Biochemical Pharmacology, Vol. 34, No. 23, pp. 4137–4143, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

EFFECT OF INTRACELLULAR OXYGEN-FREE RADICALS ON THE FORMATION OF LIPID DERIVED MEDIATORS IN RAT RENOMEDULLARY INTERSTITIAL CELLS

ISABELLE MARIDONNEAU-PARINI, ARMEL FRADIN, LHOUSSEINE TOUQUI and FRANÇOISE RUSSO-MARIE*

Unité des Venins, Unité associée Institut Pasteur/INSERM U285, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France

(*Received* 10 *April* 1985; *accepted* 18 *June* 1985)

Abstract—An intracellular generation of oxygen free radicals was induced by phenazine methosulfate (PMS) in rat renomedullary interstitial cells (RMIC) in culture. This response was associated with an increase in PGE_2 and 15 HETE production. The synthesis of cyclooxygenase and lipoxygenase derivatives in PMS-treated cells was inhibited by indomethacin and NDGA respectively. Inhibitors of PLA_2 such as mepacrine and dexamethasone were able to inhibit partially the PGE_2 synthesis induced by PMS. The formation of lyso-platelet activating factor, a product of membrane-bound phospholipid, by a PLA_2 catalyzed reaction was also stimulated in PMS-treated cells.

Superoxide dismutase added to the incubation medium enhanced the PMS-dependent PGE_2 synthesis whereas catalase decreased it, suggesting the involvement of H_2O_2 in this process. In addition, a depletion of soluble thiol groups was observed in PMS-treated cells. Treatment of RMIC by the thiol oxidative agent, diamide, mimicked the effect of PMS on PGE_2 synthesis, whereas diamide did not increase the formation of lyso-PAF indicating its inability to stimulate PLA_2. These results suggest that cyclooxygenase may be involved in this process, indeed added arachidonate, bypassing PLA_2, enhanced PGE_2 synthesis in PMS-treated cells further supporting the involvement of cyclooxygenase. In conclusion, generation of oxygen free radicals by PMS in RMIC enhanced the synthesis of lipid derived mediators. A decrease in the cellular thiol content is partially involved in cyclooxygenase activation but does not appear to be involved in PLA_2 activation.

The response of activated inflammatory cells to various agonists involves, among other complex phenomena, the activation of two types of enzymes: (i) phospholipase A_2 (PLA_2)† which leads to the liberation of arachidonic acid, the common precursor of cyclooxygenase and lipoxygenase derivatives; and of lyso-PAF, precursor of platelet activating factor (PAF) [1–5]; (ii) NADPH-oxidase which generates oxygen free radicals such as superoxide anions (O_2^-) [6–8].

Activation of these two enzymatic pathways during the inflammatory response has suggested a possible inter-relationship. In addition, interactions between oxygen free radicals and arachidonic acid metabolism could occur in various physiopathological conditions in which oxygen-free radicals are generated. Recently, it was reported that an extracellular generation of oxygen-free radicals induced a stimulation of PLA_2 via H_2O_2 in rat renal glomeruli [9]. Taylor *et al.* have described a PLA_2 stimulation and a modulation of the prostaglandin synthesis as a function of the organic hydroperoxide concentration, *t*-butyl hydroperoxide, in lung fibroblasts [10]. On the other hand, it was described that oxygen-free radicals formed in the course of arachidonic acid cascade activation are responsible for deactivation of different enzymes (i.e. cyclooxygenase, prostacyclin synthetase) [11–14]. We proposed to study the effects of intracellular oxygen free radicals on the synthesis of lipid-derived mediators using phenazine methosulfate (PMS), which induced oxygen-free radical production [16, 17].

The effect of PMS was studied in rat renomedullary interstitial cells (RMIC) where the metabolism of phospholipids has been well characterized [15]. In various cell types PMS reacts cyclically with intracellular NAD(P)H leading mainly to the formation of superoxide anions (O_2^-) [16, 17]. Once formed, O_2^- undergoes rapid spontaneous or enzyme-catalyzed dismutation by superoxide dismutases (SOD) to produce hydrogen peroxide: H_2O_2 [18]. The enzymatic catabolism of H_2O_2 involves catalase and GSH-peroxidase [19]. Since GSH is involved in the regulation of prostaglandin synthesis, we also investigated the effect of PMS on the cellular thiol content.

* To whom all correspondence should be addressed.

† Abbreviations: DDC, diethyldithiocarbamate; DTT, 1,4-dithiotreitol; GSH, reduced glutathione; 15-HETE, 15 hydroxyeicosatetraenoic acid; NBT, nitroblue tetrazolium; NDGA, nor-dihydro-guaiaretic acid; PGE_2, prostaglandin E2; PAF, platelet activating factor; PBS, phosphate buffered saline; PMS, phenazine methosulfate; PLA_2 phospholipase A_2; RMIC, renomedullary interstitial cells.

