Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 8016–8020, August 1995
Medical Sciences

Evidence of autocrine modulation of macrophage nitric oxide synthase by α-melanocyte-stimulating hormone

(inflammation/tumor necrosis factor α/melanocortin receptors/cAMP/lipopolysaccharide)

ROBERT A. STAR*†, NILUM RAJORA‡, JIJING HUANG*, RENEE C. STOCK*, ANNA CATANIA§, AND J. M. LIPTON‡§

Departments of *Internal Medicine, ‡Physiology and Anesthesiology and Pain Management, University of Texas Southwestern Medical Center, Dallas, TX 75235; and §First Medical Clinic, University of Milan, Milan, Italy 20122

Communicated by S. M. McCann, University of Texas Southwestern Medical Center, Dallas, TX, May 8, 1995

ABSTRACT **α-Melanocyte-stimulating hormone (α-MSH) is a potent inhibitory agent in all major forms of inflammation. To identify a potential mechanism of antiinflammatory action of α-MSH, we tested its effects on production of nitric oxide (NO), believed to be a mediator common to all forms of inflammation. We measured NO and α-MSH production in RAW 264.7 cultured murine macrophages stimulated with bacterial lipopolysaccharide and interferon γ. α-MSH inhibited production of NO, as estimated from nitrite production and nitration of endogenous macrophage proteins. This occurred through inhibition of production of NO synthase II protein; steady-state NO synthase II mRNA abundance was also reduced. α-MSH increased cAMP accumulation in RAW cells, characteristic of α-MSH receptors in other cell types. RAW cells also expressed mRNA for the primary α-MSH receptor (melanocortin 1). mRNA for proopiomelanocortin, the precursor molecule of α-MSH, was expressed in RAW cells, and tumor necrosis factor α increased production and release of α-MSH. These results suggest that the proinflammatory cytokine tumor necrosis factor α can induce macrophages to increase production of α-MSH, which then becomes available to act upon melanocortin receptors on the same cells. Such stimulation of melanocortin receptors could modulate inflammation by inhibiting the production of NO. The results suggest that α-MSH is an autocrine factor in macrophages which modulates inflammation by counteracting the effects of proinflammatory cytokines.**

The neuropeptide α-melanocyte-stimulating hormone (α-MSH), a derivative of proopiomelanocortin (POMC), found in the pituitary, brain, skin, circulation, and other sites, has potent antiinflammatory activity. α-MSH reduces fever (1, 2) and the following: (*i*) acute inflammation caused by irritants or by local mediators, such as cytokines (3, 4); (*ii*) delayed hypersensitivity responses, a model of allergic reactions (5, 6); (*iii*) chronic inflammation (mycobacterium-induced arthritis) (7, 8); and (*iv*) systemic inflammation (e.g., endotoxemia and sepsis) (8, 9). Although α-MSH antagonizes the actions of proinflammatory cytokines (1, 2, 4, 9, 10), the precise mechanism of its antiinflammatory action is unknown. α-MSH was recently identified in synovial fluid of patients with rheumatoid arthritis (11). Thus, α-MSH is produced at sites of inflammation, although the cell type(s) responsible is unknown.

α-MSH functions via melanocortin (MC) receptors (12–18). There are five subtypes of MC receptors (MC-1 through MC-5). These receptors contain seven membrane-spanning domains typical of G-protein-coupled receptors, and are coupled to adenylate cyclase to generate cAMP. MC receptors are differentially expressed in a restricted distribution, with MC-1 identified in melanoma cells and melanocytes but not in brain, adrenal, or other tissues by mRNA blotting (12, 13).

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That α-MSH has a wide spectrum of antiinflammatory activity suggests that the peptide inhibits a critical agent or event that is common to multiple forms of inflammation. Nitric oxide (NO) is a likely target because it is induced in inflammatory cells by multiple proinflammatory cytokines whose actions are inhibited by α-MSH. Lipopolysaccharide (LPS) and/or cytokines can induce certain cells to produce large quantities of NO that are cytotoxic (19, 20). Expression of the inducible macrophage isoform of NO synthase (NOS) NOS-II, is increased in experimental and clinical systemic inflammatory reactions, experimental and clinical arthritis, pancreatic insulitis, and atherosclerotic lesions (see ref. 21). Some of the NO produced by activated macrophages reacts with superoxide to produce peroxynitrite, a powerful oxidant. Peroxynitrite reacts with tyrosine to form nitrotyrosine (22). Nitration of cellular proteins serves as a second "footprint" of NO and has been demonstrated in blood and synovial fluid of patients with rheumatoid arthritis (23).

