Mechanisms of Suppression of Inducible Nitric-oxide Synthase (iNOS) Expression in Interferon (IFN)-γ-stimulated RAW 264.7 Cells by Dexamethasone

EVIDENCE FOR GLUCOCORTICOID-INDUCED DEGRADATION OF iNOS PROTEIN BY CALPAIN AS A KEY STEP IN POST-TRANSCRIPTIONAL REGULATION*

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The murine macrophage cell line RAW 264.7 expresses inducible nitric-oxide synthase (iNOS) activity upon stimulation with interferon (IFN)-γ and/or bacterial lipopolysaccharide. We have studied the mechanisms by which the synthetic glucocorticoid dexamethasone suppresses IFN-γ-stimulated iNOS expression in RAW 264.7 cells. Treatment of cells with dexamethasone reduces the formation of nitrite, one of the stable end products of NO production measured in culture supernatants with an IC50 of 9 nM. The reduction of iNOS activity is caused by decreased iNOS protein levels as assessed by immunoblotting using a specific anti-iNOS antibody. Dexamethasone treatment also reduces the formation of iNOS mRNA steady state levels to about 50% in IFN-γ-stimulated cells. This is due to decreased iNOS gene transcription and iNOS mRNA stability. More importantly, dexamethasone reduces the amount of iNOS protein by two additional mechanisms: reduction of the translation of iNOS mRNA and increased degradation of the iNOS protein. Using a specific protease inhibitor for the cysteine protease calpain I, N-acetyl-Leu-Leu-norleucinal (calpain inhibitor I), the enhanced proteolysis of the iNOS protein can efficiently be blocked, whereas other protease inhibitors such as tosyl-l-lysine chloromethyl ketone have no effect. Dexamethasone does not significantly alter calpain gene expression. Northern blot analyses reveal that calpain mRNA steady state levels are virtually not affected upon incubation of the cells with IFN-γ and dexamethasone. Immunoprecipitation using a polyclonal anti-calpain antibody reveals that calpain protein levels are also not affected by the glucocorticoid. This is the first evidence that the iNOS protein is a molecular target for the cysteine protease calpain.

Nitric oxide (NO)1 is a free radical gas mediating intercellular communications in many mammalian organs. In recent years the importance of NO for the regulation of vascular homeostasis, the involvement in neurotransmission, and the defense against infectious agents has become established (1, 2). Three isoforms of NO synthase (NOS) have been identified and cloned (3, 4). The brain (type I) and endothelial (type III) enzymes are constitutively expressed and their enzymatic activity is regulated by changes in concentration of intracellular free Ca2++. The third member of the family of NOS synthases is the inducible (type II) NOS. This enzyme is regulated at the transcriptional level and the activity is present at intracellular Ca2+ levels. Inducible NOS (iNOS) is expressed in many different cell types and produces high levels of nitric oxide. Excessive formation of NO mediates the bactericidal and tumoricidal actions of macrophages. However, under pathological conditions, high output of NO is associated with tissue damage observed in arthritis, type I diabetes, septic shock, and nephritis (for review, see Refs. 1 and 2).

Glucocorticoids are a class of steroid hormones with pleiotropic effects. At pharmacological concentrations, glucocorticoids are used to prevent and suppress inflammation and the activation of the immune system. Despite their widespread medical use, the precise mechanism(s) for the effectiveness as immunosuppressive and anti-inflammatory drugs is not yet entirely understood. Steroids exert their anti-inflammatory actions mainly by modulation of the transcription of a variety of genes involved in the control of inflammatory processes. These include cytokines and their cellular receptors, adhesion molecules, and enzymes producing inflammatory mediators (for review, see Ref. 5). Inhibition of transcriptional activity of target genes occurs either by binding of the activated glucocorticoid receptor to a negative GRE within the 5′-flanking region or by cross-coupling, i.e. direct protein-protein interaction of the glucocorticoid receptor with transcription factors induced under stimulatory conditions (6, 7). Moreover, a few reports suggest that post-transcriptional mechanisms including translation or protein secretion may also be involved in the anti-inflammatory actions of steroids (8, 9).

Several recent publications document the suppression of iNOS expression by glucocorticoids in various cell types such as mesangial cells (10), murine macrophages (11), human endothelial cells (12), insulin-producing RINm5F cells (13), rat hepatocytes (14), murine fibroblasts (15), and human epithelial cells (16). We have shown that dexamethasone blocks iNOS expression by glucocorticoids.

