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European Journal of Pharmacology 339 (1997) 87–95

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Inhibition of inducible nitric oxide synthase gene expression by glucocorticoid-induced protein(s) in lipopolysaccharide-stimulated J774 cells

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Received 29 May 1997; revised 19 September 1997; accepted 23 September 1997

Abstract

Glucocorticoids inhibit inducible-type NO synthase activity in a variety of cell types. We report here that proteins recovered from the medium of dexamethasone-treated J774 macrophages (1, 10, 100 μg/ml) inhibited lipopolysaccharide-stimulated nitrite generation by $10.0 \pm 3.0\%$, $32.3 \pm 5.3\%$ and $55.0 \pm 6.0\%$, respectively, and inducible NO synthase mRNA expression in these cells. Immunoblotting analysis of crude and partially purified glucocorticoid-induced proteins with an anti-lipocortin-1 polyclonal antiserum revealed the presence of lipocortin-1-like immunoreactive species with a molecular mass of 35–37 kDa. Furthermore, inhibition of lipopolysaccharide-induced nitrite production by glucocorticoid-induced proteins in J774 cells was reversed by addition of anti-lipocortin-1 neutralizing polyclonal antibody (1:60 dilution; 4 h before lipopolysaccharide). Comparison of glucocorticoid-induced proteins inhibition of both nitrite production and inducible NO synthase mRNA expression suggests that these effects result mainly from inhibition of lipopolysaccharide-mediated inducible NO synthase gene expression. These results indicate that negative regulation of inducible NO synthase by glucocorticoids is, at least in part, mediated by glucocorticoid-induced proteins that involve also members of the lipocortin-like superfamily. © 1997 Elsevier Science B.V.

Keywords: Glucocorticoid; Nitric oxide (NO); Nitric oxide (NO) synthase, inducible; Lipocortin; J774 macrophage; Lipopolysaccharide

1. Introduction

Nitric oxide (NO), a short-lived biologic mediator, plays an important role in a number of different systems, including smooth muscle relaxation, inhibition of platelet aggregation, neurotransmission, immune responses and inflammation (Moncada et al., 1991). Production of NO from guanidino nitrogen of L-arginine and molecular oxygen is catalyzed by the enzyme NO synthase. Three isoforms of NO synthase are known to exist and can be classified into two categories, namely constitutive and inducible (Knowles and Moncada, 1994). The inducible NO synthase is expressed to significant level in numerous cell types following immunological stimuli (Sthuer and Marletta, 1987; Nathan, 1992). The NO synthesized by this enzyme is an important cytotoxic effector molecule (Nathan and Hibbs, 1991) and it is also involved in the pathophysiology of septic shock (Kilbourn et al., 1990). Accumulating evidence indicates that the expression of inducible NO synthase induced by lipopolysaccharide or cytokines is potently inhibited by glucocorticoids in vitro and in vivo (Di Rosa et al., 1990; Radomski et al., 1990; Thiemermann and Vane, 1990; McCall et al., 1991; Wright et al., 1992). Additionally, it has been reported that glucocorticoids inhibit the lipopolysaccharide-induced inducible NO synthase mRNA expression (Liu et al., 1993; Robbins et al., 1994). Glucocorticoids are the most effective drugs in controlling both the immune response and the processes of inflammation (Cohen, 1989); moreover they are protective in endotoxic shock (Sprung et al., 1984). In spite of the widespread use of glucocorticoids, the molecular mechanisms that underlie their therapeutic effects are poorly understood. Glucocorticoids exert their effects on target

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cells by binding a cytoplasmic glucocorticoid receptor, a member of the nuclear receptors superfamily that directly regulate transcription of target genes (Truss and Beato, 1993). Some effects of glucocorticoid action have been shown to be mediated in several systems by the protein lipocortin-1 (Flower and Rothwell, 1994). Recently it has been shown that the inhibition of the induction of inducible NO synthase by dexamethasone is reversed by polyclonal anti-lipocortin-1 antibody in rat and J774 cells (Wu et al., 1995). In the present work we report the identification and preliminary characterization of glucocorticoid-induced proteins that prevent endotoxin-mediated activation of inducible NO synthase gene expression in murine J774 macrophages. As these proteins can be recovered from culture media of glucocorticoid-treated macrophages, it is possible to conceive that their synthesis and release is an important aspect of the anti-inflammatory and immuno-modulatory activity of these steroids.

2. Materials and methods

2.1. Cell culture

Murine macrophages J774 (American Tissue Culture Catalogue T1B p. 231) were grown in suspension culture in Techne spinner bottles, spun at 25 rpm and incubated at 37°C in DMEM (Dulbecco's Modified Eagle's Medium; Gibco) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 25 mM Hepes, 100 UI/ml penicillin and 100 μg/ml streptomycin (Gibco). The cell viability was shown by trypan blue exclusion test to be $>95\%$.

2.2. Induction of proteins by glucocorticoids in J774 cells

Macrophages (150×10^6 cells) were incubated in Techne spinner bottles at 37°C for 24 h in 300 ml of serum-free medium (DMEM supplemented as above described but without foetal calf serum) with or without dexamethasone (1 μM; Sigma). In other experiments the cells were incubated with dexamethasone (1 μM) plus cycloheximide (1 μM; Sigma) or cycloheximide alone (1 μM). After the incubation the cells were removed by centrifugation ($180 \times g$ for 10 min) and the supernatants were dialyzed against 0.01 M $(NH_4)_2CO_3$ pH 7.4 at 4°C, lyophilyzed and stored at -70°C.

