

SUBCELLULAR LOCALIZATION OF THE PGE₂ SYNTHESIS ACTIVITY IN MOUSE RESIDENT PERITONEAL MACROPHAGES*

BY C. DARTE[‡] AND H. BEAUFAY

*From the Laboratoire de Chimie Physiologique, Université Catholique de Louvain and
International Institute of Cellular and Molecular Pathology, Brussels, Belgium*

Macrophages are a major source of arachidonic acid (20:4) metabolites (1–7). Among these metabolites, prostaglandins (PG)¹ appear of particular interest in consideration of their proinflammatory properties (8, 9) and of their potential regulative effects on the immune response through their actions on T and possibly B lymphocytes (1, 10–13). PG have also been implicated in feed-back regulation of macrophage activities (14–16).

Under in vitro conditions, macrophages have to be triggered by inflammatory stimuli, either particulate (2, 17) or soluble substances (3, 18), to synthesize PG from their endogenous 20:4 and to release them in the culture medium. The subcellular events underlying this stimulation are still poorly understood. Regarding immune complexes, binding to plasma membrane Fc receptors seems to be a sufficient stimulus for PG secretion (19). How the signal is transmitted from the cell surface to the enzymes acting in PG synthesis is still a pending question. On the basis of electron microscope studies, it has been hypothesized that membrane events bring together the lipid and enzyme constituents necessary for PG production (20). However, information on the macrophage enzymes involved in this process, particularly on their subcellular distribution, are still scarce.

To investigate the question, we have developed a method to assay the activity of the enzyme system which converts arachidonic acid into PGE₂ in homogenates and in subcellular fractions of mouse resident peritoneal (MRP) macrophages. Using this method, we have examined the distribution of PGE₂ synthesis activity by density gradient analysis. The centrifugation behavior indicates that, in unstimulated macrophage homogenates, PGE₂ synthesis is largely associated with sedimentable elements derived from the endoplasmic reticulum. The meaning of this particular location on the mechanisms of induction of PGE₂ synthesis at the subcellular level are examined.

* Supported by a grant from the Stiftung Volkswagenwerk, Federal Republic of Germany, and by grant 2.4540.80 from the Fonds de la Recherche Fondamentale Collective, Belgium.

[‡]To whom requests for reprints should be addressed at Laboratoire de Chimie Physiologique UCL 75.39, Avenue Hippocrate, 75, B 1200 Brussels, Belgium.

¹Abbreviations used in this paper: MRP macrophage, mouse resident peritoneal macrophage; PG, prostaglandins.

Materials and Methods

Harvest, Cultivation, and Fractionation of MRP Macrophages. Female mice of the NMRI strain were used as a source of MRP macrophages throughout this work. Collection of peritoneal cells, cultivation of adhering cells for 24 h in Dulbecco's modified Eagle's medium supplemented with 15% inactivated calf serum and antibiotics (200 U/ml penicillin and 0.1 mg/ml streptomycin), cell disruption, differential centrifugation giving the cytoplasmic extract, and density equilibrium centrifugation of this extract in a linear gradient of sucrose were performed as described in a previous paper (21), with minor modifications. The solutions used for cell disruption and density gradient centrifugation contained 0.15 mg/ml catalase, which enhanced PGE₂ synthesis in various ways (see Results). The experimental procedures were also adapted to assay the PGE₂ synthesis activity in subcellular fractions within hours after cell breakage; in particular the time of centrifugation at 40,000 rev/min in the E-40 rotor (22) was reduced to 60 min.

When specified, cytoplasmic extracts were supplemented with 70 µg digitonin per mg protein and 15 mM sodium pyrophosphate, pH 8, before density equilibrium centrifugation. In several experiments a particulate fraction was isolated by centrifuging cytoplasmic extracts for 30 min at 40,000 rev/min in a no. 40 rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, CA). It is designated MLP because it contains the sedimentable material otherwise recovered separately in the M, L, and P fractions after differential centrifugation (21).

Assay Methods. The PGE₂ synthesis activity was assayed by incubation for 15 min at 37°C in a shaking water bath. The reaction medium contained 7.5 nmol [1-¹⁴C]arachidonic acid, 6 µmol L-adrenaline, 30 nmol reduced glutathione, and 60 µmol phosphate buffer at pH 6.8, in a final volume of 0.3 ml. The reaction was started by addition of the enzyme in 0.1 ml, and stopped by 0.15 ml 1 N HCl. Lipids were extracted twice in 2 ml ethylacetate. The solvent was evaporated under a stream of nitrogen. The dry residue was dissolved in 0.1 ml ethanol and spotted on plastic-backed silica gel plates. Thin-layer chromatography was developed with ethylacetate/acetic acid/2,2,4-trimethylpentane/water (90:20:50:100, vol/vol, organic phase) according to Hamberg and Samuelsson (23). The PGE₂ spots were located on the chromatogram by exposure to iodine vapor, or by autoradiography. They were excised from the plates for extraction of PG in acetone and measurement of the radioactivity by liquid scintillation. A standard (³H-labeled, or nonradioactive PGE₂) was added to the incubation medium at the end of the enzyme reaction to determine the recovery of PG in the samples counted. Tritium counts, or colorimetric assays of PG by the Zimmerman reaction (24) performed according to Takeguchi et al. (25) gave recovery values usually close to 50%.

Protein (26), NAD glycohydrolase (21), galactosyltransferase (27), sulfatase C (27), cytochrome c oxidase (27), and acid α-galactosidase (27) were assayed as described in the quoted articles.

