0026-895X/02/6203-698–704$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2002 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 62:698–704, 2002

Vol. 62, No. 3
1636/1005231
Printed in U.S.A.

Dexamethasone Inhibits Inducible Nitric-Oxide Synthase Expression and Nitric Oxide Production by Destabilizing mRNA in Lipopolysaccharide-Treated Macrophages

RIKU KORHONEN, ALEKSI LAHTI, MARI HÄMÄLÄINEN, HANNU KANKAANRANTA, and EEVA MOILANEN

The Immunopharmacological Research Group, Medical School, University of Tampere, Tampere, Finland; and Tampere University Hospital, Tampere, Finland

Received January 28, 2002; accepted June 3, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Nitric oxide (NO) production through the inducible nitric-oxide synthase (iNOS) pathway is increased in inflammatory diseases and leads to cellular injury. Anti-inflammatory steroids inhibit the expression of various inflammatory genes, including iNOS. In the present study, we investigated the mechanism how dexamethasone decreased NO production in murine J774 macrophages. Dexamethasone (0.1–10 μM) inhibited the production of NO and iNOS protein in a dose-dependent manner in cells stimulated with lipopolysaccharides (LPS). In contrast, in cells treated with a combination of LPS and interferon-γ (IFN-γ), dexamethasone did not reduce iNOS expression and NO formation. Dissociated glucocorticoid RU24858 inhibited iNOS expression and NO production to levels comparable with that of dexamethasone, suggesting that the reduced iNOS expression by dexamethasone is not a GRE-mediated event. In further studies, the effect of dexamethasone on iNOS mRNA levels was tested by actinomycin assay. The half-life of iNOS mRNA after LPS treatment was 5 h 40 min, and dexamethasone reduced it to 3 h. The increased degradation of iNOS mRNA was reversed by a protein synthesis inhibitor cycloheximide. iNOS mRNA was more stabile in cells treated with a combination of LPS plus IFN-γ (half-life = 8 h 20 min), and dexamethasone had a minor effect in these conditions. In conclusion, dexamethasone decreases iNOS-dependent NO production by destabilizing iNOS mRNA in LPS-treated cells by a mechanism that requires de novo protein synthesis. Also, decreased iNOS mRNA and protein expression and NO formation by dexamethasone was not found in cells treated with a combination of LPS plus IFN-γ, suggesting that the effect of dexamethasone is stimulus-dependent.

Nitric oxide (NO) is produced by nitric-oxide synthases (NOS) (Alderton et al., 2001), and generally, constitutively expressed endothelial NOS and neuronal NOS are responsible for physiological NO production. Inducible nitric-oxide synthase (iNOS) that is normally not present in resting cells is expressed in several pathophysiological conditions, and it produces large amounts of NO in response to inflammatory signals, such as cytokines and lipopolysaccharides (LPS) (Moilanen et al., 1999). The induction of iNOS is considered to be regulated by nuclear factor κB (NF-κB), activator protein-1 (AP-1), and interferon regulatory factor 1 (Martin et al., 1994; Xie et al., 1994; Marks-Konczalik et al., 1998; Taylor et al., 1998) in response to LPS and cytokines. In addition, there are some data indicating the involvement of post-transcriptional regulation in the iNOS expression. Transforming growth factor-β and the increase of intracellular Ca^{2+} have been shown to destabilize iNOS mRNA in activated cells (Vodovotz et al., 1993; Geng and Lotz, 1995; Korhonen et al., 2001), but the detailed mechanism remains to be clarified. A recent finding indicates that the stabilization of iNOS mRNA by HuR contributes to increased iNOS expression (Rodriguez-Pascual et al., 2000).

Glucocorticoids are potent anti-inflammatory drugs affecting the production of a wide range of inflammatory mediators (Newton, 2000). It has been shown that glucocorticoids repress inducible NO production in vascular smooth muscle cells (Radomski et al., 1990), in hepatocytes (de Vera et al., 1997), and in epithelial cells (Kleinert et al., 1996). However, glucocorticoids have been ineffective in inhibiting iNOS expression in some cell types, such as colon epithelial cells (Salzman et al., 1996) and chondrocytes (Grabowski et al., 1996; Vuolteenaho et al., 2001). These data suggest that the

This study was supported by grants from the Academy of Finland, the Medical Research Fund of Tampere University Hospital, The Pirkanmaa Regional Fund of the Finnish Cultural Foundation, Finland, and Tampere Tuberculosis Foundation, Finland.

ABBREVIATIONS: NO, nitric oxide; iNOS, inducible nitric-oxide synthase; LPS, lipopolysaccharide; IFN-γ, interferon-γ; GRE, glucocorticoid-responsive element; NOS, nitric-oxide synthase; NF-κB, nuclear factor kappa B; AP-1, activator protein 1; COX2, cyclooxygenase 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; RPA, RNase protection assay; TTP, tristetraprolin; ARE, AU-rich elements.

inhibition of iNOS expression by glucocorticoids is variable, and the mechanism of action of glucocorticoids on iNOS expression may be related to cell type and/or stimulus used.

The mechanism of action of glucocorticoids on iNOS expression and NO production is not clear. In our laboratory, we had preliminary unpublished data suggesting that the effect of dexamethasone on NO production may be related to the stimulus used. The aim of the present study was to determine the mechanism by which dexamethasone suppresses iNOS expression and NO formation. We provide data showing that dexamethasone inhibits iNOS expression and NO production in LPS-treated cells by destabilizing iNOS mRNA. In cells treated with the combination of LPS plus IFN-γ, iNOS mRNA was more stable, and dexamethasone did not inhibit NO synthesis.