2.3. Thermal stability, trypsin digestion

In order to perform partial characterization glucocorticoid-induced proteins were submitted to the following treatments: (1) heating for 5 min at 70°C; (2) incubation in PBS (phosphate buffered saline) with or without 10 U/ml of immobilized trypsin (Sigma) at 37°C overnight. After these treatments the supernatants were used for nitrite assay described below.

2.4. Partial purification

Glucocorticoid-induced proteins (8 mg) were dissolved in 0.01 M $(NH_4)_2CO_3$ pH 7.4 and eluted on Hiload 200 Superdex column (60×1.6 cm; Pharmacia). The flow rate was 60 ml/h and 1 ml fractions were collected, pooled according to elution profile and lyophylized.

2.5. Incubation of J774 cells with glucocorticoids or glucocorticoid-induced proteins

Protein activity was assessed on the lipopolysaccharide-induced nitrite accumulation. Macrophages J774 (2.5×10^6 cells/ml) were plated in 24 well culture plates (Falcon) and allowed to adhere for 2 h at 37°C in 5% CO_2/95% air. Thereafter the medium was replaced with fresh medium and then the cells were activated with lipopolysaccharide (0.1 μg/ml; Difco) and incubated for 24 h with crude and purified proteins from the following incubation medium: (a) proteins (1, 10, 100 μg/ml) from cells incubated with and without dexamethasone (1 μM); in some experiments glucocorticoid receptor antagonist RU38486 (1 μM; Russell) was added; in parallel experiments the cells were incubated with dexamethasone (1 μM) with or without RU38486 (1 μM); (b) proteins (1, 10, 100 μg/ml) from cells incubated with protein synthesis inhibitor cycloheximide (1 μM) or with dexamethasone (1 μM) plus cycloheximide; (c) proteins (10 μg/ml) from incubation with or without trypsin; (d) proteins (10 μg/ml) were added to J774 cells 12 h after endotoxin challenge to determine whether those affected the activity of inducible NO synthase; in parallel experiments dexamethasone (1 μM) or N^G-monomethyl-L-arginine (L-NMMA; Sigma) (30 μM) were added; (e) partially purified proteins (2.5 μg/ml) from Hiload G 200-Superdex column; (f) crude proteins (10 μg/ml) with anti-lipocortin-1 polyclonal antibody or pre-immune serum (1:60 dilution, 4 h prior to lipolysaccharide) (kindly provided by G. Cirino) and dexamethasone (1 μM) with anti-lipocortin-1 polyclonal antibody or pre-immune serum (1:60 dilution, 4 h prior to lipopolysaccharide).

2.6. Nitrite determination

NO was measured as nitrite (NO_2^-, nmol per 10^6 cells) accumulated in the incubation medium 24 h after lipopolysaccharide challenge. A spectrophotometric assay based on the Griess reaction was used (Green et al., 1982).

2.7. Western blot analysis

Crude and purified proteins were boiled (3 min) with gel loading buffer (50 mM Tris/10% sodium dodecyl sulfate (SDS)/10% glycerol/10% 2-mercaptoetanol/2 mg of bromophenol blue per ml) at a ratio of 1:1 and centrifuged at $10{,}000 \times g$ for 10 min. Protein concentrations

from the supernatants were determined (Biorad), and protein equivalents for each sample were separated on SDS/12% polyacrylamide minigels (Biorad) and transferred to nitrocellulose membranes according to the method described by the manufacture (Biorad). Nonspecific immunoglobulin G (IgG) binding sites were blocked with 10% (w/v) dried milk protein and the filter was incubated with anti-lipocortin-1 polyclonal antibody (1:2000) raised in rabbits (a whole blood plasma fraction) against human recombinant lipocortin-1. Bands were detected with a horseradish peroxidase-conjugated secondary antibody (Sigma) and developed with 3,3′-diaminobenzidine tetrahydrochloride (Sigma).

2.8. Northern blot analysis

Expression of inducible NO synthase mRNA in 20 or 25 μg of total RNA from J774 macrophages was analyzed by Northern blot hybridization essentially as described earlier (Weisz et al., 1994). RNAs were separated by electrophoresis on 1% agarose gel (in 6% formaldehyde) and transferred to a nylon membrane. After UV cross-linking, the filter was prehybridized for 6 h at 42°C in hybridization buffer containing 50% deionized formamide, 0.65 M NaCl, 5 mM EDTA (pH 7.6), 0.1% SDS, 0.1 M piperazine-*N*′,*N*-bis(2-ethane-sulfonic-acid) (pH 6.8), 5× Denhardt's solution (0.1% Ficoll 400/0.1% PVP/1.0% bovine serum albumin-fraction IV) and 100 μg/ml denatured salmon sperm DNA, and then hybridized with a probe for 12 h at 42°C. Probes included a 700 bp fragment of the 5′ portion of the cloned rat liver inducible-type NO synthase cDNA (Adachi et al., 1993) and a 700 bp DNA fragment of the human 36B4 cDNA (kindly provided by P. Chambon) (Masiakowski et al., 1982). They were labelled with α-^{32}P-dCTP (3,000 Ci/mmol; DuPont Nen) by random priming (Amersham). Following hybridization, the filters were washed twice for 30 min in 0.5× saline sodium citrate buffer/0.1% SDS at 60°C. Autoradiography was performed by exposing the filters for 5–6 days to imaging plates; the hybridization signals were analyzed by scanning laser densitometry. Relative inducible NO synthase mRNA levels were normalized by calculating the ratio between inducible NO synthase and 36B4 hybridization signals in each lane.

