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Differential dependence on protein kinase C of arachidonic acid metabolism stimulated by hydrogen peroxide and by zymosan in the alveolar macrophage

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The dependence on protein kinase C (PKC) of arachidonic acid (AA) metabolism stimulated by the biologically important oxidant H_2O_2, as compared to zymosan particles, was investigated in the rat alveolar macrophage. The PKC inhibitor staurosporine markedly reduced AA release and eicosanoid synthesis stimulated by zymosan, but only slightly inhibited AA release and metabolism induced by H_2O_2. Furthermore, in macrophages depleted of PKC by extended exposure to phorbol 12-myristate 13-acetate, AA release in response to zymosan was greatly inhibited, whereas that stimulated by H_2O_2 was attenuated to a significantly lesser degree. Thus, zymosan-stimulated AA metabolism requires active PKC, whereas H_2O_2-induced metabolism is largely PKC-independent. This provides direct evidence for the existence of two pathways of agonist-stimulated AA metabolism, which differ in their dependence on PKC, in the alveolar macrophage.

Agonist-specific heterogeneity in the expression of arachidonic acid (AA) metabolism is well-established. This is illustrates by the macrophage, for which the regulation of AA metabolism and particular patterns of eicosanoid synthesis differ, depending on which of many effective agonists are used to stimulate the cell [1–4]. Recent evidence indicates that AA release and metabolism stimulated by zymosan in a macrophage-derived cell-line [5] and in murine peritoneal macrophages [6] are dependent on active protein kinase C (PKC; Ca^{2+}- and phospholipid-dependent protein kinase). Similarly, AA metabolism induced by Ca^{2+} ionophore A23187 has been found to require active PKC in the human neutrophil [7] and in isolated rat glomeruli [8]. On the other hand, it has recently been reported that hormone-stimulated AA release by rat pituitary cells is not mediated by or dependent on active PKC [9]. Despite these reports of PKC-dependent and PKC-independent pathways of AA metabolism in various cells and tissues, such prior studies have not focused on the PKC dependence of AA metabolism stimulated by different kinds of agonists within a particular cell type.

We have previously demonstrated that noncytolytic doses of H_2O_2, a major reactive oxygen species released by activated inflammatory cells and an important mediator of oxidant tissue injury [10], have the capacity to stimulate the release of AA and its metabolism to cyclooxygenase (but not 5-lipoxygenase) eicosanoids in the cultured rat alveolar macrophage (AM) [11,12]. We have also found that, in contrast to AA metabolism stimulated by the particulate zymosan [13,14] or by Ca^{2+} ionophore A23187 [15], H_2O_2-induced AA metabolism is not inhibited by glucocorticoids [16]. One possible explanation for the failure of glucocorticoids to inhibit H_2O_2-induced AA metabolism is that H_2O_2 might release AA from cellular phospholipids by a different enzymatic mechanism that do zymosan and A23187, both of which activate AA deacylation via Ca^{2+}-dependent phospholipase A_2 (PLA_2) [17,18]. To further elucidate the mechanism(s) by which H_2O_2 initiates macrophage AA metabolism, we studied the PKC dependence of AA release and eicosanoid synthesis in response to H_2O_2, as compared to zymosan, in the rat AM. Our results indicate that zymosan and H_2O_2 stimulate AA release and metabolism by pathways which differ markedly in their dependence on PKC.

