Inhibition of O_2^- Generation by Dexamethasone Is Mimicked by Lipocortin I in Alveolar Macrophages

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Abstract

Glucocorticoids inhibit superoxide (O_2^-) generation by phagocytes through a mechanism that remains unclear. We investigated this effect by using dexamethasone on guinea pig alveolar macrophages. O_2^- generation was induced either by the calcium ionophore A23187, a potent stimulus of phospholipase A2, or by the protein kinase C activator, phorbol myristate acetate (PMA). Dexamethasone inhibited O_2^- generation initiated by A23187 by 50–55%. This inhibition required: (*a*) more than 45 min incubation and was maximal after 2 h; (*b*) glucocorticoid receptor occupancy; and (*c*) protein synthesis. The inhibitory effect of dexamethasone could not be explained by an interaction with the respiratory burst enzyme NADPH oxidase since O_2^- generation was only weakly affected upon PMA stimulation. Lipocortin I, a glucocorticoid inducible and phospholipase A2 inhibitory protein, inhibited O_2^- generation initiated by A23187 but failed to modulate the respiratory burst activated by PMA. These results were obtained with lipocortin I purified from mouse lungs, human blood mononuclear cells, and with human recombinant lipocortin I. We propose that lipocortin I is capable of inhibiting the activation of NADPH oxidase only when membrane signal transduction involves phospholipase A2. By mimicking the effect of dexamethasone, lipocortin I may extend its potential anti-inflammatory action to the partial control of the formation of oxygen reactive species by phagocytes.

Introduction

Activation of the superoxide-generating enzyme NADPH oxidase in phagocytes can be initiated either by stimuli involving binding to specific membrane receptors or by substances bypassing receptor interaction such as phorbol esters or calcium ionophores. Transmission of activation signals to the NADPH oxidase involves at least two distinct pathways (1, 2), implying either phospholipase A2 (PLA2)[1] or protein kinase C activation (3–5). Generation of superoxide (O_2^-) by phagocytes can be inhibited by in vivo or in vitro treatment by glucocorticoids, but their mechanism of action has not been clarified (6, 7). Glucocorticoids also affect other phagocyte functions such as AA hydrolysis and, consequently, the synthesis of eicosanoids (7–9). This particular effect of glucocorticoids was first attributed to the induction of a PLA2-inhibitory protein termed lipocortin I (8–10). Other PLA2-inhibitory proteins with sequence homologies were then described and included in the lipocortin family although their induction by glucocorticoids remains to be established. Recently it has been reported that lipocortin I can inhibit cellular PLA2 of both guinea pig perfused lungs (11) and guinea pig alveolar macrophages (12). We report here that in vitro treatment of guinea pig alveolar macrophages (AM) with dexamethasone inhibits O_2^- generation. Since one of the NADPH oxidase activation pathways has been shown to involve PLA2, we investigated the possibility that lipocortin I could mimic the glucocorticoid inhibitory process using human recombinant lipocortin I or lipocortin I purified from either human blood mononuclear cells or mouse lungs.

Methods

Preparation of AM and incubation with dexamethasone. Hartley guinea pigs were anesthetized by intraperitoneal injection of sodium pentobarbitone (30 mg/kg). The trachea was cannulated and AM were obtained by repeated lung lavages with 5 ml sterile PBS, pH 7.4, at 37°C as previously described (13), except that no lidocaine was added to the medium. Cells were washed and pelleted. Viability (> 90%) was assessed by trypan blue exclusion and the cell number was adjusted to 2×10^6 viable cells/ml of MEM (Eurobio, Paris, France) without phenol red supplemented with 0.5 mM $CaCl_2$; 0.25 mM $MgCl_2$; 20 mM Hepes, and 0.5 g/liter glucose, pH 7.4, at 37°C.

AM were incubated for 2 h in the presence of 5 μM dexamethasone and stimulated. In some experiments cycloheximide (Sigma Chemical Co., St. Louis, MO) or RU486 (Roussel-Uclaf, Paris, France) was added to the cell suspension just before dexamethasone (Sigma Chemical Co.).

Superoxide measurement. Superoxide generation was assessed by the SOD-inhibiting reduction of cytochrome *c*, continuously monitored for 4 min at 550 nm with a spectrophotometer (Uvikon 860; Kontron Analytical, Redwood City, CA) thermostated at 37°C as previously described (3). Phorbol myristate acetate (PMA) and A23187 were obtained from Sigma Chemical Co.