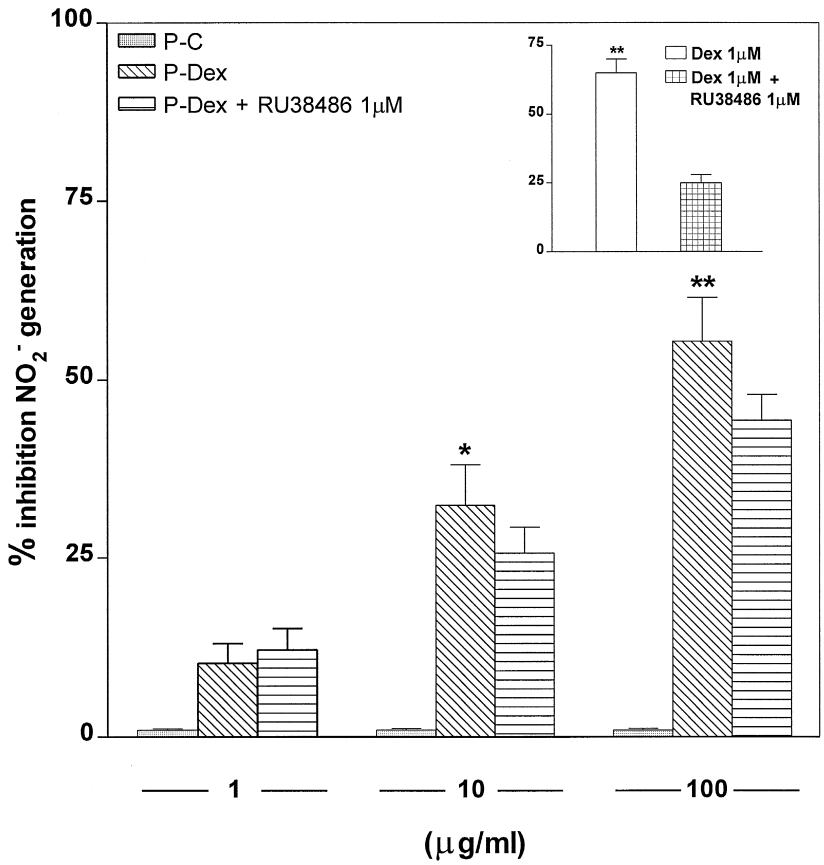

Fig. 1. Inhibition of lipopolysaccharide-mediated NO_2^- generation by conditioned medium of dexamethasone-treated J774 macrophage cultures. J774 cells were stimulated with lipopolysaccharide (0.1 μg/ml) for 24 h in the presence of varying amounts of concentrated conditioned medium containing glucocorticoid-induced proteins (P-Dex) alone or in the presence of RU38486 (P-Dex + RU38486); P-C: concentrated from medium of control, untreated J774 cells. In inset: Effect of RU38486 on direct inhibition of lipopolysaccharide-induced NO_2^- generation by dexamethasone in J774 cells. Each column represents the mean ± S.E.M. of 20 separate experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (lipopolysaccharide alone). The differences between P-Dex and P-Dex + RU38486 are not statistically significant ($n = 20$).

2.9. Statistics

Data are expressed as percent of control (mean ± S.E.M.). Comparisons were made by the unpaired two-tailed Student's *t*-test. The level of statistically significant difference was defined as $P < 0.05$.

3. Results

3.1. Effects of a protein fraction derived from the conditioned medium of dexamethasone-treated cells on lipopolysaccharide-induced nitrite production by J774 cells

Nitrite production induced by stimulation of J774 cells with lipopolysaccharide for 24 h (35.8 ± 4.1 nmol/10^6 cells; $n = 20$) was reduced by various concentrations of an extract from conditioned medium of these same cells, prepared after addition of 1 μM dexamethasone followed by incubation of the cultures for 24 h (Fig. 1). Glucocorticoid-induced proteins at 1.0, 10 and 100 μg/ml inhibited nitrite formation by $10.0 \pm 3.0\%$, $32.3 \pm 5.3\%$ and $55.0 \pm 6.0\%$, respectively ($n = 20$; $P < 0.01$). This was not the case when cells were challenged with similar amounts of extract prepared from conditioned medium of untreated cultures. The glucocorticoid receptor antagonist RU38486 (1 μM) was not able to reverse inhibition of nitrite formation when added to the cells together with glucocorticoid-induced proteins. In contrast, when J774 cells were incubated directly with dexamethasone (1 μM), the antagonism of RU38486 toward dexamethasone-mediated inhibition was easily detectable, as direct reduction of lipopolysaccharide-induced nitrite production by dexamethasone ($62.5 \pm 5.8\%$; $n = 10$, $P < 0.01$) was prevented (by about 60%) by the presence of an equimolar concentration of RU38486 ($25.0 \pm 3.5\%$; Fig. 1, inset).

