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α-MSH inhibits induction of C/EBPβ-DNA binding activity and *NOS2* gene transcription in macrophages

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Background. α-Melanocyte–stimulating hormone (α-MSH) is an endogenous tridecapeptide that exerts anti-inflammatory actions and abrogates postischemic renal injury in rodents. α-MSH inhibits lipopolysaccharide (LPS)-induced gene expression of several cytokines, chemokines, and nitric oxide synthase-2 (NOS2), but the molecular mechanisms underlying these effects have not been clearly defined. To test the hypothesis that α-MSH inhibits the expression of inducible *trans*-activating factors involved in NOS2 regulation, we used RAW 264.7 macrophage cells to examine the effects of α-MSH on the activation of nuclear factor-κB (NF-κB) and CCAAT/enhancer binding protein-β (C/EBPβ), *trans*-acting factors known to be involved in LPS + interferon (IFN)-γ induction of the *NOS2* gene.

Methods. Gel shift assays were performed to identify NF-κB and C/EBP DNA binding activities in LPS + IFN-γ–treated RAW 264.7 cells in the presence and absence of α-MSH. *NOS2* promoter assays were conducted to identify the effects of α-MSH on LPS + IFN-γ–mediated induction of *NOS2* transcription.

Results. Gel shift assays demonstrated LPS + IFN-γ induction of NF-κB and C/EBP family protein-DNA complexes in nuclei harvested from the cells. Supershift assays revealed that the C/EBP complexes were comprised of C/EBPβ, but not C/EBPα, C/EBPδ, or C/EBPε. α-MSH (100 nmol/L) inhibited the LPS + IFN-γ–mediated induction of nuclear DNA binding activity of C/EBPβ, but not that of NF-κB (in contrast to reports in other cell types), as well as the activity of a murine *NOS2* promoter-luciferase construct. In contrast, α-MSH (100 nmol/L) had no effect on the induction of *NOS2* promoter-luciferase genes harboring deletion or mutation of the C/EBP box.

Conclusions. These data indicate that α-MSH inhibits the induction of C/EBPβ DNA binding activity and that this effect is a major mechanism by which α-MSH inhibits the transcription of the *NOS2* gene. The inability of α-MSH to inhibit LPS + IFN-γ induction of NF-κB in murine macrophage cells, which contrasts with inhibitory effects of the neuropeptide in other cell types, suggests that cell-type–specific mechanisms are involved.

Key words: α-malanocyte-stimulating hormone, transcription factors, gene promoter, nitric oxide, lipopolysaccharide, interferon-γ.

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α-Melanocyte–stimulating hormone (α-MSH) is a proopiomelanocortin–derived 13 amino acid peptide released by the pituitary gland and immunocompetent cells that exerts broad anti-inflammatory actions in mammals [1]. Concentrations of this neuropeptide increase locally at sites of inflammation [2] and systemically within minutes of endotoxin injection [3]. The therapeutic administration of α-MSH has been shown to limit injury in experimental models of sepsis and inflammatory or ischemic organ failure [4–7], including ischemia-induced acute renal failure [8–10]. The salutary effects of α-MSH in these experimental models appear to be in part attributable to its ability to limit induction of genes encoding proinflammatory cytokines [tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-1 (IL-1)] [6, 11, 12] chemokines (IL-8) [13], cell adhesion molecules (ICAM-1) [8], and nitric oxide synthase-2 (NOS2) [5, 8]. By limiting these injury pathways, neutrophil infiltration, capillary congestion, and exposure of cell constituents to damaging reactive oxygen and nitrogen intermediates are reduced. In studies of a rodent model of bilateral renal ischemia/reperfusion injury, α-MSH abrogated renal injury [8, 10] and preserved the glomerular filtration rate, tubular sodium reabsorption [9], and urinary concentration [10]. The kidneys of the α-MSH–treated animals exhibited less neutrophil plugging, capillary congestion, tubular injury, and protein nitrosylation, and negligible induction of NOS2, cytokine-induced neutrophil chemoattractant (KC)/IL-8, and the adhesion molecule ICAM-1 compared with untreated controls [8]. The beneficial effect of α-MSH in ischemia-reperfusion injury to the kidney is in part mediated by neutrophil-independent mechanisms since the protective effects of the peptide were observed in ICAM-1 knockout mice, which exhibited 75% less neutrophil infiltration than background mice following renal ischemia [9]. Consequently, α-MSH is being tested as a candidate therapeutic agent for ischemic acute renal failure in humans.

The molecular details of how α-MSH restricts inducible expression of proinflammatory mediators remain incom-

pletely defined. Such information is important, since the anti-inflammatory properties of the neuropeptide may prove hazardous to immunocompromised patients, such as those with multiple organ failure, who develop acute renal failure. Macrophages represent the best studied cell type for modeling receptor-coupled signaling of α-MSH and the ability of this peptide to influence the expression of macrophage gene products, including NOS2, that contribute to inflammation and injury. It is known that cytokines induce murine and human monocyte/macrophages to secrete α-MSH and to up-regulate, in an autocrine manner, the expression of the melanocortin-1 receptor and the pro-opiomelanocortin (POMC) precursor of α-MSH [14, 15]. The increased production of α-MSH, in turn, appears to inhibit *NOS2* gene expression and limit NO production. Indeed, α-MSH limited TNF-α–mediated induction of NOS2 mRNA levels and NO production in RAW 264.7 macrophages [15]. It was not determined, however, whether this effect was the result of transcriptional repression of the *NOS2* gene.

The *NOS2* gene is typically quiescent in macrophages, as well as the majority of cell types, until transcriptionally activated in response to bacterial lipopolysaccharide (LPS) and/or certain cytokines [16]. The 5′-flanking region of the murine *NOS2* gene contains multiple *cis*-elements that bind *trans*-acting factors upon exposure to these stimuli, including κB elements [17–19] and a CCAAT/enhancer binding protein (EBP) box at nucleotide positions −150 to −142 [20] that have been shown to be functionally active and to mediate *trans*-activation of the *NOS2* gene in LPS + IFN-γ–treated cell types. The C/EBP proteins comprise a five-member family of bZIP (*b*asic region leucine *zip*per) transcription factors that regulate the expression of genes involved in diverse processes such as the acute phase response, inflammation, cell growth, and differentiation [21–23]. C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, and C/EBPε *trans*-activate target genes, whereas CHOP and the alternative translation products LIP and C/EBP-30 function as transcriptional repressors [23]. Of these, C/EBPβ and C/EBPδ have been shown to be induced by LPS or cytokines. C/EBPβ is induced in kidneys from LPS-treated [22] or hypoxic [24] mice [22], the kidneys of diabetic rats [25], the medullary thick ascending limb (mTAL) of rats subjected to renal ischemia-reperfusion injury (abstract; Price et al, *J Am Soc Nephrol* 6:987, 1995), as well as in several other cell types, including macrophages [26] and vascular smooth muscle cells [27], which are subjected to immune or hypoxic challenge.

