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INDEPENDENT REGULATION OF THROMBOXANE AND PROSTAGLANDIN SYNTHESIS IN LIVER MACROPHAGES

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Abstract—Incubation of liver macrophages with zymosan, phorbol ester and calcium ionophore A 23187 led to the formation of thromboxane, prostaglandin E_2 and prostaglandin D_2, whereas after external addition of arachidonic acid prostaglandin E_2 and prostaglandin D_2 only were found. This was confirmed by the use of labeled arachidonic acid given together with the stimuli. When the liver macrophages were prelabeled with [^{3}H]arachidonic acid, and zymosan and [^{14}C]arachidonic acid were added simultaneously, [^{3}H]-label only was found in thromboxane whereas both [^{3}H]- and [^{14}C]-labeled PGE_2 and PGD_2 were detected in the cell medium. These data suggest that in cultured rat liver macrophages externally added arachidonic acid is accessible to the cyclooxygenase supplying prostaglandin H_2 for prostaglandin E_2 and D_2 synthesis but not for thromboxane synthesis.

Resident liver macrophages (Kupffer cells) have been shown to produce mainly Tx,* PGE_2 and PGD_2 in response to phagocytotic or other stimuli [1]. These compounds are thought to exert their action by stimulating the cells to liberate arachidonic acid (AA) from endogenous stores, e.g. from phosphatides by phospholipase A_2 (phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4.) [2]; the free acid, in turn, serves as a substrate for the formation of prostanoids. Only few studies have addressed the question of regulation of prostanoid synthesis from AA. It has been shown that Kupffer cells when incubated with AA form Tx, PGE_2 and PGD_2 [1]. Differences between the patterns of eicosanoids induced by various agents have already been reported for mouse peritoneal macrophages [3, 4]. Humes *et al.* [3] showed that phorbol ester and lipopolysaccharide stimulated mainly the formation of PGE_2 whereas zymosan induced preferentially the formation of leukotrienes C_4 and B_4. Schade *et al.* [4] reported that mono- and di-hydroxy arachidonic acids were formed on incubation of mouse peritoneal macrophages with free AA whereas stimulation of the same cells with zymosan led to the release of PGI_2, PGE_2 and leukotriene C_4. Indications for an independent regulation of Tx and PGE_2 synthesis have also been obtained in studies with human monocytes [5]. The untreated cells convert added free AA mainly to PGE_2 and to a much lesser extent to TxB_2. The yields of PGE_2 and TxB_2 were reversed when the monocytes had been exposed to complement 3b or lipopolysaccharide prior to the addition of AA.

In the present study the conversion of exogenous AA by Kupffer cells was compared to that from endogenous sources. The results indicate that externally applied AA is preferentially converted to PGE_2 and PGD_2 while endogenously provided AA yields Tx in addition.

* Abbreviations used: AA, arachidonic acid; HPLC, high performance liquid chromatography; PG, prostaglandin; Tx, thromboxane.

MATERIALS AND METHODS

Chemicals. Arachidonic acid and zymosan were from Sigma (München, F.R.G.). Zymosan suspensions were kept at 95° to destroy endogenous phospholipase A_2 (PLA_2) activity. Medium RPMI 1640 was purchased from Biochrom (Berlin West) and newborn calf serum (heat-inactivated) from Gibco (Karlsruhe, F.R.G). The calcium ionophore A 23187 was obtained from Calbiochem (Giessen, F.R.G.) and the phorbol ester, phorbol 12-myristate 13-acetate, from Pharmacia (Freiburg, F.R.G.). [1-^{14}C]AA (59.6 mCi/mmol) and [5,6,8,9,11,12,14,15-^{3}H]AA (180 Ci/mmol) were purchased from Amersham Buchler (Frankfurt, F.R.G). All other chemicals were of analytical or HPLC grade.

Cell culture. Male Wistar rats (250–350 g) obtained from Interfauna Süddeutsche Versuchstierfarm (Tuttlingen, F.R.G.) were fed *ad libitum* with Altromin®. The livers were removed aseptically under Nembutal® anesthesia and the Kupffer cells isolated by the centrifugal elutriation procedure of Brouwer *et al.* [6] as modified by Eyhorn *et al.* [1]. The cells were maintained in primary culture with RPMI medium containing 30% newborn calf serum. Changes of the medium and determination of the viability and purity of the cells were performed as previously described [1]. The number of attached cells was determined microscopically. All experiments were performed with Kupffer cells kept in primary culture for 72 hr.