These results suggest that the mechanisms of action of glucocorticoid-induced proteins and dexamethasone on nitrite production in lipopolysaccharide-stimulated cells are likely to be different, only the second requiring activation of the glucocorticoid receptor. To better define the mechanisms that underlie the production of glucocorticoid-in-

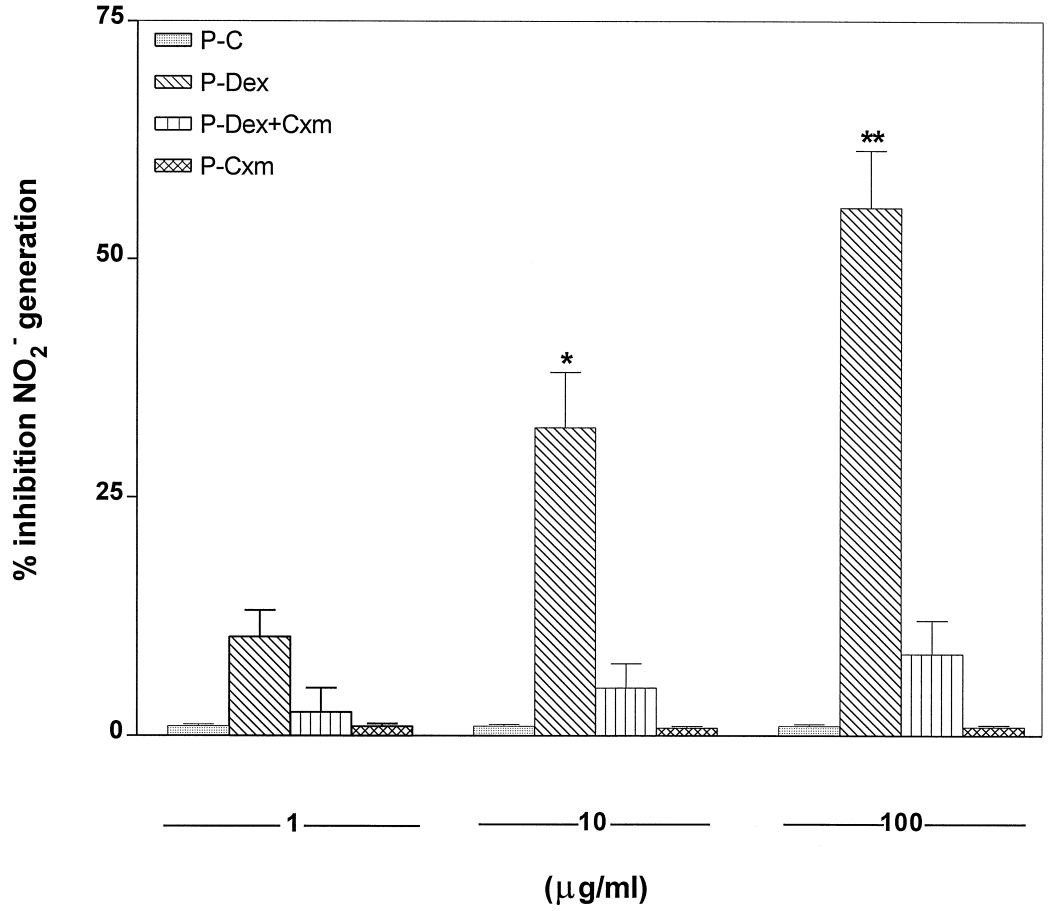

Fig. 2. Accumulation of glucocorticoid-induced proteins (P-Dex) in the medium of dexamethasone-treated J774 macrophage cultures is prevented by the protein synthesis inhibitor cycloheximide (Cxm). Extracts tested for inhibition of lipopolysaccharide-induced NO_2^- generation were from cultures of J774 cells treated as follows: P-C: control, untreated cells; P-Dex: cells treated with 1 μM dexamethasone; P-Dex + Cxm: cells treated with dexamethasone in the presence of 1 μM cycloheximide; P-Cxm: cells treated with 1 μM cycloheximide alone. Each column represents the mean ± S.E.M. of 3–6 separated experiments performed in triplicate; $^{*}P < 0.05$; $^{**}P < 0.01$ versus lipopolysaccharide alone.

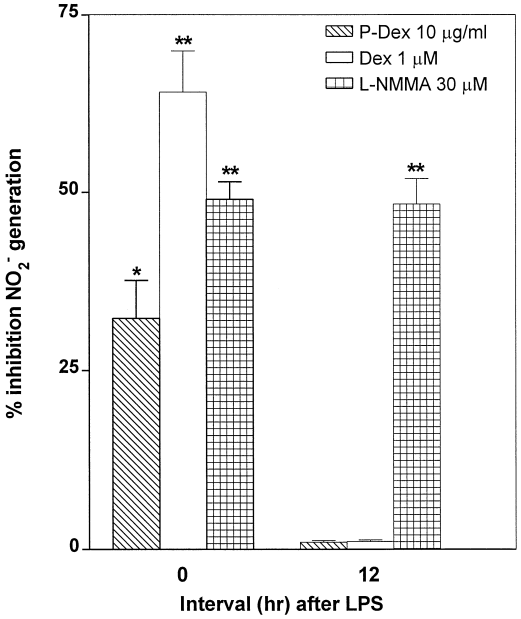

Fig. 3. Effect of glucocorticoid-induced proteins, dexamethasone or L-NMMA on basal (0) and lipopolysaccharide-induced (12 h) NO_2^- generation in J774 macrophages. Each column represents the mean ± S.E.M. of 3 separate experiments in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$ versus lipopolysaccharide alone.

duced proteins by dexamethasone-treated J774 macrophages, these cells were treated with the glucocorticoid in the presence of cycloheximide, an inhibitor of protein synthesis, and the conditioned medium so obtained was tested for its ability to prevent lipopolysaccharide effects on nitrite production (Fig. 2). No inhibition was exhibited by proteins collected from the culture medium of J774 cells incubated with 1 μM dexamethasone in presence of 1 μM cycloheximide or with cycloheximide alone, suggesting that glucocorticoid-induced de novo synthesized proteins are responsible for the activity of glucocorticoid-induced proteins.