Materials. [1-¹⁴C]arachidonic acid (50–60 mCi/mmol) and [5,6,8,11,12,14,15(n)-³H]PGE₂ (160 Ci/mmol) were purchased from The Radiochemical Centre Ltd. (Amersham, England). Arachidonic acid, PGE₂, superoxide dismutase, hemoglobin, 3-amino-1,2,4-triazole, L-adrenaline bitartrate, and reduced glutathione were products of the Sigma Chemical Co. (St. Louis, MO). Precoated thin layer chromatography-plastic sheets silica gel 60 (thickness 0.2 mm) and digitonin were products of E. Merck A.G. (Darmstadt, Federal Republic of Germany). The other reagents or products have been described previously (21).

Results

PGE₂ Synthesis by Cell-free Extracts of Cultivated MRP Macrophages. On incubation in the presence of L-adrenaline and reduced glutathione cytoplasmic extracts derived from 24 h cultivated MRP macrophages converted [1-¹⁴C]arachidonic acid into several labeled products revealed on the thin layer chromatograms by autoradiography (Fig. 1). The major reaction product accounted for ≈67% of

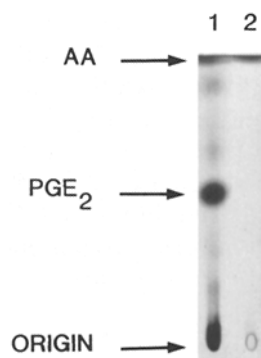


FIGURE 1. Thin layer radiochromatography of the products formed from arachidonic acid by cytoplasmic extracts derived from cultivated MRP macrophages. Lane 1: the cytoplasmic extract (0.2 mg protein) was incubated with [$1\text{-}^{14}\text{C}$]arachidonic acid as described in Materials and Methods. After 15 min at 37°C the lipid material was extracted and chromatographed on silica gel. Labeled compounds were revealed by autoradiography. Arrows indicate the position of PGE_2 and arachidonic acid (AA) standards revealed by exposure to iodine. Lane 2: control incubated exactly as the assay, except for the omission of the cytoplasmic extract.

TABLE I
Inhibition of the PGE_2 Synthesis Activity by Various Compounds

Added compound	Concentration	PGE_2 synthesis activity	
		$\mu\text{mol}/\text{min} \times \text{mg}$ protein	%
—	—	$141 \pm 16^*$	100
Indomethacin	5×10^{-4}	11	8
	0.025	3	2
Dithiothreitol	1.3	35	25
	5	54	38
	mg/ml		
Triton X-100	0.5	56	40
Triton X-405	0.5	124	88
	2.0	81	57

* Mean $\pm$ SD of three preparations.

the label associated with arachidonic acid derivatives and co-migrated with authentic PGE_2 . Consistent with this identification, synthesis of this product was abolished by indomethacin (Table I), a cyclooxygenase inhibitor (28).

The other labeled compounds seen after thin layer chromatography were present in much smaller amounts. They have not been identified, but the two minor radioactive spots found between the origin and PGE_2 probably reveal other products of the cyclooxygenase pathway, because their formation was also inhibited by indomethacin (result not shown). Judging from their respective position on the chromatogram these compounds may be 6-keto $\text{PGF}_{1\alpha}$ and $\text{PGF}_{2\alpha}$, in the order of decreasing polarity. Our subsequent studies were focused on PGE_2 synthesis because this prominent derivative was the only product formed in sufficient amount for quantitative assay.

Kinetic studies were carried out to optimize assay conditions for enzyme

92 SUBCELLULAR LOCALIZATION OF PROSTAGLANDIN E_2 SYNTHESIS

activity, and for linearity of reaction with respect to incubation time and enzyme concentration. The pH-dependency of PGE_2 synthesis showed a maximum between pH 6.5 and 7.5. The activity was very low in the absence of adrenaline (Fig. 2). It rose with the concentration of adrenaline in a manner fairly consistent with a Michaelis-Menten relationship when the activity in the absence of adrenaline was deduced from the other activity values. Adrenaline might thus act as a cosubstrate at some intermediate step in the course of PGE_2 synthesis. In other systems adrenaline and various aromatic compounds stimulate cyclooxygenase, presumably in donating electrons that reduce the hydroperoxy group of PGG_2 into the hydroxyl of PGH_2 (29–31).

Glutathione also enhanced the PGE_2 synthesis activity; the effect was complex and showed a maximum at the concentration of 0.1 mM (Fig. 3). Glutathione is known to act as a cofactor for isomerization of PGH into PGE , although its

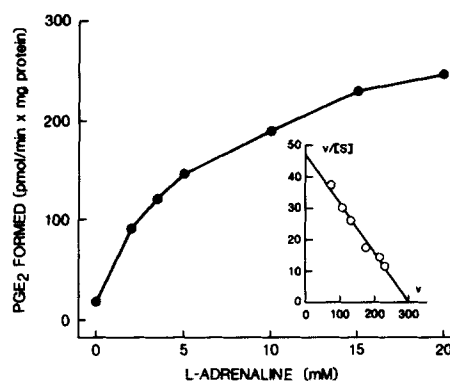


FIGURE 2. Influence of L-adrenaline concentration on PGE_2 synthesis. An MLP fraction (0.25 mg protein) was incubated with 0.05 mM $[1-^{14}C]$ arachidonic acid, in the presence of 1 mM reduced glutathione and L-adrenaline at the concentrations given in abscissa. Otherwise, PGE_2 synthesis activity was assayed as described in Materials and Methods. Inset: Hofstee plot of the values corrected for the activity in the absence of adrenaline.