Determination of radiolabeled prostanoids from [^{3}H]*AA-prelabeled cells.* The Kupffer cells (48 hr in primary culture) were incubated for 24 hr with 2 μCi [^{3}H]AA. Then the media were removed, the cells washed twice with Hanks solution and incubated in the same medium. Then zymosan (0.5 mg/ml), phorbol ester (1 μM) or A 23187 (20 μM) were added as indicated. After 1 hr the media were recovered and the labeled prostanoids extracted and separated on HPLC according to Eyhorn *et al.* [1]. Briefly, the cell supernatants were passed through a Sep-PAK ODS Cartridge (Millipore Waters Associates, Esch-

born, F.R.G.) and prostanoids eluted with methanol/isopropanol (1:1, v/v). HPLC was performed on an Orpegen C 18 column (5 μm particle size); the prostanoids were eluted with an isocratic system composed of 30.5% acetonitrile and 69.5% of an aqueous 0.05% trifluoroacetic acid solution adjusted with concentrated ammonia to pH 4.2. The radioactivity in the eluent was measured with a flow-through radiodetector (Ramona LS 4, Raytest, Straubenhardt, F.R.G.). In experiments where [^{3}H]- and [^{14}C]-labeled prostanoids were determined simultaneously, the radioactivity eluting from the column was measured in two separate channels. In channel 1, both [^{3}H] and [^{14}C] activity were measured by setting the energy regions from 0 to 998; in channel 2, [^{14}C] activity only was measured by setting the lower region limit to 250. In the region from 250 to 998, 60–70% of [^{14}C] activity but less than 0.1% of [^{3}H] activity were detected. [^{3}H] Activity was calculated by subtracting the counts of channel 2 from channel 1. HPLC pattern of released prostanoids shown are representative for at least three runs which gave essentially identical results.

Determination of radiolabeled prostanoids produced from externally added [^{14}C]AA. The Kupffer cells (72 hr in primary culture) were incubated for 1 hr with 0.5 μCi [^{14}C]AA plus 30 μM unlabeled AA; prostanoids released into the medium were extracted and separated as described above. Unlabeled AA had to be added together with radiolabeled AA to obtain measurable yields of radiolabeled prostanoids. The very small amount (8.4 nmol) of labeled AA alone would be rapidly and almost quantitatively incorporated into phospholipids and not be sufficiently available for direct eicosanoid synthesis (unpublished data). The presence of 30 μM unlabeled AA does not induce cell damage as described previously [7].

Determination of PGE_2 and TxB_2 by radioimmunoassay. The Kupffer cells (72 hr in primary culture) were incubated for 15 min in Hanks solution. Then no agent, zymosan (0.5 mg/ml) or unlabeled AA (30 μM) were added. After 1 hr the media were removed, and released PGE_2 and TxB_2 measured radioimmunologically. The antibodies raised against PGE_2 and TxB_2 were generous gifts from Drs K. Brune and M. Reinke (Erlangen, F.R.G.). Unlabeled and [^{3}H]-labeled PGE_2 and TxB_2 were from Sigma (München, F.R.G.) and Amersham Buchler (Frankfurt, F.R.G.), respectively.

RESULTS

Synthesis of radiolabeled prostanoids from AA-prelabeled cells. Treatment of [^{3}H]AA-prelabeled Kupffer cells with zymosan, phorbol ester or calcium ionophore A 23187 led to the formation of Tx, PGE_2 and PGD_2 (Fig. 1 A–C). The broad radioactive peak which coelutes with the [^{3}H]TxB_2 standard was suppressed almost totally by the thromboxane synthase inhibitor CGS 13080 [8] (unpublished results) indicating that it has been formed enzymatically by Tx synthase (EC 5.3.99.5.). The same pattern of released prostanoids as that shown in Fig. 1 was obtained when the cells were prelabeled with