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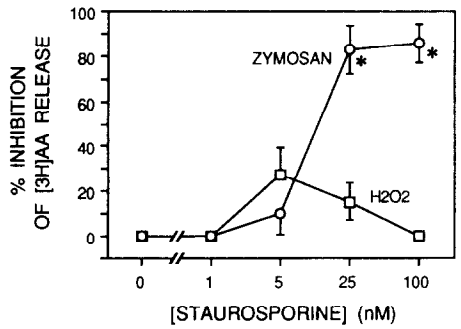

Fig. 1. Effect of staurosporine on [^{3}H]AA release stimulated by zymosan and by H_2O_2. Alveolar macrophages were prelabeled with [^{3}H]AA (0.5 μCi/well) and stimulated for 30 min with either zymosan (500 μg/ml) or H_2O_2 (0.25 mM) in the presence or absence of staurosporine, following pre-exposure for 15 min to the inhibitor or medium alone, respectively. Media plus cells were then extracted with chloroform/methanol and free [^{3}H]AA was separated by thin-layer chromatography and quantitated, as described [11]. The data represent means $\pm$ S.E. for results of three separate experiments, each performed in duplicate. * $P < 0.02$ vs. H_2O_2, by paired Student's t-test.

Alveolar macrophages were obtained from specific pathogen-free female Wistar rats (Charles River, Portage, MI) by lung lavage, plated at 5×10^5/well in 24-well tissue culture plates, adhered for 60 min at 37 ° C and cultured overnight (18 h) in medium 199 plus 10% newborn calf serum, yielding an adherent AM population > 97% pure [19]. Cellular lipids were labeled by addition of 0.5 or 1.0 μCi [^{3}H]AA (New England Nuclear, Boston, MA) per well to culture medium prior to overnight incubation. In certain experiments, macrophages were incubated in the presence and absence of 1 μM phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO), or the inactive compound 4α-phorbol 12,13-didecanoate (PDD, also 1 μM; Sigma), for 18 h following adherence. PMA pretreatment had no adverse effect on macrophage viability, as assessed by LDH release (data not shown). After overnight incubation (with or without PMA or PDD), [^{3}H]AA-prelabeled or unlabelled AMs were washed and stimulated for 30 min with H_2O_2 at 0.25 mM, or with preboiled zymosan A (Sigma) at 500 μg/ml, both diluted in serum-free medium 199. In experiments in which macrophages were not pretreated with phorbol compounds, stimulation was carried out in the presence and absence of various concentrations of staurosporine (Kamiya Biomedical, Thousand Oaks, CA), following a 15 min pre-exposure to the drug or medium alone, respectively. Quantities of radiolabeled AA and metabolites released following agonist stimulation were expressed as percentages of the [^{3}H]AA radioactivity incorporated in each experiment, in the absence or presence of PMA or PDD, as appropriate. Percent inhibition of zymosan- or H_2O_2-stimulated AA release or eicosanoid synthesis in