Furthermore, addition of glucocorticoid-induced proteins (10 μg/ml) or dexamethasone (1 μM) to cells exposed for 12 h of endotoxin challenge did not affect NO_2^- levels (Fig. 3), indicating that neither agent caused direct inhibition of inducible NO synthase enzyme activity, or interfered with the assay utilized here for nitrite detection. On the contrary, L-NMMA (30 μM), a competitive inhibitor of inducible NO synthase, suppressed nitrite production by about 49% both under basal conditions (0) or after 12 h of endotoxin challenge, when tested under comparable conditions (Fig. 3).

3.2. Effect of glucocorticoid-induced proteins on inducible NO synthase mRNA induction by lipopolysaccharide

To determine whether inhibition of lipopolysaccharide-stimulated nitrite accumulation by glucocorticoid-induced proteins was mediated by regulation of inducible NO synthase gene expression, Northern blot analysis was carried out on RNA samples extracted from J774 cells exposed to the endotoxin under various experimental conditions (Fig. 4). Upon stimulation with lipopolysaccharide (10 μg/ml) for 24 h, cells expressed a significant higher level of inducible NO synthase mRNA when compared to control, untreated cells (compare 1 and 2); while 1 μM dexamethasone induced a significant decrease of the baseline levels (compare 1 and 3). Glucocorticoid-induced proteins (10 μg/ml) inhibited lipopolysaccharide effects on inducible NO synthase mRNA levels to an extent comparable to that of dexamethasone itself (compare 5 with 4 and 2). These results, combined with those reported in Fig. 1, indicate that inhibition of endotoxin-mediated nitrite production by both dexamethasone and glucocorticoid-induced proteins results primarily from blockade of

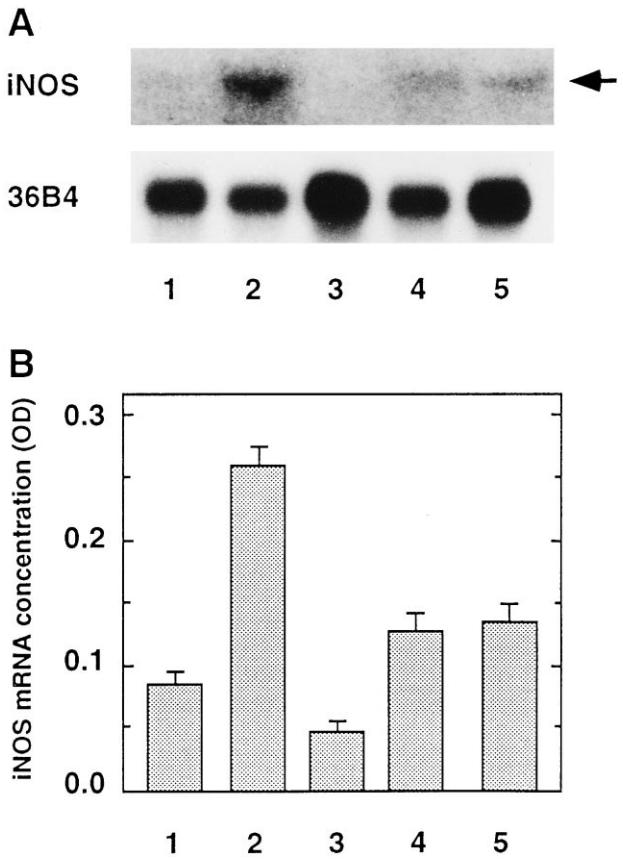

Fig. 4. Inhibition by glucocorticoid-induced proteins of lipopolysaccharide-mediated inducible NO synthase mRNA accumulation in J774 macrophages. (A) Reproduction of a representative autoradiography of a Northern blot inducible NO synthase mRNA analysis. (B) Result of quantitative densitometric scanning of results obtained in multiple ($n = 3$), separate experiments, after normalization for mRNA content in each lane based on 36B4 hybridization signals. (1) Untreated cells, (2) cells stimulated with lipopolysaccharide (0.1 μg/ml), (3) cells treated with dexamethasone (1 μM), (4) cells treated with lipopolysaccharide and dexamethasone and (5) cells treated with lipopolysaccharide and glucocorticoid-induced proteins (10 μg/ml). Cells were exposed to the indicated compounds for 24 h before lysis and RNA purification.