We tested the influence of α-MSH on NO production in a stable murine macrophage cell line known to produce NO when stimulated with proinflammatory agents. These cells have the properties of normal macrophages (24, 25), including pinocytosis, phagocytosis, secretion of lysozymes, and antibody-dependent lysis of erythrocytes and tumor targets. We also sought to determine the molecular identity of macrophage MC receptors and the signal-transduction mechanism by which α-MSH may exert its antiinflammatory effects. This cell model was tested for evidence of an autocrine influence of α-MSH.

MATERIALS AND METHODS

Cell Culture. RAW 264.7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g of glucose per liter and supplemented with 1 mM sodium pyruvate, 4 mM L-glutamine, 10% (vol/vol) heat inactivated fetal bovine serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml (GIBCO/BRL). Experiments were performed on cells between passage 15 and 25.

Nitrite Production. RAW cells were seeded in 96-well plates at a density of 2.5×10^6 cells per well. Cells were exposed to combinations of LPS [10 ng/ml; *Salmonella typhosa* LPS W (0901, Difco)], interferon γ (IFN-γ) (0.5 units of human recombinant IFN-γ per ml; Boehringer Mannheim), α-MSH, or N^{ω}-methyl-L-arginine (L-NMMA; Sigma) for 8–16 h. Nitrite concentrations were determined by using Greiss reagent (26). All assays were performed on three or four replicates and repeated in at least three independent experiments.

Abbreviations: MSH, melanocyte-stimulating hormone; MC, melanocortin; NO, nitric oxide; NOS, NO synthase; RT, reverse transcriptase; LPS, lipopolysaccharide; IFN-γ, interferon γ; POMC, proopiomelanocortin; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NMMA, N^{ω}-methyl-L-arginine; TNF-α, tumor necrosis factor α.

†To whom reprint requests should be addressed at: University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8856.

Western Blotting. Cells were washed and the cell pellet was frozen at −70°C, sonicated in ice-cold cell lysis buffer [10 mM Hepes, pH 7.4/150 mM NaCl/5 mM EDTA/100 μM leupeptin/10 μg of aprotinin per ml (United States Biochemical)/10 μM E-64 (Calbiochem)/10 μg of diisopropyl fluorophosphate per ml], and centrifuged at 200,000 × *g* for 15 min, and the supernatant fraction was frozen at −70°C. Protein (40 μg per lane) was fractionated on a 7.5% polyacrylamide gel, transferred to nitrocellulose (Schleicher & Schuell), and blotted in 5% (wt/vol) nonfat dry milk in Tris-buffered saline (TBS; 20 mM Tris·HCl, pH 7.6/137 mM NaCl) containing 0.1% Tween 20 (TBST) for 1 h. Protein content was determined by the method of Lowry with bovine serum albumin as the standard (27). The nitrocellulose filters were incubated with a primary antibody (see below), washed, and then incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham). The reaction products were visualized by using chemiluminescence (ECL; Amersham).

We used two primary antibodies: (*i*) A mouse monoclonal antibody to nitrotyrosine (Joseph Beckman, University of Alabama at Birmingham) at a dilution of 1:500. To control for specificity, we showed that the binding of antibody to antigen was blocked either by preincubation of the antibody with 10 mM nitrotyrosine for 30 min or by preincubation of the filter with 1 M dithionite (pH 9) for 1 min, which reduces nitrotyrosine to aminotyrosine, followed by neutralization in TBS. Dithionite was prepared fresh in TBS and protected from light and oxygen. In control studies, we showed that dithionite did not remove proteins from the filters. (*ii*) A mouse monoclonal antibody to mouse NOS-II (Transduction Laboratories, Lexington, Kentucky) at a dilution of 1:500.

RNA Blotting. Total cellular RNA was purified by extraction with guanidinium thiocyanate/phenol/chloroform (28). Poly(A)$^+$ RNA was selected by oligo(dT) beads (Fast-track RNA; Invitrogen), electrophoresed through agarose/formaldehyde gels, transferred onto a nylon membrane (GeneScreen, New England Nuclear), prehybridized in 50% formamide/10% (wt/vol) SDS/10% (wt/vol) dextran sulfate at 42°C, and hybridized to ^{32}P-labeled probes prepared by using hexamer primers. Blots were washed in 0.1× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) and 0.1% SDS at 50–55°C and autoradiographed for 1–5 days. The DNA template was a 1.8-kb *Nco* I restriction fragment of mouse macrophage NOS-II (29). To standardize for differences in RNA loading, the blots were reprobed for human β-cyclophilin.

cAMP Accumulation. cAMP accumulation was measured as [^{3}H]cAMP formation from [^{3}H]adenine (30, 31). Cells were incubated overnight with 2 μCi of [^{3}H]adenine per ml (1 Ci = 37 GBq) and then for 4 h with LPS and IFN-γ, washed with PBS, and incubated in 0.5 ml of serum-free medium containing 2 mM 3-isobutyl-1-methylxanthine (IBMX) and hormone as indicated. After incubation with α-MSH for 5 min at 37°C, the reaction was terminated by addition of 0.5 ml of 1 M NaOH. The cells were incubated at 4°C for 30 min, and the supernatant [^{3}H]cAMP was isolated by column chromatography (31). All values are the means of triplicate samples, expressed as cpm of [^{3}H]cAMP per 5 min, ± SEM.