The beneficial effects of α-MSH in diverse forms of inflammation and tissue ischemia, combined with α-MSH's ability to influence multiple proinflammatory genes, suggest that it acts at common, early steps in the inflammatory and injury cascades. Since transcription is the central and initial control point for the regulation of gene expression and several pleiotropic transcription factors are induced during inflammation and ischemic injury, recent attention has turned to the effects of α-MSH on inducible transcription factors. Studies in various cell types (H4 and A-172 glioma cells, U-937 myeloid cells, Jurkat T cells), and in animal models of brain inflammation showed that the neuropeptide inhibits NF-κB induction in response to cytokines or LPS administration, at least in part by preserving IκBα protein levels [28–32]. The effects of α-MSH on NF-κB induction in macrophage cells, however, were not examined. In addition, since C/EBPβ *trans*-activates many of the same proinflammatory genes (for example, NOS2, ICAM-1 [33], monocyte chemoattractant protein-1 [34], TNF-α [35], and IL-8 [13]) that α-MSH inhibits, we hypothesized that α-MSH might also block the activation or action of C/EBPβ. Using RAW 264.7 macrophage cells as a model system, the present results demonstrate a previously unrecognized action of α-MSH to inhibit the LPS + IFN-γ–mediated induction of C/EBPβ-DNA binding activity and, as a consequence, the transcriptional activation of the *NOS2* promoter. To our knowledge, these data represent the first example of neuropeptide inhibition of *NOS2* transcription, and they suggest that C/EBPβ inhibition is a central mechanism by which α-MSH exerts its broad anti-inflammatory actions and possibly its protective effects in ischemic renal failure.

METHODS

Reagents

Lipopolysaccharide from *E. coli* serotype O11:B4 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mouse recombinant IFN-γ was from Genzyme (Cambridge, MA, USA), and α-MSH was from Phoenix Pharmaceuticals, Inc. (Mountain View, CA, USA). The Dual Luciferase™ reporter assay system, pGL3-Basic, pRL-TK, and NF-κB consensus oligonucleotides were obtained from Promega Corp. (Madison, WI, USA). [γ-^{32}P]-ATP, antirabbit and antimouse IgG conjugated with horseradish peroxidase, enhanced chemiluminescence (ECL)-Western blotting reagent, and poly (dI-dC)-poly (dI-dC) were from Amersham-Pharmacia (Piscataway, NJ, USA). Dulbecco's modified Eagle's medium (DMEM), LipoFectamine Plus™ reagent, and heat-inactivated fetal bovine serum (FBS) were obtained from GIBCO/BRL (Althersburg, MO, USA). The BCA Protein Assay kit was from Pierce (Rockwood, IL, USA). Antibodies to C/EBP family proteins and NF-κB p50, as well as protein A/G PLUS-agarose, were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

Plasmids

The plasmids used in the present study have been described in our earlier publication [20]: pNOS2-luc con-

tains 1.6 kB of the 5′-flanking region and 5′-UTR of exon 1 (nucleotides −1486 to +145) of the mouse *NOS2* gene cloned into the *Hind*III and *Xho*I sites of pGL3-Basic; pNOS2-C/EBPdel-luc is identical to pNOS2-luc except that the −150 to −142 C/EBP box in the *NOS2* promoter was excised. pNOS2-ΔC/EBP-luc is identical to pNOS2-luc except that the −150 to −142 C/EBP box in the *NOS2* promoter contains a 6 bp mutation that renders the C/EBP box incompetent to bind C/EBP proteins. Plasmid preparations were made using the endotoxin-free Plasmid Maxi-prep kit (Qiagen Inc., Santa Clarita, CA, USA).

Cell culture

RAW 264.7 cells were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in complete medium (DMEM supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 10% FBS) at 37°C in a humidified incubator with 5% CO_2. Vehicle, LPS (100 ng/mL), IFN-γ (0.5 U/mL), and/or α-MSH (100 nmol/L) were added to the cells as indicated in the text and figure legends. This concentration of α-MSH was selected because it has been shown to inhibit NOS2 mRNA expression in these cells [15].

Preparation of cytoplasmic and nuclear extracts

Cytoplasmic and nuclear extracts were prepared from RAW 264.7 cells treated with vehicle, α-MSH, LPS + IFN-γ, or α-MSH + LPS + IFN-γ, as detailed in our earlier work [20]. Briefly, cells were washed twice with phosphate-buffered saline (PBS), scraped from the dish, and pelleted. The cells were resuspended in CE buffer [10 mmol/L Tris-Cl, pH 8.0, 60 mmol/L KCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 0.1% NP-40] and were allowed to swell on ice for 15 minutes. After brief vortexing, the lysed cells were centrifuged at 3000 × *g* for five minutes at 4°C. The resulting supernatant containing the cytosolic extract was collected and stored at −70°C until further use. The pellet containing the nuclear lysates was washed once with CE buffer lacking NP-40, resuspended in NE buffer (20 mmol/L Tris-Cl, pH 7.9, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 420 mmol/L NaCl, 25% glycerol, and 0.5 mmol/L PMSF), and mixed on an orbital shaker for 20 minutes at 4°C. The nuclear lysates were then centrifuged at 12,000 × *g* for 20 minutes at 4°C, and the supernatant containing the nuclear protein extract was collected and stored at −70°C. Protein contents of the extracts were assayed with the BCA-protein estimation kit.

Electrophoretic mobility shift assays (EMSAs) and supershift assays

To identify C/EBP protein-DNA complexes, binding reactions (20 μL total) were performed by incubating 10 μg nuclear extract protein with 1.75 pmol of [γ-^{32}P]-ATP end-labeled C/EBP box DNA probe corresponding to the −157 to −136 of the murine NOS2 promoter (sense strand, 5′-CACAGAGTGATGTAATCAAGCA-3′) in reaction buffer [25 mmol/L HEPES, pH 8.0, 50 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L $MgCl_2$, 1 mmol/L DTT, 10% glycerol, and 50 μg/mL poly(dI-dC)-poly (dI-dC)] for 30 minutes at room temperature [20]. For NF-κB EMSAs, 0.5 ng of [γ-^{32}P]-ATP end-labeled consensus NF-κB probe (5′-AGTTGAGGGGACTTTCC CAGGC-3′) were added to a 20 μL (final) reaction mixture containing 10 μg nuclear extract in 10 mmol/L Tris buffer (pH 7.5) with 50 mmol/L NaCl, 1 mmol/L DTT, 1 mmol/L EDTA, 5% glycerol, and 50 μg/mL of poly(dI-dC)-poly (dI-dC)] for 20 minutes at room temperature. For competition studies, a 50-fold molar excess of specific or heterologous (Oct-1) oligomer was included in the binding reaction. For supershift assays, 1 μL (10 μg) of IgG directed against C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, or NF-κB p50 was added to the binding mixture after incubating with labeled probe. The mixture was further incubated for 30 minutes at room temperature. The DNA-protein complexes were resolved on 5% polyacrylamide gels in 0.5 × Tris-Borate-EDTA buffer. The gels were dried and exposed to x-ray film for autoradiography to detect the shifted bands.

Immunoblot analysis

Twenty microgram samples of nuclear or cytoplasmic extracts were diluted in electrophoresis sample buffer, boiled for five minutes, and electrophoresed through 0.1% sodium dodecyl sulfate (SDS)-7.5% polyacrylamide gels. The proteins were electrophoretically transferred to nitrocellulose membranes (Hybond ECL; Amersham). Equality of sample loading and blotting was verified by Ponceau S staining of the membranes. The blots were first incubated in blocking solution (PBS containing 1% bovine serum albumin and 0.1% Tween 20) for one hour at room temperature and then in blocking solution containing anti-C/EBPβ antibody (5 μg/mL) for one hour at room temperature. The blots were washed extensively with a solution containing 10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, and 0.1% Tween 20. The antigen-antibody complexes were detected by the enhanced chemiluminescence protocol using horseradish peroxidase-conjugated goat antirabbit IgG as secondary antibody.