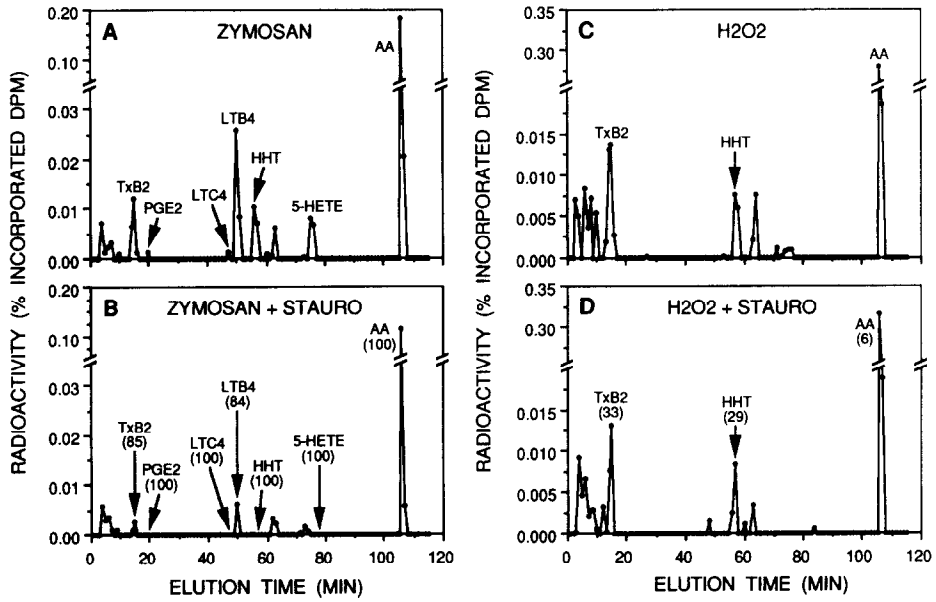

Fig. 2. HPLC analysis of effect of staurosporine on zymosan- and H_2O_2-induced eicosanoid formation and AA release. Alveolar macrophages prelabeled with [^{3}H]AA (1.0 μCi/well) were stimulated with zymosan or H_2O_2 in the absence or presence of staurosporine (25 nM), as indicated in the legend to Fig. 1. Pooled media from triplicate culture wells were extracted using Sep-pak C_{18} cartridges (Waters Associates), and subjected to reverse-phase HPLC over a Waters 5 μm Bondapak column using a gradient solvent system of acetonitrile/water/trifluoroacetic acid, as described [12]. Radioactivity in 1-min eluate fractions is expressed as a percentage of the total radioactivity incorporated per culture plate prior to stimulation. Radioactivity peaks corresponding to retention times of authentic standards are indicated by labels. Corresponding numbers in parentheses represent the percent inhibition by staurosporine of the zymosan- or H_2O_2-stimulated increase in production of each metabolite and free AA.

phorbol ester- or staurosporine-treated cultures was calculated according to the formula:

$$\left[1-\frac{(\text{agonist}-\text{control})_{+\text{inhibitor}}}{(\text{agonist}-\text{control})_{-\text{inhibitor}}}\right]\times 100$$

where 'agonist' and 'control' refer to the quantities of free AA or eicosanoid produced with and without agonist stimulation, respectively.

The first approach to determining the dependence on PKC of AA metabolism was to examine the effects of the potent PKC inhibitor staurosporine [20] in cultures of alveolar macrophages stimulated with H_2O_2 and zymosan. As in previous studies [11,13], both H_2O_2 (0.25 mM) and zymosan (500 μg/ml) significantly increased the release of free [^{3}H]AA from prelabeled AMs; no stimulus, $1.5 \pm 0.2\%$ of incorporated [^{3}H]AA dpm; H_2O_2, $4.6 \pm 0.4\%$; zymosan, $4.2 \pm 0.8\%$ ($n = 6$, $P < 0.005$ by analysis of variance for each agonist vs. unstimulated macrophages). Fig. 1 illustrates that release of [^{3}H]AA in response to zymosan was strongly inhibited by staurosporine at ≥ 25 nM, while H_2O_2-induced [^{3}H]AA release was not significantly affected by the inhibitor at concentrations up to 100 nM.

Synthesis of [^{3}H]eicosanoids by prelabeled macrophages in response to zymosan and H_2O_2 was analyzed by reverse-phase high-performance liquid chromatography (HPLC). As shown in Fig. 2, zymosan stimulated synthesis of the cyclooxygenase metabolites thromboxane (Tx)B_2, prostaglandin (PG)E_2, and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and the 5-lipoxygenase products leukotriene (LT)B_4, LTC_4, and 5-hydroxyeicosatetraenoic acid (5-HETE) (Fig. 2A), whereas H_2O_2 stimulated formation of TxB_2 and HHT and no 5-lipoxygenase products (Fig. 2C), as previously reported [11,12]. Fig. 2 also show that the effects of staurosporine on [^{3}H]eicosanoid synthesis stimulated by zymosan and H_2O_2 were similar to its effects on agonist-stimulated [^{3}H]AA release. Staurosporine (25 nM) caused virtually complete inhibition of both cyclooxygenase and 5-lipoxygenase eicosanoid synthesis stimulated by zymosan (Fig. 2B), in comparison to only weak inhibition of H_2O_2-induced formation of TxB_2 and HHT (Fig. 2D). In the experiment shown, staurosporine inhibited the release of total [^{3}H]eicosanoids stimulated by zymosan (sum of TxB_2, PGE_2, HHT, LTB_4, LTC_4, and 5-HETE) by 91%, as compared to H_2O_2-induced release (sum of TxB_2 and HHT) by only 32%. The effects of staurosporine on synthesis of [^{3}H]eicosanoids by prelabeled macrophages were confirmed by measurement of immunoreactive TxB_2 and LTB_4 in media from unlabeled cultures stimulated with zymosan and with H_2O_2 (Table I).