Detection of mRNA for MC-1 Receptor and POMC by PCR. Total RNA (200 μg) was digested with DNase (8 units; Promega) for 30 min at 37°C to remove contaminating genomic DNA. The DNase was inactivated by extraction with phenol/chloroform. cDNA was generated by using Moloney murine leukemia virus reverse transcriptase (RT). In some tubes, the RT was omitted to control for amplification from contaminating cDNA or genomic DNA. Portions of the cDNA were used for PCR with primer pairs specific for mouse MC-1 (12–14) or mouse POMC (32). The MC-1 forward primer was (all shown as 5′ to 3′) GTGAGTCTGGTGGAGAATGTGC [nt 157–178 (13)]. The MC-1 reverse primer was TTTTGTGGAGCTGGGCAATGCC (nt 664–685). The MC-1 primers were chosen in regions of low similarity among all known MC receptors. The POMC forward primer was (all shown as 5′ to 3′) TCACCACGGAGAGCAACCTG. The POMC reverse primer was GCCACCGTAACGCTTGTCCT. PCR mixtures contained the following: 1 μM primers/1.5–2.5 mM Mg^{2+}/200 μM dNTPs/1× reaction buffer/1 unit of *Taq* DNA polymerase/5 μl of dilute cDNA in 20 μl. To minimize nonspecific amplification, the *Taq* DNA polymerase was added to PCR tubes prewarmed to 80°C. The PCR temperature profile consisted of 35 cycles of 94°C for 45 sec, 60°C for 45 sec, and 72°C for 75 sec, followed by a 5-min final extension at 72°C. The PCR products were size-fractionated by agarose gel electrophoresis, transferred to nylon filters, and probed with a ^{32}P-end-labeled internal primer specific for the MC-1 receptor [5′ to 3′; CAGCATCGTCTCCAGCACCCTC (nt 629–650)] or POMC (CTTGCCCACCGGCTTGCCCCA) as described previously (33).

Determination of MC Receptors by Degenerate PCR. Degenerate PCR primers were synthesized to correspond to highly conserved sequences in the second intracytoplasmic loop and seventh transmembrane domain as used by Gantz *et al.* (14), along with extra nucleotides for cloning into a pDIRECT vector (Clontech). The correctly sized subclones were sequenced (Applied Biosystems DNA sequencer).

α-MSH Production. RAW cells were seeded in a 24-well plate and incubated with RPMI 1640 medium plus 2.5% Trasylol, Con A (10 μg/ml; Sigma), or tumor necrosis factor α (TNF-α) (500 units/ml; Cellular Products). After 24 h, the culture fluids were removed, lyophilized, and reconstituted in one-third the original volume for assays of α-MSH by RIA (Euro-Diagnostica, Malmo, Sweden; Kit #RB303). The lower limit of sensitivity is 3 pM and the cross-reactivity with other POMC peptides [adrenocorticotropic hormone (ACTH) 1–24, ACTH 1–39, β-MSH, and γ-MSH] is 0.002%.

Statistical Analysis. Data are means ± SEM. We used analysis of variance (completely randomized design) for omnibus comparisons of means followed by Tukey's test for individual comparisons between means. EC_{50} values were calculated by nonlinear curve fitting to a standard competition curve (Graph-Pad; Academic Press, New York). The null hypothesis was rejected when $P < 0.05$.

RESULTS

Effect of α-MSH on Nitrite Production. Incubation with α-MSH for 16 h decreased LPS/IFN-γ-stimulated NO pro-

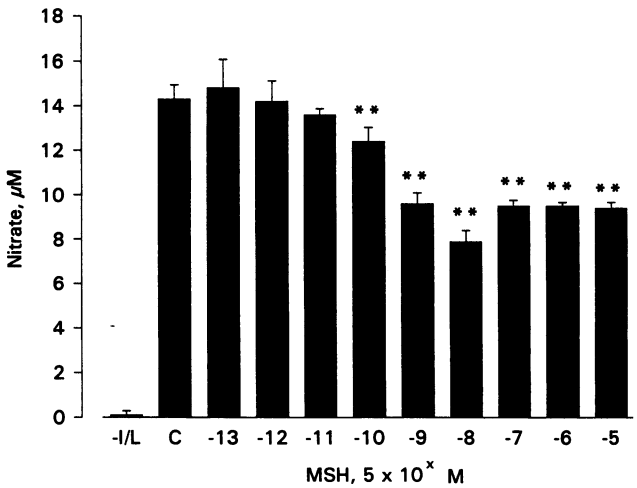

FIG. 1. α-MSH inhibits nitrite accumulation in RAW 264.7 cells stimulated with 10 ng of LPS and 0.5 unit of IFN-γ per ml. Cells were exposed to medium (−I/L), LPS and IFN-γ (C), or LPS, IFN-γ, and α-MSH (5×10^{-13} to 5×10^{-5} M) for 16 h. **, $P < 0.01$. Results are representative of four independent experiments.