Materials and Methods

Cell Cultures and Nitrite Determination. J774 murine macrophages (American Type Culture Collection, Rockville, MD) were cultured at 37°C in humidified 5% carbon dioxide atmosphere in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml) and harvested with trypsin-EDTA. Cells were seeded on 24-well plates and grown to confluence. Confluent cells were exposed to culture medium containing the compounds of interest. The culture medium was collected after 24-h incubations, and nitrite, a stable metabolite of NO in aqueous solutions, was measured by Griess reaction.

Western Blot Analysis. Cells were seeded on 6-well plates and grown to confluence, then they were stimulated for 24 h as indicated. Cell pellets from J774 cells were lysed in ice-cold extraction buffer (10 mM Tris base, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethyl sulfonyl fluoride, 2 mM Na-orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1.25 mM NaF, 1 mM Na-pyrophosphate, 10 mM *N*-octyl-β-D-glucopyranoside). After extraction by incubation on ice for 15 min, samples were centrifuged, and the resulting supernatant was boiled for 5 min in the sample buffer (62.5 mM Tris-HCl, 20% glycerol, 2% SDS, and 10 mM 2-mercaptoethanol) and stored at −70°C until needed for analysis. An aliquot of the supernatant was used to determine protein by the Coomassie blue method. Protein samples (20 μg) were separated by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose. iNOS and COX2 proteins were identified by Western blot analysis using rabbit polyclonal iNOS and rabbit polyclonal COX2 antibodies, respectively (Santa Cruz Biotechnology, Santa Cruz, CA). iNOS protein levels were quantified by densitometric analysis using SigmaGel software (SPSS Science, Chicago, IL), and COX2 levels were analyzed using FluorChem (Alpha Innotech Corporation, San Leandro, CA).

RNA Extraction and RNase Protection Assay (RPA). Cells were grown on 6-well plates to confluence and then stimulated for the time indicated. Medium was removed, and cells were washed twice with ice-cold phosphate-buffered saline and homogenized using QIAshredder (QIAGEN, Valencia, CA); extraction of total RNA was carried out with use of the RNeasy kit for the isolation of total RNA (QIAGEN). Murine iNOS and GAPDH mRNAs were detected by RNase protection assay using murine iNOS probe template (Cayman Chemical, Ann Arbor, MI) and mGAPDH probe template (BD PharMingen, San Diego, CA), respectively. In brief, 3 μg of total RNA was hybridized overnight to the ^{32}P-labeled RNA probes, which had been synthesized from the templates using T7 RNA polymerase (RiboQuant In Vitro Transcription Kit; BD PharMingen). RNase protection assay was carried out using RiboQuant Ribonuclease Protection Assay Kit (BD PharMingen). Single-stranded RNA and free probe were digested by RNase A and T1. Subsequently, protected mRNA (371 and 97 base pairs for iNOS and GAPDG, respectively) was phenolized, precipitated, and analyzed on a 6% denaturing polyacrylamide gel and then exposed on film. The levels of mRNA expression were quantified by densitometric analysis using SigmaGel software (SPSS Science). The value of iNOS expression was normalized against GAPDH.

Statistics. Results are expressed as mean ± S.E.M. Statistical significance was calculated by analysis of variance supported by Dunnett's adjusted significance levels. Statistical probabilities are expressed as ⋆, $P < 0.05$; ⋆⋆, $P < 0.01$; and ⋆⋆⋆, $P < 0.001$.

Materials. Dulbecco's modified Eagle's medium and its supplements were purchased from Invitrogen (Carlsbad, CA). Recombinant murine IFN-γ was provided by Immugenex Corporation (Los Angeles, CA). EGTA, bacterial lipopolysaccharide (*Escherichia coli* 0111:B4), actinomycin D, L-arginine, Tris-base, EDTA, NaCl, Triton X-100, phenylmethylsulfonyl fluoride, sodium orthovanadate, leupeptin, aprotinin, NaF, sodium pyrophosphate, *N*-octyl-β-D-glucopyranoside, Tris-HCl, glycerol, SDS, 2-mercapto-ethanol, sulfanilamine, and naphtalethylenediamine dihydrochloride were from Sigma (St. Louis, MO). L-*N*-iminoethyl-ornithine L-NIO (Alexis Corporation, Läufelfingen, Switzerland), dexamethasone (Orion Corporation, Espoo, Finland), and RU24858 (Aventis Pharma, Romainville Cedex, France) were obtained as indicated. 1400W was a kind gift from Dr. Richard G. Knowles (GlaxoSmithKline Research, Stevenage, United Kingdom).

Results

The Effect of Dexamethasone on NO Production in J774 Macrophages. J774 macrophages produced NO in response to LPS (10 ng/ml or 1 μg/ml) or to the combination of LPS plus IFN-γ (5 ng/ml) (Fig. 1). Dexamethasone clearly inhibited NO formation in a concentration-dependent manner in cells stimulated with LPS. The effect of dexamethasone was at its greatest when it was given to cells 1 h before LPS, and the effect was gradually decreased when dexamethasone was added 1–4 h after LPS treatment. In cells stimulated with the combination of LPS plus IFN-γ, dexamethasone (0.1–10 μM) had practically no effect on NO formation. NO production was totally abrogated with the NOS inhibitor L-NIO (1 mM) and the highly selective iNOS inhibitor 1400W (1 mM). Resting or IFN-γ–treated J774 macrophages did not produce detectable NO or express iNOS protein.

