Inhibition of Thromboxane A Synthesis in U937 Cells by Glucocorticoids

LACK OF EVIDENCE FOR LIPOCORTIN 1 AS THE SECOND MESSENGER*

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The mechanism of inhibition of eicosanoid synthesis by glucocorticoids has been investigated using differentiated U937 cells as a model. These cells synthesize thromboxane A₂ (TXA₂) in response to a variety of agonists, and synthesis of TXA₂ initiated by certain stimuli was inhibited by pretreatment of the cells with glucocorticoids. The inhibitory response was specific for glucocorticoid steroids and required receptor occupancy based on both the rate of onset of the inhibitory activity and the correlation between potency and receptor affinity of various analogs. The inhibitory response was also specific for the agonist used to initiate TXA₂ synthesis. Both lipopolysaccharide- and zymosan-induced TXA₂ synthesis were inhibited by increasing concentrations of dexamethasone (>80%, IC₅₀ 10 nM), while synthesis initiated by addition of either exogenous arachidonic acid or the Ca²⁺ ionophore A23187 was unaffected over the same concentration range. The latter result indicates that the dexamethasone block is upstream of release of esterified arachidonic acid. Attempts to localize the block more accurately showed that although dexamethasone was not acting as a generalized inhibitor of transcription or translation, its ability to inhibit TXA₂ synthesis was mimicked by the activity of actinomycin D and cycloheximide.

The role of the purported phospholipase inhibitor protein lipocortin 1 in mediating the dexamethasone inhibition of TXA₂ synthesis was studied by examining the effect of dexamethasone on lipocortin 1 metabolism. Under conditions which gave maximal inhibition of lipopolysaccharide- or zymosan-stimulated TXA₂ synthesis, dexamethasone had no effect on the steady state level of lipocortin 1 mRNA or protein, indicating that lipocortin 1 induction by dexamethasone is not responsible for the observed inhibition. Furthermore, lipocortin 1 was not secreted from the cells under any conditions examined, and the intracellular form had a relatively long half-life (>21 h). The lack of induction of lipocortin 1 by dexamethasone and the fact that it is not released from the cells are both inconsistent with the properties previously described for lipocortin-like activities and indicate that lipocortin 1 is not a glucocorticoid second messenger in this experimental model. Although the data are consistent with a mechanism involving inhibition of a factor that activates TXA₂ synthesis, we cannot rule out a mechanism involving glucocorticoid induction of a phospholipase inhibitor protein distinct from lipocortin 1.

Glucocorticoids inhibit prostaglandin synthesis in a variety of cell types but do not inhibit prostaglandin H synthase activity (1, 2). Studies on the mechanism of glucocorticoids suggest that they act by inducing the synthesis of a protein that inhibits phospholipase A₂ and hence blocks mobilization of esterified arachidonic acid (3). Dexamethasone-inducible phospholipase A₂ inhibitor proteins have been partially characterized from perfused lung and conditioned medium from macrophages (macrocortin), neutrophils (lipomodulin), renal medulary interstitial cells (renocortin), and thymus (4–8). Macrocortin, lipomodulin, and renocortin have similar physical and immunological properties (9, 10) and have been renamed lipocortin (11). Characterization of the biological properties of these inhibitor proteins has given rise to the following operational definition for lipocortin-like activity; a protein that (a) is synthesized and secreted in response to glucocorticoids, (b) inhibits pancreatic phospholipase A₂ in vitro, (c) inhibits eicosanoid synthesis when added to cells, (d) exhibits antiinflammatory activity in standard models of acute inflammation, and (e) is regulated by phosphorylation (3, 9, 12). Using inhibition of phospholipase A₂ in vitro as an assay, lipocortin has been purified from cell-free supernatants conditioned by dexamethasone-stimulated macrophages and its structure defined using a tandem protein sequencing/molecular cloning approach (13, 14). Examination of the tissue distribution of lipocortin led to the isolation and cloning of a homologous phospholipase A₂ inhibitor, lipocortin 2 (15). Lipocortins 1 and 2, as defined by molecular cloning, were isolated solely on the basis of their ability to inhibit phospholipase A₂-catalyzed hydrolysis of labeled Escherichia coli membranes. Investigations into the mechanism of this inhibition have shown that the inhibitory activity is due to association of the inhibitor with the phospholipid substrate and not due to direct binding to phospholipase A₂ (16, 17). Furthermore, this inhibitory property is shared with a number of other related proteins which exhibit Ca²⁺-dependent binding to phospholipid vesicles (18). Recombinant lipocortin 1 has been reported to inhibit leukotriene C₄-induced TXA₂ synthesis by perfused lung (19), and the lipocortin 1 gene has been

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1 The abbreviations used are: TXA₂, thromboxane A₂; TXB₂, thromboxane B₂; LPS, lipopolysaccharide; FCS, fetal calf serum; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor; EIA, enzyme immunoassay; PGH, prostaglandin H₂; EGTA, [ethylendihydroxy(nitritol)]etraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kb, kilobase pair, IL1, interleukin 1.
reported to be dexamethasone-inducible in rat peritoneal macrophages (14).