1 The abbreviations used are: NO, nitric oxide; CaM, calmodulin; CPI, calpain inhibitor I (N-acetyl-Leu-Leu-norleucinal); DEX, dexamethasone; iNOS, inducible isoform of nitric-oxide synthase; TLCK, Nω-p-tosyl-l-lysine chloromethyl ketone; TPCK, Nα-tosyl-2-phenylalana-nine chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium.

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expression in rat renal mesangial cells at transcriptional and post-transcriptional levels (17, 18).

In this report we examined the mechanisms of inhibition of iNOS expression in the IFN-γ-stimulated murine macrophage cell line RAW 264.7 by dexamethasone. The present study establishes that the glucocorticoid suppresses iNOS formation at different levels of iNOS gene expression. We observed a reduction of the transcription rate of the iNOS gene and a decrease in stability of iNOS mRNA causing reduced, but not completely abolished iNOS mRNA steady state levels. More importantly, post-transcriptional mechanisms, notably the translation of iNOS mRNA and the degradation of iNOS protein are involved in the suppression of iNOS expression even when iNOS is already induced. Moreover, we provide evidence that the enzyme mediating increased proteolysis of the iNOS protein by dexamethasone is the cysteine protease calpain I.

EXPERIMENTAL PROCEDURES

Reagents—The macrophage cell line RAW 264.7 was kindly provided by Dr. F. Erard (Ciba, Basel, Switzerland). Recombinant mouse IFN-γ was a gift from Dr. A. Adolf (Boehringer Ingelheim, Ingelheim, Austrich, Germany). Aspergillus oryzae Cellulase, N-α-p-tosyl-l-lysine chloromethyl ketone (TLCK), and 1-α-tosylamido-2-phenylalnine chloromethyl ketone (TPCK) were purchased from Sigma. N-Acetyl-Leu-Leu-norleucine (calpain inhibitor I) and actinomycin D were purchased from Boehringer Mannheim, Germany. Calpain I and II were purchased from Calbiochem-Novabiochem Corp. (Lucerne, Switzerland). Antibodies specific for iNOS were generated using an antigen peptide based on an N-terminal sequence of mouse iNOS as described (17). Polyclonal antibodies for rat calpain II were generated as described (19). The β-actin cDNA probe was a gift from Dr. U. Aebi (Maurice E. Müller Institute, Basel, Switzerland).

Cell Culture—RAW 264.7 cells were cultured in Macrophase SFM medium (Life Technologies, Basel, Switzerland) supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml). For stimulation, RAW 264.7 cells were washed twice with phosphate-buffered saline and incubated in DMEM without phenol red (Life Technologies, Basel, Switzerland) supplemented with 0.1 mg/ml fatty acid-free bovine serum albumin (Sigma), with or without agents for the indicated time periods.

Nitrile Analysis—NO activity was measured as nitrite production in RAW 264.7 cell culture supernatants. 100 µl of cell culture supernatant were mixed with 200 µl of Griess reagent (Merck, Darmstadt, Germany). The experiments were repeated four times. The plates were measured in an enzyme-linked immunosorbsent assay plate reader (KONTRON Analytical SLT 210) at 550 nm against a calibration curve with sodium nitrite standards. Nitrate was stoichiometrically reduced to nitrite by incubation of sample aliquots for 15 min at 37 °C, in the presence of 0.1 unit/ml nitrate reductase (EC 1.6.6.2; Aspergillus species, Calbiochem-Novabiochem, Munich, Germany). 50 µM NaCl was added and the volume of 160 µl. When nitrate reduction was complete, the remaining NADPH (which would interfere with nitrite determination) was oxidized with 0.1 units/ml lactate dehydrogenase (Boehringer Mannheim) and 10 mM sodium pyruvate, in a final volume of 170 µl for 5 min at 37 °C. The amount of nitrate produced in stimulated cells was approximately 30–35% of that of nitrite.

Immunoblotting—RAW 264.7 cells were cultured with IFN-γ (50 units/ml) for 12 h. Subsequently, either vehicle or dexamethasone (1 µM) was added and cells were incubated for a further 2 h. Thereafter, actinomycin D (10 µg/ml) was added, total RNA was prepared at the indicated time points and used for Northern hybridization as described above.