Dexamethasone has been shown to enhance arginase II activity (Gotoh and Mori, 1999). Therefore, we tested whether the supplementation of NO precursor L-arginine might reverse the suppressive effect of dexamethasone on NO production. L-Arginine (1 mM) slightly increased NO production in macrophages treated with LPS and LPS plus IFN-γ (12 and 9%, respectively). Addition of L-arginine did not reverse the inhibitory effect of dexamethasone on NO production, indicating that the induction of arginase II does not mediate the effect of dexamethasone on NO production in J774 cells (data not shown).

A glucocorticoid receptor antagonist mifepristone was used in 3-fold excess (3 μM) in respect to dexamethasone (1 μM). In the presence of mifepristone, dexamethasone had no inhibitory effect on NO production in cells treated with LPS. These data suggest that the inhibitory effect of dexamethasone on NO synthesis is mediated through the activation of glucocorticoid receptor (GR). Mifepristone did not alter NO formation in cells treated with the combination of LPS plus IFN-γ (data not shown).

The Effect of the Dissociated Glucocorticoid RU24858 on NO Production in J774 Macrophages. Dissociated glucorticoids are synthetic GR ligands that dissociate transactivation and transrepression properties of glucocorticoids on gene expression. In reporter gene experiments, dissociated glucocorticoid RU24858 (1 nM to 1 μM) had transrepression properties on AP-1– and NF-κB–driven transcription comparable with those of dexamethasone, whereas it did not induce glucocorticoid-responsive element (GRE)-mediated transcription (Vayssiere et al., 1997; Vanden Berghe et al., 1999). In our experiments, RU24858 inhibited NO formation in a concentration-dependent manner in cells treated with LPS, but it failed to inhibit NO in cells treated with the combination of LPS plus IFN-γ. These data suggest that dexamethasone-dependent inhibition of NO formation is probably not mediated through GRE (Fig. 2).

The Effects of Dexamethasone and Dissociated Glucocorticoid RU24858 on iNOS Protein Expression. The expression of iNOS protein after dexamethasone treatment in cells stimulated with LPS or the combination of LPS plus IFN-γ was studied by use of Western blot analysis after 24 h of incubation and quantified by densitometry. iNOS protein expression induced by LPS was reduced by 78% in cells treated with dexamethasone compared with cells not challenged with the drug. Similarly, RU24858 inhibited LPS-induced iNOS protein expression by 57%. The combination of LPS plus IFN-γ induced 2.4-fold expression of iNOS protein compared with that induced by LPS. Consistent with nitrite experiments, neither dexamethasone (−9%) nor RU24858 (+1%) inhibited iNOS protein expression stimulated with the combination of LPS plus IFN-γ (Fig. 3).

To study whether the cells stimulated with the combination of LPS plus IFN-γ remain responsive for glucocorticoids in general, the effect of dexamethasone on COX2 protein expression was investigated. Dexamethasone reduced COX2 protein levels (>50% inhibition) in cells treated with LPS in the absence or presence of IFN-γ. These results suggest that a general induction of glucocorticoid resistance by IFN-γ is not a reason for the lack of inhibition of NO production in cells treated with the combination of LPS plus IFN-γ.

The Effect of Dexamethasone on Expression and Stability of iNOS mRNA. The effect of dexamethasone on iNOS mRNA was also investigated. Stimulants were added to the cells in the beginning of the incubation, and cells were harvested for RNA extraction after 6 h of incubation. iNOS mRNA was detected by RPA. Untreated cells did not express detectable iNOS mRNA. LPS induced a marked iNOS mRNA expression, and the addition of dexamethasone (1 μM) reduced the level of iNOS mRNA slightly (18%) (Fig. 4A). The combination of LPS plus IFN-γ induced iNOS mRNA expression to twice the level induced by LPS, and dexamethasone inhibited the accumulation of iNOS mRNA by approximately 10% (Fig. 4B). This suggests that the effect of dexamethasone on iNOS mRNA expression is not a transcriptional effect.

To investigate the underlying mechanism, we measured the rate of iNOS mRNA degradation with respect to dexamethasone treatment. An inhibitor of transcription, actinomycin D (0.5 μg/ml), was added to the cells 6 h after LPS or LPS plus IFN-γ. Cells were harvested at time points of 0, 4, 8, and 12 h after the addition of actinomycin D. Dexamethasone destabilized the iNOS mRNA in LPS-treated cells: the half-lives for iNOS mRNA were 5 h 40 min and 3 h for LPS- or LPS and dexamethasone-treated cells, respectively (Fig. 4A). On the other hand, iNOS mRNA was stabilized by IFN-γ: the half-lives for iNOS mRNA in cells stimulated with the combination of LPS plus IFN-γ or LPS, IFN-γ and dexa-

