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Glucocorticoids Fail to Inhibit Arachidonic Acid Metabolism Stimulated by Hydrogen Peroxide in the Alveolar Macrophage

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We have previously demonstrated that the biologically important oxidant hydrogen peroxide (H_2O_2) triggers release and metabolism of arachidonic acid (AA) in the alveolar macrophage (AM). In this study, we evaluated the ability of glucocorticoids to inhibit rat AM AA metabolism stimulated by H_2O_2, as compared to the particulate zymosan. Methylprednisolone and other glucocorticoids failed to significantly inhibit release of AA stimulated by H_2O_2, while markedly reducing AA release in response to zymosan. Similarly, methylprednisolone only weakly inhibited synthesis of thromboxane (Tx)B_2 stimulated by H_2O_2, while inhibiting zymosan-induced eicosanoid synthesis to a marked degree. On the other hand, the phospholipase inhibitor mepacrine strongly inhibited AA release and TxB_2 formation stimulated by both H_2O_2 and zymosan, indicating that H_2O_2-induced AA metabolism is indeed susceptible to pharmacologic inhibition. The failure of glucocorticoids to inhibit AA metabolism stimulated by H_2O_2 in the AM may in part explain their inability to ameliorate oxidant-mediated lung inflammation and injury.

Key words: oxidants, zymosan, methylprednisolone, mepacrine, thromboxane, lung inflammation

INTRODUCTION

Glucocorticoid steroids are efficacious in the treatment of a variety of immune and inflammatory disorders affecting the lung and other organs. The ability of glucocorticoids to inhibit the phospholipase-catalyzed release of arachidonic acid (AA), and thereby its subsequent metabolism to bioactive eicosanoids, is believed to represent an important mechanism of their anti-inflammatory action [1–3]. The alveolar macrophage (AM), which plays a central role in regulating immune and inflammatory processes in the lung, is enriched in AA [4] and has the capacity to synthesize substantial quantities of eicosanoids via both the cyclooxygenase and the 5-lipoxygenase pathways [5–7]. In previous studies, we have examined the effects of glucocorticoids on AA metabolism in AMs challenged with the agonists zymosan and calcium ionophore A23187. Our studies demonstrated that glucocorticoids inhibit AA release and eicosanoid synthesis stimulated by the particulate zymosan in the rat AM [8,9] and by the soluble stimulus A23187 in the human AM [10]. Inhibition by glucocorticoids of eicosanoid synthesis stimulated by zymosan in the human AM has also been reported [11]. Glucocorticoid inhibition of AA release and metabolism has similarly been documented in a wide variety of other cells and tissues [1–3,12–17].

Interestingly, glucocorticoids fail to ameliorate, or actually worsen, lung inflammation and injury in animals exposed to hyperoxia [18–20] or the chemical oxidant paraquat [21]. Recently, we have demonstrated that noncytolytic doses of hydrogen peroxide (H_2O_2), a key mediator of oxidant lung injury [22–25], have the capacity to trigger the release of AA and its metabolism to cyclooxygenase (but not 5-lipoxygenase) products in the rat AM [26,27]. In the current study, we have investigated the ability of glucocorticoids to regulate rat AM AA metabolism stimulated by this biologically important oxidant. Our results indicate that, in contrast to AA metabolism stimulated by the classical agonist zymosan, glucocorticoids fail to inhibit AA release and eicosanoid synthesis triggered by H_2O_2.

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MATERIALS AND METHODS

Macrophage Isolation and Culture

Alveolar macrophages were obtained by bronchoalveolar lavage from specific pathogen-free 126–150 g female Wistar rats (Charles River, Portage, MI), as previously described [26]. Two million cells suspended in medium 199 with modified Earle's salts (M199; GIBCO, Grand Island, NY) were plated in 35 × 10 mm plastic culture dishes (Falcon Plastics, Oxnard, CA) and cultures at 37°C in a humidified atmosphere of 5% CO_2 in air. After 1 h, non-adherent cells were removed by washing twice with Hanks' balanced salt solution (HBSS; GIBCO). The resultant adherent cell population has been found to contain 95% AMs by morphologic criteria and esterase staining [8] with viability exceeding 90%. Macrophage monolayers were then cultured overnight in M199 containing 10% newborn calf serum (GIBCO), in the presence or absence of radiolabeled AA and/or glucocorticoids. Following overnight culture, these monolayers have been found to contain approximately 8.5 μg DNA [8] and 100 μg protein [26] per plate.