Transient transfection of RAW 264.7 cells and reporter gene assays

RAW 264.7 cells grown in six-well plates were transiently transfected with a total of 5 μg/well of plasmid DNAs using LipoFectamine Plus™ reagent according to the manufacturer's protocol. pNOS2-luc, pNOS2-C/EBPdel-luc, pNOS2-ΔC/EBP-luc, or the parent vector

pGL3-Basic, which lacks eukaryotic promoter and enhancer sequences, were added at 4.5 μg/well. For comparative purposes, the cells were cotransfected with the *Renilla* luciferase expression plasmid with pRL-TK (0.5 μg/well) to control for transfection efficiency and other assay-to-assay variability. Parametric studies (data not shown) revealed that the promoter activity of the pRL-TK plasmid was not affected by α-MSH or LPS + IFN-γ. Twenty-four hours after transfection, the medium was replaced with complete medium containing vehicle, α-MSH, LPS + IFN-γ, or α-MSH + LPS + IFN-γ. Twenty-four hours later, whole cell lysates were prepared using Passive Lysis Buffer (Promega) according to the manufacturer's directions. Firefly and *Renilla* luciferase activities in 100 μL lysate samples were measured in a Turner Systems 20/20 luminometer using the Dual-Luciferase™ Reporter Assay System following the manufacturer's protocol. After background subtraction, firefly luciferase activity was normalized for *Renilla* luciferase activity and protein content of the lysates. The results were recorded as "normalized *NOS2* promoter activity." Each experimental observation represents the mean of duplicate determinations.

Data analysis

Imaging and quantitation of bands were performed using an image analysis system (Bio Image, Ann Arbor, MI, USA). Quantitative data are presented as means ± SE and were analyzed for statistical significance by analysis of variance (ANOVA). $P < 0.05$ was considered significant.

RESULTS

α-MSH inhibits induction of C/EBPβ, but not NF-κB, DNA binding activity in RAW 264.7 cells

Studies in a variety of cell lines have demonstrated that LPS and/or cytokines induce nuclear activity of C/EBPβ. To determine whether induction of C/EBPβ also occurs in RAW 264.7 cells, nuclear extracts were prepared from RAW 264.7 cells that had been treated for four hours with vehicle or LPS + IFN-γ, and EMSAs with a double-stranded oligonucleotide probe corresponding to the *NOS2* promoter −150 to −142 C/EBP box were performed. Four hours of induction was selected because combinatorial interactions among *trans*-acting factors are known to be important for transcriptional regulation of the *NOS2* gene, and previous in vivo genomic footprinting studies in LPS-treated RAW 264.7 cells demonstrated that at least three hours of induction are required for the full complement of inducible transcription factors to bind to the murine *NOS2* promoter/enhancer region [36]. As seen in Figure 1A, LPS + IFN-γ induced sequence-specific DNA–protein interactions for C/EBP proteins. Scanning densitometry of the C/EBP-

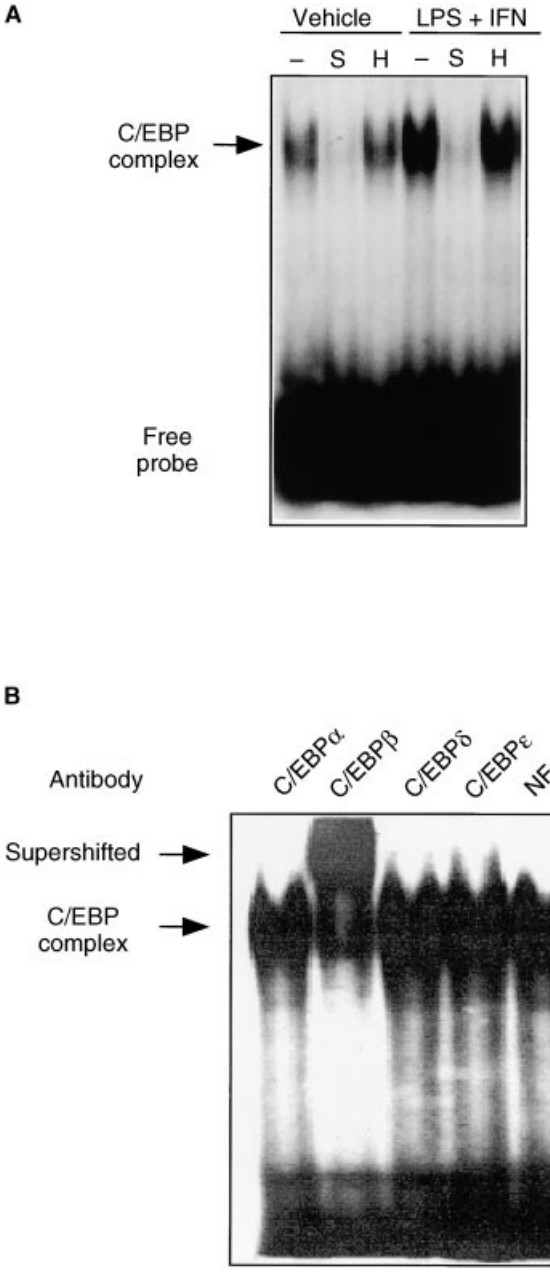

Fig. 1. Lipopolysaccharide (LPS) + interferon-γ (IFN-γ) promotes increased C/enhancer binding protein β (EBPβ) DNA binding activity in nuclear protein extracts of RAW 264.7 cells. (*A*) Nuclear proteins extracted from RAW 264.7 cells that had been exposed to vehicle or LPS (100 ng/mL) + IFN-γ (0.5 U/mL) for four hours were subjected to EMSA with a ^{32}P-labeled oligomer containing the −150 to −142 C/EBP box of the murine *NOS2* promoter. To demonstrate binding specificity, reactions were also conducted in the presence of a 50-fold molar excess of unlabeled C/EBP box oligomer (S) or heterologous (H) oligomers ($N = 4$). (*B*) Polyclonal IgGs specific for C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε and nuclear factor-κB (NF-κB) p50, p65, and c-Rel were used in supershift experiments with nuclear extracts from LPS + IFN-γ-treated (4 h) RAW 264.7 cells and the ^{32}P-labeled murine *NOS2* C/EBP box oligomer ($N = 3$).

specific protein-DNA complexes revealed an approximate fourfold induction (control, 2.6 ± 1.4 relative O.D. units; LPS + IFN-γ, 11.2 ± 1.6 relative O.D. units; $P < 0.05$). Since all C/EBP family members α, β, δ, γ, and ϵ recognize the consensus sequence of the C/EBP box probe [23] and since C/EBPβ has been shown to activate *NOS2* gene transcription [20, 37, 38], we used supershift assays with antisera for C/EBPα, C/EBPβ, C/EBPδ, and C/EBPϵ to identify the C/EBP isoform(s) that contributed to the DNA-protein complex. Figure 1B demonstrates that only the anti-C/EBPβ IgG supershifted the C/EBP-DNA complex. These results indicated that C/EBPβ was the major, if not only, C/EBP isoform bound to the DNA probe. Similarly, since NF-κB p50 is known to heterodimerize with C/EBPβ in some cell types [39], we also sought to determine whether the C/EBP-DNA gel shift complex included this protein. Supershift assays with the C/EBP box oligomer and antisera against CREB and NF-κB p50 showed no detectable supershift (Fig. 1B). Thus, C/EBPβ homodimers principally, if not exclusively, bind to the −150 to −142 C/EBP box of the *NOS2* promoter.

To evaluate whether α-MSH could inhibit the induction of C/EBP-DNA binding activity, cells were treated for 12 hours with vehicle or α-MSH and were then stimulated with LPS + IFN-γ. α-MSH (100 nmol/L) dramatically suppressed the LPS + IFN-γ induction of C/EBP-DNA binding activity (Fig. 2A, B). In contrast, direct addition of α-MSH to the binding reaction had no effect on C/EBP-DNA binding activity in the nuclear extracts from the LPS + IFN-γ–treated cells (Fig. 2C), indicating that intracellular signaling intermediates were required for the effect. Similar inhibitory effects on LPS + IFN-γ induction of C/EBP-DNA binding activity were observed when α-MSH was given simultaneously with LPS + IFN-γ ($N = 3$; data not shown).