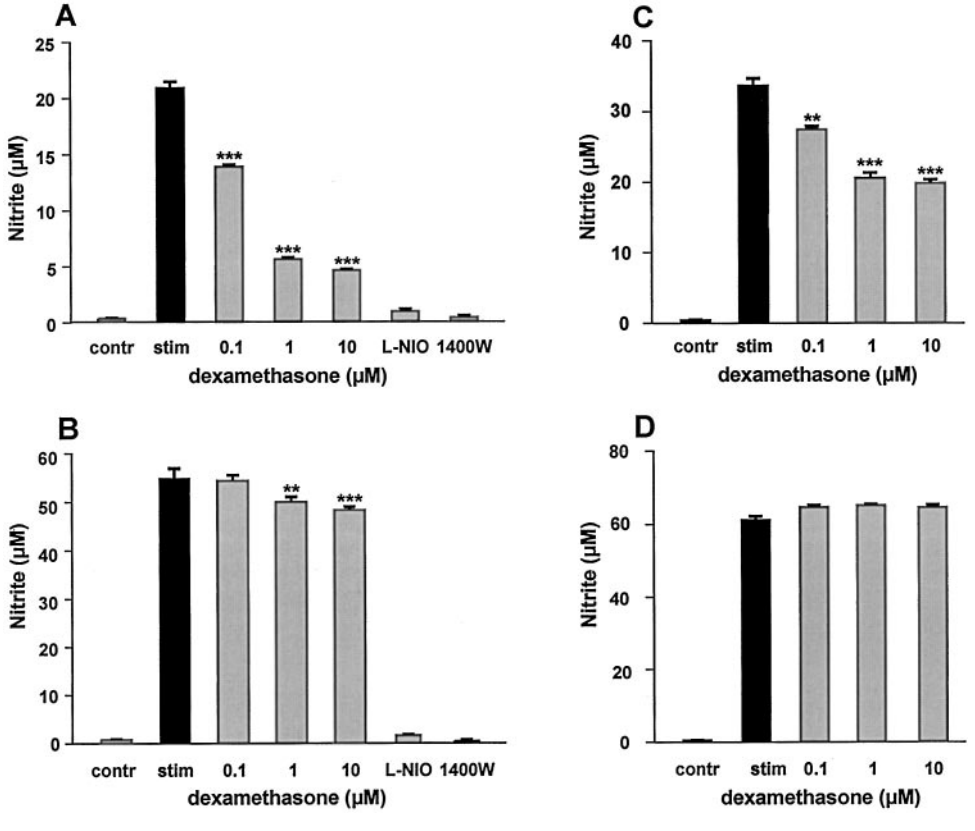

Fig. 1. The effect of dexamethasone on NO production in J774 macrophages stimulated with LPS or the combination of LPS plus IFN-γ. Cells were stimulated with LPS (10 ng/ml) in the absence (A) or presence (B) of IFN-γ (5 ng/ml), or with LPS 1 μg/ml in the absence (C) or presence (D) of IFN-γ 5 ng/ml, and dexamethasone (0.1–10 μM, ▨) was added to the cells along with the stimulants. NO formation was detected as nitrite in the culture medium after 24 h of incubation. □ (contr) represents untreated cells, and ■ (stim) represents stimulated cells in the absence of dexamethasone. Results are expressed as the mean ± S.E.M. (n = 6). ⋆⋆, $P < 0.01$, and ⋆⋆⋆, $P < 0.001$ compared with cells treated with LPS or LPS plus IFN-γ in the absence of dexamethasone.

methasone were 8 h 20 min and 6 h 20 min, respectively (Fig. 4B).

To investigate whether the mechanism of dexamethasone on iNOS mRNA expression would be a protein synthesis–dependent event, cycloheximide (1 μg/ml) was added. Cells were stimulated with LPS and incubated for 14 h. In the absence of cycloheximide, dexamethasone decreased the level of iNOS mRNA by 4% of that induced by LPS (Fig. 5A). In the presence of cycloheximide, dexamethasone did not inhibit iNOS mRNA expression. (Fig. 5A). This suggests that increased iNOS mRNA decay by dexamethasone in LPS-treated cells requires de novo protein synthesis. Cells stimulated with the combination of LPS plus IFN-γ, both cycloheximide and dexamethasone were ineffective to repress iNOS mRNA expression (Fig. 5B).

Discussion

Increased iNOS expression and NO production are involved in the pathophysiology of several inflammatory conditions such as asthma, rheumatoid arthritis, and inflammatory bowel disease. Glucocorticoids have a wide spectrum of anti-inflammatory properties, including the suppression of cytokines and their receptor synthesis, adhesion protein expression, and production of other inflammatory mediators (Newton, 2000). In the present study, we provide additional data showing that dexamethasone inhibits iNOS-dependent NO formation by destabilizing iNOS mRNA with a mechanism requiring de novo protein synthesis. In addition, the effect of dexamethasone on iNOS expression and NO production, as well as iNOS mRNA decay to some extent, is reversed by IFN-γ.

The expression of iNOS and activation of NO production was induced by LPS, and it was further increased by IFN-γ in J774 cells, suggesting synergy between these two substances in the enhanced expression of iNOS, as shown previously (Lorsbach et al., 1993; Weisz et al., 1996). Dexamethasone clearly inhibited NO production in macrophages treated with LPS alone either by lower (10 ng/ml) or higher (1 μg/ml) LPS concentrations that induced lower and higher levels of NO, respectively. On the other hand, dexamethasone did not inhibit NO formation by iNOS pathway in cells treated with a combination of LPS plus IFN-γ. This suggests that in these conditions, IFN-γ in combination with LPS has an important role in the up-regulation of NO formation through iNOS by a mechanism that is not regulated by glucocorticoids. In earlier studies, iNOS expression and NO production have been shown to decrease by glucocorticoids in some cells (Radomski et al., 1990; Kleinert et al., 1996; de Vera et al., 1997; Walker et al., 1997; Lahde et al., 2000), but this is not the case in all cell types (Salzman et al., 1996; Grabowski et al., 1996), and the mechanism of action has been unclear. Reduction in NF-κB–mediated iNOS transcription was found to take place in dexamethasone-treated lung epithelial cells after cytokine treatment (Kleinert et al., 1996), and it was associated with the concomitant induction of IκB (de Vera et al., 1997). In the present experiments, NO formation was decreased in LPS-treated cells, but not in cells treated with a combination of LPS plus IFN-γ. Reduced NO formation was associated with suppressed iNOS protein expression and increased iNOS mRNA destabilization. In contrast, cells treated with the combination of LPS plus IFN-γ expressed enhanced mRNA levels compared with LPS-treated cells, and the iNOS mRNA half-life was significantly lengthened. This suggests that the