Enzyme nomenclature: Catalase, EC 1.11.1.6; Cyclooxygenase, prostaglandin synthase, EC 1.14.99.1; Glutathione peroxidase, EC 1.8.4.2; Lipoxygenase, EC 1.13.11.12; Phospholipase A_2, EC 3.1.1.4; Superoxide dismutase, EC 1.15.1.1.

We report in this paper that PMS induced the production of oxygen-free radicals and increased the synthesis of lipid derived mediators in RMIC. The increase in prostaglandin synthesis is due in part to the stimulation of PLA_2 activity and to the cellular depletion of thiol groups which apparently enhances cyclooxygenase activity.

MATERIALS AND METHODS

Materials. Bovine serum albumin (fatty acid free), dexamethasone, diethyldithiocarbamate, nitroblue tetrazolium, 1,4 dithiotreitol, diamide, Na^+ arachidonate, PAF, porcine pancreatic PLA_2 (600 UI/mg of protein), catalase (from bovine liver, 2000 UI/mg of protein), superoxide dismutase (from bovine erythrocytes, 3100 UI/mg of protein) were purchased from Sigma Chemical Corp. (St Louis, MO). Solvents for HPLC were purchased from Carlo Erba, Milan, Italy. The antibody to PGE_2 was from Institut Pasteur Production, Paris, France. ^{3}H-PGE_2 (120 Ci/mmole) and ^{14}C-arachidonic acid (60–80 mCi/mmole) were obtained from New England Nuclear (Paris, France). PGE_2 was a gift of John Pike (The Upjohn Company, Kalamazoo, MI). A23187 was from Calbiochem (La Jolla, CA). Phosphate buffered saline, PBS, from GIBCO Biocult, Scotland. Mepacrine was from Rhone-Poulenc, Paris. Indomethacin was obtained from Merck. All other reagents were of analytical grade and obtained from local suppliers.

Methods. Monolayer tissue cultures of rat renomedullary interstitial cells were obtained as described previously [15]. Cells were grown in RPMI 1640 supplemented by 10% of heat inactivated fetal calf serum at 37° with 5% CO_2 in air atmosphere. Cultures were maintained in T75 flasks and seeded onto T25 flasks or 10-cm^2 Petri dishes for experiments. Experiments were always performed on growing cells, just before confluence. These cells synthesize mainly prostaglandin E_2 (PGE_2) after PLA_2 stimulation [20]. They were used in tissue culture up to the 25th passage. They have been shown by Muirhead [21] to retain their characteristics up to the 45th passage. For experimental procedures cells were incubated in a PBS medium (pH 7.4 at 37°) after being washed three times in this medium. The superoxide anion production was measured spectrophotometrically by the reduction of nitroblue tetrazolium (NBT) in formazan using the wavelength 560 nm [17, 22]. Cells were incubated at 37° in a PBS medium containing 75 μM NBT, in the absence or presence of 50 μg/ml SOD which inhibits the superoxide-induced NBT reduction [16]. In some experiments, the intracellular SOD was inhibited by the copper-chelator diethyldithiocarbamate [23, 24] as previously described [17]. PMS which is highly soluble in PBS was then added. Absorbance measurement was performed in the incubation medium.

Radioimmunoassay of PGE_2 was performed without extraction directly in the incubation medium (PBS, pH 7.4). We ensured that in this medium, extraction was not required for the assay. The reaction between the antibody to PGE_2 and PGE_2 was stopped by adding dextran-coated charcoal as described by Russo-Marie *et al.* [15] and the radioactivity of the supernatants counted by liquid scintillation spectrometry.

HETE were measured 20 min after addition of 75 μM PMS at 37° in a PBS solution. The reaction was stopped by adding 1 vol. cold methanol. The samples were centrifuged (3500 *g*, 4°, 15 min) and evaporated under vacuum at 56° in a water bath. Dry residues were dissolved in H_2O/methanol (1:1) and analysed by reverse phase high performance liquid chromatography (RP-HPLC) [25]. The samples were eluted at a flow rate of 1 ml/min. The solvent used was methanol/water/acetic acid (73:27:0.01) (pump 6000 A and column μ C18 Bondapak, 2.9 × 300 Waters, Milford, MA). The elution of monohydroxy acids was monitored by measurement of u.v. absorbance at 234 nm (Lamda Max 481 Waters), authentic radiolabelled standards were added. Using this system we were able to separate 15-HETE from 12-HETE. In some experiments, 0.1 μCi ^{14}C-Arachidonic acid was incubated for 2 hr with cells in RPMI 1640 medium without fetal calf serum. At the end of this incubation period, free ^{14}C-arachidonic acid was eliminated by two washings with a PBS Ca^{2+}- and Mg^{2+}-free medium containing 0.1% bovine serum albumin (BSA) and one washing without BSA. Then 75 μM PMS was added. After elution, the samples were treated as described above and standards were added. Aliquots of 1 ml were then counted by liquid scintillation spectrometry.