Nuclear factor-κB is known to be an important *trans*-activator of the *NOS2* gene in immune-activated RAW 264.7 cells [17, 18], and its induction is known to be inhibited by α-MSH in several cell types [28, 31, 32]. Therefore, we tested the effects of α-MSH on LPS + IFN-γ–mediated induction of NF-κB in RAW 264.7 cells. For cells treated with vehicle or α-MSH alone, a single NF-κB protein DNA complex was observed. LPS + IFN-γ treatment induced two additional κB-specific complexes. α-MSH given 12 hours before or simultaneously with LPS + IFN-γ had no significant effect on the induction of NF-κB protein-DNA complexes in nuclei harvested from these cells (Fig. 2D).

α-MSH does not inhibit C/EBPβ protein expression or apparent nuclear translocation

The short-term control of C/EBPβ includes regulation of translation [40], nuclear translocation [23], and phosphorylation state [41–43]. To determine whether decrements in protein abundance or nuclear translocation contributed to the effects of α-MSH to inhibit C/EBPβ DNA binding activity, immunoblot analysis was performed on cytosolic and nuclear extracts prepared from RAW 264.7 cells treated with LPS + IFN-γ in the presence or absence of α-MSH. If α-MSH inhibited nuclear translocation of C/EBPβ, more C/EBPβ protein would be observed in the cytosolic fraction and less in the nuclear fraction compared to controls. As depicted in Figure 3, neither the relative abundance nor the relative ratios of C/EBPβ in the nuclear and cytoplasmic extracts differed between the two groups. Moreover, only the full-length C/EBPβ protein was evident in the blots, indicating that the internal splice variant LIP (for liver-enriched transcriptional inhibitory protein) is not expressed under these conditions.

α-MSH inhibits LPS + IFN-γ induction of *NOS2* promoter activity

Previous work demonstrated that RAW 264.7 cells treated with α-MSH + LPS + IFN-γ exhibited lower steady-state levels of NOS2 mRNA compared with LPS + IFN-γ–treated controls [15]. However, it was not determined whether reduced rates of *NOS2* gene transcription accounted for the result. To determine directly whether α-MSH inhibits the promoter activity of the *NOS2* gene, the effects of α-MSH on the LPS + IFN-γ inducibility of the *NOS2* promoter-luciferase construct pNOS2-luc transiently transfected into RAW 264.7 cells were tested. We elected to assay promoter activities rather than nuclear run-on transcription rates, because the former is more sensitive and quantifiable in detecting changes in gene transcription. As expected from the work of others, *NOS2* promoter activity was dramatically stimulated by LPS + IFN-γ in the vehicle-cotreated cells (Fig. 4) [18]. In the presence of α-MSH, however, LPS + IFN-γ induction of *NOS2* promoter activity was only approximately 40% of that of the vehicle-cotreated cells, indicating that α-MSH dramatically inhibited *NOS2* gene transcription. Moreover, α-MSH had no measurable effect on basal *NOS2* promoter activity. In control experiments, none of these maneuvers had a significant effect on the luciferase activity of the parent vector pGL3-Basic (Fig. 4).

α-MSH inhibits LPS + IFN-γ induction of *NOS2* promoter activity via a C/EBPβ-dependent mechanism

We previously demonstrated that the −150 to −142 C/EBP box of the murine *NOS2* promoter is functionally important for *trans*-activation of the *NOS2* gene in renal epithelial cells [20]. To determine whether similar events occur in murine macrophages, RAW 264.7 cells were transfected with pNOS2-luc pNOS2-C/EBPdel-luc, which contains a mutated *NOS2* promoter lacking the C/EBP box, or pNOS2-ΔC/EBP-luc, which contains a mutated

NOS2 promoter harboring a six-nucleotide mutation in the C/EBP box. As shown in Figure 5, deletion or mutation of the −150 to −142 C/EBP box reduced *NOS2* promoter activity by roughly 60%, indicating that C/EBPβ contributes to *trans*-activation of the *NOS2* gene in RAW 264.7 cells, as has been reported in mTAL [20] and mesangial cells [37].

Given the gel shift data demonstrating no effect of α-MSH on induction of NF-κB (Fig. 2D), α-MSH would be expected to have little inhibitory effect on the LPS + IFN-γ inducibility of the *NOS2* C/EBP mutation/deletion promoter constructs if the ability of α-MSH to inhibit C/EBPβ DNA-binding activity was the principal mechanism by which the neuropeptide inhibited *NOS2* promoter activity. Indeed, as shown in Figure 5, α-MSH did not significantly alter the activity of pNOS2-C/EBPβ-del-luc or pNOS2-ΔC/EBPβ-luc in LPS + IFN-γ–treated RAW 264.7 cells. Thus, the inhibitory effect of α-MSH on *NOS2* transcriptional activity under these conditions appears to be principally the result of inhibition of C/EBPβ *trans*-activation of the *NOS2* gene.

DISCUSSION

Using a murine macrophage cell line to dissect the mechanisms by which α-MSH inhibits *NOS2* gene expression, we discovered that this neuropeptide inhibits LPS + IFN-γ induction of C/EBPβ DNA-binding activity and severely limits transcriptional activation of the *NOS2* gene by these stimuli. The parallel reductions in C/EBPβ-DNA binding activity (Fig. 2) and *NOS2* promoter activity (Fig. 4), coupled with the failure of α-MSH to inhibit LPS + IFN-γ inducibility of nuclear NF-κB DNA binding activity and of *NOS2* promoter constructs bearing a disrupted −150 to −42 C/EBP box (Fig. 5) strongly suggest that α-MSH inhibits C/EBPβ-mediated *trans*-activation of the *NOS2* gene. These results extend mechanistically previous studies that demonstrated an effect of α-MSH to limit the expression of steady-state levels of NOS2 mRNA in RAW 264.7 cells [15], and to our knowledge, they represent the first confirmed example of peptide inhibition of *NOS2* gene transcription in mammalian cells. Since α-MSH did not affect LPS + IFN-γ–mediated induction of NF-κB in RAW 264.7 cells, the effect of the neuropeptide on C/EBPβ is not a generalized effect on inducible transcription factors. Our results also predict that α-MSH may inhibit the induction of other C/EBPβ-responsive genes, such as cyclooxygenase-2 [44], CD11C/CD18 integrin [46], IL-4 [47], IL-6 [48], macrophage inflammatory protein 1α [49], involved in inflammation, injury, and immunity.