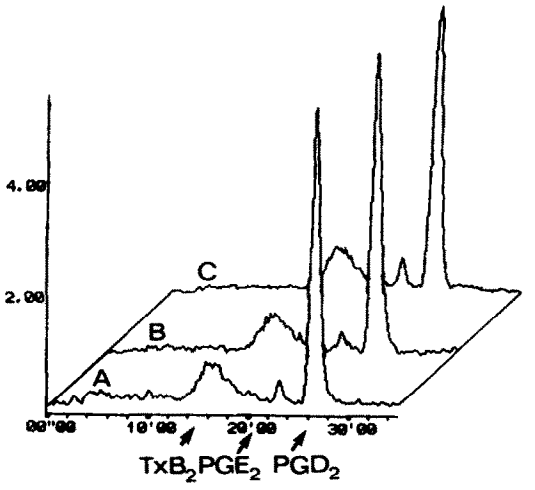

Fig. 1. HPLC profile of [^{3}H]-labeled prostanoids produced by [^{3}H]AA-prelabeled rat Kupffer cells after stimulation with zymosan (A), phorbol ester (B) and calcium ionophore A 23187 (C). Kupffer cells (48 hr in primary culture) were incubated with 2 μCi [^{3}H]AA for 24 hr. Then the media were replaced by Hanks solution and zymosan (0.5 mg/ml), phorbol ester (1 μM) or A 23187 (20 μM) added; 60 min thereafter the media were removed for processing and analysis by HPLC. A typical set of data is shown which was reproduced at least three times. On the abscissa the retention time (min), on the ordinate the radioactivity (arbitrary units) are displayed. The positions of the reference substances TxB_2, PGE_2 and PGD_2 are indicated by arrows.

[^{14}C]AA or when unlabeled AA (30 μM) was added simultaneously with the stimuli to [^{3}H]AA-prelabeled cells (data not shown).

Synthesis of radiolabeled prostanoids from externally added AA. In contrast to the prelabeled cells, unlabeled Kupffer cells converted free [^{14}C]AA (Fig. 2A) or [^{3}H]AA (data not shown) added to the medium mainly to PGD_2 and PGE_2 whereas Tx was found in the cell medium in trace amounts only. Addition of zymosan (Fig. 2B), phorbol ester or A 23187 (data not shown) to the cells together with free [^{14}C]AA did not alter the pattern of labeled prostaglandins; PGD_2 and PGE_2 but no Tx was detected in the cell media under these conditions.

The absolute amounts of labeled TxB_2, PGE_2 and PGD_2 released from [^{3}H]AA-prelabeled cells or formed from free [^{14}C]AA added to the medium were almost the same (Table 1). With zymosan, phorbol ester or A 23187, PGD_2 was the major prostanoid (65%–68%) found, followed in quantity by TxB_2 (21%–28%) and PGE_2 (7%–11%). Similar amounts of PGD_2 and PGE_2 but only very small amounts of TxB_2 (about 3%) were formed from added free AA. This observed difference in the production of TxB_2 is clearly demonstrated by the ratio of PGD_2 to TxB_2; it was significantly higher if the prostanoids were formed from added free AA than from stimulus-triggered endogenous sources (Table 1).

Synthesis of radiolabeled prostanoids from AA-prelabeled cells and from radiolabeled AA. These

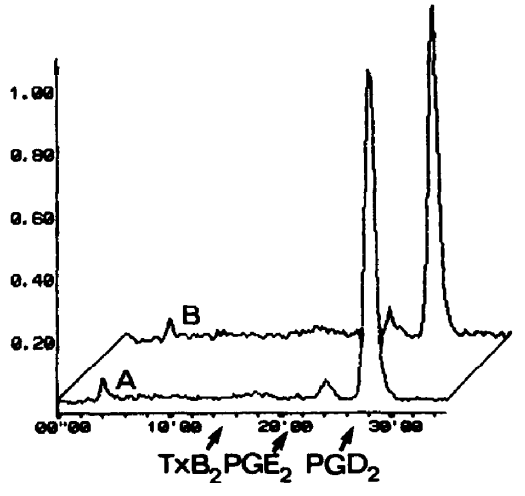

Fig. 2. HPLC profile of [^{14}C]-labeled prostanoids formed by rat Kupffer cells from added free [^{14}C]AA in the absence (A) and presence of zymosan (B). Kupffer cells (72 hr in primary culture) were incubated in Hanks solution with 0.5 μCi [^{14}C]AA (plus 30 μM unlabeled AA) without or with zymosan (0.5 mg/ml); 60 min thereafter the media were removed for processing and analysis by HPLC. A typical set of data is shown which was reproduced at least three times. On the abscissa the retention time (min), on the ordinate the radioactivity (arbitrary units) are displayed. The positions of the reference substances TxB_2, PGE_2 and PGD_2 are indicated by arrows.