To assess the possibility that staurosporine's poor ability to inhibit H_2O_2-induced AA metabolism might be the result of its direct chemical inactivation by H_2O_2, we incubated staurosporine at 25 nM with 0.25 mM H_2O_2 in culture medium for 10 min, next removed the H_2O_2 by adding catalase (10 U/ml for 30 min; Sigma), then tested the capacity of the H_2O_2-exposed drug to inhibit zymosan-induced [^{3}H]AA release from prelabeled macrophages. Exposure of staurosporine to H_2O_2 in this fashion caused no diminution of its ability to markedly inhibit zymosan-induced AA release (data not shown), indicating that the failure of staurosporine to inhibit H_2O_2-stimulated AA metabolism did not result from chemical inactivation of the drug by H_2O_2.

TABLE I

Effect of staurosporine (50 nM) on synthesis of immunoreactive TxB_2 and LTB_4 stimulated by H_2O_2 and by zymosan *

Agonist	pg/well (% inhibition)			
	TxB_2		LTB_4	
	− stauro	+ stauro	− stauro	+ stauro
None	33	52	< 9	< 9
Zymosan 500 μg/ml	160	43 (100)	314	11 (99)
H_2O_2 0.25 mM	143	138 (22)	< 9	< 9 –

* Unlabeled AMs were stimulated with either zymosan or H_2O_2 in the absence or presence of staurosporine as described in the legend to Fig. 1. TxB_2 and LTB_4 in media from these cultures were quantitated by radioimmunoassay [11,12]. Data represent means for duplicate samples from one of two similar experiments.

A second approach used to evaluate the dependence of AA metabolism on PKC was to determine the ability of zymosan and H_2O_2 to stimulate AA release in AMs depleted of PKC by prolonged exposure to PMA. Treatment with phorbol diesters for periods of 12 to 24 h has been shown to markedly deplete PKC, and in parallel to down-regulate PKC-mediated responses, in various cell types [21,22], including the murine peritoneal macrophage [6,23]. Similarly, we found that incubation of AMs with 1 μM PMA for 18 h depleted $98 \pm 1\%$ of the cells' active PKC, as determined by the histone phosphorylation assay [24]; total (cytosol plus membrane) PKC activity without PMA, 162 ± 19 pmol/mg protein/min; with PMA 4 ± 3 pmol/mg protein/min ($n = 3$, $P < 0.001$ by unpaired Student's t-test). Fig. 3 shows that [^{3}H]AA release stimulated by zymosan was markedly inhibited in AMs so depleted of PKC. In contrast, H_2O_2-induced [^{3}H]AA release, while inhibited by PMA-induced PKC depletion, was attenuated to a significantly lesser degree than [^{3}H]AA release stimulated by zymosan. On the other hand, the inactive phorbol ester PDD, which does not deplete PKC, had very little inhibitory effect on [^{3}H]AA release in response to either zymosan or H_2O_2 (Fig. 3), indicating that the inhibition seen with PMA was specifically related to its effects on PKC.