Aside from these reports, little is known concerning the relationship between lipocortin 1 (as defined by molecular cloning) and the active principle described by the operational definition above. In this report, we elected to study the regulation of TXA2 synthesis in differentiated U937 cells by glucocorticoids. Lipopolysaccharide (LPS)- or zymosan-stimulated TXA2 synthesis is inhibited by glucocorticoids by a process that requires receptor occupancy. The dexamethasone-induced block has been mapped upstream of PGH synthase and appears to be upstream from arachidonic acid mobilization (phospholipase). Attempts to correlate the dexamethasone inhibitory response with induction of lipocortin 1 (mRNA or protein) or lipocortin 1 secretion, were unsuccessful. The evidence accumulated to date suggest that dexamethasone does not act by inducing lipocortin 1 but rather by suppressing the expression of a LPS/zymosan-inducible gene(s) that in turn activates the cells to synthesize TXA2.

Alternatively, a mechanism involving glucocorticoid induction of an inhibitor protein cannot be ruled out based on the present data.

**EXPERIMENTAL PROCEDURES**

**Materials**

U937 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 medium supplemented with Fungizone and 10% FCS (Irvine Scientific) in 5% CO2 at 37°C. Fhorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (E. coli serotype 055:B5), zymosan A, dexamethasone, hydrocortisone, furocinoole acetone, aldosterone, testosterone, 17,20 estradiol, cychloheximide, emetine, actinomycin D, acetylthiol cyclomide, and 5,6-dihydro (2-nitrobenzoic acid) were from Sigma. [1,2,4(n)-3 H]Dexamethasone (47 Ci/mmol), [5,6,7 H]Juridine (40 Ci/mmol), [L-4,5,6-H]uracil (57 Ci/mmol), [32p]Thioadenosine (125 Ci/mmol), [32p]Protein A (30 mCi/mg), deoxyadenosine 5'-[32p]Triphosphate (3000 Ci/mmol), deoxyxytidine 5'-[32p]Triphosphate (800 Ci/mmol), and adenosine 5'-[32p]Triphosphate (>5000 Ci/mmol) were obtained from Amersham Corp. Arachidonic acid was from NuChech Preps. Ca2+ ionophore A23187 was from Boehringer Mannheim. Oligo(dT) cellulose, oligo(dt)(25s), random oligonucleotide primers, deoxyxucleotide triphosphates, deoxynucleotide triphosphates, RNase H, T4 DNA polymerase, Klenow fragment of DNA polymerase I, and T4 polynucleotide kinase were from Pharmacia. Restriction endonucleases, EcoRI methylase, T4 DNA ligase, and EcoRI linkers were from New England Biolabs. Diphosphorylated EcoRI digested a 10, and GigaPak packaging extracts were from Stratagene. Nitrocellulose and Nytran filters were from Schleicher & Schuell. TXB2 EIA kit was from AIA Reagents Bio-Gel A-50m was from Bio-Rad.

**Methods**

**Differntiation of U937 Cells—**U937 cells were maintained in suspension culture in RPMI 1640 supplemented with 1% FCS for various times and either harvested or chased in medium containing excess unlabeled methionine. For harvest, the monolayers twice with serum-free RPMI 1640 followed by addition of the appropriate agonist, and TXAZ synthesis quantified as described above.

**Glucocorticoid Receptor Determination—**Glucocorticoid receptors were quantified using a whole cell binding assay. PMA-differentiated U937 cells (1.5 x 106 cells/well) were incubated with RPMI 1640 medium containing 1% FCS and various amounts of [3H]dexamethasone or [3H]dexamethasone plus 250-fold molar excess of unlabeled steroid for 2 h at 21°C. The medium was removed, the cell layer washed three times with phosphate-buffered saline (Ca2+/Mg2+-free), and the cells collected on Fitergels filters. Bound [3H]dexamethasone was quantified by liquid scintillation counting.

**Effect of Transcription and Translation Inhibitors on TXAZ Synthesis—**Dose-response curves for actinomycin D and cycloheximide/eminine were determined to establish the optimum dose for inhibition of transcription and translation, respectively. PMA-differentiated U937 cells were pretreated with various concentrations of drug in RPMI 1640, 1% FCS for 30 min, and the rate of transcription (for actinomycin D) or translation (cycloheximide/eminine) was measured. For transcription, the cells were labeled with [3H]Juridine (10 μCi/ml) in the same medium for 1 h while translation was measured by labeling with [3H]leucine (10 μCi/ml in leucine-free medium) for 1 h. In each case, the labelings were performed in the continued presence of drug and the rates estimated based on incorporation of 3H into total cellular trichloroacetic acid-insoluble material. The effect of these inhibitors on TXAZ synthesis was tested by pretreating the cells with the appropriate dose of drug in RPMI 1640, 1% FCS for 30 min followed by stimulation with the desired agonist in the continued presence of drug. TXB2 levels were then determined by EIA as above.