The fact that the supershift with the anti-C/EBPβ antibody was virtually complete (Fig. 1B), that antisera against C/EBPα, C/EBPδ, and C/EBPε failed to supershift the C/EBP box complexes (Fig. 1B), and that LIP protein

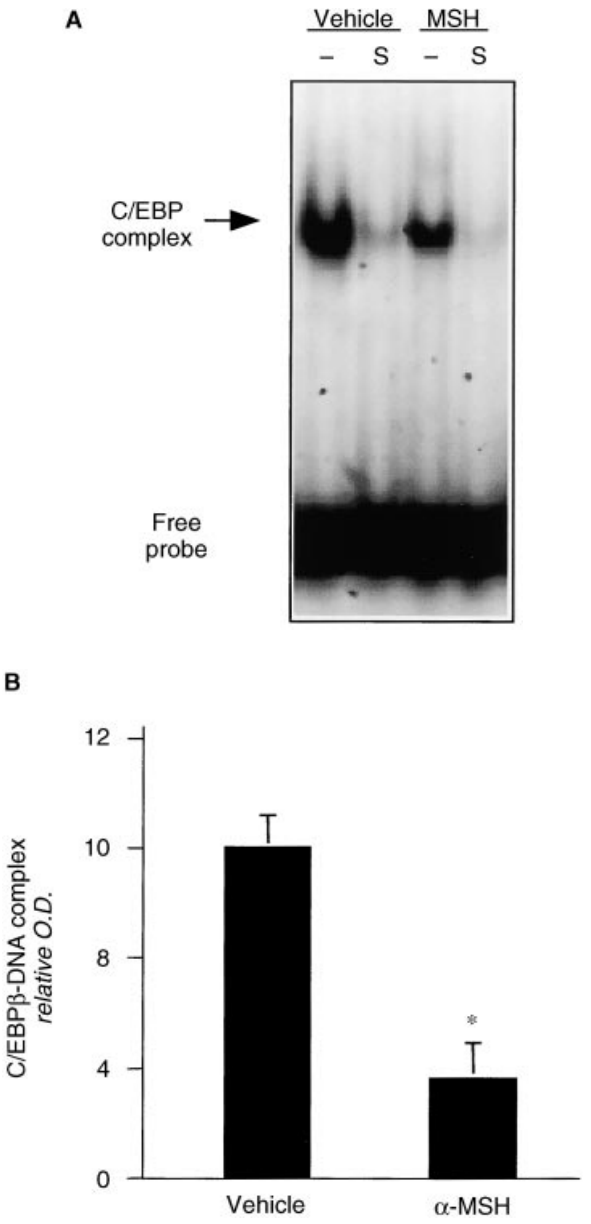

Fig. 2. α-Melanocyte-stimulating hormone (α-MSH) inhibits the DNA-binding activity of C/EBPβ, but not NF-κB, in nuclear protein extracts of RAW 264.7 cells. (*A*) Nuclear proteins extracted from RAW 264.7 cells that had been exposed to vehicle, α-MSH, LPS + IFN-γ, or LPS + IFN-γ + α-MSH were subjected to EMSA with ^{32}P-labeled oligomers for the −150 to −142 C/EBP box of the murine *NOS2* promoter ($N = 5$). In the representative autoradiograph shown, vehicle or α-MSH was given 12 hours before a 4-hour incubation with LPS + IFN-γ. Similar results were obtained when vehicle or α-MSH was given simultaneously with the LPS + IFN-γ. (*B*) Histogram showing results of densitometric analysis of EMSAs of experiments depicted in (A). The relative O.D. of the C/EBP-specific bands are plotted. $^*P < 0.05$ vs. control ("α-MSH"). (*C*) Nuclear extracts were prepared from RAW 264.7 cells treated with LPS + IFN-γ for four hours. EMSAs were performed by incubating the ^{32}P-labeled C/EBP box oligomer and the nuclear extracts directly with 100 nmol/L α-MSH ($N = 4$). (*D*) Nuclear extracts were prepared from RAW 264.7 cells treated with LPS + IFN-γ (L + I) for four hours. α-MSH (MSH, 100 nmol/L) was given either 12 hours (−12) before or simultaneously (0) with LPS + IFN-γ. EMSAs were performed by incubating the ^{32}P-labeled NF-κB consensus oligomer with the nuclear extracts ($N = 2$). Competition studies were performed with a 50-fold molar excess of unlabeled NF-κB oligomer (S).

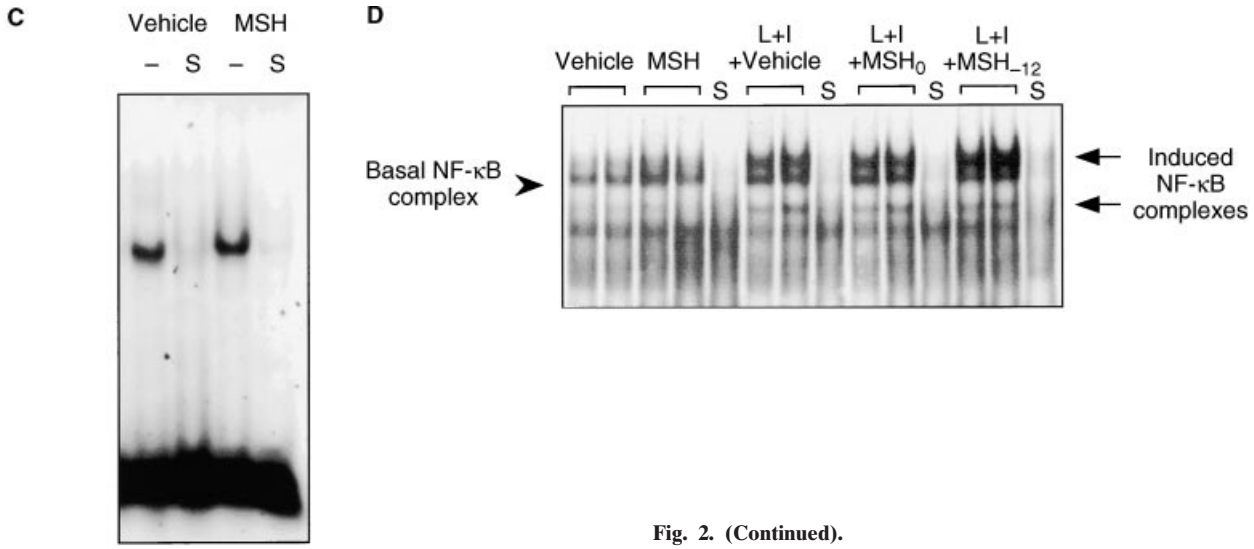

Fig. 2. (Continued).

was not observed on immunoblots of nuclear extracts suggests that other C/EBP family members did not contribute significantly to the DNA binding activity we measured. The C/EBP proteins consist of an N-terminal *trans*-activating domain, a basic DNA-binding region, and a C-terminal leucine-zipper. Dimerization through the leucine-zipper leads to formation of homodimers and heterodimers, which then bind via their basic regions to DNA sequences in the promoter/enhancer regions of a variety of genes, including acute phase response genes in liver [50], cytokine genes in monocytes/macrophages [35], and *NOS2* in various cell types. Heterodimers formed between C/EBPβ and NF-κB p50 in vitro have been reported [39], but in supershift assays, we found no evidence for NF-κB protein family immunoreactivity in the C/EBP-specific complexes (Fig. 1B). Thus, the observed protein-DNA complexes in RAW 264.7 cells contained principally, if not exclusively, C/EBPβ homodimers.

The short-term regulation of C/EBPβ is complex and incompletely defined, and includes controls of translation, phosphorylation state, interaction with regulatory nuclear proteins, and transport to the target genes [23]. The fact that α-MSH had no effect on C/EBPβ-DNA binding activity when added directly to the DNA-binding reaction (Fig. 2C) indicates that intracellular signaling intermediates and mechanisms are required for the inhibition of C/EBPβ DNA-binding activity. Theoretically, the inhibitory actions of α-MSH on C/EBPβ-DNA binding activity and *NOS2 trans*-activation could arise from reduced C/EBPβ protein expression, impaired nuclear translocation, up-regulation of LIP, or a novel post-translational mechanism that results in reduced DNA-binding capacity of the transcription factor. Since α-MSH altered neither the relative abundance nor the apparent partitioning of C/EBPβ protein in the cytoplasmic and nuclear extracts (Fig. 3), it seems unlikely that the neuropeptide exerts its actions by inhibiting C/EBPβ protein expression or nuclear translocation. Similarly, since we did not observe LIP protein on immunoblots, α-MSH does not up-regulate this transcriptional inhibitor. We conclude then that α-MSH must act at a more downstream mechanism(s) that impairs the DNA-binding capacity of C/EBPβ. The specific mechanism remains to be defined. It should be noted, however, that our methods did not allow us to distinguish a separate effect of α-MSH to inhibit the transcriptional efficacy of C/EBPβ. Phosphorylation by ras-dependent MAPKs [43], protein kinase C [42], Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) [51], and certain oncogene kinases [52] is known to enhance the *trans*-acting potential of C/EBPβ in vitro. Similarly, phosphorylation by MAPKs and kinase oncogenes neutralizes the inhibitory domains of C/EBPβ, unmasking latent transcriptional efficacy of the factor [52]. Further studies are needed to directly test the effects of α-MSH on these pathways.