For measurement of soluble reduced thiol groups, cells were washed three times with a PBS solution and scrapped. After centrifugation (5 min, 900 *g*), the pellets were diluted in PBS in order to reach a final protein concentration of approx. 200 μg/ml. Cells were then incubated for 20 min at 37° with 25 μM PMS or 0.2 mM diamide. The reaction was stopped by addition of 1 vol. of trichloroacetic acid (6.5%). Samples were then centrifuged and the supernatants removed for the spectrophotometric measurement of soluble thiol groups at 535 nm according to the method described by Saville [26]. Sample values were compared to a GSH standard curve. The diamide concentration used was not able to affect the cell viability during the time of the experiment.

Lyso-platelet activating factor (lyso-PAF) was measured as an index of endogenous PLA_2 activity. Cells were incubated in absence or in presence of PMS, diamide or A23187 for 20 min at 37°. The reaction was stopped by adding methanol–chloroform (2:1). Lipids were extracted according to Bligh and Dyer [27] and the chloroformic phases containing lipids were reduced to dryness under oxygen-free nitrogen. Aliquots from each extract were submitted to thin layer chromatography and lyso-PAF was purified as described previously [28]. Then, lyso-PAF was converted by acetylation to a physiological active product, PAF, which was measured by its ability to aggregate rabbit platelets [29] prepared according to Aıdlie *et al.* [30]. We ensured that the platelet aggregation was not caused by PMS since the latter was completely eliminated by the extraction procedure.

PLA_2 activity was measured *in vitro* using *E. coli* membranes as described in detail by Rothhut *et al.* [31]. Briefly, ^{3}H-oleic acid was incubated with *E.*

coli during exponential growth phase. Then, the labelled membranes were used as the substrate for porcine pancreatic PLA_2 (100 ng of enzyme per assay). The PLA_2 activity was reflected by the liberation of free ^{3}H-oleic acid in the incubation medium (pH 8 at 4°), and measured by liquid scintillation spectrometry. Under these conditions the PLA_2 activity was at the initial rate.

After the experiments, flasks were rinsed thoroughly with PBS to remove dead cells, and 1 N NaOH was added. Protein measurement was performed using the technique described by Lowry *et al.* [32]. Cell viability was evaluated by the number of attached cells to the flask, dead cells being unattached. This was determined by microscopic observations and by monitoring cell protein content of attached cells.

Cells were incubated for 20 hr with 1 μM dexamethasone in RPMI 1640 medium containing 10% FCS. In these conditions, dexamethasone induces the synthesis of "lipocortin" an antiphospholipase protein [31, 33]. Cells were then washed three times with PBS, in order to eliminate fetal calf serum and dexamethasone. They were then left for an additional 4-hr incubation period in a MEM Eagles modified medium. During this second incubation period, lipocortin was still synthesized and present in medium and membranes [31]. Experiments with PMS were performed directly in this medium. Control experiments without dexamethasone were run in parallel.

Indomethacin, an inhibitor of cyclooxygenase, and NDGA, an inhibitor of lipoxygenase, used both at a final concentration of 10 μM, were dissolved in ethanol provided the final ethanol concentration in the medium was less than 0.1%. Control experiments were performed in the presence of the same ethanol concentration. We ensured that ethanol at this concentration had no effect per se on the free radical measurement and PGE_2 synthesis. Mepacrine (1 μM), an inhibitor of PLA_2 and DDC (0.5 to 1 mM) an inhibitor of SOD, were dissolved in PBS. All these drugs (indomethacin, NDGA, mepacrine and DDC) were incubated for 30 min at 37° with the cells before the addition of PMS. In experiments performed with SOD, catalase, diamide or 1,4 dithiotreitol (DTT), cells were pre-incubated for 5 min at 37° with these compounds, before addition of PMS.

The ability of cells to synthesize arachidonic acid metabolites was always examined by stimulating the cells with 1 μM A23187, by comparison with PMS.