observations were corroborated by a set of experiments where [^{3}H]AA-prelabeled Kupffer cells were exposed simultaneously to zymosan and free [^{14}C]AA. Under these experimental conditions, [^{3}H]-labeled prostanoids could have been derived from endogenously liberated AA only whereas [^{14}C]-labeled prostanoids could only arise from added free AA. Figure 3A shows the pattern of the [^{14}C]-labeled prostanoids. [^{14}C] label was detected in PGD_2 and PGE_2 but only very little in TxB_2. This pattern of labeled prostanoids changed when the [^{3}H]-labeled prostanoids were determined (Fig. 3B). While the amounts of PGE_2 and PGD_2 released into the medium were very similar to those in Fig. 3A, TxB_2 became much more prominent. Integration of the peak areas and the respective ratios of PGD_2/TxB_2 revealed this difference most clearly (Table 2).

Determination of prostanoids by radioimmunoassay. The amounts of PGE_2 and TxB_2 found in the medium of rat Kupffer cells were also measured radioimmunologically (Table 3). Unstimulated Kupffer cells released very small amounts of PGE_2 and TxB_2. Addition of zymosan to the cells stimulated formation of both PGE_2 and TxB_2, whereas externally added AA led to an increase of PGE_2 but not of significant amounts of TxB_2.

DISCUSSION

Treatment of cultured rat Kupffer cells with zymosan, phorbol ester or A 23187 leads to the formation of PGD_2, TxB_2 and PGE_2. These stimuli act most probably by triggering the liberation of AA from endogenous stores which then serves as substrate for cyclooxygenase (arachidonate:oxygen oxidoreductase (cyclizing), EC 1.14.99.—). It was shown in a previous communication that zymosan induces the liberation of AA mainly from phosphatidyl choline [9]. Furthermore, we presented evidence that the synthesis of prostanoids induced by some of these

Table 1. Prostanoids produced from [^{3}H]AA-prelabeled Kupffer cells (A) and from unlabeled Kupffer cells after the addition of free [^{14}C]AA (B)

Addition	Prostanoid released (cps/10^6 cells) TxB_2	PGE_2	PGD_2	Ratio of PGD_2/TxB_2
(A)				
None	100 ± 70 (N = 3)	22 ± 11 (N = 3)	148 ± 61 (N = 3)	1.5
Zymosan	2404 ± 861 (N = 6)	1194 ± 410 (N = 6)	7686 ± 2679 (N = 6)	3.2
Phorbol ester	2438 ± 769 (N = 6)	966 ± 378 (N = 6)	6406 ± 2089 (N = 6)	2.6
A 23187	2836 ± 781 (N = 4)	731 ± 287 (N = 4)	6673 ± 2620 (N = 4)	2.4
(B)				
AA	405 ± 118 (N = 6)	1617 ± 446 (N = 6)	12380 ± 3673 (N = 6)	30.6

(A) Kupffer cells (48 hr in primary culture) were incubated with 2 μCi [^{3}H]AA for 24 hr. Then the media were replaced by Hanks solution and no agent or zymosan (0.5 mg/ml), phorbol ester (1 μM) or A 23187 (20 μM) added; 60 min thereafter the media were removed for processing and analysis by HPLC.

(B) Kupffer cells (72 hr in primary culture) were incubated in Hanks solution with 0.5 μCi [^{14}C]AA plus 30 μM unlabeled AA; 60 min thereafter the media were removed for processing and analysis on HPLC.

The data represent values of integrated peak areas corresponding to TxB_2, PGE_2 and PGD_2.

