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Diverse functional coupling of cyclooxygenase 1 and 2 with final prostanoid synthases in liver macrophages

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Abstract

Lipopolysaccharide (LPS) treatment of resident liver macrophages resulted in a coordinated enhanced expression of cytosolic phospholipase A_2 ($cPLA_2$), cyclooxygenase (COX)-2 and prostaglandin E_2-synthase. LPS-pretreated liver macrophages showed a higher release of PGE_2 after zymosan, phorbol ester and A23187, of $PGF_{2\alpha}$ after zymosan and A23187, whereas the release of thromboxane B_2 and PGD_2 was unchanged. Inhibition of COX-1 and -2 by specific inhibitors (SC560, SC236) inhibited the prostanoid release between 50–80% and 20–40%, respectively, indicating a predominant role for COX-1. In detail (1) the zymosan-induced release of all prostanoids was inhibited to a similar degree by the COX-1 inhibitor (about 70%) and the COX-2 inhibitor (20–30%), (2) PGE_2 release after all stimuli was inhibited to a greater extent by SC560 (70–90%) compared to SC236 (5–30%), (3) the phorbol ester- and A23187-induced release of $PGF_{2\alpha}$ and PGD_2 was inhibited equally (40–50%) by both inhibitors, (3) TxB_2 release after phorbol ester and A23187 was inhibited by SC560 by 50 and 30%, and by SC236 by 50 and 70%, respectively. $cPLA_2$, COX-1 and -2, and the final prostanoid synthases were found in different subcellular fractions. These results indicate, that the functional coupling of COX-1 and -2 to final prostanoid synthases depends on the stimulation of the cells.

Keywords: Arachidonic acid; Cyclooxygenase; Kupffer cell; Lipopolysaccharide; Macrophages; Prostaglandin

1. Introduction

Liver macrophages (Kupffer cells) respond to a variety of agents with the release of biologically active mediators, including prostaglandin (PG) E_2, PGD_2, $PGF_{2\alpha}$, and thromboxane (Tx) A_2 [1–6]. The current model of the regulation of prostanoid synthesis includes several steps: (1) liberation of arachidonic acid (AA) from phospholipids by different phospholipases (PLs), (2) conversion of free AA into PGG_2/PGH_2 by COX (also known as PGH synthase or prostaglandin endoperoxide synthase), and (3) conversion of PGH_2 into the different prostanoids by discrete final prostanoid synthases [7]. In resident liver macrophages, we provided evidence for a regulation of prostanoid synthesis by the calcium-dependent $cPLA_2$, the combined action of a PLC and a diacylglycerol (DAG) lipase, and COX-1 [5,8]. In contrast to this immediate response, which is induced by, e.g. zymosan, calcium mobilizers or phorbol ester, LPS induces a delayed release (after a lag phase of several hours) of AA and prostanoids [5]. The delayed release of prostanoids in liver macrophages is paralleled by a phosphorylation and an enhanced expression of $cPLA_2$, COX-2 and prostaglandin E_2 (PGE_2)-synthase, whereas COX-1, TxA_2-, $PGF_{2\alpha}$- and PGD_2-synthase are unchanged [5,6,8,9]. Concomitantly, LPS induces a change in the profile of released prostanoids, with an enhanced formation of PGE_2 [10]. Secretory PLA_2 isoenzymes IIA and V are not involved in the release of prostanoids in these cells [11].

It has been suggested that the two COX isoenzymes are differently coupled with the specific terminal prostanoid synthases. In general, the immediate response seems to depend more on COX-1, while COX-2 is more involved in the delayed response [12]. It has been reported that the coupling of COX-1 and -2 with the terminal prostanoid

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Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; DAG, diacylglycerol; LPS, lipopolysaccharide; PG, prostaglandin; PK, protein kinase; PL, phospholipase; Tx, thromboxane.

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synthases might be affected by the concentrations of AA [12]. These results have been obtained with cells, which have been transfected with cDNAs for different prostanoid synthases. However, it has not been demonstrated that the coupling of the overexpressed prostanoid synthases with COX-1 and -2 is similar as in non-transfected cells [12].