In contrast to reports in other cell lines [28, 31, 32] and brain tissue [29], in which cytokines or LPS were used to provoke NF-κB activation, α-MSH did not inhibit LPS + IFN-γ induction of NF-κB in the nucleus of RAW 264.7 cells. The reasons for this difference are unclear and require further investigation. The activation of NF-κB complexes is achieved through phosphorylation and subsequent polyubiquitination and degradation of the inhibitory component IκB. The released NF-κB complexes then translocate to the nucleus where they *trans*-activate target genes. One possibility is that α-MSH fails to inhibit IκBα degradation in RAW 264.7 cells, as it does in other cell types [28, 32], because of selective effects on specific, and differentially expressed IκB ki-

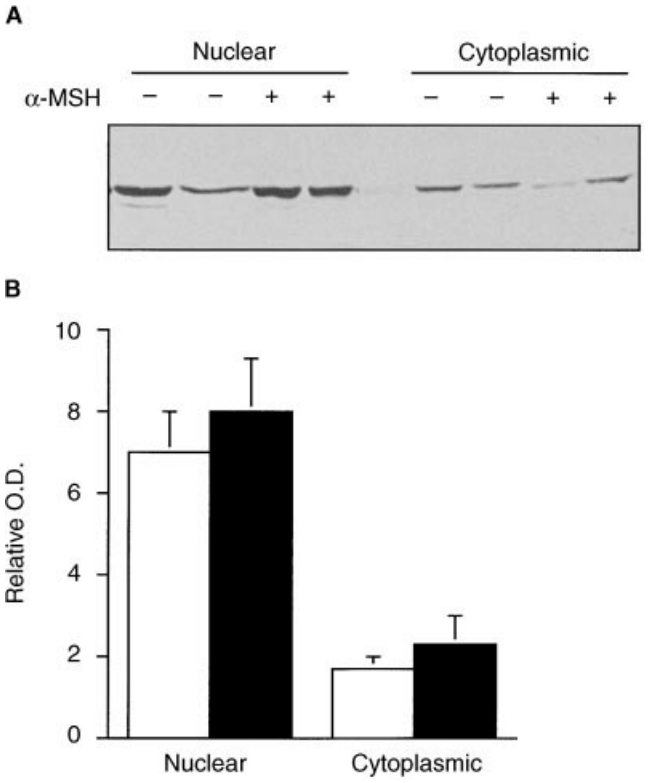

Fig. 3. Effects of α-MSH on C/EBPβ protein expression in cytosolic and nuclear extracts of LPS + IFN-γ-treated RAW 264.7 cells. (*A*) Immunoblots of nuclear and cytoplasmic extracts prepared from RAW 264.7 cells treated with LPS + IFN-γ or LPS + IFN-γ + α-MSH were probed with anti-C/EBPβ IgG and peroxidase-conjugated goat antirabbit IgG. The α-MSH was given 12 hours before the 4-hour incubation with LPS + IFN-γ. Signal was detected by the ECL method. (*B*) Densitometric analysis of immunoblots. The relative O.D. of the C/EBPβ bands are plotted ($N = 3$). Symbols are: (□) vehicle; (■) α-MSH.

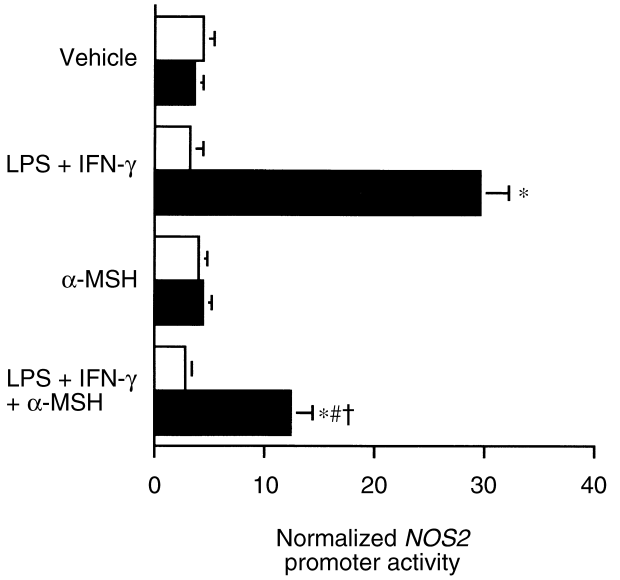

Fig. 4. α-MSH inhibits LPS + IFN-γ–mediated activation of the *NOS2* promoter. (*A*) RAW 264.7 cells were transiently cotransfected with pNOS2-luc, containing the wild-type murine *NOS2* promoter (□) or the promoterless parent vector, pGL3-Basic together with pTK-RL (■; to normalize for transfection efficiency). The transfected cells were treated for 24 hours with LPS + IFN-γ + vehicle or LPS + IFN-γ + α-MSH given simultaneously. Firefly and *Renilla* luciferase activities in lysates of the cells were then determined in a luminometer, normalized to cell protein content, and reported as "normalized promoter activity." The mean (bars) + SE of four separate experiments is shown. $*P <$ 0.05 vs. vehicle-treated; $\#P < 0.05$ vs. LPS + IFN-γ treated; $\dagger P < 0.05$ vs. α-MSH treated.

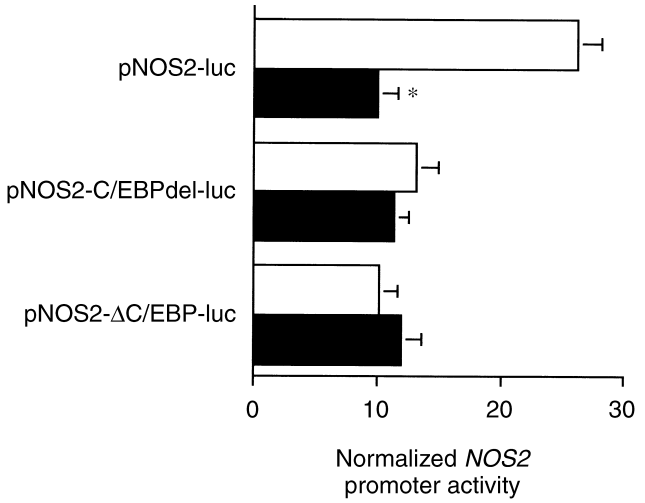

Fig. 5. α-MSH inhibits LPS + IFN-γ–mediated activation of the *NOS2* promoter via a C/EBP-dependent mechanism. RAW 264.7 cells were transiently cotransfected with pTK-RL together with one of the following three promoter-luciferase constructs: pNOS2-luc, pNOS2-C/EBP-del-luc, containing a mutated *NOS2* promoter lacking the −150 to −142 C/EBP box; or pNOS2-ΔC/EBP-luc, containing the *NOS2* promoter bearing a six-nucleotide mutation in the −150 to −142 C/EBP box. The transiently transfected cells were stimulated for 24 hours with LPS + IFN-γ + vehicle or LPS + IFN-γ + α-MSH given simultaneously, and normalized promoter activity was measured ($N = 4$). Symbols are: (□) vehicle; (■) α-MSH ; $*P < 0.05$ vs. pNOS2 transfected, LPS + IFN-γ + vehicle treated.