RESULTS

Oxygen free radical generation in PMS-treated RMIC

Reduction of NBT in formazan was observed in PMS-treated RMIC, whereas addition of vehicle did not result in formation of reduced NBT. The presence of 50 μg/ml SOD in the external medium abolished the PMS-induced NBT reduction. The involvement of superoxide anions in this process was further indicated by the enhanced reduction of NBT when intracellular SOD was inhibited by DDC. We ensured that PMS was not able to directly reduce NBT in a cell-free medium. In addition, no reduced NBT was formed by DDC-treated cells when PMS was not added to the medium. We established that the arachidonic acid metabolism was not involved in the NBT reduction. Formation of formazan was not inhibited in PMS-treated cells by indomethacin, NDGA, mepacrine and dexamethasone, inhibitors of cyclooxygenase, lipoxygenase and PLA_2 respectively. In addition, activation of the arachidonic acid cascade by the calcium ionophore A23187 did not result in NBT reduction, further indicating that NBT was reduced by superoxide anions generated by the PMS reaction and not by the arachidonic acid metabolism.

Effect of PMS on arachidonic acid derivatives

PGE_2, which is the major metabolite of the cyclooxygenase pathway in these cells, was measured in control and PMS-treated cells. Figure 1 shows that PGE_2 synthesis is PMS-dose related until a concentration of 0.1 mM. Cell death occured when PMS concentration was above 1 mM. The stimulation of PGE_2 synthesis by PMS is time-dependent. After a 30-min incubation period, PGE_2 released in the external medium decreased in both control and PMS experiments (Fig. 2). The stimulatory effect of PMS on the PGE_2 synthesis was partially suppressed by the presence of various inhibitors of cyclooxygenase and PLA_2. 10 μM indomethacin inhibited, to the same extent, PGE_2 synthesis both in control and PMS-treated cells. The decrease in PGE_2 synthesis after inhibiting PLA_2 by dexamethasone-induced "lipocortin" and by mepacrine was greater in control cells than in PMS-treated cells, suggesting already a stimulatory effect of PMS on cyclooxygenase (Table 1).

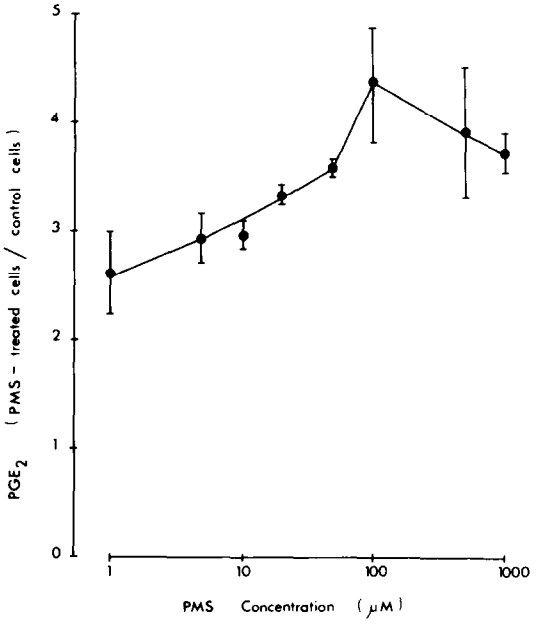

Fig. 1. Dose–response curve of PGE_2 synthesis as a function of PMS concentration. Cells were incubated in a PBS medium for 20 min at 37 and released PGE_2 were measured by radioimmunoassay. Results are expressed as mean ± S.D. of three to five experiments.

Table 1. Inhibition of PGE_2 synthesis in PMS-treated cells by cyclooxygenase and phospholipase A_2 inhibitors

	PGE_2 synthesis (% of inhibition)	
	No PMS	Plus PMS
Indomethacin (10^{-5} M)	72.9 ± 7.1	81.2 ± 5.4
Mepacrine (10^{-6} M)	63.6 ± 2.8	34.6 ± 5.5
Dexamethasone (10^{-6} M)	89.2 ± 3.6	60.6 ± 5.2

Cells were incubated with various inhibitors as described in Materials and Methods. Released PGE_2 were measured by radioimmunoassay after a 20-min incubation period of RMIC in a PBS medium in absence or in presence of 75 μM PMS. The percentage of inhibition was measured as PGE_2 released in treated cells vs control cells. Values are expressed as mean ± S.D. of three to five experiments.

The effect of PMS on the synthesis of lipoxygenase derivatives was also investigated. The HPLC profile of PMS-treated RMIC revealed that these cells released a product which co-eluted with a 15-HETE standard. In the presence of 10 μM NDGA, the amount of HETE was significantly reduced. When RMIC were pre-labelled with ^{14}C-arachidonic acid (0.1 μCi/flask), a radioactive product with a similar retention time was detected. In PMS-treated cells the labelled peak amounted to 6.0 ± 0.5% (mean ± S.D., N = 3) of the total radioactivity recovered whereas, in non-treated cells, it amounted to 2.7 ± 0.3% (mean ± S.D., N = 3). The radioactivity incorporated in the cells represented 4 ± 1% (mean ± S.D., N = 3) of the added arachidonic acid. Neither leukotrienes nor di-HETE were detected in control cells, PMS- or A23187-treated cells.