Here, we use specific inhibitors against COX-1 (SC560) and against COX-2 (SC236) [13,14] to investigate the coupling of distinct prostanoid synthases with COX-1 and -2 in primary cultures of liver macrophages. We present evidence that the coupling of COX-1 and -2 with discrete prostanoid synthases depends on the stimulation of the cells.

2. Materials and methods

2.1. Source of reagents

RPMI medium and newborn calf serum are obtained from Biochrom. Zymosan, phorbol ester (phorbol 12-myristate 13-acetate) and A23187 are purchased from Sigma. LPS from *Salmonella minnesota* is a gift from Galanos. SC560 and SC236 are kindly provided by Searle Company. ELISA kits for the determination of TxB_2, $PGF_{2\alpha}$, PGE_2 are purchased from Biotrend and for PGD_2 from Alexis. Antibodies against TxA_2-synthase and COX-2 are purchased from Alexis and Natutec, respectively; antibodies against $cPLA_2$, COX-1, $PGF_{2\alpha}$-, PGE_2-, and PGD_2-synthase are generous gifts from Clark, Créminon, Watanabe, Jakobsson, Kanaoke and Urade, respectively.

2.2. Cell culture

Liver macrophages are isolated from male Wistar rats (250–350 g) obtained from Charles River as described earlier [15]. The cells are maintained in primary culture with RPMI medium containing 30% newborn calf serum for 72 hr. After 48 hr, 500 ng/mL LPS (LPS-pretreated cells) or vehicle (resident cells) is added for 24 hr.

2.3. Determination of the release of prostanoids

The amounts of TxB_2, $PGF_{2\alpha}$, PGE_2 and PGD_2 in the cell media are determined by specific ELISAs [6].

2.4. Preparation of cell-free extracts and Western blot analysis

Cell homogenates are prepared [5] and centrifuged at 350,000 *g* for 30 min. The soluble and membrane fraction are separated and corresponding proteins (~50 μg) obtained as described previously [5]. The blots are probed with antibodies against $cPLA_2$, COX-1, COX-2, TxA_2-, $PGF_{2\alpha}$-, PGE_2- and PGD_2-synthase. For Western blot imaging, membranes are exposed in the high performance chemiluminescence system "Genegnome" (Syngene) (16-bit cooled camera, 0-65536 grey level performance, resolution of 694 × 494 pixel); the image series capture is used and exposing time varied between 2 and 20 min. For semiquantitative analysis, Western blot images are exported as TIFF-files into ImageQuaNT® software Version 5.0 (Molecular Dynamics®/Amersham Biosciences). For quantification, rectangles or polygons are set to identify specific bands; volumes are calculated as the integrated intensity of all pixels inside these set objects. Background correction is performed using background values, such as object average (average pixel value of a selected rectangle or polygon), or local average (average of all pixel values in the set object).

2.5. RT-PCR studies

RNA is isolated with RNeasy kit (Quiagen) containing DNA digestion with RNAse free DNAse or/and RNAsin (Promega). Reverse transcription (1 μg RNA) is performed with Superscript (Gibco), dNTP's and oligo dT15 primer (Promega). For PCR analysis Taq polymerase (Gibco) and specific primers (MWG Biotech) are used.