Preparation of lipocortin I. Lipocortin I was purified from human blood mononuclear cells or from mouse lungs. The procedure for isolation of human mononuclear cells (70% lymphocytes, 30% monocytes) is reported in reference 14. Mouse lungs were isolated from thoracic cages, homogenized using a teflon potter, and sonicated. Proteins were extracted (14) and purified as previously described for human blood mononuclear cells (15) or mouse lungs (12). The peak corresponding to lipocortin I was selected and analyzed in SDS-PAGE (14). A single protein band at 40 kD was obtained with the preparation from mouse lungs and a single band at 35–38 kD with the protein from mononuclear cells. The two proteins exhibited PLA2 inhibitory activ-

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1. *Abbreviations used in this paper:* AM, alveolar macrophages; PAF, platelet activating factor; PLA2, phospholipase A2; PMA, phorbol myristate acetate.

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ity as assessed on porcine pancreatic PLA2 using membranes of *Escherichia coli* labeled with [^{3}H]oleic acid (16). Western blot analyses were performed as described (14, 17). The two proteins were recognized by the lipocortin I antiserum (15, 18) raised against the recombinant protein (19) kindly provided by Biogen Corp. (Cambridge, MA). Antisera directed against lipocortin II, III, and 32 kD (now referred to as lipocortin V; Rothhut, B., C. Coméra, S. Cortial, P. Y. Haumont, K. H. Diep-le, J. C. Cavadore, J. Connard, F. Russo-Marie, and F. Lederer, manuscript submitted for publication) were also tested; the 32-kD and lipocortin III antisera were shown to be monospecific (20), and the lipocortin II antiserum (provided by Biogen Corp.) has a low titer of antibodies that recognize lipocortin I (18). The lipocortin II and the 32-kD antisera (14) did not immunoreact with recombinant-, mouse lung- (12), or human blood mononuclear cell-lipocortin I (20). The lipocortin III antiserum tested on recombinant lipocortin I and lipocortin I from human blood mononuclear cells did not immunoreact with these proteins (20). Finally, our purified proteins cannot be similar to lipocortin IV since the lipocortin I antiserum raised against the recombinant protein does not immunoreact with lipocortin IV (R. B. Pepinsky, personal communication).

Before the use of the two purified lipocortin I's in biological experiments, the chromatographic buffer was removed by sequential microconcentrations on Centricon 10 (Amicon Corp., Danvers, MA). The human recombinant lipocortin I (19) was kindly provided by Biogen Corp.

RIA of eicosanoids. Measurement of PGE_2 and TXB_2 were performed as previously described (21).

Statistical analysis. The results were statistically analyzed using a paired *t* test.

Results

It has been reported that glucocorticoid treatment may modify the number of various membrane receptors (22–24). Therefore, we decided to stimulate dexamethasone-treated AM with substances bypassing membrane receptors, the calcium ionophore A23187 and the protein kinase C activator PMA.

Generation of O_2^- induced by A23187 in AM was inhibited by dexamethasone (Table I). The inhibitory effect was detected 45 min after dexamethasone addition and was maximal 2 h later. The dexamethasone effect was abolished when the protein synthesis inhibitor, cycloheximide, or the glucocorticoid receptor antagonist, RU486, was present (25; Table I). RU486 or cycloheximide did not affect the generation of O_2^- per se and did not enhance it when AM were stimulated with A23187 in the absence of dexamethasone (Table I). At 10 μg/ml cycloheximide abolished the protein synthesis both in control and dexamethasone-treated AM as examined by autoradiography of [^{35}S]methionine-labeled proteins separated by SDS-PAGE (21; data not shown).

Table I. Effect of Cycloheximide and RU486 on Dexamethasone-induced Inhibition of O_2^- Generation by Guinea Pig Alveolar Macrophages

	Control	Dexamethasone
	nmol O_2^-/min per 10^6 cells	
None	1.92±0.35	0.99±0.22
RU486	1.51±0.12	2.43±0.41
Cycloheximide	1.96±0.45	2.11±0.33

AM were preincubated for 2 h with 5 μM dexamethasone, stimulated with 8 μM A23187, and generation of O_2^- was measured. 5 μM RU486 or 10 μg/ml cycloheximide were added just before dexamethasone. The results are expressed as mean±SD of two experiments performed in triplicate. O_2^- production before A23187 was not detectable.