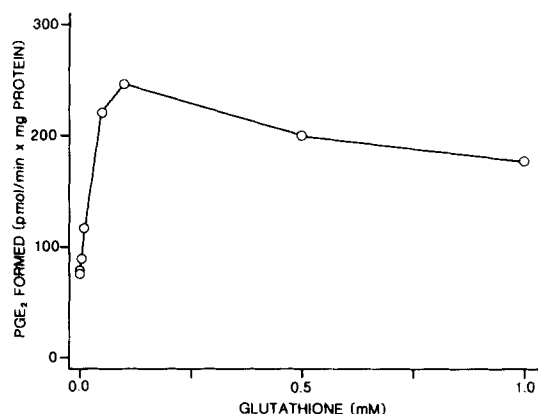


FIGURE 3. Influence of reduced glutathione on PGE_2 synthesis. An MLP fraction (0.22 mg protein) was incubated with 0.05 mM $[1-^{14}C]$ arachidonic acid, in the presence of reduced glutathione at various concentrations (abscissa). Otherwise, PGE_2 synthesis activity was assayed as described in Materials and Methods.

function is still a matter of controversy (32).

The dependence of the PGE₂ synthesis activity on the concentration of arachidonic acid was biphasic, with a sharp optimum at 25 μ M (Fig. 4). Inhibition at higher values may be linked to the amphipathic properties of the substrate. Triton X-100 and Triton X-405 also inhibited the reaction (Table I).

Under our assay conditions, the rate of PGE₂ formation was constant for at least 15 min, and linearly related with the amount of protein added to the reaction medium (Fig. 5).

The properties of the PGE₂ synthesizing enzymes in cultivated MRP macrophages are thus similar to those reported for the cyclooxygenase-isomerase pathway in studies on other cells and tissues: inhibition by indomethacin and activation by L-adrenaline and reduced glutathione. Dithiothreitol also inhibited the reaction (Table I), whereas EDTA and Ca²⁺ had no effect to speak of (not shown).

The PGE₂ synthesis activity decreased rapidly in cytoplasmic extracts kept at 0°C in the medium used for homogenization and fractionation (Fig. 6). The

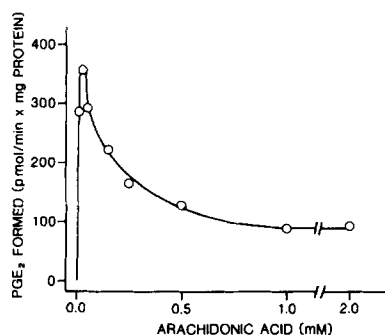


FIGURE 4. Dependence of the PGE₂ synthesis activity on the concentration of arachidonic acid. An MLP fraction (0.08 mg protein) was incubated under the conditions given in Materials and Methods, except that the concentration of arachidonic acid was varied to reach the values given in abscissa.

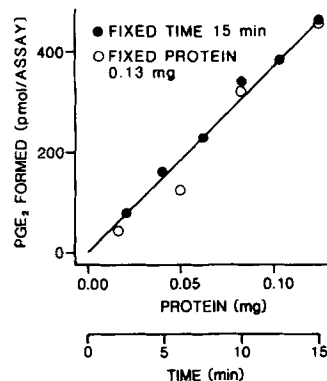


FIGURE 5. Kinetics of PGE₂ synthesis and dependence of the activity on enzyme concentration. PGE₂ were formed by incubation of radiolabeled arachidonic acid with cytoplasmic extracts as described in Materials and Methods. Reactions were run for various times in the presence of 0.13 mg protein, or for 15 min in the presence of various amounts of protein.

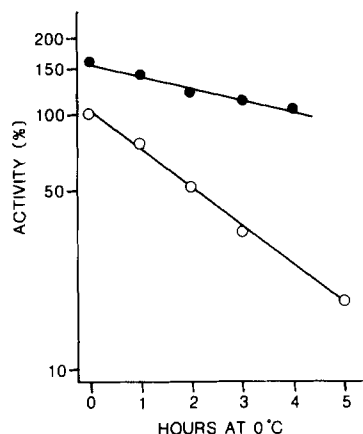
94 SUBCELLULAR LOCALIZATION OF PROSTAGLANDIN E₂ SYNTHESIS

FIGURE 6. Decay of PGE₂ synthesis activity in cytoplasmic extracts derived from cultivated MRP macrophages. A cytoplasmic extract freshly prepared in the standard suspension medium was divided into two parts. At time 0, one volume of the suspension medium was added to one part (○), and one volume of the suspension medium supplemented with 0.30 mg/ml catalase was added to the other part (●). Both preparations were stored at 0°C and assayed at the designated times for PGE₂ synthesis activity, under the conditions given in Materials and Methods. Activities are expressed in percent of the activity measured at time 0 in the absence of added catalase (0.156 nmol PGE₂ formed/min × mg protein). The half-life of PGE₂ synthesis activity was 1.9 h in the standard medium, and 6.3 h in the presence of 0.15 mg/ml catalase.

decay was exponential, with a half-life of ≈ 2 h. It precluded attempts to investigate the subcellular distribution unless conditions that better preserve the enzyme activity be found.

Effects of Catalase. Substances currently used to stabilize cyclooxygenase and PGE₂ isomerase purified from other tissues, including glycerol, ethyleneglycol, EDTA, and glutathione (32, 34, 35) did not give satisfactory results. In contrast, catalase augmented noticeably the stability (half-life ≈ 6 h) and to some extent the initial activity of the enzyme in cytoplasmic extracts (Fig. 6).