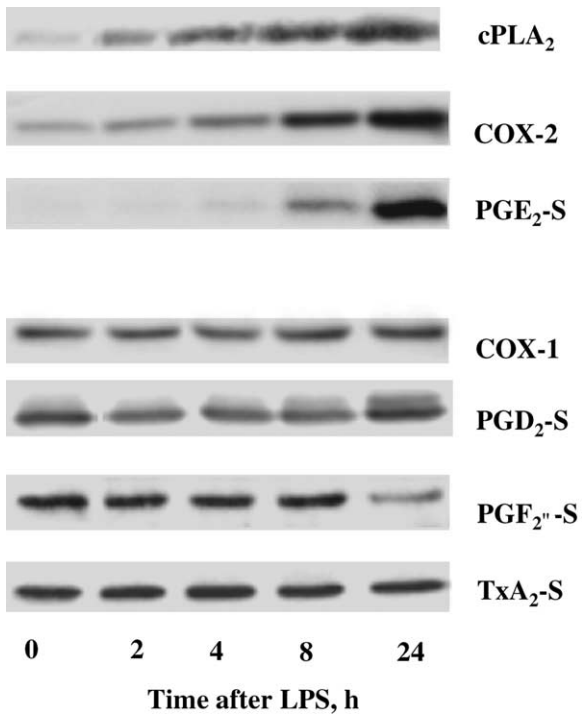

Fig. 1. RT-PCR of $cPLA_2$, COX-1, COX-2, PGE_2-, PGD_2-, $PGF_{2\alpha}$-, and TxA_2-synthase in liver macrophages. Macrophages were incubated in RPMI medium containing 10% newborn calf serum with LPS (500 ng/mL). At indicated time points, RNA was isolated and RT-PCR was performed as described in Section 2. A representative set of experiments is shown, which was reproduced at least three times. Densitometric analysis of the three experiments revealed the following data (0 hr = 100): 2 hr/4 hr/8 hr/24 hr: $cPLA_2$ (180 ± 30/260 ± 50/380 ± 20/500 ± 90), COX-2 (140 ± 30/220 ± 40/450 ± 30/650 ± 150), PGE_2-synthase (110 ± 30/140 ± 50/300 ± 50/2000 ± 600), COX-1 and PGD_2-synthase (no significant change), $PGF_{2\alpha}$-synthase (2–8 hr, no significant change, 24 hr, 50 ± 25) TxA_2-synthase (no significant change).

β-Actin (CTCCTTAATGTTCACGCACGATTTC/GTGGGGCGCCCCAGGCACCA) 94°/2 min, 52°/5 min–72°/45 s–72°/1 min (30 cycles), 72°/4° (610 bp). $cPLA_2$ (CTTACGCCACAGAAAGTTAAAAGAT/TCCAAACAAGTCAGGAGTCATAAA) 94°/2 min, 52°/5 min–60°/45 s–72°/1 min (30 cycles), 72°/4° (309 bp). COX-1 (TGCATGTGGCTGTGGATGTCATCAA/CACTAAGACAGACCCGTCATCTCCA) 94°/2 min, 63°/5 min–65°/45 s–72°/1 min (30 cycles), 72°/4° (450 bp). COX-2 (ACTCACTCAGTTTGTTGAGTCATTC/TTTGATTAGTACTGTAGGGTTAATG) 94°/2 min, 52°/5 min–55°/45 s–72°/1 min (35 cycles), 72°/4° (583 bp). PGE_2-synthase (CACACTGCTGGTCATCAAGA/CGGTTCTAATCGGACACATC) 94°/2 min, 52°/5 min–57°/45 s–72°/1 min (35 cycles), 72°/4° (603 bp).

TxA_2-synthase (ACCCAAGCTGATAGCAGACA/GTGACCATGTCAAAGGCTTC) 94°/2 min, 57°/5 min–57°/45 s–72°/1 min (30 cycles), 72°/4° (555 bp).

PGD_2-synthase (CAGAATAGAACAAGCTGACTGGCCT/CCAAAAGTGTGGTGCTGCAGATATC) 94°/2 min, 63°/5 min–63°/45 s–72°/1 min (30 cycles), 72°/4° (388 bp).