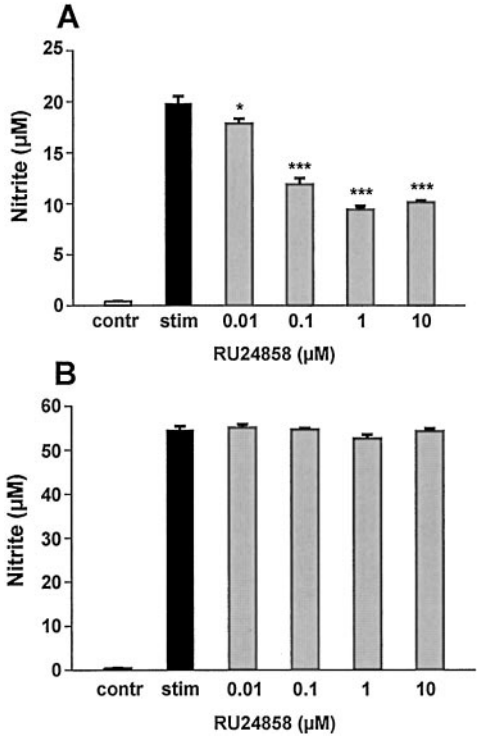

Fig. 2. The effect of dissociated glucocorticoid RU24858 on NO production in J774 macrophages stimulated with LPS or the combination of LPS plus IFN-γ. Cells were stimulated with (A) LPS (10 ng/ml), and (B) the combination of LPS (10 ng/ml) plus IFN-γ (5 ng/ml), and RU24858 (0.01–10 μM, ▨) was added to the cells along with the stimulants. NO formation was detected as nitrite in the culture medium after 24 h of incubation. □ (contr) represents untreated cells, and ■ (stim) represents stimulated cells in the absence of RU24858. Results are expressed as the mean ± S.E.M. ($n = 6$). ⋆, $P < 0.05$, ⋆⋆, $P < 0.01$, and ⋆⋆⋆, $P < 0.001$ compared with cells treated with LPS or LPS plus IFN-γ in the absence of RU24858.

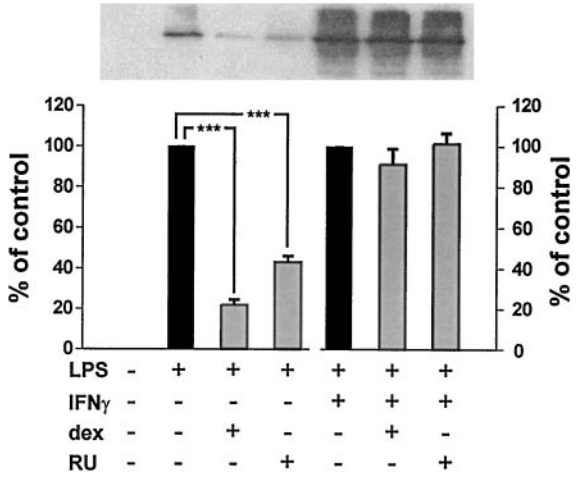

Fig. 3. The effect of dexamethasone and a dissociated glucocorticoid RU24858 on iNOS protein expression in J774 macrophages stimulated with LPS or the combination of LPS plus IFN-γ. Cells were stimulated with LPS (10 ng/ml) or the combination of LPS (10 ng/ml) plus IFN-γ (5 ng/ml). The concentration of dexamethasone (dex) and RU24858 (RU) was 1 μM. Cells were incubated for 24 h and then harvested for protein extraction. iNOS protein was detected by Western blot. Untreated cells were used as the negative control. iNOS protein levels were determined densitometrically, and results are expressed as the mean ± S.E.M. ($n = 8$, except for RU24858-treated cells, $n = 4$). ⋆⋆⋆, $P < 0.001$ compared with cells treated with LPS in the absence of dexamethasone.

effect of dexamethasone on iNOS expression and NO formation is stimulus-dependent, not just a cell type-dependent phenomenon.

Arginase II catalyzes the conversion of L-arginine to L-ornithine. Arginase II is coinduced with iNOS by LPS, and it has been reported to be further induced by dexamethasone. Induction of arginase II led to decreased NO production because of the depletion of L-arginine (Gotoh and Mori, 1999). In our experiments, the addition of L-arginine did not reverse the inhibitory action of dexamethasone on NO production, suggesting that in these conditions, the induction of arginase II does not explain the decrease in NO formation by dexamethasone.

iNOS mRNA was induced despite protein synthesis inhibition in LPS-stimulated cells, suggesting that the induction of iNOS mRNA synthesis does not require de novo protein