Although the mechanism through which catalase acts is still unclear, our results show that distinct effects enhance the activity and slow down its decay. Indeed, the enhancement of PGE₂ synthesis was conditioned by the enzyme activity of catalase, and did not occur when aminotriazole-inhibited catalase was used (Table II). It may be that peroxides are formed in the cell-free extracts and lower the PGE₂ synthesis activity. Addition of hydrogen peroxide to the reaction medium inhibited PGE₂ synthesis, and active catalase prevented this inhibition, at least partly (results not shown). The catalase concentration of 0.15 mg/ml was optimal; higher values lowered the activity of PGE₂ synthesis. In contrast, the greater stability found in the presence of catalase did not require the activity of this enzyme. Aminotriazole-inhibited catalase was as powerful as the active enzyme in slowing the loss of PGE₂ synthesis activity (Table II). Hemoglobin and, to a lesser extent cytochrome *c*, acted like inhibited-catalase, whereas superoxide dismutase had no perceptible stabilizing effect.

Density Distribution of PGE₂ Synthesis Activity. The combined protective and activating effects of catalase, jointly with the characteristic of the E-40 rotor to equilibrate the organelles rapidly in the gradient (22) made it possible to inves-

TABLE II
Effect of Various Proteins on the Activity and Stability of the PGE₂ Synthesis Activity

Added protein	Concentration in storage medium	PGE ₂ synthesis activity		
		Initial	After 2 h at 0°C	
	mg/ml	pmol/min × mg protein		%
<i>Expt. 1</i>				
None		270	150	56
Catalase	0.15	460	400	87
Inhibited catalase	0.15	300	270	90
<i>Expt. 2</i>				
None		190	70	37
Cytochrome <i>c</i>	0.5	170	110	65
Hemoglobin	0.5	200	160	80
<i>Expt. 3</i>				
None		130	60	46
Superoxide dismutase	0.2	140	70	50
Superoxide dismutase	1.0	150	70	47

The experimental conditions were similar to those described in Fig. 6. PGE₂ synthesis activities were assayed as described under Materials and Methods immediately after the additions (initial activity) and after 2 h storage at 0°C. Catalase was inhibited (>99%) by aminotriazole according to Margoliash and Novogrodsky (33). Percent values refer to initial activities.

tigate the subcellular distribution of the PGE₂ synthesis activity by density equilibrium analysis in a linear gradient of sucrose. Cytoplasmic extracts of cultivated MRP macrophages were prepared and fractionated by centrifugation in a sucrose gradient in the medium used previously (21), supplemented with 0.15 mg catalase per ml. The resulting fractions were assayed immediately for PGE₂ synthesis activity, and subsequently for a number of reference enzymes.

The density distributions of reference enzymes are shown by solid lines in separate sections of Fig. 7. They are similar to those obtained after equilibrium centrifugation in the absence of catalase (21). Thus, addition of this enzyme had no perceptible effect on the density of subcellular components. The distribution of PGE₂ synthesis activity is given by the shaded histograms superimposed on the density profile of each reference enzyme for the sake of making the comparison easy. The profile of PGE₂ synthesis activity fits fairly well with the distribution of sulfatase C, although a slight excess is noted in the high density subfractions. It markedly dissociates from the density patterns of galactosyltransferase and NAD glycohydrolase, which occur in subfractions of lower density, and from cytochrome *c* oxidase, which shows a much narrower density profile. The dissociation from acid α -galactosidase is also apparent, although the excess over sulfatase C in high density subfractions could reflect association of part of the PGE₂ synthesis activity with lysosomal elements. As specified in the legends of Figs. 7 and 8, the recovery of PGE₂ synthesis activity in the gradient fractions was ~60%. Apparently, the decay of this activity was somewhat faster in the gradient than in the sample kept to establish the balance sheet.

Sulfatase C belongs to a group of enzymes associated with subcellular components that show two typical properties in density equilibrium centrifugation: in

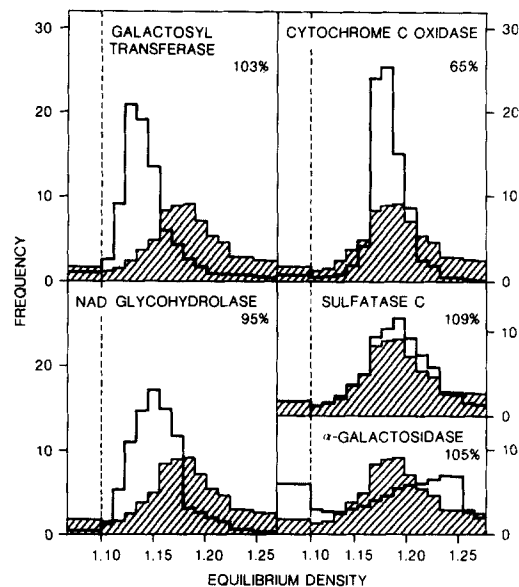
96 SUBCELLULAR LOCALIZATION OF PROSTAGLANDIN E_2 SYNTHESIS

FIGURE 7. Density distribution of PGE_2 synthesis activity after isopycnic centrifugation in sucrose gradient. Results of two experiments in which cytoplasmic extracts have been layered over a linear gradient of sucrose and centrifuged for density equilibration as described in Materials and Methods and with more experimental details in reference 21. The frequency histograms obtained have been normalized and averaged as described elsewhere (22). The represented portion of the density distribution is divided into 15 fractions of identical density increment and extends from 1.10 to 1.27 in density. On the left-hand side of the dashed line a single block gives the activity found in the sample layer (below 1.1 density units); it has been constructed assuming a density increment of 0.03 units. Solid lines give the density distribution of reference enzymes specified in each section. These density profiles are compared to that of the PGE_2 synthesis activity (shading), which is superimposed on each plot. Percentage values give the average recoveries of reference enzymes in the gradient fractions. Recovery of PGE_2 synthesis activity was 55%.