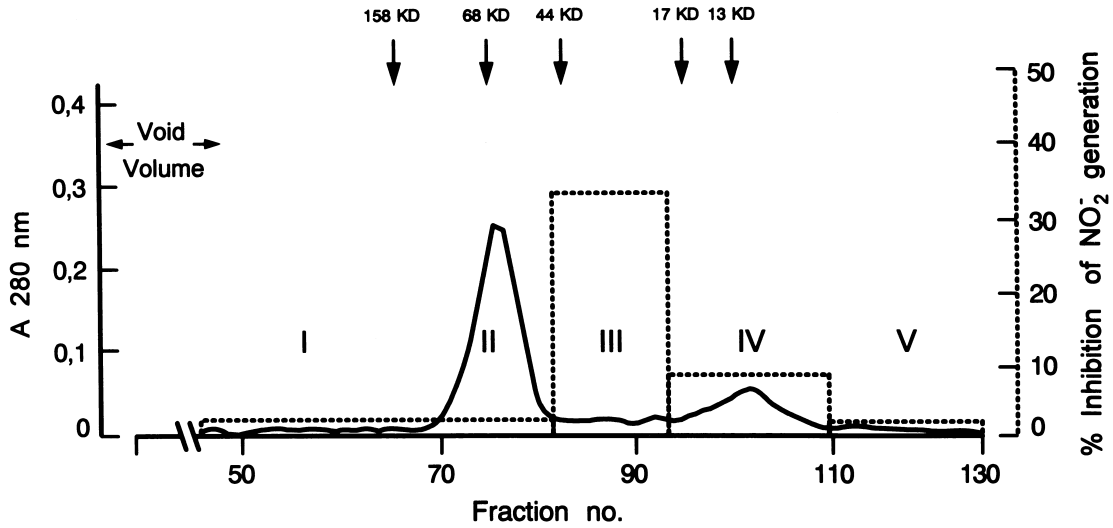

Fig. 5. Partial purification of glucocorticoid-induced proteins from culture medium of dexamethasone-treated J774 macrophages by molecular sieve chromatography. A Hiload 200 Superdex column (60 × 1.6 cm) was equilibrated in $(NH_4)_2CO_3$ 0.01 M, pH 7.4. The flow rate was 60 ml/h and a 1 ml fraction was collected, pooled as indicated (I to V) and lyophylized. Aliquots of each pool were then assayed for inhibition of lipopolysaccharide-induced NO_2^- generation as described in the text. Results are reported as inhibition of NO_2^- generation relative to the inhibition induced by 10 μg/ml of glucocorticoid-induced proteins (100%). Elution of standard proteins (ribonuclease: 13.7 kDa, chymotrypsin: 25 kDa, ovalbumin: 43 kDa, collagenase: 105 kDa) is marked by vertical arrows.

inducible NO synthase mRNA accumulation within the cell.

3.3. Partial characterization and purification of glucocorticoid-induced proteins

Glucocorticoid-induced proteins are thermostable, being still active following heating at 70°C for 5 min: 10 μg/ml glucocorticoid-induced proteins inhibited nitrite formation by 35.1 ± 7.3% after heat treatment versus 32.3 ± 5.3% when untreated ($P < 0.01$; $n = 3$). On the contrary, complete loss of its activity is observed after incubation with 10 UI/ml trypsin (5.1 ± 4.3% of inhibition of nitrite formation; $n = 3$). This indicates that either the active component(s) of glucocorticoid-induced proteins are polypeptide(s) that loose their activity upon a short treatment with this proteolytic enzyme, or that trypsin digestion generates products that interfere with the action of gluco-

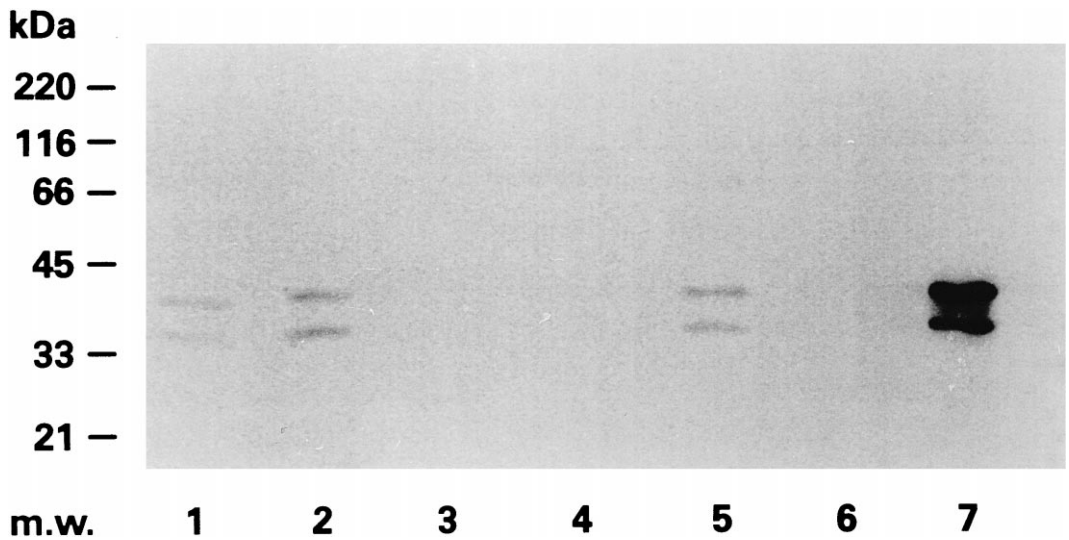

Fig. 6. Western blot analysis of crude and partially purified glucocorticoid-induced proteins from J774 macrophages. Lane 1: crude P-C (30 μg), lane 2: crude P-Dex (30 μg), lanes 3–6: purified P-Dex (pools I–IV; 15 μg) and lane 7: rhlipocortin-1 (20 ng). On the left-hand side is reported the position of migration of molecular size markers in the same gel. For each lane, the results shown are representative of at least two immunoblotting experiments.

corticoid-induced proteins active component(s). To start addressing this question, a first fractionation of glucocorticoid-induced proteins was carried out by molecular sieve liquid chromatography, followed by analysis of the biological activity of the different fractions obtained as described above. Results, reported in Fig. 5, indicate the presence of two bioactive pooled fractions (III and IV), appearing like a major peak (fraction III) with a broad shoulder toward the lower molecular weight region (fraction IV). The first pool includes molecules of apparent MW ranging between 17 and 44 kDa and the second includes smaller molecules (MW < 17 kDa): fraction III and IV (2.5 μg/ml) inhibited nitrite formation by $33.0 \pm 2.5\%$ and $9.3 \pm 1.8\%$, respectively ($n = 6$). This result suggests that this crude preparation include multiple active components even if further purification by FPLC, now in progress, is required to better characterize the nature of all glucocorticoid-induced proteins components active on inducible NO synthase.