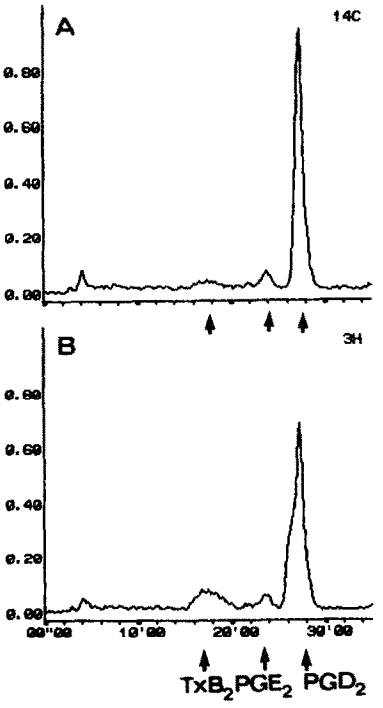

Fig. 3. HPLC profile of [^{3}H]-labeled and [^{14}C]-labeled prostanoids released from [^{3}H]AA-prelabeled Kupffer cells after the simultaneous addition of [^{14}C]AA and zymosan. Kupffer cells (48 hr in primary culture) was incubated with 2 μCi [^{3}H]AA for 24 hr. Then the media were replaced by Hanks solution and 0.5 μCi [^{14}C]AA (plus 30 μM unlabeled AA) and zymosan (0.5 mg/ml) added; 60 min thereafter the media were removed for processing and analysis by HPLC. [^{3}H] and [^{14}C] activities were determined as described in Materials and Methods. A typical set of data is shown which was reproduced at least two times. On the abscissa the retention time (min), on the ordinate the radioactivity (arbitrary units) is displayed. The position of the reference substances TxB_2, PGE_2 and PGD_2 are indicated by arrows.

agents in rat Kupffer cells include an activated Na^+/H^+ exchange [10] and Ca^{2+}-dependent cellular reactions [11] whereas prostaglandin formation from externally provided free AA was shown to be independent of a Na^+/H^+ exchange [10] and was not influenced by the removal of intracellular Ca^{2+} [11]. Here it is shown that, in contrast to endogenously provided AA, added free AA is not converted to Tx. The presence of zymosan, phorbol ester or A 23187 did not induce formation of Tx from externally added AA, indicating (a) that Tx synthase does not require cellular processes which are elicited by these agents, and (b) that incorporation of the added free AA in cellular lipids and subsequent liberation of this incorporated AA during the time of incubation with the stimuli does not contribute significantly to the amounts of released prostanoids. On the other hand, addition of free AA together with one of the stimuli did not suppress formation of Tx from endogenously liberated AA. This indicates that added AA does not suppress Tx synthase activity.

Table 2. Prostanoids produced by [^{3}H]-prelabeled rat Kupffer cells in the presence of zymosan and added free [^{14}C]AA

Radioactivity	Prostanoid released (cps/10^6 cells) TxB_2	PGE_2	PGD_2	Ratio of PGD_2/TxB_2
[^{3}H] Exp. A	1542	489	9290	6.0
Exp. B	1934	758	8099	4.2
[^{14}C] Exp. A	456	576	12745	27.9
Exp. B	616	681	15965	25.9

Kupffer cells (48 hr in primary culture) were incubated with 2 μCi [^{3}H]AA for 24 hr. Then the media were replaced by Hanks solution and 0.5 μCi [^{14}C]AA (plus 30 μM unlabeled AA) and zymosan (0.5 mg/ml) added, 60 min thereafter the media were removed for processing and analysis by HPLC. [^{3}H] and [^{14}C] activities were determined as described in Materials and Methods. The data represent values of integrated peak areas corresponding to TxB_2, PGE_2 and PGD_2.

Table 3. Release of immunoreactive PGE_2 and TxB_2 from rat Kupffer cells

Addition	Release of PGE_2 (pmol/10^6 cells)	TxB_2
None	0.8 ± 1.6 (N = 2)	4.6 ± 4.6 (N = 2)
Zymosan	42.0 ± 9.8 (N = 5)	63.9 ± 7.1 (N = 5)
AA	39.1 ± 3.0 (N = 3)	8.6 ± 3.1 (N = 3)

Kupffer cells (72 hr in primary culture) were incubated in Hanks solution. After 15 min either zymosan (0.5 mg/ml), AA (30 μM) or no agent was added; 60 min thereafter the amounts of PGE_2 and TxB_2 in the cell medium were determined by radioimmunoassay.

At present it is not known why AA liberated from endogenous sources but not exogenously provided free AA leads to the formation of Tx. It may be due to independently regulated cellular compartments for Tx and PG syntheses. The cellular localization of these enzymes in rat Kupffer cells is not yet known. In other cells cyclooxygenase, PGE_2 and Tx synthase have been found to be associated with the endoplasmic reticulum [12, 13] whereas the conversion of PGH_2 to PGD_2 has been reported to be cytosolic as well as associated with microsomal membranes [12, 13].

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