contrast to NAD glycohydrolase and other enzymes associated with the cell periphery they do not acquire a higher density by treatment with digitonin, but they shift to a lower density when sodium pyrophosphate has been added to cytoplasmic extracts (21). Because the density distribution of PGE_2 synthesis activity correlates with sulfatase C in untreated preparations, it was of interest to examine its centrifugation behavior in preparations treated with digitonin and pyrophosphate. Density distributions of reference enzymes were established using cytoplasmic extracts supplemented with digitonin and sodium pyrophosphate. As expected from our previous studies (21) the density profiles of NAD glycohydrolase and sulfatase C were shifted in opposite directions. The median equilibrium density of NAD glycohydrolase was 1.153 in untreated preparations (Fig. 7) and became 1.184 after treatment (Fig. 8), whereas that of sulfatase C decreased from 1.185 to 1.160 as a result of the treatment. The behavior of the PGE_2 synthesis activity was similar to that of sulfatase C, except that some activity remained in the high density subfractions. Median values of the equilibrium density were 1.189 and 1.171 in the control and the treated preparation, respectively. Cytochrome *c* oxidase was not perceptibly influenced by the treatment, and acid α -galactosidase was largely released in the suspension medium

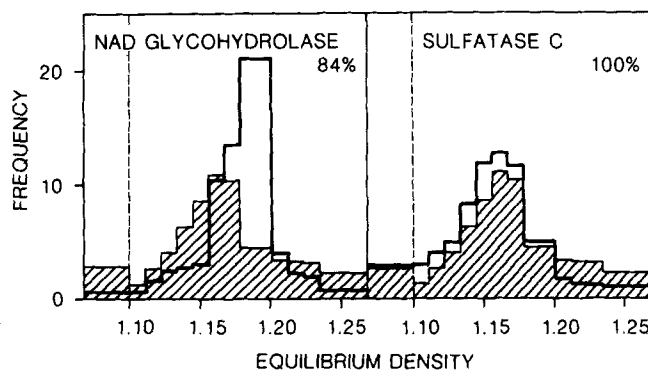


FIGURE 8. Density distribution of PGE_2 synthesis activity, sulfatase C and NAD glycohydrolase after treatment with digitonin and pyrophosphate. The cytoplasmic extract has been brought into a medium which contained digitonin and sodium pyrophosphate, as described in Materials and Methods, and subsequently layered on a linear gradient of sucrose and centrifuged exactly as in the experiments reported in Fig. 7. Solid lines give the distribution of NAD glycohydrolase and sulfatase C; the shading is the distribution of PGE_2 synthesis activity. Histograms were constructed as described in Fig. 7. Recovery of PGE_2 synthesis activity was 65%.

(not shown), in agreement with the properties of the mitochondrial and lysosomal enzymes reported earlier (21, 27).

Discussion

In cell-free extracts derived from MRP macrophages the general properties of the enzymes that convert arachidonic acid into PGE_2 are similar to those reported for the enzymes from several other sources (36). In particular the reaction is strongly inhibited by indomethacin and activated synergistically by reduced glutathione and L-adrenaline.

The dependency on reduced glutathione is of special interest, because it may have a direct physiological significance. Recent work has shown that macrophages depleted of intracellular glutathione have a reduced capacity for PGE_2 secretion (37). This was interpreted as indicating a cofactor role for reduced glutathione in PGE_2 synthesis by macrophages, by analogy with the properties of enzymes from other sources. The finding that reduced glutathione stimulates the PGE_2 synthesis activity in cell-free extracts from MRP macrophages brings a direct support to this view.

In contrast, the absolute requirement for L-adrenaline in PGE_2 synthesis by cytoplasmic extracts from MRP macrophages reflects the need for a reducing agent to convert PGG_2 into PGH_2 through cyclooxygenase (36). The actual electron donor in living cells has not yet been identified; L-adrenaline, which acts at relatively high concentration under our assay conditions, is likely to be a mere substitute.

Because the PGE_2 synthesis activity is labile in cell-free extracts from MRP macrophages, studies on its subcellular distribution were hampered until this difficulty was largely overcome by addition of catalase to the suspension medium. The presence of catalase resulted in a stimulated activity (by 1.7-fold) in fresh homogenates, and in a distinctly greater stability (by more than threefold) of the

98 SUBCELLULAR LOCALIZATION OF PROSTAGLANDIN E_2 SYNTHESIS

active enzyme. Our goal was not to elucidate the mechanisms responsible for these effects of catalase, which are still unclear. The enhancement of the PGE_2 synthesis activity and its stabilization are apparently independent phenomena. The former is conditioned by the catalase activity, which suggests that peroxides formed in cytoplasmic extracts from MRP macrophages reversibly inhibit the PGE_2 synthesis. According to Hemler and Lands (38) hydroperoxides initiate the cyclooxygenase reaction, but may also lead to unproductive oxidized forms of the enzyme, susceptible to be reduced back to the active form.