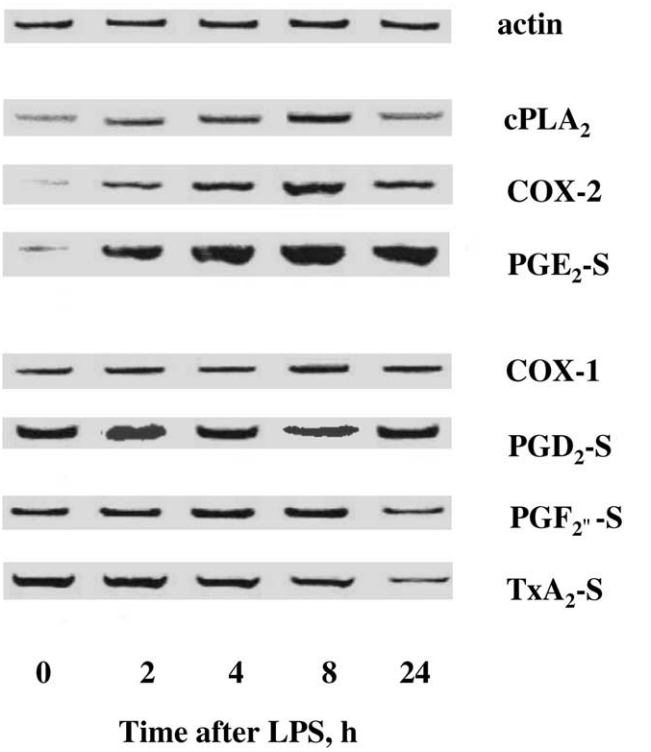

Fig. 2. Western blot analysis of $cPLA_2$, COX-1, COX-2, PGE_2-, PGD_2-, $PGF_{2\alpha}$-, and TxA_2-synthase in liver macrophages. Macrophages were incubated in RPMI medium containing 10% newborn calf serum with LPS (500 ng/mL). At indicated time points, protein was isolated and Western blot analysis was performed as described in Section 2. A representative set of experiments is shown, which was reproduced at least three times. Densitometric analysis revealed the following data (0 hr = 100): 2 hr/4 hr/8 hr/24 hr: $cPLA_2$ ($160 \pm 30/150 \pm 40/300 \pm 100/130 \pm 40$), COX-2 ($210 \pm 30/320 \pm 40/530 \pm 80/260 \pm 50$, PGE_2-synthase (>1500), COX-1 (no significant change), PGD_2-synthase (no significant change), $PGF_{2\alpha}$-synthase (2–8 hr, no significant change, 24 hr, 50 ± 30). TxA_2-synthase (2–8 hr, no significant change, 24 hr 30 ± 20).

$PGF_{2\alpha}$-synthase (CATTGCTATGGAAGAGTCAACA/CCTGCCTTCTGAAAAGATGTG) 94°/2 min, 57°/5 min–58°/45 s–72°/1 min (30 cycles), 72°/4° (314 bp).

3. Results

To investigate the relationship between $cPLA_2$, COX-2 and PGE_2-synthase, a time-course study on mRNA and protein expression is performed in liver macrophages after treatment with LPS (Figs. 1 and 2). LPS addition induces a time-dependent coordinated enhanced expression of mRNA's encoding all three enzymes with a maximum at about 8 hr; thereafter, mRNA's encoding $cPLA_2$ and COX-2 decline, whereas mRNA encoding PGE_2-synthase remains at an elevated level up to 24 hr (Fig. 1). $cPLA_2$, COX-2 and PGE_2-synthase proteins increase almost linearily from 4 to 24 hr after the addition of LPS (Fig. 2). LPS exerts only minor effects on mRNA and protein levels of