**Lipocortin 1 Metabolism—**Lipocortin 1 metabolism was studied by metabolic labeling of PMA-differentiated U937 cells with [32p]Chloroacetate or by immunoprecipitation with a lipocortin 1-specific polyclonal antiserum (21). Cells were labeled with [32p]Methionine (100-500 μCi/ml) in methionine-free RPMI 1640 medium containing 10% FCS for various times and either harvested or chased in medium containing excess unlabeled methionine. For harvest, the monolayers were removed, dialyzed at 4°C against 25 mM HEPES/CO2 (pH 7.8), and lyophilized. The washed cells were lysed in 2.0 ml of 25 mM Tris-HCl (pH 7.4), 0.1% Nonidet P-40, 5 mM MgCl2, 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin at 4°C for 20 min, centrifuged (1000 x g, 10 min), and the supernatant used for immune precipitation. An aliquot of the cell lysate or culture medium fraction (0.3 ml) was incubated with 10 μl of lipocortin 1-specific rabbit antihuman lipocortin 1 antiserum for 30 min on ice, and the immune complexes were then precipitated using activated Pansorbin (Calbiochem). The washed pellets were dissociated in SDS-PAGE loading buffer, run on 15% Lamelli gels and the bands detected using 9-protein A followed by autoradiography.

**Isolation of Human Lipocortin, 1 cDNA—**Two lipocortin 1-specific oligonucleotide probes, complementary to nucleotides 254-278 (CTTCCAGGAACACAGGAGAACCCCTG) or 910-934 (TGAAT-CATTCGAGCCCAATTCAGA) of the human lipocortin 1 cDNA sequence were synthesized using an Applied Biosystems Instruments 3805 synthesizer. The oligonucleotides were purified by preparative denaturing PAGE, scaled on C18 Sep Pak cartridges, and labeled using T4 polynucleotide kinase and [γ-32p]ATP. U937 cells were treated with dexamethasone (1.0 μM) and PMA (0.1 μM) for 4 h and total cellular RNA prepared as described. Poly(A) RNA was prepared by two rounds of chromatography on oligo(dt) cellulose and used to synthesize cDNA by oligo(dt) primed first strand synthesis using avian myeloblastosis virus reverse transcriptase followed by Ranase H/avian myeloblastosis virus reverse transcriptase-mediated second strand synthesis (23). The cDNA was then sequenced by hybridization with the lipocortin 1-specific oligonucleotide probes. A 1.3-kb EcoRI fragment, corresponding to nucleotides 16-1326 of the human lipocortin 1 cDNA sequence was subcloned into pUC19 and completely sequenced by the dideoxy chain termination method.
Quantification of Lipocortin 1 mRNA—Analytical RNA isolations (10^6 cells/sample) were performed by cell lysis in 4 M guanidine isothiocyanate, 8% β-mercaptoethanol exactly as described (26). Aliquots of total RNA (10 μg) were denatured by treatment with glyoxal, fractionated on a 1% agarose gel with buffer recirculation, electroblotted to Nytran, and immobilized on the filter by UV irradiation. Lipocortin 1 cDNA (1.3-kb EcoRI fragment) was labeled using Klenow fragment and random primers (27) and hybridized (10^7 dpm/ml) to the blot as described by Church and Gilbert (28). Labeled bands were detected by autoradiography and quantified by densitometry.

RESULTS

Characterization of Cellular Model—Differentiated U937 cells were chosen as a cellular model to study the effect of glucocorticoids on arachidonic acid mobilization. Since the validity of this model depends on the presence of glucocorticoid receptors in these cells, receptors were quantified by [3H]dexamethasone binding in a whole cell receptor binding assay. U937 cells or PMA-differentiated U937 cells (dU937) were incubated with various amounts of [3H]dexamethasone in the absence or presence of a 500-fold molar excess of unlabeled dexamethasone for 3 h at room temperature and the cell-associated steroid quantified by liquid scintillation counting. The binding curve shown in Fig. 1 indicates a specific binding component in dU937 cells that saturates at about 5 nM. Undifferentiated U937 cells had approximately 3-fold less specific binding capacity (data not shown). Scatchard analysis of the binding data obtained from dU937 cells reveals a single class of glucocorticoid receptors with a K_d of 1.9 nM and a receptor density of 16,600 receptors/cell. Because dU937 cells have a higher receptor density, they were used in all subsequent experiments. Preliminary metabolism experiments using dU937 cells pre-labeled with [3H]arachidonic acid followed by stimulation with a variety of agonists showed that thromboxane A (TXA_2) was the major eicosanoid synthesized, while smaller amounts of prostaglandin E were also detected (40% of the TXA_2 level). Undifferentiated U937 cells produced only 2% of the TXA_2 synthesized by dU937 cells, again requiring the use of dU937 cells for all subsequent experiments. The kinetics of agonist-stimulated TXA_2 synthesis by dU937 cells are shown in Fig. 2. Cells were treated with either lipopolysaccharide (10 μg/ml), zymosan (500 μg/ml), A23187 (1 μg/ml), or exogenous arachidonic acid (10 μM) for various times and TXA_2 synthesis was quantified by its hydrolysis product TXB_2 by enzyme immunoassay. TXA_2 synthesis induced by LPS or zymosan (top panel) occurred following a lag of 2 h and continued at a near linear rate for 7 h. The rates of TXA_2 synthesis were more rapid when either A23187 or arachidonic acid were used (bottom panel), exhibiting linear release for 5 min followed by a slow release lasting for 1 h. Based on these results, a 4-h stimulation (LPS and zymosan) or a 15-min stimulation (A23187 and arachidonic acid) was used in all subsequent experiments.