Table II. Effect of Dexamethasone Removal on O_2^- Generation

	Control	Dexamethasone	*P*
	nmol O_2^-/min per 10^6 cells		
8 μM A23187	1.89±0.21	0.87±0.11	<0.02
10 μM A23187	2.63±0.23	1.09±0.17	<0.02
50 ng/ml PMA	1.81±0.24	1.65±0.26	<0.05

AM were incubated for 2 h with 5 μM dexamethasone, washed, and incubated for an additional 2-h period without dexamethasone. The cells were stimulated with A23187 or PMA, and O_2^- generation was recorded. The results expressed as mean±SD of four experiments were statistically analyzed using a paired *t* test.

To assess whether inhibition of O_2^- generation persists even after removal of glucocorticoids, cells were exposed to dexamethasone, then washed and incubated for an additional 2 h in the absence of dexamethasone before stimulation. During this particular procedure, which has been previously used for the study of the inhibitory effect of glucocorticoids on PLA2, the cells were still synthesizing proteins induced by glucocorticoids for 3–5 h (25). Under these conditions, generation of O_2^- by AM stimulated with A23187 was inhibited (Table II) to a similar extent as in the presence of dexamethasone (Table I), indicating that preincubation with dexamethasone is sufficient to induce the inhibitory process. Stimulation of AM with PMA was only inhibited 9% by dexamethasone, whereas 50 ng/ml PMA or 8 μM A23187 provoked cell responses of similar magnitude (Table II). This indicates that NADPH oxidase was probably not directly inhibited since O_2^- generation was weakly modified upon PMA stimulation. Therefore, the inhibitory factor(s) seems most likely to interfere with the NADPH oxidase activation pathway initiated by A23187.

The calcium ionophore A23187 initiates AA hydrolysis which mainly results from PLA2 activation (26, 27). Synthesis of a derivative of AA, PGE_2, was measured after incubation of AM for 2 h with or without dexamethasone, and with or without stimulation with A23187 for 15 min (picograms/10^6 cells in 1 ml, mean±SD, $n = 4$): (*a*) unstimulated cells: control, 671.7±119.5; dexamethasone, 344.2±27.2, $P < 0.03$; (*b*) A23187 stimulated cells: control, 1038.2±53.1; dexamethasone, 774.2±66.7, $P < 0.02$. In contrast, PMA, which is a potent activator of the respiratory burst, is unable, at the concentration and the stimulation length we used, to initiate the release of AA from membrane phospholipids (3, 27, data not shown) and, therefore, to modify the basal synthesis of PGE_2 (control, 684±125; PMA, 709±86 pg/10^6 cells in 1 ml; mean±SD, $n = 3$) or TXA_2 (measurement of its stable metabolite TXB_2, control, 19.8±4.3; PMA, 17.1±3.9 ng/10^6 cells in 1 ml) as measured by RIA 15 min after stimulation of AM with 50 ng/ml PMA. These results agree with previous reports on the absence of PLA2 involvement in the transmission of activation signals to NADPH oxidase upon PMA stimulation (3, 28). Therefore, we examined whether the effect of dexamethasone observed upon A23187 stimulation could be mimicked by the PLA2 inhibitory- and glucocorticoid-induced protein, lipocortin I.

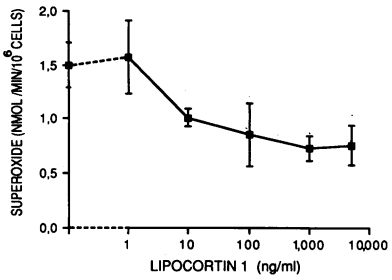

Figure 1. Effect of increasing concentrations of lipocortin I on O_2^- generation. Lipocortin I purified from mouse lungs was added at the indicated concentration to the cell suspension and incubated at 37°C for 30 min. Macrophages were stimulated with 8×10^{-6} M A23187 and O_2^- generation was recorded. Shown are the means±SD of three experiments. The statistical analysis was performed using a paired *t* test. From 10 ng/ml to 5 μg/ml lipocortin I, the differences in superoxide production were significative when compared with control cells with $P < 0.04$.