Effect of superoxide dismutase and catalase on the PMS-induced PGE_2 synthesis

Addition of SOD in the incubation medium enhanced the stimulatory effect of PMS on the PGE_2 production whereas catalase decreased it (Table 2). This indicates that H_2O_2 is the likely oxygen by-produce involved in the increase in PGE_2 synthesis.

Role of soluble thiol groups on the PGE_2 synthesis in PMS-treated RMIC

A loss of intracellular soluble thiol groups was observed in PMS-treated cells. Measurement of PGE_2 at the same time showed that thiol oxidation caused by diamide, a thiol oxidative agent [34, 35], or by PMS, an oxygen free radical generator, induced a stimulation of the PGE_2 synthesis. This effect was partially diminished by DDT, a thiol protective agent [35, 36] (Table 3).

Table 2. Effect of superoxide dismutase (SOD) and catalase on the PGE_2 synthesis in PMS-treated cells

		PGE_2 Synthesis (ng/mg protein)	
		Control cells	PMS-treated cells
None		1.95 ± 0.52	6.75 ± 0.81
SOD (μg/ml)	20	1.84 ± 0.31	9.83 ± 0.8
	50	1.66 ± 0.09	10.86 ± 0.38
	100	1.70 ± 0.17	11.76 ± 1.18
Catalase (μg/ml)	5	1.91 ± 0.67	3.83 ± 0.53
	10	1.57 ± 0.43	3.00 ± 0.26
	20	1.90 ± 0.58	3.02 ± 0.07
	50	1.97 ± 0.52	2.87 ± 0.35
	100	1.70 ± 0.56	3.08 ± 0.28

Cells were incubated in absence or presence of 75 μM PMS for 20 min at 37° after a preliminary incubation time of 5 min with the indicated enzyme.
Results are expressed as mean ± S.D. of three experiments.

Table 3. Effects of PMS, diamide and dithiotreitol (DTT) on the synthesis of PGE_2 and the content of soluble thiol groups in RMIC

Compounds	Concentration (μM)	PGE_2 (× control value)	Soluble SH-groups (nmoles/mg of protein)
None		1	7.1 ± 0.98
PMS	25	4.54 ± 0.17	3.1 ± 1.20
Diamide	200	7.09 ± 0.61	1.2 ± 0.48
DTT	200	1.65 ± 0.1	—
DTT + PMS	200 + 25	3.71 ± 0.21	—

Cells were pre-incubated for 5 min with diamide or DTT and PMS was added for an additional 20-min incubation period. PGE_2 were detected by RIA and soluble thiol groups were spectrophotometrically measured at 535 nm as described in Materials and Methods. Reported values are expressed as mean ± S.D. of three to five experiments.

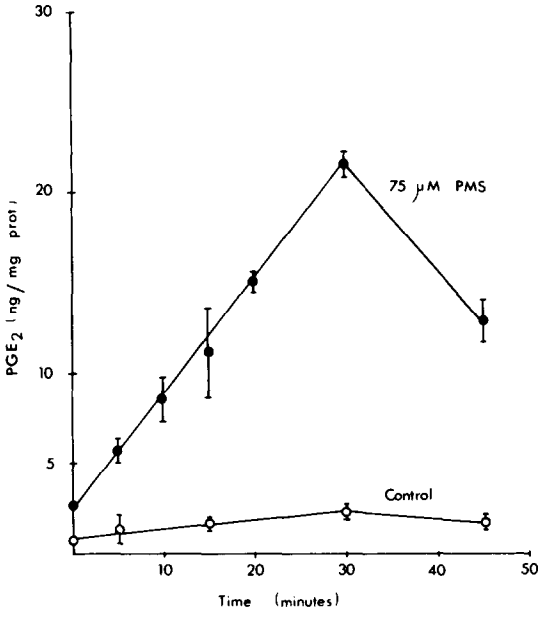

Fig. 2. PGE_2 synthesis as a function of time in PMS-treated cells. See legend Fig. 1.