Northern Blot Analysis—For the nuclear run-on transcription assay, a nuclei suspension was prepared and mixed with 0.2 ml of 2 × reaction buffer (100 mM Hapes, pH 8.0, 10 mM MgCl₂, 300 mM KCl, 200 units of RNasin (Boehringer Mannheim) per ml per 1 ml each ATP, GTP, and CTP per 150 µg (1 µCi = 37 kBq) of [3²P]UTP (300 Ci/mmol; Amersham, Dübendorf, Switzerland) and incubated for 30 min at 30 °C. Transcription was stopped by adding 20 µg of Dnase I, followed by 80 µg of protease K. The [3²P]-labeled RNA was purified by extraction with phenol/chloroform and two subsequent precipitations with ammonium acetate. Equal amounts of [3²P]-labeled RNA were hybridized in 50% formamide, 5 × SSC, 5 × Denhardt’s solution, 1% SDS (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) at 42 °C for 72 h. Filters contained 10 µg each of linearized plasmids immobilized on GeneScreen membranes (DuPont de Nemours International, Regensdorf, Switzerland) after blotting in 12 × SSSPE with a dot-blot apparatus. After hybridization filters were rinsed for 30 min in 2 × SSC at 60 °C, for 5 min in 2 × SSC containing 10 µg of RNase A/ml at 37 °C, and finally for 1 h in 2 × SSC at 37 °C. Filters were air dried and exposed to Kodak X-Omat XAR film for 2 to 4 days using intensifying screens. Densitometrical analyses were performed on a Molecular Dynamics densitometer. A quantitative differential superhybridization with a radioactive probe representing the whole human iNOS gene, and a filter was used for normalization.

Metabolic Labeling and Immunoprecipitation—RAW 264.7 cells were cultured in 150-mm (diameter) culture dishes and stimulated with IFN-γ (50 units/ml) for 3.5 h. Then vehicle, dexamethasone (100 nM), or a combination of dexamethasone (100 nM) and RU-486 (1 µM) was added for 0, 2, and 4 h, as indicated. Thereafter the medium was aspirated and replaced with DMEM without L-methionine. 5 µM Nω-Nitro-L-arginine methyl ester (L-NAME) was added and the cells were incubated at 37 °C for 45 min for depletion of intracellular stores of methionine. 1-[35S]Methionine (300 µCi/dish) in pulse medium was added for 30 min. The medium was removed, the dishes were washed three times with phosphate-buffered saline, and the cells were scraped into 1 ml of buffer A (50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 µM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride). Cells were homogenized in a Dounce homogenizer and centrifuged at 200,000 × g for 30 min at 4 °C. The protein concentration of the lysate was determined by the Bradford protein assay (Bio-Rad). Equal amounts of protein lysate were incubated overnight with 100 µl of a 1:1(v/v) slurry of a specific polyclonal anti-iNOS antibody coupled to protein A-Sepharose CL-4B. The beads were washed twice with buffer B (50 mM Tris, pH 7.5, 150 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.1% SDS) with buffer B plus 500 mM NaCl, and once with buffer B. Proteins were solubilized from the beads by heating for 5 min at 95 °C in 0.1 ml of Laemmli buffer and subjected to SDS-PAGE (7.5% (w/v) acrylamide gel). Immunoblotting was performed as described (17) using an anti-iNOS antibody at a dilution of 1:1000 (v/v).
used in the presence of stimuli for 4.5 h. Lysates were prepared as described above. Immunoprecipitation was performed using a specific polyclonal anti-calpain II antibody coupled to protein A-Sepharose CL-4B and proteins were separated on SDS-PAGE (9% (w/v) acrylamide gel).

Pulse-Chase Analyses—RAW 264.7 cells were stimulated with IFN-γ (50 units/ml) for 6 h. Then dexamethasone (1 μM) was added for 1 h. The medium was aspirated and replaced with 10 ml of pulse medium (DMEM without L-methionine) containing IFN-γ (50 units/ml) and dexamethasone (1 μM). The cells were starved for L-methionine for 45 min to deplete intracellular stores of methionine. Subsequently the cells were pulsed for 2 h with [35S]methionine (300 μCi/dish). Thereafter, pulse medium was aspirated and replaced by 10 ml of chase medium (pulse medium containing 15 mg of unlabeled L-methionine per liter). The cells were further incubated for 0, 2, 4, 8, and 12 h, as indicated. Subsequently, cells were harvested and processed for immunoprecipitation and SDS-PAGE as described above.