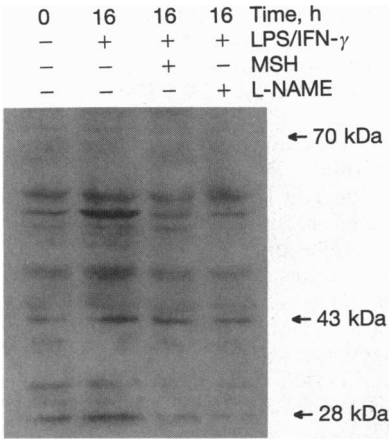

FIG. 2. α-MSH inhibits nitration of macrophage proteins. RAW cells were incubated with (+) or without (−) LPS and IFN-γ, or with LPS/INF-γ and either α-MSH (50 nM) or N^{ω}-nitro-L-arginine methyl ester (L-NAME) (5 mM). Additional α-MSH was added at 8 h. The intensity of all bands was reduced by either α-MSH or L-NAME. Results are representative of two independent experiments.

duction by RAW cells in a dose-related fashion (Fig. 1). The maximal inhibition by α-MSH was 33%; the EC_{50} was 0.6 nM. In additional experiments, the magnitude of inhibition caused by α-MSH (5×10^{-11} to 5×10^{-7} M) was similar to that of equivalent concentrations of L-NMMA (40–50% inhibition). However, 50 μM L-NMMA was more effective (90% inhibition) than 50 μM α-MSH (50% inhibition). To determine if the incomplete inhibition of NO production was a result of the short half-life of α-MSH, we added additional α-MSH midway through the incubation (at 8 h) in two experiments. Under these conditions, 100 nM α-MSH inhibited NO production by 45%. Therefore, the subsequent studies were performed with α-MSH added every 8–12 h.

Effect of α-MSH on Protein Nitration. Stimulation of RAW cells with LPS/IFN-γ for 24 h increased nitration of 60-, 56-, 46-, 41-, 32-, and 30-kDa proteins (Fig. 2). All bands were blocked by preincubation of antibody with excess nitrotyrosine or by incubation of the filter with dithionite, which reduces nitrotyrosine to aminotyrosine (data not shown). Both α-MSH and L-NAME inhibited LPS/IFN-γ-stimulated nitration of RAW cell proteins (Fig. 2) at 24 h. Similar changes were seen after 13 h (data not shown).

Effect of α-MSH on NOS-II Protein. To determine the locus of the effect of α-MSH on nitrite production, we performed Western blotting with a monoclonal antibody to murine NOS-II. Exposure to α-MSH for 8–16 h markedly inhibited induction of NOS-II protein (Fig. 3). In an additional experiment, NOS-II was measured at 16 h, without application of additional α-MSH at 8 h. In this case, NOS-II protein and nitrite accumulation returned toward that of the LPS/IFN-γ control.

Effect of α-MSH on NOS-II mRNA. To begin to determine if α-MSH affects gene transcription or gene translation, we hybridized an mRNA Northern blot with a mouse NOS-II

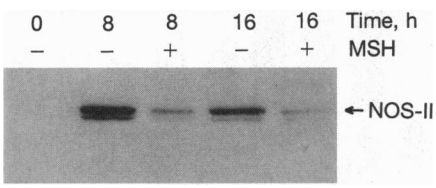

FIG. 3. Western blot showing that α-MSH inhibits expression of NOS-II protein in RAW cells stimulated with LPS/IFN-γ. Cells were stimulated for 8 or 16 h with LPS/IFN-γ and vehicle and with (+) or without (−) α-MSH (additional α-MSH added at 8 h). Data shown are representative of three independent experiments.

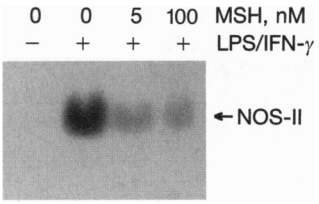

FIG. 4. Northern blot showing the inhibitory influence of 5 nM α-MSH on production of NO-II mRNA in RAW cells stimulated with LPS and IFN-γ. Cells, either unstimulated or stimulated with LPS + IFN-γ for 6 h, were treated with α-MSH (0, 5, and 100 nM). Data shown are representative of two independent experiments.

probe. Exposure to α-MSH for 6 h markedly inhibited induction of NOS-II mRNA (Fig. 4).

