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Prostaglandin E_2 inhibits and indomethacin and aspirin enhance, A23187-stimulated leukotriene B_4 synthesis by rat peritoneal macrophages

[1]Graham R. Elliott, Adrianus P.M. Lauwen & Ivan L. Bonta

Department of Pharmacology, Medical Faculty, Erasmus University, P.O.B. 1738, 3000 DR, Rotterdam, The Netherlands.

1 The calcium ionophore, A23187, stimulated leukotriene B_4 (LTB_4), thromboxane B_2 (TxB_2) and prostaglandin E_2 (PGE_2) synthesis by 4 day carrageenin-elicited rat peritoneal macrophages.

2 At concentrations of 2×10^{-7}–2×10^{-5} M indomethacin and aspirin enhanced A23187-stimulated LTB_4 synthesis and inhibited PGE_2 and TXB_2 formation.

3 PGE_2 inhibited A23187-stimulated LTB_4 and TXB_2 formation as well as the augmentation of LTB_4 release caused by aspirin and indomethacin. However, PGE_2 was ineffective when the cells were challenged with arachidonic acid (AA).

4 Dibutyryl adenosine 3′ : 5′-cyclic monophosphate (db-cyclic AMP) partially inhibited A23187-stimulated LTB_4 production.

5 Our results suggest that PGE_2 inhibits macrophage LTB_4 synthesis by limiting the availability of AA. Indomethacin and aspirin, possibly by removing the regulatory effect of PGE_2, promote the synthesis of the pro-inflammatory LTB_4.

Introduction

It is becoming increasingly apparent that eicosanoid products of arachidonic acid (AA) metabolism are important modulators of macrophage cyclo-oxygenase and lipoxygenase pathways. For example, mouse resident peritoneal macrophage cyclo-oxygenase and 5′-lipoxygenase activities were inhibited by hydroperoxy- and hydroxy-eicosatetraenoic acid metabolites of the lipoxygenase pathway (Chang *et al.*, 1985; Humes *et al.*, 1986). Synthesis of a cyclo-oxygenase metabolite, prostaglandin E_2 (PGE_2) by rat peritoneal macrophages was stimulated by the lipoxygenase product, leukotriene C_4 (LTC_4) (Schenkelaars & Bonta, 1986), while PGE_2 inhibited synthesis of the cyclo-oxygenase metabolites, thromboxane B_2 (TXB_2) and 6-keto-$PGF_{1\alpha}$ (Elliott *et al.*, 1985). Such interactions between eicosanoids may be important for regulation of macrophage functions, as demonstrated by Schenkelaars & Bonta (1986) who found that LTC_4 stimulated the secretion of the lysosomal enzyme β-glucuronidase (GUR). This secretory response was enhanced by the non-steroidal anti-inflammatory drugs (NSAIDs) indomethacin and aspirin which possess cyclo-oxygenase inhibitory activity. Exogenously added PGE_2 prevented this stimulation of enzyme release. Of relevance to these interactions are reports showing that PGE_1 inhibited, and indomethacin stimulated, human neutrophil LTB_4 formation (Ham *et al.*, 1983; Docherty & Wilson, 1987). Therefore, in the light of published data, it appeared conceivable that indomethacin and aspirin stimulated macrophage GUR release by promoting synthesis of leukotrienes, as a consequence of the inhibition of PGE_2 synthesis. In order to investigate this possibility we examined the effect of PGE_2, indomethacin and aspirin on A23187-stimulated LTB_4 release from carrageenin-elicited rat peritoneal macrophages.

Methods

Experimental animals

Male Wistar rats (170–200 g) were injected with 2 ml carrageenin (1 mg ml^{-1}, i.p.) on day 1 and the elicited peritoneal macrophages were isolated on day 4. Animals were ordered 12 at a time and divided into

[1] Author for correspondence.

groups of 3 or 4. Experiments were repeated on animals from the same batch.