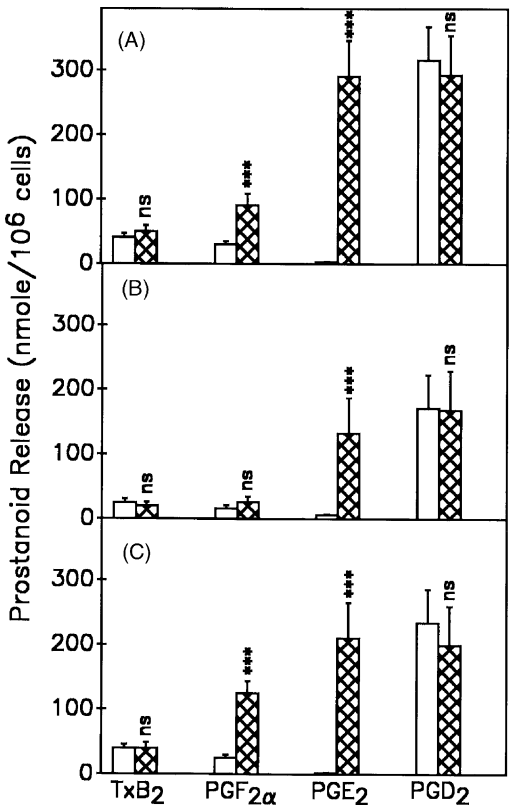

Fig. 3. Release of TxB_2, $PGF_{2\alpha}$, PGE_2 and PGD_2 from resident and LPS-pretreated liver macrophages after stimulation with zymosan, phorbol ester and A23187. Cells were kept in primary culture for 72 hr. After 48 hr, either LPS (500 ng/mL, crosshatched bars) or vehicle (open bars) were added for another 24 hr. Then, the cells were washed and incubated without or with zymosan (0.5 mg/mL, A), phorbol ester (1 μM, B) and A23187 (1 μM, C) for 60 min. Released TxB_2, $PGF_{2\alpha}$, PGE_2 and PGD_2 were measured by specific ELISAs. The basal release of prostanoids (TxB_2, $PGF_{2\alpha}$, PGE_2, PGD_2) in the absence of stimuli was <10 nmol/10^6 cells in resident and in LPS-pretreated cells. Data shown are means ± SD of four to seven experiments. *P*-values were calculated using Student's *t*-test (−LPS vs. +LPS: $^{***}P < 0.0001$; ns: not significant).

COX-1, PGD_2-, $PGF_{2\alpha}$- and TxA_2-synthases (Figs. 1 and 2); only, $PGF_{2\alpha}$- and TxA_2-synthase show a decrease after 24 hr (Figs. 1 and 2).

Fig. 3 shows the release of TxB_2, $PGF_{2\alpha}$, PGE_2 and PGD_2 from resident and from LPS-pretreated liver macrophages after the addition of zymosan, phorbol ester and A23187. The release of TxB_2 and PGD_2 after all stimuli is similar in resident and LPS-pretreated cells (Fig. 3A–C). In contrast, the release of $PGF_{2\alpha}$ is higher in LPS-pretreated cells after zymosan and A23187 (Fig. 3A and C). The release of PGE_2 is higher in LPS-pretreated cells after all stimuli (Fig. 3A–C).

The functional coupling of the two COX isoenzymes with discrete final prostanoid synthases is determined by measuring prostanoid formation in the presence and absence of specific inhibitors of COX-1 (SC560) and COX-2 (SC236) [13,14]. Recently, we showed, that in liver macrophages SC560 and SC236 specifically inhibit COX-1 and -2 at concentrations of 0.1 and 1 μM, respectively [9]. Furthermore, we demonstrated that the formation of prostanoids in resident liver macrophages is only inhibited by the specific COX-1 inhibitor SC560, but not by the COX-2 inhibitor SC236 [9]. Here, we show that in LPS-pretreated liver macrophages, the COX-1 inhibitor SC560 exhibits (a) a higher inhibitory potency for prostanoid release (TxB_2, $PGE_{2\alpha}$, PGE_2, PGD_2) after stimulation with zymosan (Fig. 4A) and (b) for the release of PGE_2 after phorbol ester (Fig. 4B) and A23187 (Fig. 4C). An almost identical inhibition with both inhibitors is observed for the release of TxB_2, $PGF_{2\alpha}$ and PGD_2 after the addition of phorbol ester (Fig. 4B), and for the release of $PGF_{2\alpha}$ and PGD_2 after the addition of A23187 (Fig. 4C). A higher inhibition with SC236 is only seen for the release of TxB_2 after the addition of A23187 (Fig. 4C).