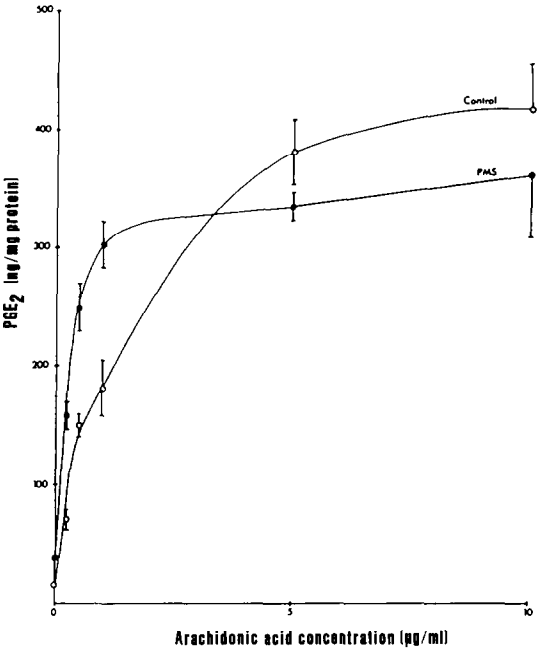

Fig. 3. Effect of PMS on the prostaglandin synthesis stimulated by added arachidonic acid. Cells were incubated for 20 min at 37° in presence of various arachidonic acid concentrations added concomitantly to 75 μM PMS. Similar incubations with arachidonic acid were carried out in absence of PMS. The release of PGE_2 was measured by RIA. Results are expressed as mean ± S.D. of three experiments.

PLA_2 activity in PMS-treated RMIC

In order to determine PLA_2 activity in PMS-treated cells, we measured the formation of lyso-Platelet-activating factor (lyso-PAF). The production of lyso-PAF in PMS-treated cells showed a 3-fold increase over control cells, whereas diamide had no effect (Table 4). A similar production of lyso-PAF was obtained with 75 μM PMS and 1 μM A23187 which activates PLA_2 by increasing cellular calcium content.

A direct effect of the above mentioned compounds on PLA_2 could not be excluded. They were therefore tested on a purified porcine pancreatic PLA_2 incubated in presence of ^{3}H-oleic acid pre-labelled *E. coli* membranes. Under conditions, none of the tested compounds (PMS; PMS + NADH; DDC; GSH; DTT; diamide; H_2O_2) altered PLA_2 activity.

Table 4. Increase in lyso-PAF formation by PMS and A23187 in RMIC

		Lyso-PAF formation (% of control)
PMS	(75 μM)	293 ± 29
A23187	(1 μM)	253 ± 35
Diamide	(200 μM)	84 ± 12

Cells were treated with PMS, A23187 and diamide at the indicated concentrations for 20 min at 37°. The reaction was stopped by addition of chloroform–methanol and lipids were extracted as indicated in Materials and Methods. The chloroformic phase containing lyso-PAF was evaporated and submitted to chemical acetylation. For lyso-PAF measurement, see the procedure described in Materials and Methods. The absolute value of lyso-PAF in control cells was: 6.57 ± 0.73 ng/mg of protein. Values are expressed as mean ± S.D. of three to five experiments.

Cyclooxygenase activity in PMS-treated cells

We studied the effect of PMS on the cyclooxygenase activity in the presence of exogenously added arachidonate in order to bypass its release from lipids by the PMS-stimulated PLA_2. At low arachidonic acid concentrations, the enzyme activity was enhanced when PMS was present in the medium, whereas for high arachidonic acid concentrations (10 μg/ml), values of PGE_2 in control and PMS experiments were not significantly different (Fig. 3). Such a stimulating effect of PMS for low arachidonic acid concentrations was not reproduced when PMS (75 μM) was substituted by A23187 (1 μM), which activates PLA_2 to an extent similar to PMS (Table 4). This suggests that PLA_2 stimulation cannot totally explain the increase in cyclooxygenase activity in PMS-treated cells.

DISCUSSION

In rat renomedullary interstitial cells, PMS enhances the formation of inflammatory lipid derivatives such as lyso-PAF, PGE_2 and 15-HETE. This effect is associated with generation of superoxide anions which is partially masked by the very active intracellular SOD. Indeed, the presence of DDC, an inhibitor of SOD [23, 24], reveals a more significant production of superoxide anions. A relationship between oxygen-free radical production and PGE_2 synthesis was indicated by alterations of the PMS-

effect provided by exogenous SOD and catalase. PMS induces oxygen free radical formation within the cells. Oxygen-free radicals can cross plasma membrane (at least O_2^- and H_2O_2) [37, 17] and can be scavenged by SOD and catalase added in the external medium. Under these conditions, the effect of PMS was enhanced by SOD whereas catalase decreased it. This strongly suggests that H_2O_2 is the oxygen by-product involved in the PMS-induced PGE_2 synthesis. The implication of H_2O_2 in this process is in agreement with previous reports [9, 10].