Effect of Glucocorticoids on TXA_2 Synthesis—Since LPS was the strongest agonist for initiating TXA_2 synthesis, the effect of representative steroids on the LPS response was determined, and the results are shown in Fig. 3. dU937 cells were pre-treated with either dexamethasone, aldosterone, testosterone, or 17β-estradiol at concentrations ranging from 10^{-10} to 10^{-4} M for 2 h, stimulated with LPS in the absence of steroid, and TXA_2 release quantified by EIA. Pretreatment with the glucocorticoid dexamethasone caused a dose-dependent inhibition of TXA_2 synthesis with an IC_50 of 10 nM and a maximal inhibition of 82% (top panel). Aldosterone, testosterone, or 17β-estradiol were without effect over the same dose range. To further test the specificity of the glucocorticoid effect, we examined a series of glucocorticoid analogs for their...
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FIG. 3. Effect of representative steroids on LPS-stimulated TXA₂ synthesis in dU937 cells. U937 cells were differentiated and cultured as described in Fig. 1. Triplicate wells were then pretreated with representative steroids (top panel) or various glucocorticoid analogs (bottom panel) over the concentration range 10⁻⁸ to 10⁻¹⁹ M for 2 h. The medium was then removed, replaced with steroid-free medium containing 10 μg/ml LPS, and the medium harvested after 4 h. The TXB₂ content of the medium was then determined by EIA, and the results are expressed as mean ± S.E. *Top panel, dexamethasone (O), aldosterone (Δ), testosterone (A), or 17β-estradiol (□). Bottom panel, dexamethasone (O), methylprednisolone (Δ), fluocinolone acetonide (A), or hydrocortisone (□).*

Inhibitory potency, and the results are shown in Fig. 3 (bottom panel). This experiment was performed as described above using fluocinolone acetonide, dexamethasone, methylprednisolone, and hydrocortisone in the pretreatments. Fluocinolone acetonide was the most potent analog with an IC₅₀ of 0.8 nM, while the other analogs were less active. The fact that the dose-response relationships parallel the known receptor affinities for these analogs indicates that the inhibitory effect is specific for glucocorticoids and requires receptor occupancy. Furthermore, the data shown in Fig. 3 show that the glucocorticoid effect is irreversible for at least 4 h after steroid removal. Whether this is due to the steroid remaining associated with the cells or the synthesis of a factor that is mediating the inhibitory response is unknown.

Mechanism of the Glucocorticoid Inhibition—The mechanism of the glucocorticoid inhibition of TXA₂ synthesis was further investigated by initiating TXA₂ synthesis using a series of agonists that activate different portions of the TXA₂ biosynthetic pathway. dU937 cells were pretreated with dexamethasone at various concentrations for 2.0 h and then stimulated with either LPS or zymosan (4 h), or A23187 or exogenous arachidonic acid (15 min) in the absence of steroid. As shown in Fig. 4, when stimulated with either LPS or zymosan, the cells produced nearly equivalent amounts of TXA₂ and the dexamethasone dose-response curves for inhibition of TXA₂ synthesis were parallel. When dexamethasone-treated cells were challenged with either the Ca²⁺ ionophore (A23187) or exogenous arachidonic acid for 15 min, only synthesized about 40% as much TXA₂ as LPS- or zymosan-treated cells. The amount of TXA₂ synthesized upon treatment with either A23187 or exogenous arachidonic acid was weakly inhibited by pretreatment with dexamethasone (<10%) over the same concentration range where >80% inhibition was noted for either LPS or zymosan. These results indicate that the dexamethasone block appears to be upstream from PGH synthase (reversed by exogenous arachidonic acid) and possibly upstream from release of esterified arachidonic acid (reversed by A23187).