Our finding that both staurosporine and PMA pretreatment inhibit AA release and that staurosporine

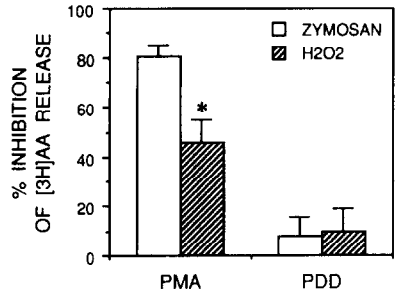

Fig. 3. Effect of pretreatment with PMA or PDD on [^{3}H]AA release stimulated by zymosan and by H_2O_2. After incubation for 18 h in the absence or presence of 1 μM PMA or 1 μM PDD, [^{3}H]AA-prelabeled AMs were stimulated with zymosan (500 μg/ml) or H_2O_2 (0.25 mM) for 30 min. Free [^{3}H]AA was quantitated as indicated in the legend to Fig. 1. Data for PMA pretreatment represent means $\pm$ S.E. for results of four separate experiments, each performed in duplicate; data for PDD pretreatment are means $\pm$ S.E. for results of three separate experiments, each performed in duplicate or triplicate. * $P < 0.05$ vs. zymosan, by paired Student's t-test.

also inhibits eicosanoid synthesis in response to zymosan confirm that zymosan-induced AA metabolism requires active PKC in the rat AM, as previously demonstrated for other macrophage populations [5,6]. In contrast, however, the release of free AA stimulated by H_2O_2 was not affected by staurosporine, even at a concentration (100 nM) 4-fold greater than that which maximally inhibited zymosan-induced AA release (Fig. 1) and 5-fold greater than that shown to maximally inhibit partially purified rat brain PKC [20]. This result could not be attributed to direct chemical inactivation of staurosporine by H_2O_2. In addition, PMA-induced down-regulation of PKC reduced H_2O_2-stimulated AA release to a significantly lesser degree than that stimulated by zymosan (Fig. 3). Taken together, these results indicate the H_2O_2-stimulated AA release and eicosanoid synthesis are much less dependent on active PKC than zymosan-induced AA metabolism, implying that H_2O_2 triggers AA release, at least in part, by a pathway which does not require active PKC. Thus, we have provided direct evidence for the existence of two pathways of agonist-stimulated AA metabolism, which differ in their dependence on PKC, in the AM. This finding extends the concept of agonist-specific heterogeneity in macrophage AA metabolism to regulation by PKC.

Since PKC plays an important role in regulating Ca^{2+}-dependent cellular processes [25], and zymosan is believed to release AA in macrophages via a Ca^{2+}-dependent PLA_2 [17], the strong PKC dependence of zymosan-induced AA metabolism suggests that PKC may be required for activation of PLA_2 in the macrophage. On the other hand, the relative lack of PKC dependence of H_2O_2-induced AA metabolism may mean that H_2O_2 initiates AA metabolism, at least in part, by an enzymatic mechanism not involving PLA_2. One such alternative mechanism could be that H_2O_2 releases AA via phospholipase C, the activity of which is not increased, but rather inhibited, by PKC [26]. Another potential mechanism is that, instead of (or perhaps in addition to) activating phospholipases to increase deacylation, H_2O_2 might active macrophage AA metabolism by interfering with AA reacylation. This is plausible in view of the fact that the rate of constitutive AA turnover in macrophage phospholipids is very high [27], and that sulfhydryl reactive chemicals have the capacity to stimulate AA release and prostaglandin synthesis in macrophages by inhibiting reacylation, without affecting the activity of PLA_2 [28,29]. Moreover, recent evidence indicates that PKC activation itself inhibits AA incorporation into phospholipids [30,31]; similar inhibition of AA reacylation by H_2O_2 would thus explain the lack of requirement for active PKC in order for H_2O_2 to trigger accumulation of free AA. In any case, the relative PKC independence of H_2O_2-stimulated AA release is of interest in view of the biological importance of H_2O_2 as an oxidant in the setting of inflammation and tissue injury, and the fact that eicosanoid metabolites of AA have the capacity to influence both the pathogenesis and resolution of inflammatory lesions. Further studies to clearly define the enzymatic mechanism(s) by which H_2O_2 initiates AA release and metabolism, and the specific regulation of these enzymes by PKC, are currently underway in our laboratory.

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