In the experiments using protease inhibitors, cells were incubated as above. N-Acetyl-Leu-Leu-norleucinal (calpain inhibitor I, 100 μM) or TLCK (100 μM) were added during the pulse period of 2 h with [35S]methionine (300 μCi/dish). Thereafter, pulse medium was aspirated and replaced by 10 ml of chase medium (pulse medium containing 15 mg of unlabeled L-methionine per liter). The cells were further incubated for 0 and 2 h as indicated. Subsequently, cells were harvested and processed for immunoprecipitation and SDS-PAGE as described above.

Statistics—Statistical analysis was done by Student’s t test, and p < 0.05 was used as the criterion for statistical significance.

RESULTS

Dexamethasone Dose Dependently Inhibits NO Release from RAW 264.7 Cells—Incubation of RAW 264.7 cells for 24 h with IFN-γ (50 units/ml) increases the production of nitrite, one of the stable end products of NO formation, about 66-fold, from 2 ± 0.2 nmol/mg of protein in unstimulated cells to 138 ± 15 nmol/mg of protein (means ± S.D., n = 3). Simultaneous incubation of the cells with IFN-γ (50 units/ml) and dexamethasone for 24 h dose dependently inhibits the formation of nitrite in cell culture supernatants as shown in Fig. 1. Half-maximal inhibition is observed at 9 ± 0.5 nM dexamethasone (means ± S.D., n = 4).

Dexamethasone Inhibits the Formation of iNOS Protein Levels—To assess whether the reduced production of nitrite in dexamethasone-treated RAW 264.7 cells is due to a decrease in iNOS protein levels, immunoblotting experiments using a specific anti-iNOS antibody were performed. Cells were stimulated with IFN-γ (50 units/ml) in the presence of vehicle or dexamethasone at concentrations of 100 nM and 1 μM or a combination of dexamethasone (100 nM) plus a 10-fold molar excess of the glucocorticoid receptor antagonist RU-486 (1 μM) for 12 h. Subsequently cells were lysed and extracts were used for immunoblotting. As shown in Fig. 2, in unstimulated RAW 264.7 cells no iNOS protein is detected, whereas IFN-γ strongly induces the formation of detectable iNOS protein of approximately 130 kDa. This induction is blocked under the action of dexamethasone at concentrations of 1 μM and 100 nM, respectively. The effect is reversed upon the simultaneous incubation of the cells with dexamethasone (100 nM) and the glucocorticoid receptor antagonist RU-486 (1 μM; Fig. 2).

Dexamethasone Treatment Reduces the Formation of iNOS mRNA Steady State Levels—Northern blot analyses were performed to assess whether the glucocorticoid affects the formation of iNOS mRNA steady state levels. RAW 264.7 cells were stimulated for 24 h with IFN-γ (50 units/ml) in the presence of vehicle or dexamethasone at concentrations of 1 nM, 10 nM, 100 nM, and 1 μM. As shown in Fig. 3, strong induction of iNOS mRNA is observed upon stimulation of the cells with IFN-γ (50 units/ml). Dexamethasone (1 μM) decreases the formation of iNOS mRNA, however, only to about 50% when compared with levels obtained with IFN-γ alone (Fig. 3). The application of lower concentrations of dexamethasone such as 10 and 1 nM does not block the induction of iNOS mRNA levels in IFN-γ-stimulated RAW 264.7 cells. Thus, suppression of iNOS protein levels is only in part caused by a decrease in iNOS mRNA steady state levels.

Dexamethasone Inhibits iNOS mRNA Formation When Administered before IFN-γ, However, Fails to Inhibit When Added after IFN-γ—We further were interested in determining whether dexamethasone would inhibit the formation of iNOS mRNA levels when the drug is administered at different time points relative to IFN-γ. RAW 264.7 cells were stimulated with dexamethasone (1 μM) for 1, 8, and 15 h prior to addition of IFN-γ (50 units/ml). Subsequently incubations were continued for 24 h. Total cellular RNA was isolated and subjected to Northern blot hybridization. As shown in Fig. 4, dexamethasone efficiently blocks the induction of iNOS mRNA only when administered 15 h prior to IFN-γ. Furthermore, RAW 264.7
cells were pretreated with IFN-γ (50 units/ml) for 1 and 8 h. Subsequently, dexamethasone (1 μM) was added and incubations were continued for a total of 24 h. As shown in Fig. 4, dexamethasone fails to inhibit the induction of iNOS mRNA steady state levels when administered after IFN-γ.