3.4. Immunoblotting with anti-lipocortin-1 antibodies indicates lipocortin-1 as one of the components of glucocorticoid-induced proteins

As lipocortin-1 is one of the factors secreted by glucocorticoid-stimulated macrophages, we performed a control experiment to verify the possibility that this protein is present in glucocorticoid-induced proteins. For this, Western blot (WB) analysis was performed both on 30 μg crude and on 15 μg partially purified protein pooled fractions (I, II, III and IV), using a polyclonal anti-lipocortin-1 antiserum that recognizes mouse lipocortin-1 in this assay. Results of a representative WB are reported in Fig. 6A, to show that two protein species recognized by the antibody selected are present in crude and purified protein pooled fraction III (lanes 2 and 5, respectively), but not in purified protein pooled fractions I, II, IV (lanes 3, 4 and 6). The two protein species show a molecular weight of about 35 and 37 kDa corresponding to lipocortin-1 (lane 7). These same bands are slightly detectable in extracts from control, untreated cells (lane 1). No protein species of all above samples mentioned are recognized by pre-immune serum (data not shown).

3.5. Anti-lipocortin-1 neutralizing antibodies block glucocorticoid-induced proteins-mediated inhibition of nitrite accumulation in endotoxin-treated macrophages

As it was recently reported that inhibition of inducible NO synthase expression by dexamethasone can be prevented by polyclonal anti-lipocortin-1 antibodies (Wu et al., 1995), we tested the possibility whether the anti-lipocortin-1-immunoreactive species present in crude and partially purified glucocorticoid-induced proteins are responsible, at least in part, of its biological activity described here.

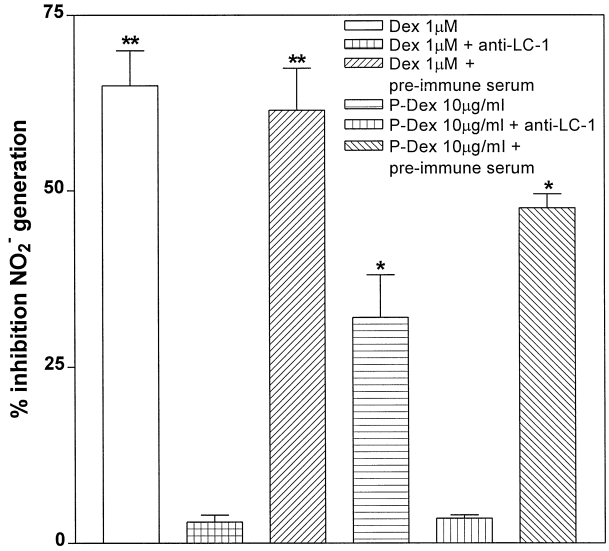

Fig. 7. Effect of anti-lipocortin-1 polyclonal antibodies (1:60 dilution, at 4 h prior to lipopolysaccharide) on the inhibition by dexamethasone (1 μM) or crude glucocorticoid-induced proteins (10 μg/ml) of nitrite generation by J774 macrophages stimulated with lipopolysaccharide (0.1 μg/ml). Each column represents the mean ± S.E.M. of 3 separate experiments performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$ versus lipopolysaccharide alone.

For this, a neutralization experiment was carried out in glucocorticoid-induced proteins-treated J774 cells. As shown in Fig. 7, addition of an excess of anti-lipocortin-1 antibodies (1:60 dilution) 4 h prior to the endotoxin greatly diminishes inhibition of lipopolysaccharide-mediated nitrite accumulation by both dexamethasone and glucocorticoid-induced proteins, whereas a control pre-immune serum was without effect when used at the same final concentration.

4. Discussion

The aim of the present study was to test the possibility that factors produced by glucocorticoid-stimulated macrophages are involved in the inhibitory action of these steroids on endotoxin-induced nitrite accumulation and inducible NO synthase gene activation. Results clearly indicate that extracts from conditioned media of glucocorticoid-treated J774 mouse cells present such inhibitory activity. A preliminary biochemical characterization of these extracts and partial purification of their active components, shows that they include multiple, small molecular weight proteins, that are synthesized and subsequently released from the cells in response to the glucocorticoid. Indeed, the biological activity of glucocorticoid-induced proteins was abolished when the conditioned medium was prepared from cultures stimulated with dexamethasone in the presence of the protein synthesis inhibitor cyclohex-

imide, indicating that glucocorticoid-induced proteins are either part of glucocorticoid-induced proteins itself or are required for secretion of active glucocorticoid-induced proteins components in the medium.