The mechanism of the dexamethasone-induced inhibition was further investigated by determining the kinetics of the inhibitory response as a function of dexamethasone concentration using LPS as the stimulus. dU937 cells were treated with various concentrations of dexamethasone for 0–4 h and then stimulated with LPS for 4 h in the absence of steroid. TXA₂ synthesis was quantified, and the results are shown in Fig. 5. The rate of induction of the inhibitory effect was directly proportional to the initial dexamethasone concentration in the medium. Maximal inhibition of TXA₂ synthesis...
was observed after 15 min (1 μM), 60 min (0.1 μM), or 120 min (0.01 μM), while maximal inhibition at 0.001 μM was not achieved even after 4 h of treatment. At near physiological concentrations of glucocorticoid (10^-7-10^-8 M) the inhibitory effect requires 1-2 h to reach a maximum, a time frame consistent with the requirement for protein synthesis for expression of the inhibitory response. To determine whether transcription or translation was required for the dexamethasone response, the effect of various macromolecular synthesis inhibitors on both LPS-stimulated TXA2 synthesis and the dexamethasone inhibition of the LPS response was determined. The results summarized in Table I show that concentrations of cycloheximide or emetine that give 94 and 99% inhibition of translation, respectively, completely inhibited the LPS-stimulated TXA2 synthesis in the absence of dexamethasone. A concentration of actinomycin D that inhibits transcription 89% also completely inhibited LPS-stimulated TXA2 synthesis, independent of dexamethasone addition. Because the LPS response was blocked by transcription and translation inhibitors in the absence of dexamethasone, it was not possible to demonstrate a reversal of the glucocorticoid response. Although dexamethasone and the macromolecular synthesis inhibitors both prevented LPS-stimulated TXA2 synthesis, dexamethasone did not inhibit bulk translation and only weakly inhibited transcription at a dose that maximally inhibited TXA2 synthesis.

Because the LPS-stimulated cells had been treated with cycloheximide for a total of 4.5 h, it was necessary to establish that the inhibition of LPS-stimulated TXA2 synthesis was due to inhibition of translation and not a loss of biosynthetic enzymes. The half-life of TXA2 synthesizing capacity was estimated by comparing TXA2 synthesis stimulated by LPS versus A23187 as a function of time of cycloheximide treatment. U937 cells were pretreated with cycloheximide for varying amounts of time (ranging from 30 to 240 min), and then TXA2 synthesis was initiated with either LPS or A23187 (added 15 min prior to harvest of the LPS-treated cultures) in the continued presence of cycloheximide. TXB2 levels were then determined, and the results are summarized in Table II. The half-life of TXA2 synthesizing capability (the sum of TXA2 synthase, PGH synthase, and phospholipase) for A23187 was approximately 270 min while, aside from a slight stimulation at early times, the LPS-induced response was inhibited at all times tested. From these results we conclude that the observed inhibition of LPS-stimulated TXA2 synthesis by protein synthesis inhibitors is not due to a loss of TXA2 synthase, PGH synthase, or even phospholipase but is due to the requirement of protein synthesis for expression of the TXA2 synthesis aspect of the LPS response.

Role of Lipocortin 1 in Mediating the Dexamethasone Response—Previous studies have suggested that dexamethasone inhibits arachidonic acid mobilization by inducing the synthesis of a phospholipase A2 inhibitor protein, and lipocortin 1 has been proposed as a candidate for the glucocorticoid second messenger. The possible role of lipocortin 1 in mediating the dexamethasone response in U937 cells was investigated by studying the effect of dexamethasone on lipocortin 1 metabolism. U937 cells were metabolically labeled with [35S]methionine for either 2 or 4 h in the absence or presence of dexamethasone (10^-8 M). The incorporation of [35S]methionine into protein was not affected by the addition of dexamethasone to the labeling medium (Table I). The lipocortin 1 content of both the culture medium and cell layer was then determined by immune precipitation using a lipocortin 1-specific rabbit polyclonal antiserum under conditions of antibody excess. The immune precipitated lipocortin 1 was analyzed by SDS-PAGE, detected by fluorography, and the results are shown in Fig. 6 (top panel). A single 37-kDa band was detected in samples derived from the cell layer (lanes 1-4) but not the culture medium (lanes 5-8). Furthermore, the

### Table I

**Effect of various inhibitors on macromolecular synthesis and LPS-stimulated TXA2 synthesis**

| Inhibitor | Transcription (a) | Translation (b) | LPS-stimulated TXA2 synthesis |
|-----------|-------------------|-----------------|-----------------------------|
| 10 μg/ml actinomycin D | 89 | ND | 100 |
| 10 μg/ml cycloheximide | ND | 94 | 97 |
| 10 μM emetine | ND | 99 | 100 |
| 0.1 μM dexamethasone | 18 | 0 (c) | 80 |
| 10 μg/ml actinomycin D + 0.1 μM dexamethasone | ND | ND | 100 |
| 10 μg/ml cycloheximide + 0.1 μM dexamethasone | ND | ND | 94 |