**Dexamethasone Reduces iNOS mRNA Stability**—We performed mRNA stability experiments to assess whether dexamethasone would affect the half-life of IFN-γ-induced iNOS mRNA. RAW 264.7 cells were stimulated with IFN-γ (50 units/ml) for 12 h. Then vehicle or dexamethasone (1 μM) was added for an additional 2 h. Thereafter actinomycin D (10 μg/ml) was added and RNA was harvested at the indicated time points. Samples of 20 μg of total RNA were blotted onto GeneScreen membranes which were hybridized to 32P-labeled iNOS cDNA. To assess for equal loading of RNA transfer, ribosomal RNAs were stained on the GeneScreen membranes using methylene blue.

**FIG. 5. Effect of dexamethasone on iNOS mRNA stability.** RAW 264.7 cells were stimulated with IFN-γ (50 units/ml) for 12 h. Then vehicle or dexamethasone (1 μM) was added for an additional 2 h. Thereafter actinomycin D (10 μg/ml) was added and RNA was harvested at the indicated time points. Samples of 20 μg of total RNA were blotted onto GeneScreen membranes which were hybridized to 32P-labeled iNOS cDNA. To assess for equal loading of RNA transfer, ribosomal RNAs were stained on the GeneScreen membranes using methylene blue.

12 h. Incubations of RAW 264.7 cells with actinomycin D (10 μg/ml) longer than 12 h were not performed because of severe disturbances of cell viability. Whereas the half-life of iNOS mRNA in this experimental setting was determined to be greater than 12 h (Fig. 5), dexamethasone (1 μM) decreased iNOS mRNA half-life to approximately 8 h.

**Dexamethasone Treatment Decreases iNOS Gene Transcrip-
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**Fig. 6.** Dexamethasone treatment reduces transcriptional activity of the iNOS gene. RAW 264.7 cells were stimulated for 6 h with vehicle (U), IFN-γ (50 units/ml), and IFN-γ (50 units/ml) plus dexamethasone (1 μM). The rate of transcription of iNOS and β-actin genes by isolated nuclei was determined by hybridizing the elongated, 32P-labeled RNA transcripts to iNOS cDNA, β-actin, or pSPTBM20 probes immobilized onto nitrocellulose membranes. Similar results were obtained in two independent experiments.

**Fig. 7.** Effects of dexamethasone on the translation of iNOS mRNA. RAW 264.7 cells were stimulated with IFN-γ (50 units/ml) for 3.5 h. Then vehicle, dexamethasone (100 nM) or a combination of dexamethasone (100 nM) plus the glucocorticoid receptor antagonist RU-486 (1 μM), was added to the cells and incubation continued for 0, 2, and 4 h as indicated. Thereafter the medium was removed and replaced with pulse medium without L-methionine for 45 min. L-[35S]Methionine (300 μCi/dish) was added for 30 min. Subsequently cells were lysed and used for immunoprecipitation with a rabbit polyclonal anti-iNOS antibody and SDS-PAGE. The position of iNOS is indicated by the arrow. The experimental design is depicted below the blot. The densitometrical evaluation of iNOS translation is shown in the bottom part of the figure. Similar results were obtained in two independent experiments.

**Fig. 8.** Dexamethasone increases the degradation of the iNOS protein. We next examined whether dexamethasone would affect the degradation of the iNOS protein. RAW 264.7 cells were incubated with IFN-γ (50 units/ml) for 6 h. Then vehicle or dexamethasone (1 μM) was added, cells were starved for L-methionine for 45 min, then pulsed with L-[35S]methionine for 2 h and finally chased with an excess of nonradioactive L-methionine for 0, 2, 4, 8, and 12 h. Subsequently cells were lysed and iNOS protein was immunoprecipitated using a rabbit polyclonal anti-iNOS antibody and separated by SDS-PAGE (Fig. 8). By the end of the pulse (a total of 3 h 45 min of dexamethasone) a difference in the amount of iNOS protein is detectable between IFN-γ-stimulated and IFN-γ plus dexamethasone-treated cells (Fig. 8). This is most probably caused by the action of dexamethasone during the pulse period. However, the rate of degradation of radioactively labeled iNOS protein during the chase period is drastically accelerated in dexamethasone-treated cells already at 2 h (Fig. 8). Thus, enhanced proteolysis of the iNOS protein, together with a decrease in translation of iNOS mRNA may account for the post-transcriptional part in the dexamethasone-dependent inhibi-
tion of IFN-γ-induced iNOS expression in RAW 264.7 cells.