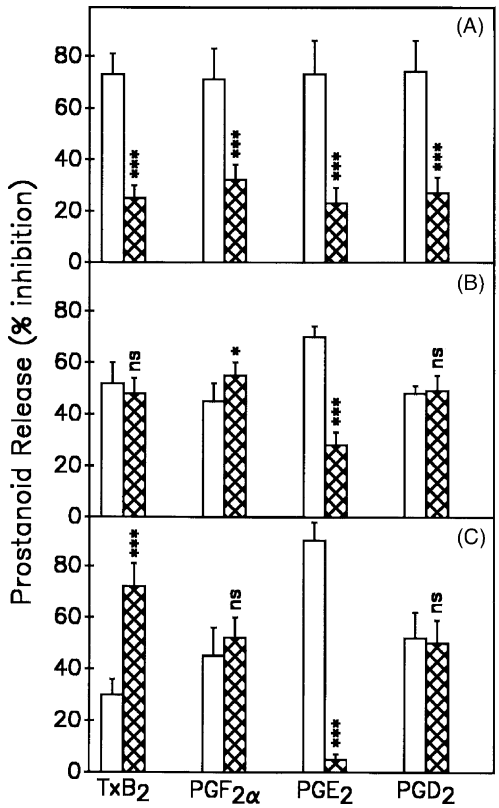

Fig. 4. Effect of specific inhibitors of COX-1 (SC560, open columns) and COX-2 (SC236, crosshatched columns) on the release of TxB_2, $PGF_{2\alpha}$, PGE_2 and PGD_2 from LPS-pretreated liver macrophages. Cells were kept in primary culture for 48 hr, thereafter LPS (500 ng/mL) was added for another 24 hr. Then, the cells were washed and incubated without or with 0.1 μM SC560 or 1 μM SC236 for 15 min; thereafter, zymosan (0.5 mg/mL, A), phorbol ester (1 μM, B) and A23187 (1 μM, C) were added for another 1 hr. Released TxB_2, $PGF_{2\alpha}$, PGE_2 and PGD_2 were measured by specific ELISAs. The basal release of prostanoids (TxB_2, $PGF_{2\alpha}$, PGE_2, PGD_2) in the absence of stimuli was <10 nmol/10^6 cells. The release of TxB_2, $PGF_{2\alpha}$, PGE_2, PGD2 is 55 ± 8, 88 ± 9, 320 ± 68, 315 ± 42 nmol/10^6 cells after zymosan, 18 ± 4, 22 ± 6, 125 ± 49, 155 ± 61 nmol/10^6 cells after phorbol ester, and 38 ± 7, 132 ± 19, 205 ± 58, 202 ± 63 nmol/10^6 cells after A23187, respectively. Data shown are means ± SD of four to seven experiments. *P*-values were calculated using Student's *t*-test (COX-1 inhibition vs. COX-2 inhibition, $^{***}P < 0.0001$; $^{*}P < 0.06$; ns: not significant).

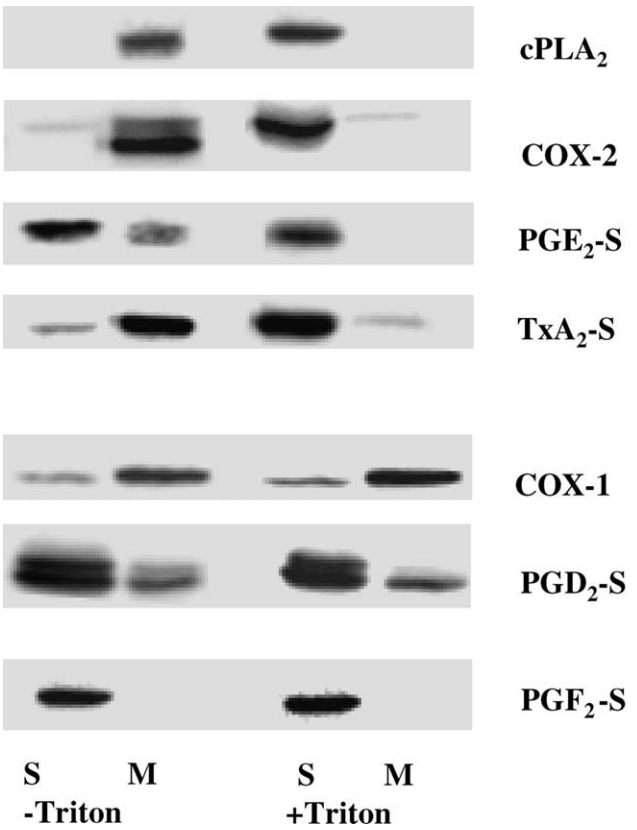

Fig. 5. Distribution of $cPLA_2$, COX-1, COX-2, and the final prostanoid synthases in soluble (S) and membrane (M) fractions obtained from LPS-pretreated liver macrophages. Cells were kept in primary culture for 48 hr, thereafter LPS was added for another 24 hr. Subcellular fractionation in the absence or presence of 1% Triton X-100 and Western blot analysis was carried out as described earlier [5]. A representative set of experiments is shown, which was reproduced at least five times. Densitometric analysis revealed the following data (−Triton/+Triton (S:M, percent (%) distribution): $cPLA_2$ (0:100/100:0), COX-2 (10:90/90:10), PGE_2-synthase (70:30/100:0), TxA_2-synthase (20:80/90:10), COX-1 (20:80/20:80), PGD_2-synthase (80:20/80:20), $PGF_{2\alpha}$-synthase (100:0/100:0).