Effect of α-MSH on cAMP Accumulation. To determine if the MC receptors are coupled to adenylate cyclase, we measured α-MSH-stimulated cAMP accumulation in cells stimulated with LPS/IFN-γ for 4 h. α-MSH increased cAMP accumulation in a dose-related fashion ($P < 0.007$; Fig. 5).

Expression of MC-1 Receptor in RAW Cells. We next sought to determine whether the melanocyte α-MSH receptor MC-1 is present in RAW cells. We found that RAW cells contain mRNA for the MC-1 melanocortin receptor by RT-PCR (Fig. 6). The sequence of the PCR product was identical to mouse MC-1. We further confirmed the presence of MC-1 by degenerate PCR with primers targeted to different portions of the MC receptor cDNA. A total of 35 clones with correctly sized inserts were found to be >99% identical to mouse MC-1 at the nt level (12). No additional MC receptor cDNAs were found.

Endogenous α-MSH Production. RAW 264.7 cells produced measurable concentrations of α-MSH even in the unstimulated state (Fig. 7). Addition of TNF-α to the cells resulted in a doubling of α-MSH production, whereas Con A had no effect. The presence of POMC mRNA in both unstimulated and TNF-α-stimulated RAW cells was confirmed by RT-PCR (Fig. 8).

DISCUSSION

The present results reveal four findings that characterize the mechanisms underlying the antiinflammatory actions of α-MSH in murine macrophages and converge to suggest an autocrine circuit through which α-MSH may act as an endog-

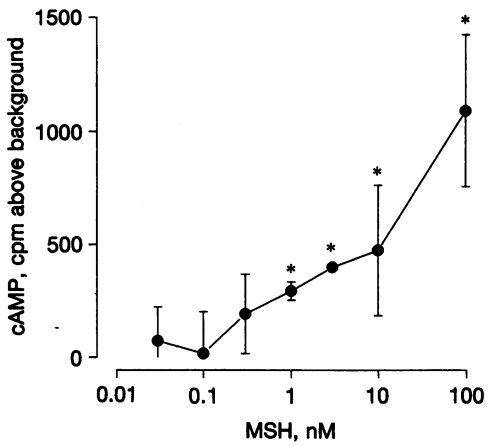

FIG. 5. α-MSH stimulates cAMP accumulation in RAW cells. Cells were metabolically labelled with [^{3}H]adenine and incubated with LPS and INF-γ for 4 h. Medium was removed, and α-MSH-stimulated cAMP accumulation was measured in the presence of 3-isobutylmethyl-1-xanthine. Data shown are mean ± SEM of triplicate determinations. *, $P < 0.05$. Results are representative of two independent experiments.

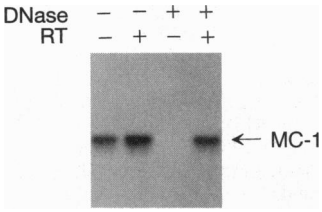

FIG. 6. Southern blot of RT-PCR product for murine MC-1 receptor in RAW cells stimulated with IFN-γ and LPS. Presence of DNase and RT as indicated. No bands were found in lanes containing reaction mixture without cells (not shown). Lanes containing mouse genomic DNA or MC-1 cDNA also contained the 529-bp product. The experiment is representative of three independent experiments.

enous modulator of inflammation (Fig. 9): (*i*) α-MSH inhibits LPS- and cytokine-stimulated nitrite production and protein nitration; (*ii*) α-MSH inhibits accumulation of LPS/cytokine-stimulated NOS-II protein and NOS-II mRNA; (*iii*) α-MSH stimulates cAMP accumulation, and macrophages contain mRNA for at least one MC receptor; and (*iv*) α-MSH is released by macrophages in response to cytokine treatment. Because cAMP has been demonstrated to inhibit a number of macrophage functions (34, 35), our data may explain the broad spectrum of antiinflammatory effects of α-MSH.

α-MSH Inhibits LPS and Cytokine-Stimulated NO Production by Preventing Induction of NOS-II. We found that α-MSH inhibited macrophage production of nitrite, a stable metabolite of NO, in a dose-related fashion (Fig. 1). That α-MSH did not completely inhibit nitrite production is characteristic of the actions of α-MSH: it generally modulates inflammation and does not completely inhibit or prevent it (2). α-MSH also inhibited LPS/cytokine-induced protein nitration (Fig. 2), which is believed to be a NO footprint in histological sections. Beckman (22) found extensive protein nitration in endothelial cells, foamy macrophages, and inflammatory cells of atheroma from human coronary arteries. We have shown that protein nitration is modulated in concert with NO production. This is important since peroxynitrite, the NO metabolite that attacks proteins, kills cells in *N*-methyl-D-aspartic acid (NMDA)-induced brain toxicity (36). We conclude that α-MSH inhibits NO production in activated inflammatory cells.