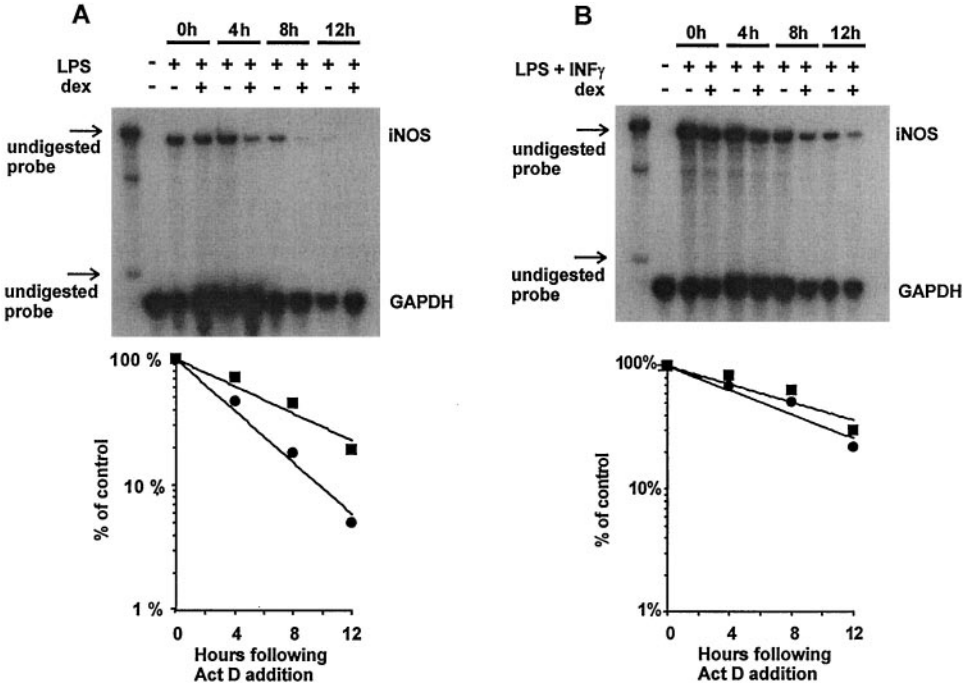

Fig. 4. The effect of dexamethasone on iNOS mRNA decay in J774 macrophages stimulated with LPS or the combination of LPS plus IFN-γ. Cells were stimulated with (A) LPS (10 ng/ml) or (B) LPS (10 ng/ml) plus IFN-γ (5 ng/ml) in the absence (■) or in the presence (●) of dexamethasone (1 μM) as indicated. Actinomycin D (0.5 μg/ml) was added to the cells after 6 h of incubation. Untreated cells were used as the negative control. Cells were harvested for RNA extraction after the incubation time indicated after actinomycin D addition, and iNOS and GAPDH mRNAs were detected by RPA and quantified densitometrically. iNOS mRNA levels were normalized against GAPDH, and results are expressed as the mean (n = 3). The gels are representatives of three separate experiments with similar results.

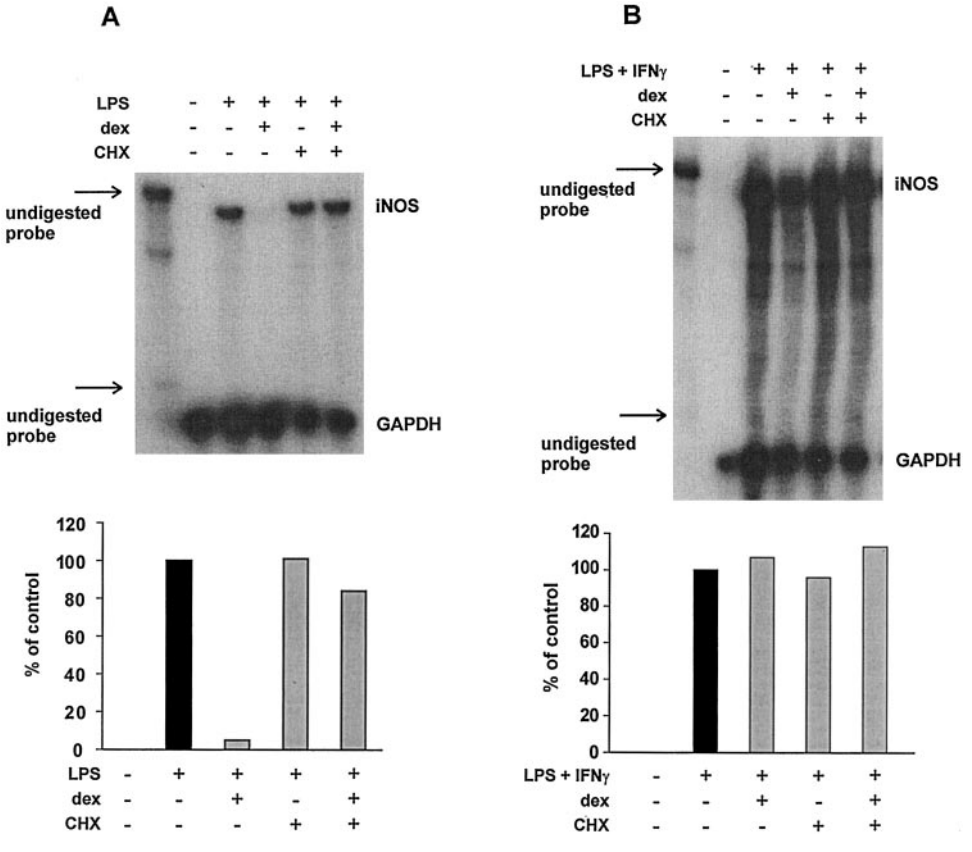

Fig. 5. The effect of inhibition of protein synthesis on the suppressive action of dexamethasone on iNOS mRNA expression in cells treated with LPS or the combination of LPS plus IFN-γ. Cells were stimulated by (A) LPS (10 ng/ml) and (B) LPS (10 ng/ml) plus IFN-γ (5 ng/ml). The concentration of dexamethasone (dex) and cycloheximide (CHX) was 1 μM. Untreated cells were used as the negative control. Cells were incubated with the compounds of interest for 12 h and then harvested for RNA extraction. iNOS and GAPDH mRNAs were detected by RPA and quantified densitometrically. iNOS mRNA levels are normalized against GAPDH, and results are expressed as the mean (n = 3). The gels are representatives of three separate experiments with similar results.

synthesis. In cells treated with LPS, the reduction of iNOS mRNA by dexamethasone was nullified by cycloheximide, suggesting that the effect of dexamethasone is a protein synthesis–mediated event in LPS-treated cells. In contrast, in cells cultured with LPS plus IFN-γ, the inhibition of protein synthesis had no effect on iNOS mRNA expression. This suggests that the mRNA stabilizing effect of IFN-γ is not mediated by de novo protein synthesis. Dexamethasone had no effect in the presence of IFN-γ, and it is thus possible that the mechanisms of the effects of IFN-γ and dexamethasone are totally distinct or that IFN-γ suppresses the induction or action of dexamethasone-induced protein that destabilizes iNOS mRNA. The data from the actinomycin D experiments support the former assumption.