Isolation and incubation of macrophages

Carrageenin-elicited peritoneal macrophages were isolated by density-gradient centrifugation over Ficoll–Isopaque as previously described (Schenkelaars & Bonta, 1986). They were then suspended (2×10^6 cells ml^{-1}) in Dulbecco's modified Eagle's Minimum Essential Medium (DMEM), and kept on ice until needed. The cell preparation was more than 95% viable, as assayed by trypan blue exclusion and consisted of greater than 85% macrophages as judged by morphological criteria under the light microscope. The rest of the cells were mainly lymphocytes; the contribution of polymorphonuclear leukocytes and mast cells was always less than 1%. Lymphocytes do not release eicosanoids and this contamination was thus not a problem in the experimental design used (Kurland & Bockman, 1978; Poubelle *et al.*, 1987). Aliquots of the macrophage preparation (1 ml) were transferred to 1 ml polypropylene reaction vials on ice and then incubated for 30 min at 37°C in a water bath (Gemsa *et al.*, 1982; Williams *et al.*, 1984). The reaction vials were then centrifuged and the supernatants analysed for LTB_4, PGE_2 and TXB_2 by radioimmunoassay (RIA) (Zijlstra & Vincent, 1984). None of the compounds used interfered with the measurement of PGE_2, TXB_2 or LTB_4. PGE_2 was not analysed in those experiments where it was added to the incubations. The PGE_2 RIA had a 100% cross reaction with PGE_1. However rat macrophages do not synthesize PGE_1 unless incubated with dihomo-γ-linolenic acid (Elliott *et al.*, 1986), its direct precursor, so that we feel justified in giving concentrations of PGE_2 rather than of immunoreactive PGE. The cell pellet was heated for 5 min at 95°C in 150 μl Tris/EDTA buffer and the reaction vials centrifuged at 12000 *g* for 1 min. The concentration of adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) in the supernatants was assayed by a modification (Bonta *et al.*, 1984) of the protein binding method of Gilman (1970).

Chemicals

Carrageenin was dissolved in physiological saline. Stock solutions of AA, PGE_2, A23187 and indomethacin in ethanol were diluted with DMEM. Aspirin and db-cyclic AMP were dissolved directly in DMEM.

A23187, aspirin, indomethacin, AA, db-cyclic AMP and PGE_2, (10 μl volumes) were added to incubations at the beginning of the 30 min period. The final concentration of ethanol resulting from the addition of A23187, AA, PGE_2 and indomethacin was 0.01% or less and had no effect on any of the parameters assayed.

Carrageenin was obtained from Marine Colloids, Inc., Springfield, N.J., USA. Standard PGE_2, TXB_2, LTB_4, cyclic AMP, AA, db-cyclic AMP and A23187 were obtained from Sigma Chemical Co., St. Louis, USA. Antisera against TXB_2 and PGE_2 were purchased from Bio-Yeda, Rehovot, Israel and LTB_4 antiserum from Wellcome Diagnostics, Beckenham. Radiolabelled cyclic AMP, PGE_2 and TXB_2 were obtained from Amersham International plc, Aylesbury, Buckinghamshire, and radiolabelled LTB_4 from Wellcome Diagnostics. Indomethacin and aspirin were purchased from the Pharmacy Department, Dijkzigt Hospital, Rotterdam, The Netherlands.

Statistical analysis

Statistical analysis was carried out by use of the Mann–Whitney U-test.

Results

The effect of indomethacin and aspirin on A23187-stimulated macrophage eicosanoid synthesis

A23187 (10^{-6} M) stimulated macrophage PGE_2, TXB_2 and LTB_4 synthesis and release (Table 1). Indomethacin and aspirin enhanced A23187-stimulated LTB_4 synthesis and inhibited A23187-stimulated PGE_2 and TXB_2 formation (Table 2).

Table 1 The effect of A23187 on rat peritoneal macrophage eicosanoid synthesis and release

Treatment	*PGE_2*	*TXB_2*	*LTB_4*
Control	4.60 ± 1.30	0.95 ± 0.35	<0.02
A23187 (10^{-6} M)	20.80* ± 4.50	6.15* ± 2.16	0.24* ± 0.17

Results are expressed as eicosanoid release in ng per 2×10^6 nucleated cells and are the mean ± s.d. in 9–11 experiments. PGE_2, prostaglandin E_2; TXB_2, thromboxane B_2; LTB_4, leukotriene B_4.
* $P < 0.05$ with respect to appropriate control values.