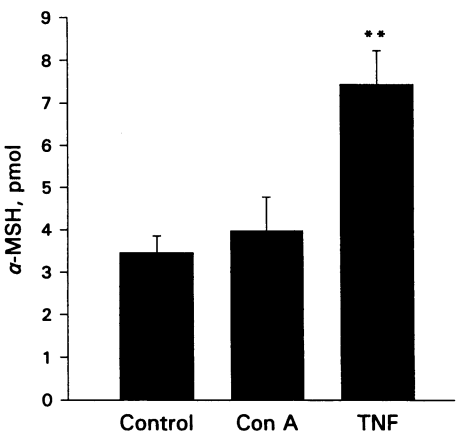

FIG. 7. Production of α-MSH in RAW cells. Cells were cultured for 24 h with vehicle (Control), Con A (10 μg/ml), or TNF-α (500 units/ml). α-MSH was measured by RIA. **, $P < 0.01$. α-MSH was not detected after a 24-h incubation of medium alone, medium plus TNF-α, or medium plus Con A. After 24 h of incubation of medium with 30 pM α-MSH, 10.2 pM α-MSH was recovered. When the latter incubation was carried out in the presence of RAW cells, only 4.8 pM α-MSH was recovered. This indicates that the cells bound, destroyed, or otherwise inactivated radioimmunoassayable α-MSH.

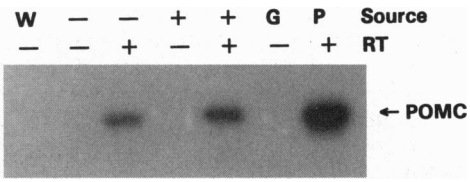

FIG. 8. Southern blot of RT-PCR product for murine POMC in RAW cells. Source: W, water; −, RAW cells; +, RAW cells with LPS and IFN-γ; G, mouse genomic DNA; P, mouse pituitary. No bands were found in control lanes lacking RT, whereas a single 512-bp product was found in the lane containing cDNA from mouse pituitary.

α-MSH also inhibited LPS/cytokine-induced accumulation of NOS-II protein (Fig. 3) and NOS-II mRNA (Fig. 4), indicating that α-MSH prevents production of NOS-II protein, with the primary locus of action at the mRNA level. The exact molecular mechanism of this effect—i.e., inhibition of transcription or message stability—is unknown. Previous data on macrophages and other tissues indicate that certain cytokines increase transcription of NOS-II mRNA (29). This suggests that α-MSH may inhibit transcription. When α-MSH was given once only at time 0, we found that it inhibited NOS-II protein at 8 h; however, the inhibition was substantially reduced at 16 h. This is likely due to the short half-life of α-MSH. Desensitization of the α-MSH receptor is less likely, since readdition of α-MSH at 8 h restored the inhibition. We have not determined if α-MSH has a direct effect on NOS-II activity.

Macrophages Express MC-1 α-MSH Receptor. We found that α-MSH increases cAMP accumulation in cultured macrophages (Fig. 5). Because α-MSH alters macrophage function—i.e., NO and cAMP production—we sought evidence of MC receptor mRNA in RAW cells. We found that cultured macrophages contain mRNA for the α-MSH receptor MC-1 (Fig. 6), the primary α-MSH receptor. Previous studies have shown that MC-1 is expressed in melanocytes; its mRNA is not detectable in brain, adrenal, and other tissues by mRNA blotting (12). Tissue macrophages are widely distributed throughout the body. Perhaps previous studies could not detect MC-1 in other organs because of inadequate sensitivity of mRNA blotting to detect a low-abundance transcript in sparsely distributed cells. The other MC receptors are expressed in the adrenal gland (MC-2), brain (MC-3, MC-4, and MC-5), and gut and placenta (MC-3) (14, 16, 37). MC-5 has a broad tissue distribution (18). However, we found no evidence

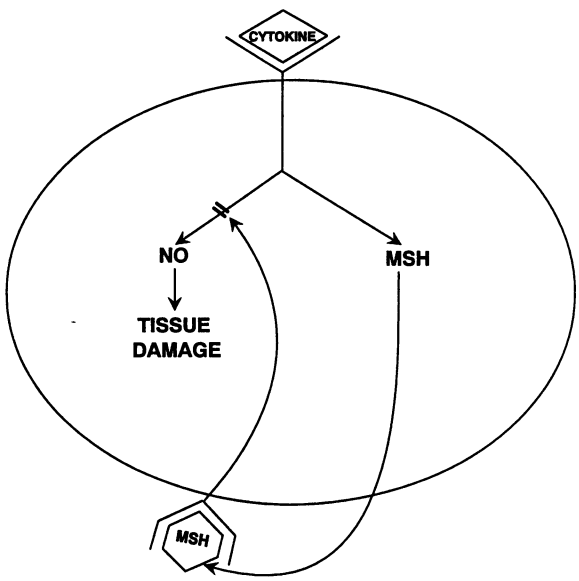

FIG. 9. Model of autocrine action of α-MSH in macrophages.

for expression of additional MC receptors in murine macrophages. Taken together, the present results indicate that the MC-1 receptor is involved in modulation of inflammatory processes, opening the possibility of manipulating inflammation via stimulation and blocking of this receptor.