Macrophage Prelabeling With [^{14}C]AA

Cellular lipids were prelabeled by including 0.2 μCi [1-^{14}C]AA (specific activity 54–57 mCi/mmol, Dupont-New England Nuclear, Boston, MA) per dish in the culture medium during overnight incubation (~16 h), as described [27]. Macrophages incorporated 158,000 ± 7,000 dpm or 32 ± 2% (mean ± SE, n = 21) of the added [^{14}C]AA, in the absence of glucocorticoid.

Pretreatment With Glucocorticoids

Following adherence, AMs were incubated overnight (~16 h) in the presence of varying concentrations of methylprednisolone, or with hydrocortisone (100 μM), dexamethasone (1 μM), or fluocinolone (0.1 μM) (all glucocorticoids from Sigma Chemical, St. Louis, MO), added in dimethylsulfoxide at a final concentration of 0.5%. We have previously shown that overnight incubation with hydrocortisone, dexamethasone, and fluocinolone at these concentrations, and with methylprednisolone at ≥1 μM, results in maximal glucocorticoid inhibition of zymosan-stimulated AA metabolism in the rat AM [8,9]. These concentrations of glucocorticoids also did not adversely affect AM viability [8,9]. Incubation with glucocorticoids caused dose-dependent reduction of macrophage [^{14}C]AA incorporation, with a maximal effect for methylprednisolone at 10^{-4} M, which reduced uptake to 69 ± 2% (n = 6) of the radiolabel incorporated by AMs in the absence of glucocorticoid. This effect was corrected for in all cases by expressing the quantities of radiolabeled AA and metabolites released by glucocorticoid-treated and untreated AMs as a percentage of the dpm of [^{14}C]AA incorporated with or without glucocorticoid, respectively, in each experiment.

Agonist Stimulation of AA Metabolism

After pretreatment with or without glucocorticoid, [^{14}C]AA-prelabeled or unlabeled AMs were washed with HBSS and stimulated for 30 min with H_2O_2 at concentrations as indicated, or with preboiled zymosan A (Sigma Chemical) at 100 μg/ml, both diluted in serum-free M199. In certain experiments (in which macrophages were not pretreated with glucocorticoid), stimulation with H_2O_2 or zymosan was carried out in the presence and absence of various concentrations of mepacrine (quinacrine; Sigma Chemical), following a 15 min pre-exposure to the drug or medium alone, respectively.

Extraction, Separation, and Quantitation of [^{14}C]AA

Following agonist stimulation, cells plus media were extracted with chloroform:methanol, and free [^{14}C]AA was separated by thin layer chromatography (TLC) on Silica Gel 60 plates (E. Merck, Darmstadt, FRG) using hexane:diethyl ether:acetic acid (70:30:2, v/v/v) and quantitated by scintillation counting, as described [26].

Extraction, Separation, and Quantitation of Eicosanoids

Following stimulation, media from both labeled and unlabeled cultures were extracted using Sep-pak C_{18} cartridges (Waters Associates, Milford, MA), and the extracts were dried under N_2 [27].

For separation of radiolabeled metabolites produced by [^{14}C]AA prelabeled AMs, extracts of pooled media from triplicate cultures were dissolved in acetonitrile: water:trifluoroacetic acid (33:67:0.1, v/v/v) and subjected to reverse-phase high performance liquid chromatography (HPLC) over a Waters 5 μm Bondapak column (30 × 0.4 cm) eluted with acetonitrile:water:trifluoroacetic acid at 1 ml/min, as previously described [9]. Using this system, cyclooxygenase metabolites are eluted during an initial isocratic phase (33:67:0.1, v/v/v), followed by lipoxygenase metabolites and free AA, which elute during a stepwise gradient increase of acetonitrile to 100:0:0.1 (v/v/v). Radiolabeled AA and its metabolites were identified on the basis of retention times corresponding to those of authentic standards as determined by UV absorbance. Authentic prostaglandin (PG) and thromboxane (Tx)B_2 standards were generous gifts of Dr. J. Pike (Upjohn Co., Kalamazoo, MI), and lipoxygenase standards of Dr. J. Rokach (Merck Frosst, Inc., Pointe Claire-Dorval, Quebec, Canada). Authentic 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) was ob-