**Dexamethasone-induced Degradation of the iNOS Protein Is Blocked by Calpain Inhibitor I, but Not by TLCK**—To assess the identity of the protease involved in the dexamethasone-induced degradation of the iNOS protein, we performed pulse-chase experiments in the presence of protease inhibitors such as calpain inhibitor I and TLCK.

TLCK is known to irreversibly inhibit serine proteases like trypsin. RAW 264.7 cells were incubated with IFN-γ (50 units/ml) for 6 h. Then vehicle or dexamethasone (1 μM) was added for 1 h. The cells were starved for L-methionine for 45 min and subsequently pulsed with L-[35S]methionine (300 μCi/dish) for 2 h. Chase medium containing an excess of unlabeled L-methionine was added for the indicated time periods. Subsequently cells were lysed and used for immunoprecipitation with a rabbit polyclonal anti-iNOS antibody and SDS-PAGE. The position of iNOS is indicated by the arrow. The experimental design is depicted below the blot. The densitometrical evaluation of iNOS degradation is shown in the bottom part of the figure. Similar results were obtained in two independent experiments.

![Fig. 8. Dexamethasone increases the degradation of the IFN-γ-induced iNOS protein.](image)

***FIG. 8.*** Dexamethasone increases the degradation of the IFN-γ-induced iNOS protein. RAW 264.7 cells were incubated with IFN-γ (50 units/ml) for 6 h. Then vehicle or dexamethasone (1 μM) was added for 1 h. The cells were starved for L-methionine for 45 min and subsequently pulsed with L-[35S]methionine (300 μCi/dish) for 2 h. Chase medium containing an excess of unlabeled L-methionine was added for the indicated time periods. Subsequently cells were lysed and used for immunoprecipitation with a rabbit polyclonal anti-iNOS antibody and SDS-PAGE. The position of iNOS is indicated by the arrow. The experimental design is depicted below the blot. The densitometrical evaluation of iNOS degradation is shown in the bottom part of the figure. Similar results were obtained in two independent experiments.

![Fig. 9. Calpain inhibitor I blocks dexamethasone-induced degradation of iNOS protein.](image)

***FIG. 9.*** Calpain inhibitor I blocks dexamethasone-induced degradation of iNOS protein. RAW 264.7 cells were incubated with IFN-γ (50 units/ml) for 6 h. Then vehicle or dexamethasone (1 μM) was added for 1 h. The cells were starved for L-methionine for 45 min and subsequently pulsed with L-[35S]methionine (300 μCi/dish) for 2 h. During the pulse period of 2 h, vehicle or calpain inhibitor I (100 μM) was added. Then chase medium containing an excess of unlabeled L-methionine was added for the indicated time periods. Subsequently cells were lysed and extracts used for immunoprecipitation with a rabbit polyclonal anti-iNOS antibody and SDS-PAGE. The position of iNOS is indicated by the arrow. The experimental design is depicted below the blot. The densitometrical evaluation of iNOS degradation is shown in the bottom part of the figure. Increase in degradation is expressed as the ratio of the effects of IFN-γ alone and IFN-γ plus drugs. Similar results were obtained in two independent experiments.

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methasone-induced degradation is inhibited as shown in Fig. 9 (IFN-γ + DEX + CPI). This effect is specific for dexamethasone-induced iNOS protein degradation and is not seen in the absence of the glucocorticoid (IFN-γ + CPI in Fig. 9). The graph (Fig. 9, bottom) shows the densitometrical analysis. The increase in iNOS protein degradation is expressed as the ratio of IFN-γ to IFN-γ plus drug.

The trypsin inhibitor TLCK does neither affect dexamethasone-induced proteolysis of the iNOS protein nor the degradation which occurs by IFN-γ alone (IFN-γ + DEX compared with IFN-γ + DEX + TLCK in Fig. 10). Similar results have been obtained using the chymotrypsin inhibitor TPCK, an irreversible inhibitor of chymotrypsin (data not shown). Interestingly TLCK treatment indicates the disappearance of the lower band while not having an effect on the dexamethasone-induced protease. This may suggest that the process of the putative post-translational modification of the iNOS protein is sensitive to a protease that is inhibited by TLCK. Alternatively, the lower band is due to proteolytic processing of iNOS protein by a protease that is inhibited by TLCK.