Macrophages Produce α-MSH. We found that macrophages express mRNA for POMC and produce and release α-MSH even under basal conditions. Furthermore, α-MSH production is increased when cells are stimulated with TNF-α (Fig. 7). Previous research has shown α-MSH production in the pituitary, central nervous system, placenta, certain endocrine organs, and "barrier" tissues of the skin and gastrointestinal tract (38, 39). α-MSH occurs in plasma (1) and synovial fluid of patients with rheumatoid arthritis (11). These results, together with previous observations that α-MSH-binding sites are widespread (40), indicate that α-MSH is widely available to modulate inflammatory reactions.

Physiological Significance. The combined evidence suggests that α-MSH exerts an autocrine regulatory influence on the inflammatory function of macrophages (Fig. 9). Stimulation of macrophages with inflammatory cytokines such as TNF-α or IFN-γ, and/or with LPS induces proinflammatory events within the cell, including production of NOS-II and NO. This stimulation also promotes production and release of α-MSH from macrophages. The α-MSH released is presumed to occupy MC receptors (MC-1) on the macrophage membrane in an "external autocrine" action (41), which increases intracellular cAMP. Although there is evidence that elevations in intracellular cAMP can increase NO synthesis in endothelial cells and macrophages, NO in murine macrophages was reduced if the elevation in cAMP was prolonged (42). Activation of this signaling pathway is therefore believed to result in inhibition of NOS-II production and subsequent inhibition of NO production. There is evidence that activation of a related MC receptor, MC-3, is coupled to both cAMP- and inositol 1,4,5-triphosphate/Ca^{2+}-mediated signal cascades (43). Whether another signal-transduction pathway is also involved in the anti-NO effect of α-MSH via MC-1 receptors is unknown. Thus, α-MSH inhibits inflammation by counteracting LPS/cytokine-induced proinflammatory influences within the cell. Such an influence of α-MSH is consistent with the general idea put forward earlier (1) that the peptide is part of an endogenous system for modulating inflammatory responses. The increases in circulating α-MSH after endotoxin injection in laboratory animals and man (1) are likely to modulate inflammatory reactions through an endocrine influence on inflammatory cells that bear α-MSH receptors.

α-MSH could operate as an immune-cell NOS inhibitor which specifically alters macrophage NO production. Whereas arginine analogues have been successfully used to treat sepsis-induced hypotension and arthritis (44, 45), complete or nonselective inhibition of NOS in endotoxemia can be detrimental, resulting in increased liver, kidney, and intestinal damage (46, 47). It may be appropriate, therefore, to modulate NO production in inflammation and not completely inhibit it. α-MSH modulates but does not completely inhibit the inflammatory reaction (1, 2, 8).

The cDNA for mouse macrophage NOS-II was provided by Dr. C. Richard Lyons, University of Texas Southwestern, Dallas. The cDNA for human MC-1 was provided by Dr. Ira Gantz, University of Michigan Medical Center, Ann Arbor. We thank Dr. R. Tyler Miller for his help with the cAMP assays. This work was done during the tenure of an established investigatorship from the American Heart Association (R.A.S.). R.A.S. was supported by National Institutes of Health Grant DK-01888 and a Grant-in-Aid from the American Heart Association. J.M.L. was supported in part by National Institutes of Health Grant NS10046. A.C. and J.M.L. were supported by North Atlantic Treaty Organization Collaborative Research Grant 200467 and by VII Progetto AIDS 1994, Istituto di Superiore di Sanita, Italy.