### Table II

**Kinetics of cycloheximide inhibition of LPS- and A23187-stimulated TXA2 synthesis**

| Cumulative treatment time | TXB2 concentration | A23187 |
|--------------------------|-------------------|--------|
|                          | LPS              | A23187 |
|                          | pg/10^6 cells     | pg/10^6 cells |
| min                      | -Cycloheximide + Cycloheximide | -Cycloheximide + Cycloheximide |
| 60                       | 143 ± 82          | 525 ± 72          | 2515 ± 79          | 1985 ± 88          |
| 90                       | 314 ± 59          | 558 ± 182         | 2269 ± 32          | 1543 ± 153         |
| 150                      | 950 ± 179         | 491 ± 27          | 2270 ± 88          | 1435 ± 64          |
| 270                      | 2989 ± 55         | 361 ± 83          | 2430 ± 60          | 1278 ± 86          |
induction by dexamethasone, the lack of secretion into the culture medium, and the long half-life of the intracellular form are not consistent with the properties of lipocortin-like activities reported previously. To verify the lack of dexamethasone induction of lipocortin 1, we attempted to measure induction under conditions that we had previously determined were maximal for inhibition of LPS-stimulated TXA2 synthesis. dU937 cells were glucocorticoid starved by treatment overnight with RPMI 1640, 10% steroid-free FCS and then treated with RPMI 1640, 1% steroid-free fetal calf serum with or without 0.1 μM dexamethasone for various times. At the appropriate time point, replicate dishes were harvested and analyzed for lipocortin 1 mRNA (Northern blot) or for lipocortin 1 protein (Western blot). The results are shown in Fig. 7. The top panel shows the steady state levels of lipocortin 1 mRNA detected by hybridization with a 32P-labeled 1.3-kb EcoRI fragment of human lipocortin cDNA as a probe. The resting level of lipocortin 1 mRNA (1.4 kb) is readily detected (lane 1) and it increased slightly with time in culture (lanes 2, 4, 6, and 8). Addition of dexamethasone to the culture medium gave results comparable to the untreated cultures (lanes 3, 5, 7, and 9). Similar results were obtained when the protein was measured directly by Western blotting of cell lysates containing equal amounts of protein (middle panel). A slight but significant decrease in the level of lipocortin 1 during the treatment was noted, and this decrease was unaffected by dexamethasone (compare even and odd numbered lanes). The densitometric quantification of both the lipocortin 1 mRNA levels (normalized to tubulin mRNA) and lipocortin 1 (by comparison to a blot containing dilutions of pure lipocortin 1) is shown in the bottom panel. Since the levels of lipocortin 1 are not changing in response to dexamethasone under conditions where maximal inhibition of LPS-stimulated TXA2 synthesis is observed, we conclude that lipocortin 1 induction is not responsible for the observed decreased arachidonic acid mobilization. An alternate possibility is that the absolute amount of lipocortin 1 is not changing but the protein is undergoing a modification (phosphorylation) during LPS stimulation that is blocked by dexamethasone. In this regard, we have not been able to detect phosphorylated lipocortin 1 in immune precipitates prepared from cells prelabeled with 32P and stimulated with either LPS or PMA (data not shown).

**DISCUSSION**

The exact mechanism by which glucocorticoids inhibit arachidonic acid mobilization and eicosanoid biosynthesis is unknown. We have developed a cellular model that is responsive to glucocorticoids in an attempt to further define the mechanism of inhibition of eicosanoid biosynthesis. The model is based on a human histiocyte lymphoma cell line (U937 cells) that can be terminally differentiated into cells possessing a variety of phenotypic markers characteristic of monocytes/macrophages by treatment with PMA (29). PMA-differentiated U937 cells were chosen over their undifferentiated counterparts for the following reasons: 1) they possess glucocorticoid receptors and have approximately 3-fold more receptors than undifferentiated cells, 2) they synthesize TXA2 as the major product in response to a variety of agonists while the undifferentiated cells produce very low amounts of eicosanoid products, and 3) they express at least two families of phospholipase A2 inhibitor proteins, namely lipocortins and antiinflammatory protein (13, 14, 30). The glucocorticoid receptor density in the PMA-differentiated U937 cells is similar to that reported for human peripheral blood monocytes (31). Increases in glucocorticoid receptor number upon...
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terminal differentiation of HL60 cells have been reported (32), and the increase is comparable to that observed in U937 cells. Arachidonic acid metabolism in these cells is also consistent with previous reports (33, 34) with regard to both the detection of TXA₂ as the major product and the large increase in biosynthetic capacity after differentiation with PMA. The increased eicosanoid biosynthetic capacity upon differentiation of U937 cells by dimethyl sulfoxide has been reported and appears to be due to increased levels of phospholipase (35). Similar studies in HL60 cells indicate that induction of PGH synthase is responsible for increased prostaglandin synthesis capacity upon terminal differentiation (36).