In contrast, the stabilizing action of catalase is unrelated to its own enzyme activity. Amino-triazole-inhibited catalase was as powerful as the active protein and other hemoproteins, e.g. hemoglobin and cytochrome *c*, led to a similar result. Hemoproteins activate PG endoperoxide synthetase in purified enzyme preparations indirectly, free hematin dissociated from hemoproteins by the interaction of arachidonic acid being then the true activating factor (39). If a loss of heme is involved in the decay of PGE_2 synthesis activity in cytoplasmic extracts of MRP macrophages, other hemoproteins may have a stabilizing influence. This explanation, however, is hardly valid for cytochrome *c*, in which heme is covalently linked to the apoprotein.

Comparison of the density distributions in a linear gradient of sucrose disclosed that PGE_2 synthesis behaves like sulfatase C, an enzyme that may be regarded as a reference for the endoplasmic reticulum in MRP macrophages (21). The distribution of PGE_2 synthesis activity clearly differed from those of galactosyl-transferase, cytochrome *c* oxidase, and NAD glycohydrolase, which are essentially associated with the Golgi apparatus, mitochondria, and plasma membrane, respectively. Thus, we have found no evidence in favor of a plasma membrane-associated activity, postulated recently (40) to account for the metabolism of exogenously supplied arachidonic acid into oxygenated products by MRP macrophages. Because catalase was added to the sucrose solutions making up the gradient it was not possible to compare the distribution of PGE_2 synthesis activity with that of the catalase-bearing organelles identified recently in these cells (21). Nevertheless, judging from the behavior of the endogenous catalase in our earlier experiments, the PGE_2 synthesis activity cannot be assigned to these organelles. The distributions shown in Figs. 7 and 8 also dissociate the bulk of this activity from lysosomes. Indeed, the density distribution of acid α -galactosidase is not perturbed like that of enzymes associated with the endoplasmic reticulum when cytoplasmic extracts are treated with pyrophosphate (21). However, the presence of some activity in excess over that of sulfatase C at high density values makes it possible that a small part of the PGE_2 synthesis activity be associated with lysosomes, or related vacuoles.

These conclusions suffer from two limitations that must be briefly examined. First, the recovery of the PGE_2 synthesis activity in the density gradient fractions was consistently around 60%, even though the experimental design takes the loss of enzyme activity during the time of centrifugation into account. Conceivably, a pool of enzyme especially labile could have a distinct subcellular distribution undetectable in our experiments. This assumption is not consistent with the finding that the loss of activity in cytoplasmic extracts follows a simple first-order kinetics (Fig. 6).

Another limitation is that the activity detected in our assays of PGE₂ synthesis involves two enzymes acting successively, cyclooxygenase and PGE isomerase (41). These enzymes could be associated with distinct subcellular components. If one of the reactions is sharply rate-limiting in the cytoplasmic extract, its distribution in the gradient cannot markedly differ from that found when arachidonic acid is transformed into PGE₂, and our conclusion is valid for the rate-limiting enzyme. Otherwise, the PGE₂ synthesis activity would be found in the fractions in which the distributions of cyclooxygenase and isomerase overlap, and the correlation between its density profile and that of sulfatase C could be nothing but a coincidence. This possibility seems unlikely because the correlation persists after treatment of the cytoplasmic extracts with pyrophosphate and digitonin. The only subcellular components known to be perturbed in their density distribution by these agents derived from the plasma membrane and from the endoplasmic reticulum (21), and they are shifted in opposite directions. Thus, the joint shifts of PGE₂ synthesis activity and of sulfatase C shows that at least the rate-limiting enzyme reaction in the transformation of arachidonic acid into PGE₂ is associated with the endoplasmic reticulum membranes.

The various phenomena that surround the synthesis of PG by macrophages include the triggering events, the release of free arachidonic acid from phospholipids by phospholipase(s), and the oxygenation and isomerization reactions that convert arachidonic acid into PGE₂. In connection with other studies our results emphasize the fact that they involve several distinct organelles of the cell, at least the plasma membrane and the endoplasmic reticulum.

It is well established that the binding of immune complexes to Fc receptors of the plasma membrane of MRP macrophages is a sufficient stimulus and that phagocytosis of the complexes is not a prerequisite for synthesis of PGE₂ to occur (19, 20). In addition, the plasma membrane Fc γ receptor for IgG₂b of a murine cell line possesses an intrinsic phospholipase A₂ activity that is expressed concomitantly with the release of PGE₂ after binding of IgG₂b (42, 43). The subcellular source of the phospholipids that supply the phospholipase-cyclooxygenase system with arachidonic acid is still uncertain, but the association of PGE₂ synthesis activity with endoplasmic reticulum membranes, rather than with plasma membranes, implies some kind of cooperation between distinct entities of the cell. Earlier studies by Brune et al. (20) have revealed that there is a striking coincidence of the PG release and the formation of large cytoplasmic vacuoles in macrophages, even when secretion of PG is induced in the presence of cytochalasin B, which inhibits phagocytosis. It was envisaged that fusion of different membranes brings the phospholipids in contact with the enzymes of the PG synthesis pathway. This concept is still a reasonable hypothesis for future work. Alternatively, phospholipids and/or arachidonic acid and its derivatives could be exchanged by specific carrier proteins analogous to the phosphatidylcholine exchange protein identified in liver cells (44).