Knowledge concerning the proteins regulating the mRNA stability of inflammatory genes is limited so far. TTP is an LPS-inducible protein that has been shown to participate in the destabilization of tumor necrosis factor-α, interleukin-3, and granulocyte-macrophage colony-stimulating factor mRNAs through AU-rich elements (ARE) located in the 3′-untranslated region (Carballo et al., 2000; Stoecklin et al., 2000; Mahtani et al., 2001). According to a very recent finding, TTP and another RNA binding protein, K-type RNA binding protein, are able to recruit ARE-containing mRNAs to exosome, which are 3′-5′-exonuclease complexes responsible for the rapid degradation of mRNA (Chen et al., 2001). In our cells, LPS enhanced TTP mRNA expression, but dexamethasone suppressed it both in the presence and in the absence of LPS (R. Korhonen et al., unpublished data). This suggests that TTP is not mediating the dexamethasone-induced destabilization of iNOS mRNA. HuR is a protein that belongs to Elav/Hu RNA binding proteins and is capable of binding to ARE sequences of mRNA (Brennan and Steitz, 2001); it is found to be involved in the stabilization of 3′-untranslated region–containing mRNAs (Fan and Steitz, 1998; Peng et al., 1998), including iNOS, in cytokine-treated colon epithelial cells (Rodriguez-Pascual et al., 2000). So far, we have not been able to detect HuR in our cells. The role of HuR in the dexamethasone-induced iNOS destabilization remains unknown.

The classic effects of glucocorticoids are mediated through GR. Mifepristone is a steroid that competitively binds to GR and inhibits the effect of glucocorticoids (Mahajan and London, 1997). In the presence of mifepristone, dexamethasone did not inhibit NO formation in LPS-treated cells, indicating that suppressed NO production by dexamethasone is a GR-mediated process. Glucocorticoid-GR complexes are able to both activate gene expression through GRE and suppress gene expression by inhibiting the action of inflammatory transcription factors. With certain glucocorticoid derivatives, namely dissociated glucocorticoids, it has been possible to separate these two properties of glucocorticoids. We tested one such compound, RU24858, that does not induce GRE-mediated transcription, but it inhibits NF-κB and AP-1 activation (Vanden Berghe et al., 1999). The effect of RU24858 was comparable with that of dexamethasone, suggesting that the inhibitory effect of dexamethasone on NO formation is probably not mediated through GRE, but may merely be caused by the inhibition of NF-κB and/or AP-1 activation.

In addition to its effect on GRE-regulated transcription and inflammatory transcription factors, dexamethasone has been shown to regulate the mRNA stability of some inflammatory genes, i.e., COX2 and interleukin-11 (Ristimäki et al., 1996; Wang et al., 1999). Dexamethasone-mediated destabilization of iNOS mRNA is a new mechanism by which glucocorticoids repress iNOS expression and NO formation. The present knowledge of the post-transcriptional mechanisms in the regulation of iNOS expression is rather limited. Data provided by the present study suggest that post-transcriptional regulation of iNOS mRNA is of significance in the overall regulation of inducible NO production.

In conclusion, dexamethasone was found to destabilize iNOS mRNA in LPS-treated cells, which led to decreased iNOS protein expression and NO production, and the effect of dexamethasone turned out to be a protein synthesis–dependent event. These data add to our knowledge of the mechanisms of actions of anti-inflammatory steroids and the regulation of inducible NO production.

Acknowledgments

We thank Niina Ikonen, Heli Määttä, and Jaana Laine for their excellent technical assistance. 1400W was a kind gift from Dr. Richard G. Knowles (GlaxoSmithKline Research, Stevenage, United Kingdom).

References

Alderton WK, Cooper CE, and Knowles RG (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem J* **357:**593–615.

Brennan CM and Steitz JA (2001) HuR and mRNA stability. *Cell Mol Life Sci* **58:**266–277.

Carballo E, Lai WS, and Blackshear PJ (2000) Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability. *Blood* **95:**1891–1899.

Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJ, Stoecklin G, Moroni C, Mann M, and Karin M (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell* **107:**451–464.

de Vera ME, Taylor BS, Wang Q, Shapiro RA, Billiar TR, and Geller DA (1997) Dexamethasone suppresses iNOS gene expression by upregulating I-κBα and inhibiting NF-κB. *Am J Physiol* **273:**G1290–G1296.

Fan XC and Steitz JA (1998) Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. *EMBO (Eur Mol Biol Organ) J* **17:**3448–3460.

Geng Y and Lotz M (1995) Increased intracellular Ca^{2+} selectively suppresses IL-1-induced NO production by reducing iNOS mRNA stability. *J Cell Biol* **129:**1651–1657.

Gotoh T and Mori M (1999) Arginase II downregulates nitric oxide (NO) production and prevents NO-mediated apoptosis in murine macrophage-derived RAW 264.7 cells. *J Cell Biol* **144:**427–434.