Inhibition of prostaglandin synthesis by glucocorticoids in macrophages (5, 37-40), neutrophils (6), renomedullary interstitial cells (7, 41), and fibroblasts (2, 42) has been well documented. A mechanism involving glucocorticoid induction of a protein that inhibits phospholipase A₂ and hence arachidonic acid mobilization and prostaglandin synthesis has been proposed (3-7). Evidence supporting this mechanism includes the release of the inhibitor from the cells by dexamethasone treatment and the ability of macromolecular synthesis inhibitors to block glucocorticoid-induced inhibitor synthesis. Several phospholipase A₂ inhibitor proteins have been isolated based on their ability to inhibit porcine pancreas phospholipase A₂ in vitro, and their complete structures have been defined by molecular cloning (14, 15, 30). Although these phospholipase A₂ inhibitor proteins have been regarded as glucocorticoid second messengers, the exact relationship between these inhibitors and inhibition of eicosanoid synthesis has not been studied systematically. We have verified the inhibitory effect of glucocorticoids in our cellular model and have further defined the mechanism by which they block TXA₂ synthesis by utilizing a variety of agonists to activate the cells to produce TXA₂. Inhibition of TXA₂ synthesis was specific for glucocorticoid steroids and also for the nature of the stimulus. Agonists that cause general cell activation (LPS or zymosan) stimulated maximal synthesis of TXA₂ by a process that was strongly inhibited by glucocorticoid analogs of various potencies (43). Incomplete inhibition of agonist-stimulated TXA₂ synthesis by dexamethasone was due to background synthesis of TXA₂ when the culture medium was changed. The quantity synthesized in control cultures, presumably due to synthesis initiated by exogenous arachidonic acid and/or serum factors, consistently matched the amount of TXA₂ that was not blocked by maximal concentrations of dexamethasone (data not shown). Agents which activate only a portion of the eicosanoid biosynthetic pathway (L23187 or arachidonic acid) gave lower yields of TXA₂, but the synthesis was not affected by dexamethasone. One interpretation of these results is that the block occurs upstream from both PGH synthase and release of esterified arachidonic acid. Alternatively, the LPS/zymosan-stimulated activation of TXA₂ synthesis may be coupled to a different set of biosynthetic pathways.

FIG. 7. Lack of induction of lipocortin 1 by dexamethasone. Lipocortin 1 induction by dexamethasone was monitored by Northern (top panel) or Western blotting (middle panel) at various times after steroid treatment. U937 cells (1.0 × 10⁸ cells/T-75 flask) were differentiated as described in Fig. 1 and then cultured in medium containing steroid-free FCS (10%) for 18 h. The medium was removed and replaced with fresh medium containing steroid-free FCS (1%) (lanes 1, 2, 4, 6, and 8) or the same containing 0.1 μM dexamethasone (lanes 3, 5, 7, and 9). After 0 h (lane 1), 0.5 h (lanes 2 and 3), 1.0 h (lanes 4 and 5), 2.0 h (lanes 6 and 7), or 4.0 h (lanes 8 and 9), sister dishes were harvested for RNA (top panel) or for total protein (middle panel). In the top panel, 10-μg aliquots of total cellular RNA were run on a denaturing gel (glyoxal), electrotransferred to a Nytran membrane, and probed with a ³²P-labeled 1.3-kb EcoRI fragment of human lipocortin 1 as described under "Experimental Procedures." Lipocortin 1 transcripts were detected by autoradiography and the signal normalized to an internal standard mRNA (tubulin). In the middle panel, postnuclear supernatants containing 20 μg of protein/ lane were run on 15% SDS-PAGE, electoblotted to nitrocellulose, and probed with lipocortin 1-specific polyclonal antiserum (1/400 dilution). Antigen-antibody complexes were visualized using ¹²⁵I-protein A followed by autoradiography. Quantification was performed by running dilutions of pure lipocortin 1 from placenta in adjacent lanes followed by densitometry of the autoradiography signals. The normalized quantification of both lipocortin 1 mRNA (C, ■) and protein (O, ◆) in the absence (C, O) or presence (■, ◆) of dexamethasone is summarized in the bottom panel.
thetic enzymes (phospholipase/PGH synthase) than the enzymes that make TXA₂ when given exogenous arachidonic acid or A23187. In the second case, the LPS/zymosan-coupled system would be dexamethasone-sensitive, while the other set of enzymes would be insensitive to the inhibitory effects of dexamethasone. At present, it is not possible to distinguish between these two mechanisms.