Table 2 The effect of indomethacin and aspirin on A23187-stimulated eicosanoid synthesis and release from rat peritoneal macrophages

Net eicosanoid release	*Indomethacin/Aspirin* (M)				
	0	*2×10^{-8}*	*2×10^{-7}*	*2×10^{-6}*	*2×10^{-5}*
Indomethacin					
PGE_2	8.85 ± 1.00	6.68 ± 1.46	5.58* ± 1.56	2.68* ± 1.04	2.41* ± 1.56
TXB_2	10.12 ± 2.84	8.70 ± 1.34	4.50* ± 1.26	1.19* ± 0.19	0.82* ± 0.39
LTB_4	0.38 ± 0.08	0.48 ± 0.08	0.62* ± 0.06	0.66* ± 0.07	0.53* ± 0.04
Aspirin					
PGE_2	22.87 ± 3.15	13.95* ± 6.19	11.98* ± 5.54	9.11* ± 5.75	2.05* ± 0.67
TXB_2	5.39 ± 1.85	5.90 ± 3.04	2.17* ± 0.80	1.89* ± 0.64	0.92* ± 0.45
LTB_4	0.15 ± 0.05	0.16 ± 0.04	0.26* ± 0.05	0.47* ± 0.11	0.44* ± 0.11

Results are expressed as ng net eicosanoid release from 2×10^6 nucleated cells. Data shows the mean ± s.d. in 7–9 experiments.
* $P < 0.05$ with respect to appropriate control.

These NSAIDs also inhibited the basal formation of cyclo-oxygenase metabolites (data not shown), but had no detectable stimulatory effect on the basal synthesis of LTB_4, which was below the level of detection of the RIA (0.02 ng ml^{-1}).

The effect of PGE_2 and NSAIDs on A23187-stimulated macrophage eicosanoid synthesis

PGE_2 (2.8×10^{-10} M–2.8×10^{-5} M) inhibited the A23187-stimulated synthesis and release of TXB_2

Table 3 The effect of prostaglandin E_2 (PGE_2) on A23187-stimulated eicosanoid synthesis and release in rat peritoneal macrophages

Net eicosanoid release	*PGE_2* (M 2.8)				
	0	*10^{-10}*	*10^{-8}*	*10^{-6}*	*10^{-5}*
TXB_2	18.4 ± 4.2	18.2 ± 1.6	9.7* ± 4.3	4.8* ± 0.5	3.5* ± 0.3
LTB_4	0.20 ± 0.06	0.23 ± 0.03	0.10* ± 0.03	0.05* ± 0.03	0.04* ± 0.04

Results are expressed as net eicosanoid release from 2×10^6 nucleated cells. Data shows the mean ± s.d. in 6 experiments.
* $P < 0.05$ with respect to appropriate control values.

Table 4 The effect of prostaglandin E_2 (PGE_2) on the indomethacin- and aspirin-dependent enhancement of A23187-stimulated leukotriene B_4 (LTB_4) synthesis and release

Treatment	*Indomethacin/Aspirin* (M)				
	0	*2×10^{-8}*	*2×10^{-7}*	*2×10^{-6}*	*2×10^{-5}*
Indomethacin					
A23187 (10^{-6} M)	100	118 ± 32	177* ± 19	194* ± 28	153* ± 18
A23187 + PGE_2 (2.8×10^{-5} M)	100	106 ± 12	113† ± 6	77† ± 13	67† ± 34
Aspirin					
A23187 (10^{-6} M)	100	110 ± 40	190* ± 21	366* ± 120	339* ± 59
A23187 + PGE_2 (2.8×10^{-5} M)	100	124 ± 30	170* ± 26	161*† ± 4	218*† ± 39

Results are expressed as the percentage of values obtained without indomethacin or aspirin and were calculated from three separate experiments.
* $P < 0.05$ control vs indomethacin or aspirin treated.
† $P < 0.05$ A23187 vs A23187 + PGE_2
Absolute values for LTB_4 release, expressed as ng per 2×10^6 nucleated cells, in the absence of indomethacin or aspirin, were as follows for the indomethacin experiments: A23187, 0.27 ± 0.05. A23187 + PGE_2, 0.20 ± 0.02 ($P < 0.05$ vs A23187); and for the aspirin experiments: A23187, 0.19 ± 0.09. A23198 + PGE_2, 0.14 ± 0.03.