Summary

The aim of this work was to establish, on a quantitative basis, the subcellular distribution of the enzyme system that converts arachidonic acid into prostaglandin (PG) E₂ in mouse resident peritoneal (MRP) macrophages. Kinetic studies

100 SUBCELLULAR LOCALIZATION OF PROSTAGLANDIN E₂ SYNTHESIS

were conducted on cell-free extracts derived from cells cultivated for 1 d, using [1-¹⁴C]arachidonic acid as substrate and measuring the label in PGE₂ after extraction and thin layer chromatography. The activity was synergistically enhanced by L-adrenaline and reduced glutathione, inhibited by indomethacin, and linearly related to the concentration of the cell-free extract. It was labile at 0°C in the medium used for homogenization and fractionation of the cells (half-life <2 h). Addition of catalase (0.15 mg/ml) to the suspension medium increased the initial activity (by ≈70%) and the stability (half-life ≈6 h) of the enzyme in cytoplasmic extracts. It enabled us to establish the density distribution after isopycnic centrifugation in a linear gradient of sucrose. The sample centrifuged consisted of untreated cytoplasmic extracts, or cytoplasmic extracts treated with digitonin and Na pyrophosphate. Comparison of the centrifugation behavior of PGE₂ synthesis activity with that of various enzymes used as reference for the major subcellular entities has revealed that PGE₂ synthesis fairly fits the density profile of sulfatase C in each case. The conclusion is that at least the rate-limiting reaction in the conversion of arachidonic acid into PGE₂ is catalyzed by an enzyme associated with the endoplasmic reticulum.

We gratefully acknowledge the expert technical assistance of Beatrice Paulus-Clotuche.

Received for publication 11 July 1983.

References

1. Gordon, D., M. A. Bray, and J. Morley. 1976. Control of lymphokine secretion by prostaglandins. *Nature (Lond.)*. 262:401.
2. Humes, J. L., R. J. Bonney, L. Pelus, M. E. Dahlgren, S. J. Sadowski, F. A. Kuehl, Jr., and P. Davies. 1977. Macrophages synthesise and release prostaglandins in response to inflammatory stimuli. *Nature (Lond.)*. 269:149.
3. Kurland, J. I., and R. Bockman. 1978. Prostaglandin E production by human blood monocytes and mouse peritoneal macrophages. *J. Exp. Med.* 147:952.
4. Scott, W. A., J. M. Zrike, A. L. Hamill, J. Kempe, and Z. A. Cohn. 1980. Regulation of arachidonic acid metabolites in macrophages. *J. Exp. Med.* 152:324.
5. Valone, F. H., M. Franklin, F. F. Sun, and E. J. Goetzl. 1980. Alveolar macrophage lipoxygenase products of arachidonic acid: isolation and recognition as the predominant constituents of the neutrophil chemotactic activity elaborated by alveolar macrophages. *Cell. Immunol.* 54:390.
6. Doig, M. V., and A. W. Ford-Hutchinson. 1980. The production and characterisation of products of the lipoxygenase enzyme system released by rat peritoneal macrophages. *Prostaglandins*. 20:1007.
7. Rouzer, C. A., W. A. Scott, Z. A. Cohn, P. Blackburn, and J. M. Manning. 1980. Mouse peritoneal macrophages release leukotriene C in response to a phagocytic stimulus. *Proc. Natl. Acad. Sci. USA*. 77:4928.
8. Vane, J. R. 1976. Prostaglandins as mediators of inflammation. *Advances in Prostaglandin and Thromboxane Research*. 2:791.
9. Wedmore, C. V., and T. J. Williams. 1981. Control of vascular permeability by polymorphonuclear leukocytes in inflammation. *Nature (Lond.)*. 289:646.
10. Zimecki, M., and D. R. Webb. 1976. The regulation of the immune response to T-independent antigens by prostaglandins and B cells. *J. Immunol.* 117:2158.
11. Goodwin, J. S., and D. R. Webb. 1980. Regulation of the immune response by