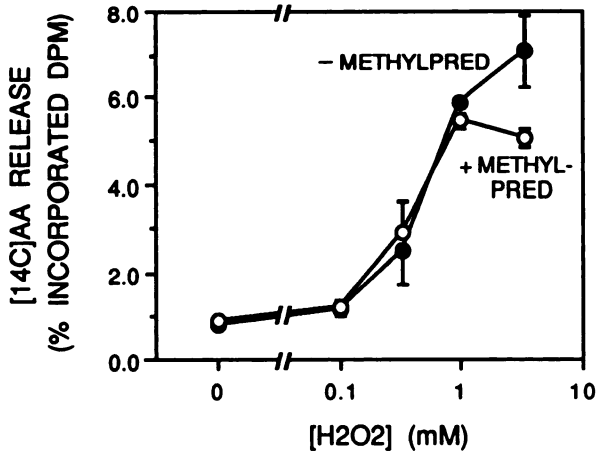

Fig. 1. Effect of 1 μM methylprednisolone on AA release by alveolar macrophages stimulated with various doses of H_2O_2. After incubation for ~16 h in the absence or presence of 1 μM methylprednisolone, monolayers of [^{14}C]AA-prelabeled AMs were washed, media containing the indicated concentrations of H_2O_2 were added, and cultures were incubated for 30 min. Media plus cells were then extracted, and free [^{14}C]AA was separated by TLC and quantitated by scintillation counting. The results represent means ± SE of data from 3 individual experiments, each performed in duplicate.

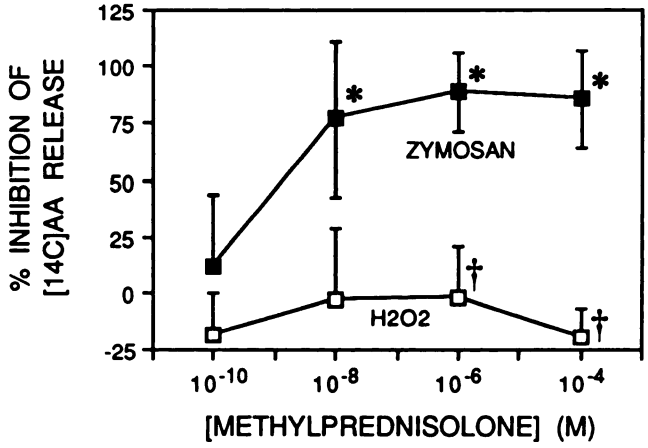

Fig. 2. Effect of methylprednisolone concentration on AA release by alveolar macrophages stimulated with H_2O_2 and zymosan. After incubation for ~16 h in the absence or presence of the indicated concentrations of methylprednisolone, monolayers of [^{14}C]AA-prelabeled AMs were stimulated with 1 mM H_2O_2 or 100 μg/ml zymosan for 30 min. Free [^{14}C]AA was then separated and quantitated as described in the legend to Figure 1. The results represent means ± SE of data from 3 or 4 individual experiments at each methylprednisolone concentration. *P <0.005 vs. zymosan without methylprednisolone, by analysis of variance and the Newman-Keuls multiple range test. † P <0.02 vs. zymosan at the corresponding methylprednisolone concentration, by paired t-test. H_2O_2-induced [^{14}C]AA released was not significantly affected by methylprednisolone at any concentration (P > 0.05 by analysis of variance and the Newman-Keuls multiple range test).

tained from Cayman Chemical Co. (Ann Arbor, MI), and the AA standard from Nu-Chek Prep (Elysian, MN). Eluate fractions of 1 ml were collected, and radioactivity quantitated in 6 ml of scintillant.

Thromboxane A_2 produced by unlabeled macrophages was quantitated as the stable metabolite TxB_2 by radioimmunoassay [26]. Dried Sep-pak extracts were redissolved in 1 ml of phosphate-buffered saline containing 0.1% gelatin, and 0.1 ml aliquots were assayed in duplicate. Results were corrected for recovery of each sample during extraction.

H_2O_2 Assay

H_2O_2 was measured by the homovanillic acid-horseradish peroxidase assay of Ruch et al. [28]. The $t_{1/2}$ of H_2O_2 was determined from the linear regression equation for a plot of log [H_2O_2] vs. time.