Table 5 The effect of prostaglandin E_2 (PGE_2) on arachidonic acid (AA)-stimulated eicosanoid synthesis by rat peritoneal macrophages

Treatment	*TXB_2*	*LTB_4*
Control	0.98 ± 0.37	<0.02
AA (8×10^{-6} M)	11.48* ± 3.60	0.21* ± 0.10
AA + PGE_2 (2.8×10^{-5} M)	12.48* ± 2.20	0.28* ± 0.11

Results are expressed as mediator release in ng per 2×10^6 nucleated cells. Data are the mean ± s.d. in 8 experiments.
* $P < 0.05$ control vs AA or AA + PGE_2.

and LTB_4 in a concentration-related manner (Table 3).

In addition to inhibiting the eicosanoid release elicited by A23187 PGE_2 (2.8×10^{-5} M) also reversed the enhancing effect of indomethacin and aspirin on A23187-stimulated macrophage LTB_4 formation (Table 4). However, it had no effect on the synthesis of LTB_4 and TXB_2 following challenge with 8×10^{-6} M AA (Table 5).

Challenge of the cells with A23187 resulted in an elevation in intracellular cyclic AMP concentration and this was inhibited in a concentration-dependent manner by indomethacin (2×10^{-7} M–2×10^{-5} M) (Table 6). When cells were incubated for 30 min with db-cyclic AMP (5×10^{-7} and 5×10^{-5} M) during activation with A23187 there was an inhibition of the release of TXB_2 and LTB_4 (Table 7).

Discussion

For our experiments we used a non-physiological agent, A23187, to stimulate calcium flux. A23187 was used primarily as a leukotriene releasing agent so that we could investigate regulatory events associ-

Table 6 The effect of indomethacin on basal and A23187-stimulated rat peritoneal macrophage cycle AMP concentrations

	Indomethacin (M)			
Treatment	*0*	*2×10^{-7}*	*2×10^{-6}*	*2×10^{-5}*
Control	1.42 ± 0.16	1.10* ± 0.14	0.83* ± 0.19	0.79* ± 0.21
A23187 (10^{-6} M)	2.81† ± 0.04	1.96† ± 0.60	1.69* ± 0.64	1.01* ± 0.26

Results are expressed as pmol cyclic AMP per 2×10^6 cells.
Data are the mean ± s.d. in 6 experiments.
* $P < 0.05$ control vs indomethacin treated.
† $P < 0.05$ A23187 treated vs appropriate control.

Table 7 The effect of db-cyclic AMP on basal and A23187-stimulated thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4) release

	db-Cyclic AMP (M)		
Eicosanoid	*0*	*5×10^{-7}*	*5×10^{-5}*
TXB_2			
Basal	100	105 ± 10	81* ± 7
A23187 (10^{-6} M)	100	75* ± 15	64* ± 11
LTB_4			
Basal	N/D	N/D	N/D
A23187 (10^{-6} M)	100	84* ± 8	73* ± 4

Results are expressed as the percentage of release obtained in the absence of db-cyclic AMP and were calculated using the percentage changes measured in 3 separate experiments. Data are the mean ± s.d.
* $P < 0.05$ control vs db-cyclic AMP treated.
Control values, expressed in ng per 2×10^6 nucleated cells, for basal and A23187-stimulated eicosanoid release were for TXB_2: basal 0.65 ± 0.27; A23187 19.71 ± 8.2; and for LTB_4: basal not done (ND); A23187 1.06 ± 0.48.

ated with LTB_4 formation, specifically the role played by PGE_2 in the mobilization and subsequent metabolism of AA to LTB_4, events thought to be associated with an increase in calcium flux. We feel that it is reasonable to assume that PGE_2 and the NSAIDs used would also modify the effect of other mediators which similarly stimulated AA turnover.