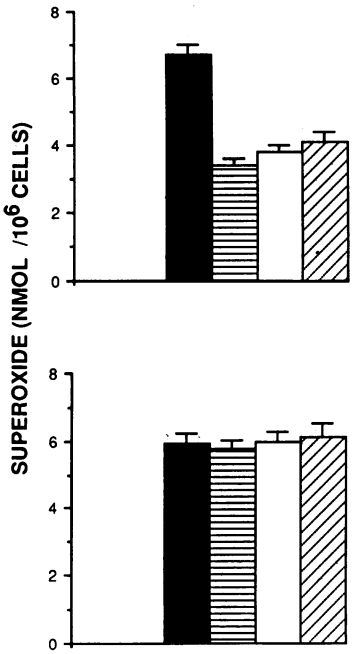

Figure 2. Effect of lipocortin I on generation of O_2^- by AM stimulated with A23187 (*top*) or PMA (*bottom*). Alveolar macrophages were incubated for 30 min with each lipocortin I (1 μg/ml) before stimulation with 8 μM A23187 or 50 ng/ml PMA, and O_2^- generation was recorded for 200 s. Shown are the means±SD of triplicate determinations from one experiment representative of two to seven separate experiments. This particular experiment is shown because the three lipocortin I's were tested in parallel on the same cells. ■, control; ▤, mouse lung; □, recombinant; ▨, mononuclear cells. Seven experiments performed with mouse lung lipocortin I were statistically analyzed using a paired *t* test. The results are expressed in nmol $O_2^-/10^6$ cells per 200 s (mean±SD); A23187 stimulation: control, 5.54±2.51; lipocortin I, 2.52±1.35 ($P < 0.02$). PMA stimulation: control, 5.26±2.43; lipocortin I, 5.59±3.71 (NS).

As shown in Fig. 1, O_2^- generation by AM stimulated with A23187 was inhibited by lipocortin I purified from mouse lungs in a concentration-dependent manner from 10 ng/ml (2.5×10^{-10} M) to 1 μg/ml, which provided the maximal effect. The inhibitory effect was detected 15 min after addition of lipocortin I, was maximal at 30 min, and was not changed by the concomitant addition of 10 μg/ml cycloheximide or by supplementing the calcium-containing incubation medium with 1 mM $CaCl_2$. When 1 μg/ml of the protein was boiled for 10 min, its inhibitory effect was abolished (53.6±15.5% inhibition vs. 7.8±8.3%). Control proteins, BSA and ovalbumin (10 μg/ml), tested in the same conditions as lipocortin I, did not modify O_2^- generation. The inhibitory effect of dexamethasone (53.8±10.5%, $n = 3$) was not potentiated by addition of 1 μg/ml lipocortin I to the cell suspension 30 min before stimulation (47.7±12.3%, $n = 3$).

Inhibition of PLA2 by extracellular lipocortin I was examined. Since inhibition of the release of AA by AM has been published elsewhere using the same preparations of lipocortin I as in the present work (see reference 12), here we only report the effect of 1 μg/ml lipocortin I on PGE_2 synthesis (pg/10^6 cells in 1 ml, mean±SD, $n = 3$): (*a*) unstimulated cells: control, 345.3±35; lipocortin I, 222.3±23.6, $P < 0.03$; (*b*) A23187-stimulated cells: control, 865.3±79; lipocortin I, 611±45.6, $P < 0.01$).

The effects of three lipocortin I's purified from different sources were then compared. The human recombinant-, the mouse lung-, and the human blood mononuclear cell lipocortin I exhibited similar inhibitory ranges on the O_2^- generation initiated by A23187 (Fig. 2). Upon PMA stimulation, none of the tested lipocortins inhibited O_2^- generation, suggesting that lipocortins did not interact with NADPH oxidase.

The effect of the mouse lung lipocortin I was investigated in the presence of another agonist that stimulates PLA2 (12, 29), platelet-activating factor (PAF), a stimulus binding specific membrane receptors. As observed with A23187, lipocortin I inhibited O_2^- generation initiated by 5×10^{-7} M PAF (0.71 vs. 0.37 nmol O_2^-/min per 10^6 cells, mean of duplicate determinations).