Inhibition by glucocorticoid-induced proteins of nitrite production was not consequent to direct inhibition of cellular inducible NO synthase activity, consistent with previous reports indicating that glucocorticoids inhibition of inducible NO synthase activity in macrophages is not mediated by interference with enzyme kinetics (Thiemermann and Vane, 1990; Wright et al., 1992; Robbins et al., 1994). On the contrary, glucocorticoid-induced proteins prevented inducible NO synthase mRNA synthesis in lipopolysaccharide-stimulated cells. Comparison of the inhibitory effect of glucocorticoid-induced proteins on nitrite accumulation versus inducible NO synthase mRNA expression suggests that the main target for the effect of the active glucocorticoid-induced proteins components is the iNOS gene itself. Interestingly, inhibition of lipopolysaccharide-mediated nitrite production, or inducible NO synthase mRNA accumulation, by dexamethasone or glucocorticoid-induced proteins are quantitatively comparable, suggesting that they both exert similar effects within the cell. It is possible that they affect the same molecular targets, to include regulatory sites within the inducible NO synthase gene (Radomski et al., 1990) and components of the signal transduction pathways that converge on these sites. The mechanism of action of glucocorticoid and glucocorticoid-induced proteins on the inducible NO synthase gene is likely to be quite different, however, as only glucocorticoid activity is inhibited by the glucocorticoid receptor antagonist RU38486. Purification to homogeneity of all the active components of glucocorticoid-induced proteins, followed by a detailed characterization of their mechanism of action and regulation by glucocorticoids, is the prerequisite to elucidate the full role of these newly-found molecules on inducible NO synthase, as well as on other genes and cellular components that are target for regulation by glucocorticoids in macrophages and other cell types of the immune system.

Previous studies have shown that glucocorticoid inhibition of lipopolysaccharide-mediated inducible NO synthase expression can be reversed in the rat in vivo by co-injection of polyclonal anti-lipocortin-1 antibodies (Wu et al., 1995). In addition, human recombinant lipocortin-1 (rhlipocortin-1: fragment 1.188) mimics glucocorticoid effects on inducible NO synthase in J774 macrophages activated with lipopolysaccharide, an effect that was abrogated by pretreatment of these cells with anti-lipocortin-1 (Wu et al., 1995). We now present evidence that proteins recovered from the culture medium of glucocorticoid-treated J774 macrophages and mediating glucocorticoid inhibition of nitrite production and inducible NO synthase mRNA expression are likely to include also lipocortin-1, as: (i) like lipocortin-1, active proteins recovered from glucocorticoid conditioned medium are resistent to heating; (ii) immunoblotting analysis with anti-lipocortin-1 antibodies shows in both crude and partially purified active fractions, two molecular species of 35 and 37 kDa, respectively, corresponding to the size reported for lipocortin-1 in mouse cells; (iii) pre-treatment of crude glucocorticoid-induced proteins fractions with anti-lipocortin-1 neutralizing antibodies reduces their inhibitory action toward nitrite production by lipopolysaccharide-stimulated J774 cells. Previous studies have shown that the lipocortin/annexin superfamily consists of at least 12 distinct proteins and several members of the family present important phosphorylation sites especially within the N-terminal domains (Flower and Rothwell, 1994). Lipocortin-1 and its N-terminal derived peptides have been reported to exert potent inhibitory actions in various models of acute inflammation (Flower and Rothwell, 1994). A recent report, suggesting that rhlipocortin-1 fragment does not inhibit lipopolysaccharide-stimulated release of tumour necrosis factor-α and prostaglandin E_2 from human peripheral blood mononuclear cells, nevertheless shows that anti-lipocortin-1 antibody reverses the suppressive effect of dexamethasone in these cells. In addition, when human rhlipocortin-1 and purified human annexins 2, 4 and 6 were immunoblotted with an anti-lipocortin-1 polyclonal antibody, only rhlipocortin-1 was detected (Sudlow et al., 1996). Comparing glucocorticoid-induced proteins inducible NO synthase inhibitory activity with the immunoblotting experiments, our results demonstrate that: (i) the pooled fraction III contains lipocortin-1 like immunoreactive proteins that inhibit nitrite production by lipopolysaccharide-stimulated J774 cells; (ii) the pooled fraction IV inhibits nitrite production, but does not exhibit lipocortin-1 like immunoreactivity. Therefore, the neutralizing effect of anti-lipocortin-1 antibody on inducible NO synthase inhibitory activity of glucocorticoid-induced proteins could not be necessary associated with the appearance of lipocortin-1 in immunoblotting experiments. Lipocortin-1 like proteins have been shown to be the products of proteolytic degradation of full sequence lipocortin-1 (Flower, 1988). We suggest that lipocortin-1 derived peptides not recognized by anti-lipocortin-1 polyclonal antibody in immunoblot experiments (pooled fraction III and IV) are also responsible for the inhibitory effect of glucocorticoid-induced proteins on nitrite production by lipopolysaccharide-stimulated J774 cells. These data, indicating that lipocortin-1 or lipocortin-1-derived peptides are present in glucocorticoid-induced proteins, support the conclusion that the crude fractions prepared as described in the present report contains bioactive mediators of glucocorticoid action on macrophages, i.e. cells involved in inflammation and immune response. Furthermore, these observations support the idea that glucocorticoids directly regulate inducible NO synthase activity primarily by controlling inducible NO synthase mRNA expression, a phenomenon which may be likely mediated by lipocortin-1 and/or derived peptides.

Acknowledgements

We are thankful to P. Chambon for the 36B4 cDNA probe and G. Cirino for anti-lipocortin-1 antibody. Research was supported by the Italian Ministry of the University and Scientific Research (M.U.R.S.T. 60%; R.C., A.W.).

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