In this article we have shown that added PGE_2 inhibited A23187-stimulated LTB_4 synthesis. The lowest effective concentration of PGE_2 used (2.8×10^{-8} M) inhibited A23187-stimulated LTB_4 formation by 50% (Table 3). Basal and ionophore-stimulated rat peritoneal macrophages released about 10^{-8} M and 4×10^{-8} M PGE_2 respectively (calculated from data given in Table 1) indicating that endogenously formed PGE_2 could also play a role in regulating leukotriene synthesis. This extends our previous finding that PGE_2 inhibits the synthesis and release of TXB_2 and 6-keto-$PGE_{1\alpha}$ induced by carrageenin (Elliott *et al.*, 1985). PGE_2 also inhibited the further increase in LTB_4 formation observed when cells were incubated with A23187 together with indomethacin or aspirin. The finding that the NSAIDs promoted A23187-stimulated LTB_4 synthesis supports our contention that endogenously formed PGE_2 could have a regulatory function. However, we cannot say to what extent the stimulatory effect of the cyclo-oxygenase inhibitors on LTB_4 formation was due to removal of the inhibitory PGE_2. A switching of AA from the cyclo-oxygenase to the lipoxygenase path i.e. 'substrate shunting', could also have contributed to the increase observed. Basal synthesis of LTB_4 was too low to assay, even in the presence of aspirin and indomethacin. It would appear therefore that cyclo-oxygenase inhibitors can influence leukotriene formation only if the lipoxygenase enzyme is stimulated by some other agent, i.e. they are not direct activators of the lipoxygenase.

Interestingly, Docherty & Wilson (1987) found that neither aspirin nor ibuprofen (a NSAID with cyclo-oxygenase inhibitory activity), had an effect on A23187-stimulated LTB_4 formation by human neutrophils, although indomethacin had a stimulatory action. Human neutrophil LTB_4 release is sensitive to the inhibitory action of PGE_1 (Ham *et al.*, 1983), so that the reason for the lack of effect of aspirin and ibuprofen on LTB_4 production is not clear.

In our experiments PGE_2 inhibited both lipoxygenase (LTB_4) and cyclo-oxygenase (TXB_2) metabolite release. Furthermore, PGE_2 had no effect on AA stimulated LTB_4 or TXB_2 synthesis. It is unlikely therefore that PGE_2 acted on specific enzymes within the AA cascade. A more likely explanation is that PGE_2 limited the availability of AA. PGE_2 is thought to exert its immunosuppressive effects by stimulating cyclic AMP synthesis (Bonta & Parnham, 1982) and we found that db-cyclic AMP partially inhibited A23187-stimulated LTB_4 and TXB_2 formation. In support of this interpretation, carrageenin-stimulated eicosanoid synthesis has also been shown to be inhibited by db-cyclic AMP (Elliott *et al.*, 1985). Furthermore, we show in this paper that both PGE_2 and cyclic AMP synthesis were decreased when macrophages were incubated with indomethacin. This finding is consistent with the proposal that endogenously formed PGE_2 is important for the maintenance of macrophage cyclic AMP concentrations (Lim *et al.*, 1983). There are two conceivable mechanisms by which cyclic AMP and db-cyclic AMP, could reduce the amount of AA available to the different enzymes, stimulation of AA reacylation and inhibition of phospholipase (PL) activity. Indeed, Lapetina *et al.* (1981) reported that cyclic AMP stimulated the reincorporation of AA into platelet phosphatidylinositol and Hirata *et al.* (1984) demonstrated that cyclic AMP blocked deactivation of the PLA_2 inhibitory polypeptide, lipocortin, by agents such as A23187 and phorbol esters.

Interestingly, most of the NSAIDs, such as aspirin and indomethacin, which are used to treat certain chronic inflammatory conditions are thought to act, at least in part, by inhibiting the cyclo-oxygenase pathway (Brune & Rainsford, 1979). Schenkelaars & Bonta (1986) demonstrated that both aspirin and indomethacin enhanced leukotriene-stimulated macrophage lysosomal enzyme release. We have now shown that these two NSAIDs also enhance A23187-stimulated LTB_4 synthesis and that this effect is reversed by added PGE_2. Indomethacin has also been shown to promote neutrophil superoxide production although the authors suggested that this was due to an inhibition of diacylglycerol lipase activity (Dale & Penfield, 1987). Our results, together with other findings (Docherty & Wilson, 1987; Schenkelaars & Bonta, 1986) provide experimental evidence for the theoretical proposal of Rang & Dale (1987) that NSAIDs could, by inhibiting PGE_2 synthesis and stimulating leukotriene production, exacerbate tissue damage in the long term.

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