Data Analysis

Throughout the study, duplicate culture plates were utilized for each experimental condition and the resulting values averaged to yield a single data point (except in experiments where HPLC was employed, in which case material from triplicate cultures was combined for a single analysis). All data for which n ≥ 3 are expressed as the mean ± SE. The significance of differences between group means was assessed by paired or unpaired Student's t-tests, or by one-way analysis of variance and the Newman-Keuls multiple range test, as appropriate [29]. In all cases, $P < 0.05$ was considered significant.

RESULTS

Effect of Methylprednisolone on H_2O_2- and Zymosan-Induced AA Metabolism

In our previous study [9], methylprednisolone maximally inhibited zymosan-induced rat AM eicosanoid synthesis at a concentration of 1 μM, by ~80%. We therefore examined the effect of methylprednisolone at 1 μM on the release of [^{14}C]AA stimulated by H_2O_2 in prelabeled AMs. Figure 1 shows that at this concentration methylprednisolone did not inhibit the dose-dependent release of free [^{14}C]AA stimulated by H_2O_2. Indeed, as illustrated in Figure 2, methylprednisolone from 10^{-10} to 10^{-4} M failed to inhibit [^{14}C]AA release induced by 1 mM H_2O_2, in contrast to its ability to markedly inhibit [^{14}C]AA release stimulated by zymosan. It was similarly demonstrated that the additional glucocorticoids hydrocortisone (100 μM), dexamethasone (1 μM), and fluocinolone (0.1 μM) failed to inhibit or only slightly inhibited [^{14}C]AA release triggered by H_2O_2,

TABLE 1. Effect of Hydrocortisone (100 μM), Dexamethasone (1 μM), and Fluocinolone (0.1 μM) on Release of [^{14}C]AA by Alveolar Macrophages Stimulated With H_2O_2 and With Zymosan[a]

	% inhibition of [^{14}C]AA release		
Agonist	Hydrocortisone	Dexamethasone	Fluocinolone
H_2O_2 (1 mM)	14	20	3
Zymosan (100 μg/ml)	66	55	80

[a]Data represent the average of duplicates from one experiment.

while, as previously reported [8], strongly inhibiting that stimulated by zymosan (Table 1).

The effect of methylprednisolone on eicosanoid synthesis by [^{14}C]AA-labeled AMs was analyzed using reverse-phase HPLC. Figure 3A shows that 1 mM H_2O_2 triggers synthesis of the cyclooxygenase metabolites TxB_2 and HHT, as well as release of a large amount of unmetabolized free [^{14}C]AA, although it does not stimulate 5-lipoxygenase metabolism, as we have previously demonstrated [26,27]. Methylprednisolone (1 μM) had only a minor inhibitory effect on H_2O_2-induced synthesis of these cyclooxygenase metabolites and release of free [^{14}C]AA (Fig. 3B), such that the total release of radiolabeled AA plus metabolites (calculated as the sum of the radioactivity in all HPLC fractions) was reduced by the glucocorticoid by only 16.5%. As shown in Figure 3C, the profile of metabolites synthesized in response to zymosan differs from that stimulated by H_2O_2, and includes substantial amounts of the 5-lipoxygenase metabolites LTB_4, LTC_4, and 5-HETE, in addition to cyclooxygenase metabolites and a lesser amount of free [^{14}C]AA. Importantly, synthesis of all eicosanoids and release of free AA stimulated by zymosan were markedly inhibited by methylprednisolone (Fig. 3D), such that total radiolabeled AA plus metabolites were reduced by 76.2%. Of note, the total release of radiolabeled AA plus metabolites stimulated by H_2O_2 (4.17% of incorporated dpm) was very similar to that stimulated by zymosan (4.28% of incorporated dpm), indicating that the difference in inhibition by methylprednisolone of H_2O_2- and zymosan-induced AA metabolism was not the result of differing potencies of the two agonists as stimuli for the liberation of esterified AA.

We also used radioimmunoassay to measure release of TxB_2 by unlabeled AMs stimulated with H_2O_2 or zymosan. In these experiments, methylprednisolone (1 μM) only weakly inhibited synthesis of immunoreactive TxB_2 stimulated by H_2O_2, while strongly inhibiting TxB_2 formation induced by zymosan (Table 2). Thus, results obtained by radioimmunoassay from unlabeled cultures corroborated those obtained using [^{14}C]AA-prelabeled cells and analysis by HPLC.