- prostaglandins. *Clin. Immunol. Immunopathol.* 15:106.
12. Leung, K. H., and E. Mihich. 1980. Prostaglandin modulation of development of cell-mediated immunity in culture. *Nature (Lond.)*. 288:597.
 13. Mertin, J., and A. Stackpoole. 1981. Anti-PGE antibodies inhibit *in vivo* development of cell-mediated immunity. *Nature (Lond.)*. 294:456.
 14. Schultz, R. M., N. A. Pavlidis, W. A. Stylos, and M. A. Chirigos. 1978. Regulation of macrophage tumoricidal function: a role for prostaglandins of the E series. *Science (Wash. DC)*. 202:320.
 15. Cantarow, W. D., H. T. Cheung, and G. Sundharadas. 1978. Effects of prostaglandins on the spreading, adhesion and migration of mouse peritoneal macrophages. *Prostaglandins*. 16:39.
 16. Snyder, D. S., D. I. Beller, and E. R. Unanue. 1982. Prostaglandins modulate macrophage Ia expression. *Nature (Lond.)*. 299:163.
 17. Bonney, R. J., P. Naruns, P. Davies, and J. L. Humes. 1979. Antigen-antibody complexes stimulate the synthesis and release of prostaglandins by mouse peritoneal macrophages. *Prostaglandins*. 18:605.
 18. Humes, J. L., P. Davies, R. J. Bonney, and F. A. Kuehl, Jr. 1978. Phorbol myristate acetate (PMA) stimulates the release of arachidonic acid and its cyclooxygenation products by macrophages. *Fed. Proc.* 37:1318.
 19. Rouzer, C. A., W. A. Scott, J. Kempe, and Z. A. Cohn. 1980. Prostaglandin synthesis by macrophages requires a specific receptor-ligand interaction. *Proc. Natl. Acad. Sci. USA*. 77:4279.
 20. Brune, K., M. Glatt, H. Kälin, and B. A. Peskar. 1978. Pharmacological control of prostaglandin and thromboxane release from macrophages. *Nature (Lond.)*. 274:261.
 21. Dart, C., and H. Beaufay. 1983. Analytical subcellular fractionation of cultivated mouse resident peritoneal macrophages. *J. Exp. Med.* 157:1208.
 22. Beaufay, H., and A. Amar-Costesec. 1976. Cell fractionation techniques. In *Methods in Membrane Biology*. E. D. Korn, editor. Plenum Press, New York-London. 6:1.
 23. Hamberg, M., and B. Samuelsson. 1966. Prostaglandins in human seminal plasma. Prostaglandins and related factors 46. *J. Biol. Chem.* 241:257.
 24. Wilson, H. 1954. Chromogenic values of various ketosteroids in a micro modification of the Zimmermann reaction: comparison with the macro procedure. *Arch. Biochem. Biophys.* 52:217.
 25. Takeguchi, C., E. Kohn, and C. J. Sih. 1971. Mechanism of prostaglandin biosynthesis. I. Characterization and assay of bovine prostaglandin synthetase. *Biochemistry*. 10:2372.
 26. Böhlen, P., S. Stein, W. Dairman, and S. Udenfriend. 1973. Fluorometric assay of proteins in the nanogram range. *Arch. Biochem. Biophys.* 155:213.
 27. Canonico, P. G., H. Beaufay, and M. Nyssens-Jadin. 1978. Analytical fractionation of mouse peritoneal macrophages: physical and biochemical properties of subcellular organelles from resident (unstimulated) and cultivated cells. *J. Reticuloendothel. Soc.* 24:115.
 28. Vane, J. R. 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biol.* 231:232.
 29. Miyamoto, T., N. Ogino, S. Yamamoto, and O. Hayaishi. 1976. Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J. Biol. Chem.* 251:2629.
 30. Egan, R. W., J. Paxton, and F. A. Kuehl, Jr. 1976. Mechanism for irreversible self-deactivation of prostaglandin synthetase. *J. Biol. Chem.* 251:7329.
 31. Hemler, M. E., C. G. Crawford, and W. E. M. Lands. 1978. Lipooxygenase activity of purified prostaglandin-forming cyclooxygenase. *Biochemistry*. 17:1772.

102 SUBCELLULAR LOCALIZATION OF PROSTAGLANDIN E₂ SYNTHESIS

32. Ogino, N., T. Miyamoto, S. Yamamoto, and O. Hayaishi. 1977. Prostaglandin endoperoxide E isomerase from bovine vesicular gland microsomes, a glutathione-requiring enzyme. *J. Biol. Chem.* 252:890.
33. Margoliash, E., and A. Novogrodsky. 1958. A study of the inhibition of catalase by 3-amino-1:2:4-triazole. *Biochem. J.* 68:468.
34. Hemler, M., W. E. M. Lands, and W. L. Smith. 1976. Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. *J. Biol. Chem.* 251:5575.
35. Roth, G. J., C. J. Siok, and J. Ozols. 1980. Structural characteristics of prostaglandin synthetase from sheep vesicular gland. *J. Biol. Chem.* 255:1301.
36. Samuelsson, B., M. Goldyne, E. Granström, M. Hamberg, S. Hammarström, and C. Malmsten. 1978. Prostaglandins and thromboxanes. *Annu. Rev. Biochem.* 47:997.
37. Rouzer, C. A., W. A. Scott, O. W. Griffith, A. L. Hamill, and Z. A. Cohn. 1982. Arachidonic acid metabolism in glutathione-deficient macrophages. *Proc. Natl. Acad. Sci. USA.* 79:1621.
38. Hemler, M. E., and W. E. M. Lands. 1980. Evidence for a peroxide-initiated free radical mechanism of prostaglandin biosynthesis. *J. Biol. Chem.* 255:6253.
39. Ueno, R., T. Shimizu, K. Kondo, and O. Hayaishi. 1982. Activation mechanism of prostaglandin endoperoxide synthetase by hemoproteins. *J. Biol. Chem.* 257:5584.
40. Scott, W. A., N. A. Pawlowski, M. Andreach, and Z. A. Cohn. 1982. Resting macrophages produce distinct metabolites from exogenous arachidonic acid. *J. Exp. Med.* 155:535.
41. Miyamoto, T., S. Yamamoto, and O. Hayaishi. 1974. Prostaglandin synthetase system—Resolution into oxygenase and isomerase components. *Proc. Natl. Acad. Sci. USA.* 71:3645.
42. Suzuki, T., T. Saito-Taki, R. Sadasivan, and T. Nitta. 1982. Biochemical signal transmitted by Fc γ receptors: phospholipase A₂ activity of Fc γ 2b receptor of murine macrophage cell line P388D₁. *Proc. Natl. Acad. Sci. USA.* 79:591.
43. Nitta, T., and T. Suzuki. 1982. Fc γ 2b receptor-mediated prostaglandin synthesis by a murine macrophage cell line (P388D₁). *J. Immunol.* 128:2527.
44. Kamp, H. H., K. W. Wirtz, and L. L. Van Deenen. 1973. Some properties of phosphatidylcholine exchange protein purified from beef liver. *Biochim. Biophys. Acta.* 318:313.