**DISCUSSION**

In recent years it has become evident that high output production of nitric oxide by iNOS is responsible for the development of a variety of diverse pathological events in mammalian organs. Recent research has focused on the development of selective inhibitors of iNOS catalytic activity. The goal is to
Inhibition of iNOS expression by Dexamethasone

Fig. 12. Dexamethasone does not alter IFN-γ-induced calpain protein levels. RAW 264.7 cells were incubated with vehicle (Un), IFN-γ (50 units/ml), IFN-γ (50 units/ml) plus dexamethasone (1 μM), or dexamethasone (1 μM) for 12 h in 10 ml of pulse medium (DMEM without t-methionine). The medium was aspirated and replaced with pulse medium containing t-[35S]methionine (300 μCi/dish) and incubations were continued in the presence of stimuli for 4.5 h. Subsequently cells were lysed and used for immunoprecipitation with a rabbit polyclonal anti-calpain antibody and SDS-PAGE. The position of calpain is indicated by the arrow. For control, molecular weight marker (HMW, Bio-Rad) and purified calpain proteins were subjected to the SDS-PAGE. Subsequently this part of the gel was used for Coomassie staining.

We found that glucocorticoid acts at different levels of iNOS expression to inhibit the formation of NO without interfering with the production of small quantities of NO generated by endothelial and neuronal isoforms (for review, see Refs. 24 and 25). Understanding the mechanisms involved in the induction and modulation of iNOS gene expression is important for the development of pharmacological strategies aiming to selectively inhibit NO formation. Among the most widely used drugs in anti-inflammatory therapies, glucocorticoids are highly effective in controlling inflammation and this may be in part due to their capability to inhibit iNOS expression.

Glucocorticoid inhibition of NO production was first described in cytokine-stimulated mesangial cells (10, 26). Di Rosa et al. (11) first demonstrated that dexamethasone and hydrocortisone also inhibit the production of NO in the lipopolysaccharide and IFN-γ-stimulated macrophage cell line J774. In the present report we have focused on studying the mechanisms by which the synthetic glucocorticoid dexamethasone modulates iNOS expression in IFN-γ-stimulated RAW 264.7 cells.

We found that glucocorticoid acts at different levels of iNOS expression to inhibit the formation of NO. Our data suggest that a combination of decreased transcriptional activity of the iNOS gene and stability of iNOS mRNA causes the reduction of iNOS mRNA steady state levels observed under the action of dexamethasone. A very interesting task is the identification of the transcription factors which are involved in the regulation of the iNOS gene by IFN-γ and which may be the target of drug action. Recent publications of several groups have shown that the transcription factor IRF-1 is required for the synergistic induction of the iNOS gene in response to lipopolysaccharide and IFN-γ (27). The contribution of IFN-γ to iNOS gene transcription in RAW 264.7 cells requires binding of IRF-1 to IRF-E, its cis-acting element within the murine iNOS promoter (27). Furthermore, in IRF-1−/− gene knockout mice, iNOS is not expressed (28). However, using a gene knockout model Meraz et al. (29) demonstrated the importance of STAT-1 for the induction of iNOS in macrophages. The authors show that activation of STAT-1 is required for IFN-γ-dependent IRF-1 and iNOS expression, suggesting that STAT-1 has functionally to be placed upstream of IRF-1 and most likely is the main mediator of IFN-γ-dependent signal transduction. Preliminary data obtained in our laboratory by electrophoretic mobility shift analyses using a radioactively labeled STAT-1 consensus oligonucleotide and nuclear extracts from RAW 264.7 cells strongly suggest that dexamethasone interferes with the DNA binding activity of this transcription factor.2 This may cause the reduction of iNOS gene transcription observed in IFN-γ-stimulated RAW 264.7 cells.