Effect of Mepacrine on H_2O_2- and Zymosan-Induced AA Metabolism

We also examined the effects of mepacrine [30], a phospholipase inhibitor unrelated to glucocorticoids, on H_2O_2- and zymosan-induced AA metabolism. As shown in Figure 4, mepacrine markedly inhibited [^{14}C]AA release stimulated by both H_2O_2 and zymosan, in dose-dependent fashion. Also, 1 mM mepacrine almost completely blocked both H_2O_2- and zymosan-induced immunoreactive TxB_2 synthesis by unlabeled macrophages (Table 3). To determine whether inhibition of H_2O_2-stimulated AA metabolism by mepacrine might merely be the result of the inhibitor scavenging H_2O_2, we measured the rate of consumption of 1 mM H_2O_2 added to macrophage cultures in the absence and presence of mepacrine at 1 mM (the highest concentration used). H_2O_2 was consumed at identical rates in AM cultures with ($t_{1/2}$ = 13.8 ± 1.8 min, n = 3) and without mepacrine ($t_{1/2}$ = 14.4 ± 2.1 min, n = 3), indicating that mepacrine did not inhibit H_2O_2-induced AA metabolism by scavenging H_2O_2. Thus, mepacrine indeed differed from methylprednisolone in its ability to actually inhibit release of AA and cyclooxygenase metabolism stimulated by H_2O_2.

DISCUSSION

In this report, we have demonstrated that while methylprednisolone and other glucocorticoids potently inhibited alveolar macrophage AA release and eicosanoid formation stimulated by the particulate zymosan, they failed to inhibit AA metabolism triggered by the oxidant H_2O_2. These findings indicate that inhibition of AA metabolism by glucocorticoids represents an agonist-specific regulatory interaction. On the other hand, the phospholipase inhibitor mepacrine, which acts by a mechanism unrelated to that of glucocorticoids (involving formation of mepacrine-phospholipid complexes, as well as possible direct interaction with phospholipase enzyme [30]) blocked AA metabolism stimulated not only by zymosan, but by H_2O_2 as well. Thus, H_2O_2-induced AA metabolism is indeed susceptible to pharmacologic inhibition, although not by glucocorticoids.

Glucocorticoids have been proposed to exert their inhibitory effect on AA metabolism by inducing synthesis of phospholipase inhibitory proteins [16,31], termed lipocortins [32]. Lipocortins have been shown to inhibit phospholipase A_2 in cell-free assays by binding its phospholipid substrate, rather than by directly interacting with the enzyme itself [33,34]. Although purified mouse lung lipocortins [35] and recombinant human lipocortin [36,37] have been shown to inhibit AA metabolism in several tissues (including the AM [35]), lipocortin's role

