytes through the microsomal cytochrome P450 system [35–37]. A prerequisite for prostanoid degradation is their facilitated uptake into the cells, since in the absence of carriers they diffuse only poorly across biological membranes [12]. In 1995, the first PG transporter was identified as a novel organic anion transporter [13] with high affinity for prostanoids, as demonstrated for PGE_2, PGD_2, $PGF_{2\alpha}$ and TXB_2 [12,13,38]. Besides the rat PGT [13], also the human [39] and mouse [40] PGT have been cloned. So far, it is not clear whether prostanoid transport into hepatocytes is mediated by the PGT or possibly any other transport system. However, indirect evidences argue for an involvement of the PGT:

(i) Even though the PGT has been postulated to be involved in the release of newly synthesized PGs, in their transepithelial transport, and in their metabolic clearance [12], experimental evidence so far has only been provided for prostanoid uptake [41]. The finding that in rat liver PGT mRNA was exclusively expressed in hepatocytes but not in nonparenchymal cells (own unpublished observations) supports a possible involvement of the PGT in prostanoid uptake into these cells.

(ii) The kinetics of PGD_2 uptake into the hepatocytes—with a maximum at 10 min and thereafter reaching an equilibrium between uptake vs. efflux and degradation (Fig. 4)—were very similar to those observed for PGE_2 uptake into HeLa cells transfected with PGT cDNA [13].

(iii) The TX analogue U46619 was not transported into HeLa cells transiently transfected with the rat PGT, but blocked the PGT by binding to it with high affinity [38]. This proposes, that a common TX-like structural motif of the different inhibitors as well as the TX mimetic U46619 might interact with the prostanoid binding site itself or with an exosite of the PGT, and thereby block the transport of any prostanoid.

(iv) Besides PGT also the liver-specific organic anion transporter (LST-1) has been reported to transport eicosanoids (including PGE_2 and TXB_2). However, its main *in vivo* function seems to be the clearance of bile acids [42,43]. Of the other members of the organic anion transporter (OATP) family, which are expressed in the liver [44–46], so far only moat1 has been reported to be involved in the transport of prostanoids.

Taken together, our data suggest that the TX-receptor antagonists daltroban and ifetroban as well as the TX synthase inhibitor furegrelate inhibit PGD_2 clearance by hepatocytes thereby causing the strongly enhanced C5a-induced prostanoid overflow in the perfused liver.

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