Most importantly, dexamethasone has pronounced inhibitory effects on iNOS expression in IFN-γ-stimulated RAW 264.7 cells at the post-transcriptional level, notably on the translation of iNOS mRNA and the degradation of iNOS protein. Several reports demonstrate that post-transcriptional mechanisms are involved in the suppression of cytokine synthesis by dexamethasone. In human monocytes, dexamethasone affects multiple levels of interleukin-1β production. It slightly increases the interleukin-1β mRNA half-life, causes a moderate inhibition of translation of the mRNA, and has profound effects on the release of mature interleukin-1β. Similarly, endotoxin-induced tumor necrosis factor-α expression in monocytes is inhibited by dexamethasone at the level of translation of tumor necrosis factor-α mRNA whereas the transcription rate of the tumor necrosis factor-α gene is only marginally decreased (9).

The first data suggesting that post-transcriptional mechanisms are involved in the regulation of iNOS expression were provided by Vodovotz et al. (30). The authors demonstrated that transforming growth factor β1 inhibits IFN-γ-induced iNOS expression in mouse peritoneal macrophages at multiple levels, including translation of the iNOS mRNA and stability of the iNOS protein. Interestingly, dexamethasone seems to activate the same post-transcriptional mechanisms as transforming growth factor β1 to exert its anti-inflammatory effects. We show that dexamethasone reduces the translation of iNOS mRNA and, in addition, increases the degradation of the iNOS protein. These results contrast well with our recent findings obtained in interleukin-1β-stimulated rat renal mesangial cells (18). Thus we conclude that the effects of dexamethasone on the translation of iNOS mRNA and stability of the iNOS protein are independent of the cell type or the stimulus used for induction. Furthermore, dexamethasone inhibits iNOS expression even after iNOS has already been expressed. This may have important implications for the clinical treatment of diseases associated with overproduction of NO and strongly argues for the effectiveness of dexamethasone in the acute anti-inflammatory therapy.

To identify the degrading enzyme we performed pulse-chase experiments in the presence of protease inhibitors. Our results strongly suggest that the cysteine protease calpain I is involved in the increased degradation of the iNOS protein caused by dexamethasone. Calpain constitutes a large family of proteolytic enzymes and is classified into two classes, ubiquitous and tissue specific. By limited proteolysis, calpain alters the activity or function of substrate proteins. Thus calpain is regarded as a biomodulator involved in the regulation of many physio-

2 G. Walker, J. Pleischiffer, and D. Kunz, unpublished results.
logical processes such as cell division, signal transduction, and long-term potentiation. Various molecular targets for calpain-dependent proteolysis have been identified. Among them are cytoskeletal proteins, membrane proteins, enzymes and transcription factors (for review, see Refs. 31 and 32). Glucocorticoid-induced expression of calpain was reported in human lymphoid cells (33). In rat L6 myotube cultures, dexamethasone increases the expression of a number of proteases including calpain. These changes may account for the ability of glucocorticoids to induce increased proteolysis in skeletal muscle (34). Moreover, the involvement of calpain in dexamethasone-induced programmed cell death is discussed (35). Our results (34). Moreover, the involvement of calpain in dexamethasone-induced programmed cell death is discussed (35). Our results obtained by Northern blot analyses (Fig. 11) and immunoprecipitation using a polyclonal anti-calpain antibody (Fig. 12) suggest that the amount of calpain mRNA steady state levels and calpain protein levels are considerably high in control RAW 264.7 cells and are not changed upon IFN-\(\gamma\) exposure. Moreover, these levels are not altered by treatment of the cells with dexamethasone. Therefore, activation of iNOS proteolysis by calpain most likely does not occur by glucocorticoid-dependent calpain gene induction. We speculate that the calmodulin (CaM)-binding site in the iNOS protein may provide the starting point for the enhanced degradation triggered by dexamethasone. A common structural feature of a number of different substrates of calpain is the presence of a calmodulin binding motif. Molinari et al. (36) demonstrated that in the plasma membrane Ca\(^{2+}\)-ATPase, an accessible CaM-binding region appears to be critical for substrate recognition and proteolysis by calpain. Occupation of the CaM-binding site by CaM significantly decreases the rate of proteolysis (36). Although the iNOS protein has long been demonstrated to bind CaM (37), the function is not yet entirely understood. Recently, Stevens et al. (38) provided data suggesting that CaM is tightly but reversibly bound to iNOS protein in a fashion different from other known CaM-enzyme interactions and that it may be required for enzymatic activity. Our observation may provide a new aspect to understand the function of CaM binding to the iNOS protein. Furthermore, it is of particular interest whether transforming growth factor \(\beta\) also activates the cysteine protease calpain I to increase degradation of iNOS protein. Such studies are currently being performed in our laboratory.

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