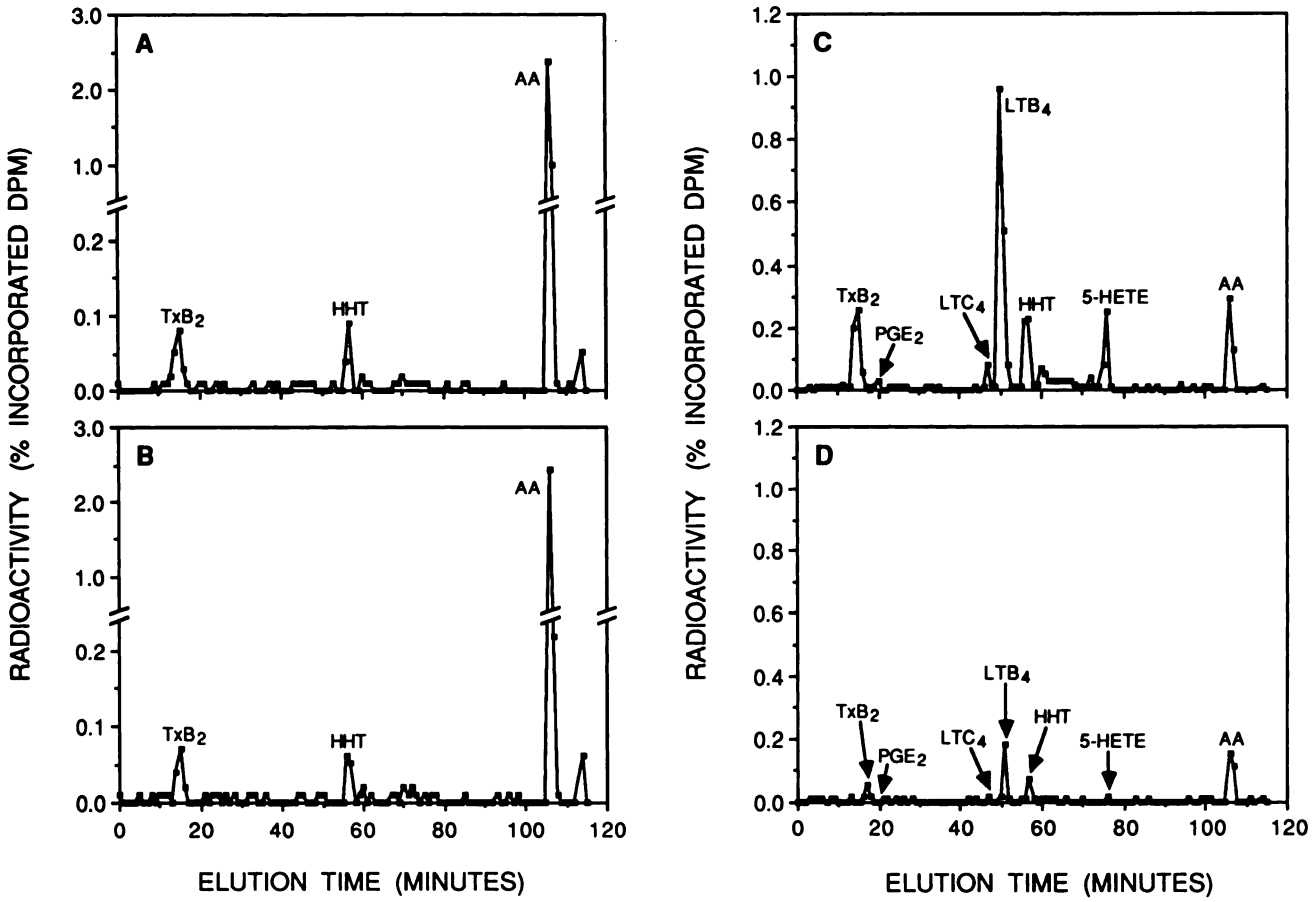

Fig. 3. HPLC analysis of effect of methylprednisolone on H_2O_2- and zymosan-induced eicosanoid formation and AA release by alveolar macrophages. After incubation for ~16 h in the absence or presence of 1 μM methylprednisolone, monolayers of [^{14}C]AA-prelabeled AMs were stimulated with 1 mM H_2O_2 or 100 μg/ml zymosan for 30 min. Pooled media from triplicate cultures were extracted using Sep-pak C_{18} cartridges, and subjected to reverse-phase HPLC as described in Materials and Methods. Radioactivity in 1 min eluate fractions was quantitated, and 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. A: H_2O_2. B: H_2O_2 after pretreatment with methylprednisolone. C: Zymosan. D: Zymosan after pretreatment with methylprednisolone.

TABLE 2. Effect of Methylprednisolone (1 μM) on Synthesis of Immunoreactive TxB_2 by Alveolar Macrophages Stimulated With H_2O_2 and With Zymosan

		TxB_2 synthesis (pg/plate)		
Agonist	(n)	Without methylprednisolone	With methylprednisolone	% inhibition
None	(10)	310 ± 50	185 ± 24	
H_2O_2 (1 mM)	(6)	1,988 ± 435	1,402 ± 208*	22 ± 7***
Zymosan (100 μg/ml)	(8)	2,188 ± 373	548 ± 86**	82 ± 4

*P not significant (>0.05) vs. H_2O_2 without methylprednisolone, by paired t-test.
**P <0.0025 vs. zymosan without methylprednisolone, by paired t-test.
***P <0.0001 vs. zymosan, by unpaired t-test.

as the actual endogenous second messenger responsible for glucocorticoid inhibition of AA metabolism has recently been challenged [38–40], raising the possibility that another glucocorticoid-induced protein(s) may serve this function physiologically.