Grabowski PS, Macpherson H, and Ralston SH (1996) Nitric oxide production in cells derived from the human joint. *Br J Rheumatol* **35:**207–212.

Kleinert H, Euchenhofer C, Ihrig-Biedert I, and Forstermann U (1996) Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-κB. *Mol Pharmacol* **49:**15–21.

Korhonen R, Kankaanranta H, Lahti A, Lahde M, Knowles RG, and Moilanen E (2001) Bi-directional effects of the elevation of intracellular calcium on the expression of inducible nitric oxide synthase in J774 macrophages exposed to low and to high concentrations of endotoxin. *Biochem J* **354:**351–358.

Lahde M, Korhonen R, and Moilanen E (2000) Regulation of nitric oxide production in cultured human T84 intestinal epithelial cells by nuclear factor-κB-dependent induction of inducible nitric oxide synthase after exposure to bacterial endotoxin. *Aliment Pharmacol Ther* **14:**945–954.

Lorsbach RB, Murphy WJ, Lowenstein CJ, Snyder SH, and Russell SW (1993) Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-γ and lipopolysaccharide. *J Biol Chem* **268:**1908–1913.

Mahajan DK and London SN (1997) Mifepristone (RU486): a review. *Fertil Steril* **68:**967–976.

Mahtani KR, Brook M, Dean JL, Sully G, Saklatvala J, and Clark AR (2001) Mitogen-activated protein kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability. *Mol Cell Biol* **21:**6461–6469.

Marks-Konczalik J, Chu SC, and Moss J (1998) Cytokine-mediated transcriptional induction of the human inducible nitric oxide synthase gene requires both activator protein 1 and nuclear factor κB-binding sites. *J Biol Chem* **273:**22201–22208.

Martin E, Nathan C, and Xie QW (1994) Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *J Exp Med* **180:**977–984.

Moilanen E, Whittle BJR, and Moncada S (1999) Nitric oxide as a factor in inflammation, in *Inflammation: Principles and Clinical Correlations* (Gallin JI and Snyderman R eds) pp 787–800, Lippincott Williams & Wilkins, Philadelphia.

Newton R (2000) Molecular mechanisms of glucocorticoid action: what is important? *Thorax* **55:**603–613.

Peng SS, Chen CY, Xu N, and Shyu AB (1998) RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. *EMBO (Eur Mol Biol Organ) J* **17:**3461–3470.

Radomski MW, Palmer RM, and Moncada S (1990) Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc Natl Acad Sci USA* **87:**10043–10047.

Ristimäki A, Narko K, and Hla T (1996) Down-regulation of cytokine-induced cyclooxygenase-2 transcript isoforms by dexamethasone: evidence for post-transcriptional regulation. *Biochem J* **318:**325–331.

Rodriguez-Pascual F, Hausding M, Ihrig-Biedert I, Furneaux H, Levy AP, Forstermann U, and Kleinert H (2000) Complex contribution of the 3′-untranslated region to the expressional regulation of the human inducible nitric-oxide synthase gene. Involvement of the RNA-binding protein HuR. *J Biol Chem* **275:**26040–26049.

Salzman A, Denenberg AG, Ueta I, O'Connor M, Linn SC, and Szabo C (1996) Induction and activity of nitric oxide synthase in cultured human intestinal epithelial monolayers. *Am J Physiol* **270:**G565–G573.

Stoecklin G, Ming XF, Looser R, and Moroni C (2000) Somatic mRNA turnover mutants implicate tristetraprolin in the interleukin-3 mRNA degradation pathway. *Mol Cell Biol* **20:**3753–3763.

Taylor BS, de Vera ME, Ganster RW, Wang Q, Shapiro RA, Morris SMJ, Billiar TR, and Geller DA (1998) Multiple NF-κB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J Biol Chem* **273:**15148–15156.

Vanden Berghe W, Francesconi E, De Bosscher K, Resche-Rigon M, and Haegeman G (1999) Dissociated glucocorticoids with anti-inflammatory potential repress interleukin-6 gene expression by a nuclear factor-κB-dependent mechanism. *Mol Pharmacol* **56:**797–806.

Vayssiere BM, Dupont S, Choquart A, Petit F, Garcia T, Marchandeau C, Gronemeyer H, and Resche-Rigon M (1997) Synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression exhibit antiinflammatory activity *in vivo*. *Mol Endocrinol* **11:**1245–1255.

Vodovotz Y, Bogdan C, Paik J, Xie QW, and Nathan C (1993) Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor β. *J Exp Med* **178:**605–613.

Vuolteenaho K, Moilanen T, al-Saffar N, Knowles RG, and Moilanen E (2001) Regulation of the nitric oxide production resulting from the glucocorticoid-insensitive expression of iNOS in human osteoarthritic cartilage. *Osteoarthritis Cartilage* **9:**597–605.

Walker G, Pfeilschifter J, and Kunz D (1997) 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. *J Biol Chem* **272:**16679–16687.

Wang J, Zhu Z, Nolfo R, and Elias JA (1999) Dexamethasone regulation of lung epithelial cell and fibroblast interleukin-11 production. *Am J Physiol* **276:**L175–L185.

Weisz A, Cicatiello L, and Esumi H (1996) Regulation of the mouse inducible-type nitric oxide synthase gene promoter by interferon-γ, bacterial lipopolysaccharide and N^G-monomethyl-L-arginine. *Biochem J* **316:**209–215.

Xie QW, Kashiwabara Y, and Nathan C (1994) Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J Biol Chem* **269:**4705–4708.

Address correspondence to: Professor Eeva Moilanen, Medical School, FIN-33014 University of Tampere, Finland. E-mail: eeva.moilanen@uta.fi