Discussion

In the present paper we report that in vitro treatment of AM with dexamethasone is able to inhibit O_2^- generation through a mechanism involving glucocorticoid receptor occupancy and protein synthesis. The PLA2-inhibitory protein lipocortin I mimicked the inhibitory effect of dexamethasone. Like dexamethasone, extracellular application of lipocortin I resulted in a decrease in both O_2^- generation and PGE_2 synthesis in response to A23187. In contrast, the calcium- and PLA2-independent NADPH oxidase activation pathway initiated by PMA (1, 3, 4) was not modified. This indicates that NADPH oxidase was not directly inhibited. More likely, activation of NADPH oxidase appeared to be affected only when membrane signal transduction involved PLA2. This was also supported by the results obtained with PAF, which stimulates PLA2 (12, 29) and NADPH oxidase (13); both enzyme activities were inhibited by lipocortin I (12; this paper). Moreover, dexamethasone and lipocortin I inhibitory effects were not additive, suggesting that they may affect the same step of the activation pathway of NADPH oxidase. The differences observed between the mechanisms of action of lipocortin I and dexamethasone strengthened the hypothesis that lipocortin I could account for the dexamethasone effect; i.e., lipocortin I inhibitory effect occurred more rapidly than dexamethasone and it did not require protein synthesis. Nevertheless, it cannot be excluded that other glucocorticoid-inducible proteins may operate in that process.

Although this study further supports the involvement of PLA2 in the activation pathway of NADPH oxidase (3, 5, 30), the relationship between these two enzymes was not investigated. It has been previously reported that AA and eicosanoids play a role in the activation of NADPH oxidase (3, 5, 28, 30, 31), but their mechanisms of action are still unclear.

As previously described (8–12), neither lipocortin I nor dexamethasone provided a total inhibition of O_2^- generation or AA hydrolysis. This may be explained either by the existence

of a pool of lipocortin I- and dexamethasone-insensitive PLA2, or by the activation of several distinct phospholipid-degrading processes in macrophages stimulated with A23187 or PAF (26, 29). Therefore, it is possible that the remaining fraction of O_2^- formation results from the hydrolysis of AA by enzymatic processes insensitive to lipocortin I and/or from the liberation of diacylglycerol by phospholipase C (although it is weakly stimulated by A23187 [26]) which is involved in NADPH oxidase activation through protein kinase C stimulation (1, 4, 32).

It has been shown that treatment of macrophages with glucocorticoids induces the release of lipocortin (8, 33). However, induction of its synthesis by glucocorticoids is still controversial although an increase in lipocortin I mRNA was reported (19). Since lipocortin I is also a constitutive protein, another possible effect of glucocorticoids would be to provoke its secretion through a mechanism involving protein synthesis. When purified lipocortin I is applied extracellularly it may, therefore, mimic the effect of glucocorticoids. Indeed, some recent reports demonstrate an inhibition of PLA2 by extracellular lipocortin I (11, 12). These and our results are, however, in disagreement with the data of Northup and co-workers (34), who reported that extracellular lipocortin I does not inhibit PLA2 of mouse peritoneal macrophages, in contrast to what they observe by treating the cells with dexamethasone. In their experiments, lipocortin I was applied to the cells for only 15 min; therefore, we can question whether longer incubation time would have favored the action of lipocortin I.

The mechanism of action of extracellular lipocortin I has not yet been defined. It seems unlikely that a strongly polar 40-kD protein could enter the plasma membrane. The simplest model would be that lipocortin I interacts with a putative membrane receptor which could be PLA2 itself, assuming that PLA2 is a transmembrane protein. However, it has been proposed that the inhibition of PLA2 is dependent on the ability of lipocortin I to bind negatively charged phospholipids (14, 35). Whether the low concentration of lipocortin I providing a biological effect (11, 12, this report) is compatible with this hypothesis remains to be elucidated. Albumin, which has also the ability to bind phospholipids, did, however, not modify the generation of O_2^-, further suggesting a specific effect of lipocortin I.

Glucocorticoids are by far the most potent anti-inflammatory molecules active against virtually every type of inflammatory disease. Lipocortin, which has been recently defined as a "second messenger" of glucocorticoids (33), can account in part for their anti-inflammatory action by controlling the synthesis of eicosanoids and PAF (33). From the present study, the action of lipocortin I can be extended to the partial control of proinflammatory and tissue damaging oxygen reactive species.

In conclusion, we report that lipocortin I mimics the effect of dexamethasone and we propose that it could account for part of the action of glucocorticoids. We also confirm that lipocortin I is biologically active when applied extracellularly (11, 12). In addition, our data further support the previously proposed critical role of PLA2 in the transduction of activation signals to NADPH oxidase (3, 5, 30) together with the involvement of a PLA2-independent activation pathway of the oxidase (1–3, 32).

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