nases. Recent work in RAW 264.7 cells, for example, identified a novel LPS-inducible IκB kinase, termed IKK-*i*, that is differentially expressed among LPS-treated cell types and tissues [53]. It is highly induced by LPS in RAW 264.7 cells, spleen, and peripheral blood leukocytes, but is only weakly induced in brain, a tissue in which the inhibitory effects of α-MSH on the induction of NF-κB have been observed [29]. It is also noteworthy that Manna and Aggarwal observed significant differences among the cell types they tested in the ability of the neuropeptide to inhibit TNF-dependent induction of NF-κB [32]. α-MSH almost completely abolished NF-κB induction in U-937, HeLa, and H4 glioma cells, but only partially inhibited NF-κB induction in Jurkat cells. In the aggregate, the existing data suggest that cell-type–specific mechanisms define the molecular means by which α-MSH elicits its anti-inflammatory actions.

Our finding that α-MSH inhibits LPS + IFN-γ induction of *NOS2* promoter activity establishes that α-MSH

exerts major effects on transcription of the murine *NOS2* gene. Although the magnitude of the reduction in *NOS2* promoter activity could solely account for the decrements in NOS2 mRNA levels observed by Star et al [15], further studies are required to test whether α-MSH also destabilizes NOS2 mRNA in RAW 264.7 cells. For example, Weisz et al reported that changes in both *NOS2* gene transcription and mRNA half-life are important determinants of the steady-state levels of NOS2 mRNA in RAW 264.7 cells subjected to LPS + IFN-γ treatment [54]. While much is known about how *NOS2* is transcriptionally induced, very little is known about how the gene, once stimulated, is inactivated. The present data, together with earlier work [15], disclose a host mechanism by which this endogenous neuropeptide can restrict or terminate NO biosynthesis in activated macrophages. Such a mechanism might serve to preserve the functional integrity of macrophages during the inflammatory response, since high NO concentrations are known to be autotoxic to macrophages. Macrophages, then, have the capacity to secrete under immune challenge two peptides, α-MSH and atrial natriuretic peptide [55], that inhibit induction of NOS2 expression, apparently by targeting different regulatory controls important for *NOS2* gene activation. In addition, since macrophage infiltration is a hallmark of ischemia-reperfusion injury to the kidney [56], the present results disclose a possible molecular mechanism by which α-MSH attenuates postischemic acute renal failure in rodents.

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REFERENCES

1. Lipton JM, Catania A: Mechanisms of antiinflammatory action of the neuroimmunomodulatory peptide α-MSH. *Ann NY Acad Sci* 840:373–380, 1998
2. Catania A, Gerloni V, Procaccia S, Airaghi L, Manfredi MG, Lomater C, Grossi L, Lipton JM: The anticytokine neuropeptide α-melanocyte-stimulating hormone in synovial fluid of patients with rheumatic diseases: Comparisons with other anticytokine molecules. *Neuroimmunomodulation* 1:321–328, 1994
3. Catania A, Suffredini AF, Lipton JM: Endotoxin causes release of α-melanocyte stimulating hormone in normal human subjects. *Neuroimmunomodulation* 2:258–262, 1995
4. Ceriani G, Diaz J, Murphree S, Catania A, Lipton JM: The neuropeptide α-melanocyte stimulating hormone inhibits experimental arthritis in rats. *Neuroimmunomodulation* 1:28–32, 1994
5. Chiao H, Foster S, Thomas R, Lipton J, Star RA: α-Melanocyte-stimulating hormone reduces endotoxin-induced liver inflammation. *J Clin Invest* 97:2038–2044, 1996
6. Rajora N, Boccoli G, Burns D, Sharma S, Catania AP, Lipton JM: α-MSH modulates local and circulating tumor necrosis factor-α in experimental brain inflammation. *J Neurosci* 17:2181–2186, 1997
7. Rajora N, Boccoli G, Catania A, Lipton JM: α-MSH modulates experimental inflammatory bowel disease. *Peptides* 18:381–385, 1997
8. Chiao H, Kohda Y, McLeroy P, Craig L, Housini I, Star RA: α-Melanocyte-stimulating hormone protects against renal injury after ischemia in mice and rats. *J Clin Invest* 99:1165–1172, 1997
9. Chiao H, Kohda Y, McLeroy P, Craig L, Linas S, Star RA: α-Melanocyte-stimulating hormone inhibits renal injury in the absence of neutrophils. *Kidney Int* 54:765–774, 1998
10. Kwon T-H, Frokiaer J, Fernandez-Llama P, Knepper MA, Nielsen S: Reduced abundance of aquaporins in rats with bilateral ischemia-induced acute renal failure: Prevention by α-MSH. *Am J Physiol* 277(3 Pt 2):F413–F427, 1999
11. Taylor AW, Streilein JW, Cousins SW: α-Melanocyte stimulating hormone suppresses antigen stimulated T cell production of gamma interferon. *Neuroimmunomodulation* 1:188–194, 1994
12. Robertson BA, Gahring LC, Daynes RA: Neuropeptide regulation of interleukin 1 activities: Capacity of alpha melanocyte stimulating hormone to inhibit interleukin 1 inducible responses in vivo and in vitro exhibits target cell selectivity. *Inflammation* 10:371–385, 1986
13. Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, Akira S: Transcription factors NF-IL6 and NF-κB synergistically activate transcription of the inflammatory cytokines, interleukin-6 and interleukin-8. *Proc Natl Acad Sci USA* 90:10193–10197, 1993
14. Rajora N, Ceriani G, Catania A, Star RA, Murphy MT, Lipton JM: α-MSH production, receptors, and influence on neopterin in a human monocyte/macrophage cell line. *J Leukoc Biol* 59:248–253, 1996
15. Star RA, Rajora N, Huang J, Stock RC, Catania A, Lipton JM: Evidence of autocrine modulation of macrophage nitric oxide synthase by α-melanocyte-stimulating hormone. *Proc Natl Acad Sci USA* 92:8016–8020, 1995
16. Kone BC: Nitric oxide in renal health and disease. *Am J Kidney Dis* 30:311–333, 1997
17. Xie QW, Kashiwabara Y, Nathan C: Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J Biol Chem* 269:4705–4708, 1994
18. Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, Russell SW, Murphy WJ: Macrophage nitric oxide synthase gene: Two upstream regions mediate induction by interferon-γ and lipopolysaccharide. *Proc Natl Acad Sci USA* 90:9730–9734, 1993
19. Kone BC, Schwobel J, Turner P, Mohaupt MG, Cangro CB: Role of NF-κB in the regulation of inducible nitric oxide synthase in an MTAL cell line. *Am J Physiol* 269:F718–F729, 1995
20. Gupta AK, Kone BC: CCAAT/enhancer binding protein-β transactivates murine nitric oxide synthase-2 gene in an MTAL cell line. *Am J Physiol* 276:F599–F605, 1999
21. Cao Z, Umek RM, McKnight SL: Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev* 5:1538–1552, 1991
22. Alam T, An MR, Papaconstantinou J: Differential expression of three C/EBP isoforms in multiple tissues during the acute phase response. *J Biol Chem* 267:5021–5024, 1992
23. Wedel A, Ziegler Heitbrock HW: The C/EBP family of transcription factors. *Immunobiology* 193:171–185, 1995
24. Yan S-F, Zou YS, Mendelsohn M, Gao Y, Naka Y, Yan SD, Pinsky D, Stern D: Nuclear factor-IL6 motifs mediate tissue-specific gene transcription in hypoxia. *J Biol Chem* 272:4287–4294, 1997
25. Zador IZ, Hsieh CC, Papaconstantinou J: Renal CCAAT/enhancer binding proteins in experimental diabetes mellitus. *Nephron* 79:312–316, 1998
26. Sonoki T, Nagasaki A, Gotoh T, Takiguchi M, Takeya M, Matsuzaki H, Mori M: Coinduction of nitric-oxide synthase and arginase I in cultured rat peritoneal macrophages and rat tissues in vivo by lipopolysaccharide. *J Biol Chem* 272:3689–3693, 1997
27. Hecker M, Preiss C, Schini-Kerth VB: Induction by staurosporine of nitric oxide synthase expression in vascular smooth muscle cells:

Role of NF-κB, CREB and C/EBPβ. *Br J Pharmacol* 120:1067–1074, 1997
28. Ichiyama T, Zhao H, Catania A, Furukawa S, Lipton JM: α-Melanocyte-stimulating hormone inhibits NF-κB activation and IκBα degradation in human glioma cells and in experimental brain inflammation. *Exp Neurol* 157:359–365, 1999
29. Ichiyama T, Sakai T, Catania A, Barsh GS, Furukawa S, Lipton JM: Systemically administered α-melanocyte-stimulating peptides inhibit NF-κB activation in experimental brain inflammation. *Brain Res* 836:31–37, 1999
30. Ichiyama T, Sakai T, Catania A, Barsh GS, Furukawa S, Lipton JM: Inhibition of peripheral NF-κB activation by central action of α-melanocyte-stimulating hormone. *J Neuroimmunol* 99:211–217, 1999
31. Ichiyama T, Campbell IL, Furukawa S, Catania A, Lipton JM: Autocrine α-melanocyte-stimulating hormone inhibits NF-κB activation in human glioma. *J Neurosci Res* 58:684–689, 1999
32. Manna SK, Aggarwal BB: α-Melanocyte-stimulating hormone inhibits the nuclear transcription factor NF-κB activation induced by various inflammatory agents. *J Immunol* 161:2873–2880, 1998
33. Chini BA, Fiedler MA, Milligan L, Hopkins T, Stark JM: Essential roles of NF-κB and C/EBP in the regulation of intercellular adhesion molecule-1 after respiratory syncytial virus infection of human respiratory epithelial cell cultures. *J Virol* 72:1623–1626, 1998
34. Hu HM, Baer M, Williams SC, Johnson PF, Schwartz RC: Redundancy of C/EBP α, -β, and -δ in supporting the lipopolysaccharide-induced transcription of IL-6 and monocyte chemoattractant protein-1. *J Immunol* 160:2334–2342, 1998
35. Pope RM, Leutz A, Ness SA: C/EBPβ regulation of the tumor necrosis factor-α gene. *J Clin Invest* 94:1449–1455, 1994
36. Goldring CE, Reveneau S, Algarte M, Jeannin JF: *In vivo* footprinting of the mouse inducible nitric oxide synthase gene: Inducible protein occupation of numerous sites including October and NF-IL6. *Nucleic Acids Res* 24:1682–1687, 1996
37. Eberhardt W, Pluss C, Hummel R, Pfeilschifter J: Molecular mechanisms of inducible nitric oxide synthase gene expression by IL-1β and cAMP in rat mesangial cells. *J Immunol* 160:4961–4969, 1998
38. Sakitani K, Nishizawa M, Inoue K, Masu Y, Okumura T, Ito S: Synergistic regulation of inducible nitric oxide synthase gene by CCAAT/enhancer-binding protein β and nuclear factor-κB in hepatocytes. *Genes Cells* 3:321–330, 1998
39. Leclair KP, Blanar MA, Sharp PA: The p50 subunit of NF-κB associates with the NF-IL6 transcription factor. *Proc Natl Acad Sci USA* 89:8145–8149, 1992
40. Lincoln AJ, Monczak Y, Williams SC, Johnson PF: Inhibition of CCAAT/enhancer-binding protein α and β translation by upstream open reading frames. *J Biol Chem* 273:9552–9560, 1998
41. Trautwein C, Caelles C, van der Geer P, Hunter T, Karin M, Chojkier M: Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. *Nature* 364:544–547, 1993
42. Trautwein C, van der Geer P, Karin M, Hunter T, Chojkier M: Protein kinase A and C site specific phosphorylations of LAP (NF-IL6) modulate its binding affinity to DNA recognition elements. *J Clin Invest* 93:2554–2561, 1994
43. Nakajima T, Kinoshita S, Sasagawa T, Sasaki K, Naruto M, Kishimoto T, Akira S: Phosphorylation at threonine 235 by a ras-dependent mitogen activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc Natl Acad Sci USA* 90:2207–2211, 1993
44. Sorli CH, Zhang H-J, Armstrong MB, Rajotte RV, Macloufs J, Robertson RP: Basal expression of cyclooxygenase-2 and nuclear factor-interleukin-6 are dominant and coordinately regulated by interleukin-1 in the pancreatic islet. *Proc Natl Acad Sci USA* 95:1788–1793, 1998
45. Deleted in proof.
46. Lopez Rodriguez C, Botella L, Corbi AL: CCAAT enhancer binding proteins (C/EBP) regulate the tissue specific activity of the CD11c integrin gene promoter through functional interactions with Sp1 proteins. *J Biol Chem* 272:29120–29126, 1997
47. Davydov IV, Krammer PH, Li Weber M: Nuclear factor-IL6 activates the human IL-4 promoter in T cells. *J Immunol* 155:5273–5279, 1995
48. Ray A, Ray BK: Lipopolysaccharide mediated induction of the bovine interleukin-6 gene in monocytes requires both NF-κB and C/EBP binding sites. *DNA Cell Biol* 14:795–802, 1995
49. Grove M, Plumb M: C/EBP, NF-κB, and c-Ets family members and transcriptional regulation of the cell specific and inducible macrophage inflammatory protein 1 alpha immediate early gene. *Mol Cell Biol* 13:5276–5289, 1993
50. Moshage H: Cytokines and the hepatic acute phase response. *J Pathol* 181:257–266, 1997
51. Wegner M, Cao Z, Rosenfeld MG: Calcium-regulated phosphorylation within the leucine zipper of C/EBPβ. *Science* 256:370–373, 1992
52. Kowenz-Leutz E, Twamley G, Ansieau S, Leutz A: Novel mechanism of C/EBPβ (NF-M) transcriptional control: Activation through depression. *Genes Dev* 8:2781–2791, 1994
53. Shimada T, Kawai T, Takeda K, Matsumoto M, Inoue J-i, Tatsumi Y, Kanamaru A, Akira S: IKK-*i*, a novel lipopolysaccharide-inducible kinase that is related to IκB kinases. *Int Immunol* 11:1357–1362, 1999
54. Weisz A, Oguchi S, Cicatiello L, Esumi H: Dual mechanism for the control of inducible type NO synthase gene expression in macrophages during activation by interferon-γ and bacterial lipopolysaccharide: Transcriptional and post-transcriptional regulation. *J Biol Chem* 269:8324–8333, 1994
55. Kiemer AK, Vollmar AM: Autocrine regulation of inducible nitric-oxide synthase in macrophages by atrial natriuretic peptide. *J Biol Chem* 273:13444–13451, 1998
56. Takada M, Nadeau KC, Shaw GD, Marquette KA, Tilney NL: The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney: Inhibition by a soluble P-selectin ligand. *J Clin Invest* 99:2682–2690, 1997