Assuming that A23187 activates phospholipase indirectly by Ca²⁺ influx, the lack of inhibition by dexamethasone was unexpected in the context of a phospholipase-inhibitor protein complex, unless complex formation is reversed by increased levels of intracellular Ca²⁺. Kinetic analysis of the inhibitory response revealed that at physiological concentrations of arachidonic acid or A23187, the agents also blocked TXA₂ synthesis initiated by either LPS or zymosan. The lipocortin hypothesis predicts that induction of a phospholipase inhibitor protein gene (lipocortin 1) and concomitant inhibition of arachidonic acid mobilization is responsible for the observed inhibition of eicosanoid synthesis. We did not observe any correlation between induction of the steady state levels of lipocortin 1 mRNA or lipocortin 1 protein and the glucocorticoid inhibition. Expression of the lipocortin 1 gene has been reported to be induced severalfold in rat peritoneal macrophages from dexamethasone-treated rats, but the increase was not correlated with a block in eicosanoid synthesis or increases in lipocortin 1 protein levels (14). Under conditions where maximal inhibition of TXA₂ synthesis occurs, there are no changes in the lipocortin 1 content of the cells. We could not detect lipocortin 1 secretion (constitutive or dexamethasone-induced) from dU937 cells or release from human neutrophils upon degranulation (data not shown). The lipocortin 1 cDNA sequence (14) does not predict a signal sequence as would be expected for a secretory protein. Addition of a purified lipocortin 1 preparation that was active as a phospholipase A₂ inhibitor in the in vitro assay to dU937 cells did not block LPS-induced TXA₂ synthesis in the dose range 0.25–25 μg/ml. These results are not consistent with points a and c of the operational definition presented in the Introduction. Our results are consistent with a recent report demonstrating the lack of correlation of lipocortin 1 as an inhibitor of zymosan-stimulated arachidonic acid release from macrophages under conditions where glucocorticoids are active (44). Alternatively, recombinant lipocortin 1 has been reported to inhibit leukotriene C₄-induced TXA₂ synthesis from perfused lung (19) and prostacyclin synthesis by arterial rings (49). The reasons for the discrepancy between inhibitory activity of recombinant lipocortin 1 and the apparent lack of activity of natural lipocortin 1 purified from placenta are unknown since they have not been compared in parallel (21, 44). Finally, the significance of pancreatic phospholipase A₂ inhibition in vitro (point b) and the antiinflammatory activity of lipocortin 1 has been questioned (16, 18, 44). Since the original lipocortin-like activities were detected in cell-free supernatants from steroid-treated cells, the relationship between lipocortin 1 (as defined by molecular cloning) and previously reported phospholipase inhibitor proteins is unclear. Involvement of other members of the lipocortin family in mediating the glucocorticoid inhibition in this system cannot be ruled out. Inclusion of these proteins into the lipocortin family is based on amino acid sequence homology with lipocortin 1 (46) and the ability to inhibit phospholipase A₂-catalyzed hydrolysis of E. coli membranes by binding to the substrate micelles (16). Since lipocortin 1 was the first member of this family and does not appear to be a glucocorticoid second messenger, it is unlikely that other members of the lipocortin family are responsible for the inhibitory action of glucocorticoids on eicosanoid synthesis. If the mechanism of glucocorticoid suppression of eicosanoid synthesis does involve a steroidal inducible gene, the possibility remains that the protein factor has yet to be defined.

Although the dexamethasone block has been mapped upstream from phospholipase, the lack of understanding of the signal transduction pathways involved in macrophage activation by either LPS or zymosan has complicated our efforts to map the inhibition more accurately. LPS stimulation of the murine macrophage cell line RAW 264.7 leads to the synthesis of prostaglandins and the accumulation of lysophosphatidylcholine (47) suggesting that cell activation by LPS may be coupled to a phosphoinositide-specific phospholipase A₂. The LPS-induced accumulation of lysophosphatidylcholine is partially blocked by cycloheximide, consistent with our observation that protein synthesis is required for LPS-induced TXA₂ production (47). Activation of macrophage eicosanoid synthesis by zymosan has been studied in more detail (48). Initiation of synthesis requires binding of the zymosan particles to the cell surface (presumably via mannose receptors) but can be dissociated from phagocytosis (49). As with LPS, stimulation of eicosanoid synthesis by zymosan is blocked by inhibitors of transcription or translation (50). In fact, eicosanoid synthesis initiated by both LPS and zymosan is inhibited by both dexamethasone and protein synthesis inhibitors, while neither of these agents affect A23187- or arachidonic acid-induced TXA₂ synthesis. The parallel activities of dexamethasone and transcription/translation inhibitors suggests that glucocorticoids may be acting by suppression of transcription and/or translation. Dexamethasone does not inhibit bulk transcription or translation under conditions where it is maximally active against eicosanoid synthesis, but it may act by inhibiting the expression of a single or discrete set of genes that are required for autocrine stimulation of the cells to produce TXA₂. LPS-inducible macrophage products include lymphokines such as IL₁ and TNF and both of these factors are known to stimulate prostaglandin synthesis in a number of cell types. In mouse peritoneal macrophages, IL₁ production leads to autocrine stimulation of prostaglandin E synthesis which in turn down-regulates IL₁ and TNF synthesis (51). Furthermore, it has been recently reported that dexamethasone inhibits LPS induction of IL₁β in both peripheral blood monocytes or U937 cells (52, 53) and of TNF induction in mouse macrophages (54, 55). We have demonstrated dexamethasone inhibition of LPS-induced increases in both IL₁β and TNFα transcripts in our system, and the concentration dependence is similar to the inhibition of TXA₂ synthesis. Down-regulation of IL₁/TNF production by glucocorticoids and a resultant loss of prostaglandin synthesis could account for the observed inhibition of TXA₂ synthesis and would simultaneously prevent the synthesis of two families of inflammatory mediators, IL₁/TNF and eicosanoids. Although our results are consistent with dexamethasone inhibition of the synthesis of an activator of TXA₂ synthesis, based on the available data we cannot rule out the induction of an inhibitor of TXA₂ synthesis. Further investigations of the dexamethasone inhibition of TXA₂ synthesis and its relationship to both
of these mechanisms are in progress.

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