Based on this model of glucocorticoids' inhibitory ac-

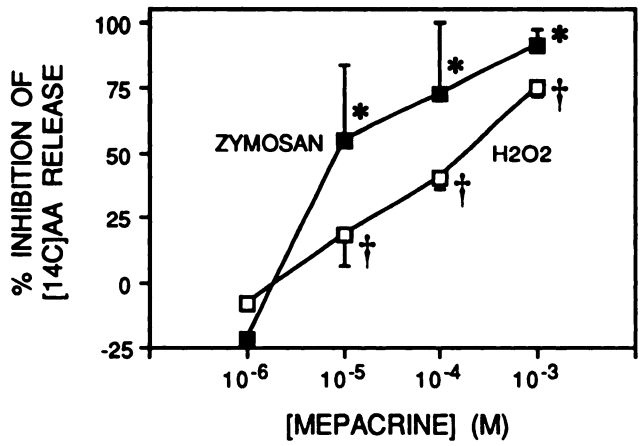

Fig. 4. Effect of mepacrine on AA release by alveolar macrophages stimulated with H_2O_2 and zymosan. Alveolar macrophages prelabeled with [^{14}C]AA were stimulated with 1 mM H_2O_2 or 100 μg/ml zymosan for 30 min in the presence and absence of the indicated concentrations of mepacrine, following a 15 min pre-exposure to the drug or medium alone, respectively. Free [^{14}C]AA was then separated and quantitated as described in the legend to Figure 1. The results represent means ± SE of data from 3 to 6 individual experiments, each performed in duplicate (except for [mepacrine] = 10^{-6} M, for which the means of duplicates from a single experiment are shown). *P <0.05 vs. zymosan without mepacrine, by analysis of variance and the Newman-Keuls multiple range test. †P <0.025 vs. H_2O_2 without mepacrine, by analysis of variance and the Newman-Keuls multiple range test.

TABLE 3. Effect of Mepacrine (1 mM) on Synthesis of Immunoreactive TxB_2 by Alveolar Macrophages Stimulated With H_2O_2 and With Zymosan

		TxB_2 synthesis (pg/plate)		
Agonist	(n)	Without mepacrine	With mepacrine	% inhibition
None	(4)	138 ± 19	14 ± 12	
H_2O_2 (1 mM)	(3)	821 ± 34	116 ± 27*	83 ± 4
Zymosan (100 μg/ml)	(4)	1,452 ± 324	51 ± 35**	95 ± 5

*P <0.0025 vs. H_2O_2 without mepacrine, by paired t-test.
**P <0.05 vs. zymosan without mepacrine, by paired t-test.

tion, a number of potential explanations for their inability to inhibit AA metabolism induced by H_2O_2 may be proposed. 1) H_2O_2 might oxidatively inactivate lipocortin, or some other glucocorticoid-induced second messenger protein. 2) Peroxidation of membrane lipids by H_2O_2 might alter lipocortin binding to phospholipids, and thereby impair the ability of the putative inhibitor protein to block phospholipase action. 3) Rather than stimulating AA release via phospholipase A_2, H_2O_2 might cause AA to be released via a pathway involving phospholipase C, which is much less sensitive than phospholipase A_2 to glucocorticoid inhibition [41]. 4) Since macrophages exhibit very high rates of fatty acyl turnover in their phospholipids [42], the increases in free AA and metabolites generated in response to H_2O_2 may in large part result from oxidative inhibition of reacylation of deacylated AA back to phospholipids, rather than from activation of phospholipase(s), the enzymatic step which is the putative locus of glucocorticoid inhibition. Further investigation will be required to determine the importance of these or other possible mechanisms which may be responsible for the failure of glucocorticoids to inhibit AA metabolism stimulated by H_2O_2.

Differences in the susceptibility of AA metabolism triggered by various stimuli to inhibition by glucocorticoids may be an important determinant of the degree to which certain experimental or clinical disease processes do or do not respond to glucocorticoid pharmacotherapy. In particular, the failure of glucocorticoids to inhibit AA metabolism stimulated by H_2O_2 may in part explain their inability to ameliorate oxidant-mediated lung injury in animals [18–21], or to improve survival in the human adult respiratory distress syndrome [43,44], in which oxidants appear to also play an important role [45,46].

ACKNOWLEDGMENTS

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