Once formed, O_2^- and H_2O_2 are involved in the generation of hydroxy radical $^{\cdot}OH$ which oxidizes thiol groups and unsaturated lipids [38]. Therefore, cellular depletion in thiol groups observed in PMS-treated cells may be the result of $^{\cdot}OH$ formation and GSH-peroxidase activation which metabolized low concentrations of H_2O_2 and lipid peroxides at the expense of GSH [39]. Cellular depletion in thiol groups mediated by diamide induced an increase in PGE_2 synthesis mimicking the effect of PMS. PLA_2 activation was not involved in this process since diamide was not able to stimulate the release of lyso-PAF. In physiological conditions, the cyclooxygenase activity is controlled by GSH-peroxidase which removes both H_2O_2 and part of the lipid peroxides available to the prostaglandin synthesis [40, 41]. A decrease in the cellular GSH content impairs the GSH-peroxidase activity and leads to the accumulation of peroxides with a subsequent stimulating effect of cyclooxygenase activity [42]. Thus, in PMS- or diamide-treated cells, a thiol group depletion could increase the PGE_2 synthesis through cyclooxygenase activation. Moreover, addition of arachidonic acid was associated with an enhanced cyclooxygenase activity in PMS-treated cells which could not be mimicked simply by increasing PLA_2 activity because this effect was not reproduced with the calcium ionophore A23187 (data not shown). Taken together, these results strongly suggest that, in PMS-treated cells, activation of cyclooxygenase may occur through an alternative mechanism than the activation of the upper substrate-limiting enzyme: PLA_2. Furthermore, the lower inhibitory effect of two PLA_2 inhibitors, mepacrine and dexamethasone-induced lipocortin, in PMS-treated cells, is in favor of a partial dissociation between the activation of cyclooxygenase and that of PLA_2.

Although the decrease in soluble thiols such as GSH could be involved in the enhancement of cyclooxygenase activity, it remains unclear how this phenomenon could be compatible with the synthesis of PGE_2. Indeed, the transformation of PGH_2 into PGE_2 requires a GSH-dependent enzyme: the prostaglandin E isomerase. A possible explanation could be that the loss in thiol groups was sufficient to stimulate the cyclooxygenase without inducing disturbances in prostaglandin E isomerase activity.

However, thiol group-depletion is not the only factor involved in the stimulation of the PGE_2 synthesis as suggested by the poor reversibility of the PMS effect when DTT, a thiol protective agent, was present in the medium. Thus, activity of PLA_2 in PMS-treated cells was investigated and we found that formation of lyso-PAF by a PLA_2 catalysed reaction was enhanced. The mechanism for PLA_2 activation in presence of oxygen-free radicals is not well understood. The reported ability of intracellular oxygen-free radicals to increase cytosolic calcium concentration [43] could be involved in this process, especially because this effect was recently observed in PMS-treated isolated rat hepatocytes (unpublished result).

Activation of the lipoxygenase pathway was also observed in presence of PMS. This may reflect an effect on PLA_2 activity and/or of a direct lipoxygenase activation as suggested by the previously reported similarity of this enzyme with cyclooxygenase [42]. Our results do not allow us to hypothesize the mechanism involved in this activation. Further experiments are required to understand the exact effect of oxygen-free radicals on the lipoxygenase pathway.

In conclusion, we found that generation of intracellular oxygen-free radicals enhanced the synthesis of lipid derived mediators through different mechanisms of enzyme activations. The increase in PGE_2 synthesis results in part from PLA_2 activation and in other part from cellular depletion in soluble thiol groups. The latter apparently suppresses the inhibition of cyclooxygenase by GSH-peroxidase.

Acknowledgements—We wish to thank Merck Sharp and Dohme-Chibret Laboratory, France, for its financial support. The help of B. Rothhut in the studies with porcine pancreatic PLA_2 is gratefully acknowledged.

REFERENCES

1. R. J. Flower, *Adv. Prostaglandin Thromboxane Res.* **3**, 105 (1978).
2. A. J. Marcus, *J. Lipid Res.* **19**, 793 (1978).
3. P. Borgeat and B. J. Samuelsson, *J. biol. Chem.* **254**, 7865 (1979).
4. D. H. Albert and F. Snyder, *J. biol. Chem.* **258**, 97 (1981).
5. M. Chignard, J. P. LeCouedic, E. Coeffier and J. Benveniste, *Biochem. biophys. Res. Commun.* **124**, 637 (1984).
6. P. P. Patriarca, P. Cramer, S. Monocalvo, F. Rossi and D. Romeo, *Archs Biochem. Biophys.* **145**, 255 (1971).
7. B. M. Babior, J. T. Curnutte and R. S. Kipnes, *J. clin. Invest.* **56**, 1035 (1975).
8. N. Borregaard and A. I. Tauber, *J. biol. Chem.* **259**, 47 (1984).
9. L. Baud, M. P. Nivez, D. Chansel and R. Ardaillou, *Kidney Int.* **20**, 332 (1981).
10. L. Taylor, M. J. Menconi and P. Polgar, *J. biol. Chem.* **258**, 6855 (1983).
11. E. A. Ham, R. W. Egan, D. D. Soderman, P. H. Gale and F. A. Kuehl, Jr., *J. biol. Chem.* **254**, 2191 (1979).
12. L. J. Marnett, P. Wlodawer and B. Samuelsson, *J. biol. Chem.* **250**, 8510 (1975).
13. R. W. Egan, J. Paxton and F. A. Kuehl, Jr., *J. biol. Chem.* **251**, 7329 (1976).
14. B. Kalyanaraman, R. P. Mason, B. Tainer and T. E. Eling, *J. biol. Chem.* **257**, 4764 (1982).
15. F. Russo-Marie, M. Paing and D. Duval, *J. biol. Chem.* **254**, 8498 (1979).
16. M. Nishikimi, N. A. Rao and K. Yagi, *Biochem. biophys. Res. Commun.* **46**, 849 (1972).
17. I. Maridonneau, P. Braquet and R. P. Garay, *J. biol. Chem.* **258**, 3107 (1983).
18. R. K. Poot and J. A. Metcalf, *J. clin. Invest.* **60**, 1266 (1977).