Fig. 5 shows the distribution of $cPLA_2$, the two COX isoenzymes and the final prostanoid synthases in soluble and membrane fractions of LPS-pretreated liver macrophages. $cPLA_2$, COX-2, PGE_2- and TxA_2-synthase show a different distribution between the soluble and membrane fraction in the absence and presence of Triton X-100, indicating a loose association of these enzymes to cellular membranes. COX-1 and PGD_2-synthase are found in both fractions, and $PGF_{2\alpha}$-synthase is exclusively found in the soluble fraction.

4. Discussion

In the present study, we demonstrate that treatment of liver macrophages with LPS induces a coordinated enhanced expression of $cPLA_2$, COX-2 and PGE_2-synthase on mRNA and on protein level (Figs. 1 and 2). LPS exerts only minor effects on the expresssion of COX-1, $PGF_{2\alpha}$-, PGD_2- and TxA_2-synthase in these cells (Figs. 1 and 2) [8,9]. Furthermore, we show here that LPS-pretreated liver macrophages possess a higher capacity to release PGE_2 and $PGF_{2\alpha}$, while the release of TxB_2 and PGD_2 is unchanged (Fig. 3). The higher release of PGE_2 is independent of the stimulation, whereas the enhanced release of $PGF_{2\alpha}$ is seen only after a stimulation with zymosan and A23187. The higher capacity of LPS-pretreated liver macrophages to release PGE_2 might therefore be a consequence of the higher expression of the terminal PGE_2-synthase.

Furthermore, we demonstrate that the functional coupling of COX-1 and -2 with the final prostanoid synthases is diverse and depends on the stimulation of the cells: (1) COX-1 couples preferentially with the PGE_2-synthase after zymosan, phorbol ester, A23187 (Fig. 4) and AA [9]; (2) after zymosan, COX-1 is predominantly coupled with the $PGF_{2\alpha}$-, PGD_2- and TxA_2-synthase (Fig. 4A); (3) the two COX isoenzymes couple about equally with the $PGF_{2\alpha}$- and PGD_2-synthase after phorbol ester (Fig. 4B) and AA [9]; (4) TxA_2-synthase is equally coupled with COX-1 after phorbol ester (Fig. 4B) and AA [9]; after A23187, the TxA_2-synthase is preferentially coupled with COX-2 (Fig. 4C).

Here, we present first evidence for a different subcellular localization of $cPLA_2$, COX-1 and -2, and the final prostanoid synthases (Fig. 5). $PGF_{2\alpha}$-synthase is exclusively found in a soluble fraction, COX-1 and PGD_2-synthase are found in soluble and membrane fractions. The distribution of $cPLA_2$, COX-2, PGE_2- and TxA_2-synthase between the soluble and membrane fraction depends on the absence or presence of Triton X-100, indicating a loose association of these enzymes to cellular membranes. It is known that