19. S. J. Weiss and A. F. LoBuglio, *Lab. Invest.* **47**, 5 (1982).
20. M. J. Dunn, R. S. Staley and M. Harrison, *Prostaglandins* **12**, 37 (1976).
21. E. E. Muirhead, G. S. Germain, B. E. Leach, J. A. Pitcock, P. Stephenson, B. Brooks, W. L. Brosius, E. G. Daniels and J. W. Hinman, *Circ. Res.* **30/31**, 161 (1972).
22. R. L. Baehner, S. K. Murrmann, J. Davis and R. B. Jr. Johnston, *J. clin. Invest.* **56**, 571 (1975).
23. R. E. Heikkila, F. S. Cabbat and G. Cohen, *J. biol. Chem.* **251**, 2182 (1976).
24. S. Tsunawaki and C. F. Nathan, *J. biol. Chem.* **259**, 4305 (1984).
25. R. A. Lewis, J. M. Mencia-Huerta, R. J. Soberman, D. Hoover, A. Marfat, E. J. Corey and K. F. Austen, *Proc. natn. Acad. Sci. U.S.A.* **79**, 7904 (1982).
26. B. Saville, *Analyst* **83**, 670 (1958).
27. E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.* **37**, 911 (1959).
28. L. Touqui, C. Jacquemin, C. Dumarey and B. B. Vargaftig, *Biochim. biophys. Acta* **833**, 111 (1985).
29. J. Benveniste, M. Chignard, J. P. LeCouedic and B. B. Vargaftig, *Thromb. Res.* **25**, 375 (1982).
30. N. G. Ardlie, M. A. Packham and J. F. Mustard, *Br. J. Haemat.* **19**, 7 (1970).
31. B. Rothhut, F. Russo-Marie, J. Wood, J. DiRosa and R. J. Flower, *Biochem. biophys. Res. Commun.* **117**, 878 (1983).
32. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
33. J. F. Cloix, O. Colard, B. Rothhut and F. Russo-Marie, *Br. J. Pharmac.* **79**, 313 (1983).
34. J. L. Plummer, B. R. Smith, H. Sies and J. R. Bend, *Meth. Enzymol.* **77**, 50 (1981).
35. S. N. Kosower and E. M. Kosower, in *Functions of Glutathione: Biochemical, Physiological, and Clinical Aspects* (Eds. W. A. Larsson *et al.*), p. 307. Raven Press, New York (1983).
36. G. Bellomo, S. A. Jewell, H. Thor and S. Orrenius, *Proc. natn. Acad. Sci.* **79**, 6842 (1982).
37. R. E. Lynch and I. Fridovich, *J. biol. Chem.* **253**, 1838 (1978).
38. B. A. Freeman and J. D. Crapo, *Lab. Invest.* **47**, 412 (1982).
39. I. Fridovich, in *Free Radicals in Biology* (Ed. W. A. Pryor), p. 239. Academic Press, New York (1976).
40. M. E. Hemler, H. W. Cook and W. E. M. Lands, *Archs Biochem. Biophys.* **193**, 542 (1979).
41. M. E. Hemler and W. E. M. Lands, *J. biol Chem.* **255**, 6253 (1980).
42. W. E. M. Lands, R. J. Kulmacz and P. J. Marshall, in *Free Radicals in Biology* (Ed. W. A. Pryor), 6, p. 39. Academic Press, New York (1984).
43. D. Di Monte, G. Bellomo, H. Thor, P. L. Nicotera and S. Orrenius, *Archs Biochem. Biophys.* **235**, 343 (1984).