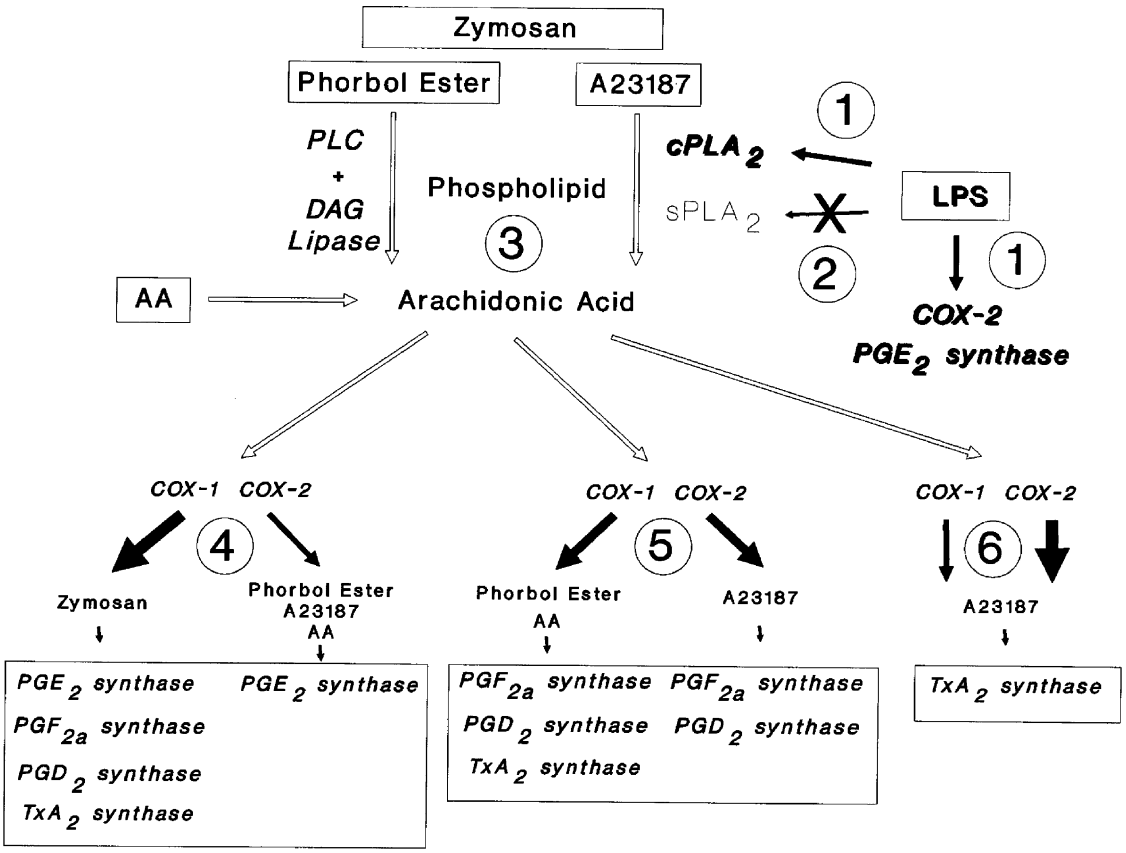

Fig. 6. A tentative scheme of the AA cascade in LPS-pretreated liver macrophages.

activation of $cPLA_2$ induces a translocation of the enzyme from the cytosol to membranes and a loose association to the membrane [5]. If a similar mechanism is true for COX-2, PGE_2- and TxA_2-synthase, is unknown.

There exists little information about the coupling of the two COX isoenzymes to the final prostanoid synthases in other cells. Recently, it has been suggested that the immediate response depends more on COX-1, whereas COX-2 is more involved in the delayed response [12]. Also, the concentration of AA seems to affect the coupling process [12]. For instance, in liver macrophages (this paper) and in rat brain [16], PGE_2-synthase couples preferentially with COX-1, whereas in rat peritoneal macrophages and osteoblasts, COX-2 couples predominantly with the PGE_2-synthase [17–19].

Fig. 6 shows a tentative scheme of the AA cascade in LPS-pretreated liver macrophages: (1) LPS treatment induces a coordinated enhanced expression of $cPLA_2$, COX-2 and PGE_2-synthase (Figs. 1 and 2); (2) $sPLA_2$ isoenzymes are not involved in prostanoid release in these cells [11]; (3) prostanoid release is induced via different intracellular signal pathways by phorbol ester, A23187, zymosan and AA [4–6] (Fig. 3); (4) COX-1 is preferentially coupled with all terminal prostanoid synthases after zymosan, and after A23187, phorbol ester and AA [9] with the PGE_2-synthase (Fig. 4); (5) COX-1 and -2 are equally coupled with $PGF_{2\alpha}$-, PGD_2-, and TxA_2-synthase after phorbol ester and AA [9], and with the $PGF_{2\alpha}$- and PGD_2-synthase after A23187 (Fig. 4); (6) COX-2 is preferentially coupled with the TxA_2-synthase after A23187 (Fig. 4). Further experiments, including a detailed determination of the cellular localization and a possible cellular reorganization of the enzymes during stimulation, is necessary to clarify the molecular mechanisms of the distinct and specific coupling process of COX-1 and -2 with the final prostanoid synthases.

Acknowledgments

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