1. Catania, A. & Lipton, J. M. (1993) *Endocr. Rev.* **14,** 564–576.
2. Catania, A. & Lipton, J. M. (1994) *NeuroImmunoModulation* **1,** 93–99.
3. Hiltz, M. E., Catania, A. & Lipton, J. M. (1992) *Cytokine* **4,** 320–328.
4. Ceriani, G., Macaluso, A., Catania, A. & Lipton, J. M. (1994) *Neuroendocrinology* **59,** 138–143.
5. Hiltz, M. E. & Lipton, J. M. (1990) *Peptides* **11,** 979–982.
6. Rheins, A., Cotleur, A. L., Kleier, R. S., Hoppenjans, W. B., Saunder, D. N. & Nordlund, J. J. (1989) *J. Invest. Dermatol.* **93,** 511–517.
7. Ceriani, G., Diaz, J., Murphree, S., Catania, A. & Lipton, J. M. (1994) *NeuroImmunoModulation* **1,** 28–32.
8. Lipton, J. M., Ceriani, G., Macaluso, A., McCoy, D., Carnes, K., Biltz, J. & Catania, A. (1994) *Ann. N.Y. Acad. Sci.* **741,** 137–148.
9. Catania, A. & Lipton, J. M. (1993) *Ann. N.Y. Acad. Sci.* **680,** 412–423.
10. Watanabe, T., Hiltz, M. E., Catania, A. & Lipton, J. M. (1993) *Brain Res. Bull.* **32,** 311–314.
11. Catania, A., Gerloni, V., Procaccia, S., Airaghi, L., Manfredi, M. G., Lomater, C., Grossi, L. & Lipton, J. M. (1994) *NeuroImmunoModulation* **1,** 321–328.
12. Mountjoy, K. G., Robbins, L. S., Mortrud, M. T. & Cone, R. D. (1992) *Science* **257,** 1248–1251.
13. Chhajlani, V. & Wikberg, J. E. (1992) *FEBS Lett.* **309,** 417–420.
14. Gantz, I., Konda, Y., Tashiro, T., Shimoto, Y., Miwa, H., Munzert, G., Watson, S. J., DelValle, J. & Yamada, T. (1993) *J. Biol. Chem.* **268,** 8246–8250.
15. Roselli-Rehfuss, L., Mountjoy, K. G., Robbins, L. S., Mortrud, M. T., Low, M. J., Tatro, J. B., Entwistle, M. L., Simerly, R. B. & Cone, R. D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8856–8860.
16. Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S. J., DelValle, J. & Yamada, T. (1993) *J. Biol. Chem.* **268,** 15174–15179.
17. Gantz, I., Yamada, T., Tashiro, T., Konda, Y., Shimoto, Y., Miwa, H. & Trent, J. M. (1994) *Genomics* **19,** 394–395.
18. Desarnaud, F., Labbe, O., Eggerickx, D., Vassart, G. & Parmentier, M. (1994) *Biochem. J.* **299,** 367–373.
19. Moncada, S. (1992) *Acta Physiol. Scand.* **145,** 201–227.
20. Moncada, S. & Higgs, A. (1993) *N. Engl. J. Med.* **329,** 2002–2012.
21. Nussler, A. K. & Billiar, T. R. (1993) *J. Leukocyte Biol.* **54,** 171–178.
22. Beckman, J. S., Ye, Z. Y., Anderson, P. G., Chen, J., Accavitti, M. A., Tarpey, M. M. & White, C. R. (1994) *Biol. Chem. Hoppe-Seyler* **375,** 81–88.
23. Kaur, H. & Halliwell, B. (1994) *FEBS Lett.* **350,** 9–12.
24. Ralph, P. & Nakoing, I. (1977) *J. Immunol.* **119,** 950–954.
25. Raschke, W. C., Baird, S., Ralph, P. & Nakoing, I. (1978) *Cell* **15,** 261–267.
26. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
28. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. & Tannenbaum, S. R. (1982) *Anal. Biochem.* **126,** 131–138.
29. Lyons, C. R., Orloff, G. J. & Cunningham, J. M. (1992) *J. Biol. Chem.* **267,** 6370–6374.
30. Kitamura, K. & Miller, R. T. (1994) *J. Clin. Invest.* **94,** 328–336.
31. Solomon, Y., Londos, C. & Rodbell, M. (1974) *Anal. Biochem.* **58,** 541–552.
32. Uhler, M. & Herbert, E. (1983) *J. Biol. Chem.* **258,** 257–261.
33. Ujiie, K., Drewett, J. G., Yuen, P. S. T. & Star, R. A. (1993) *J. Clin. Invest.* **91,** 730–734.
34. Borune, H. R., Lichenstein, L. M., Melmon, K. L., Henney, C. S., Weinstein, Y. & Shearer, G. M. (1974) *Science* **184,** 19–28.
35. Coffey, R. G. & Hadden, J. W. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44,** 112–117.
36. Dawson, V. L., Dawson, T. M., Longon, E. D., Bredt, D. S. & Snyder, S. H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 6368–6371.
37. Schmouder, R. L., Strieter, R. M. & Kunkel, S. L. (1993) *Kidney Int.* **44,** 43–49.
38. Eberle, A. N. (1988) *The Melanotrophins* (Karger, Basel).
39. Schauer, E., Trautinger, F., Köck, A., Schwarz, A., Bhardwaj, R., Simon, M., Ansel, J. C., Schwarz, T. & Luger, T. A. (1994) *J. Clin. Invest.* **93,** 2258–2262.
40. Tatro, J. B. & Reichlin, S. (1987) *Endocrinology* **121,** 1900–1907.
41. Sporn, M. B. & Roberts, A. B. (1992) *Ann. Intern. Med.* **117,** 408–414.
42. Bulut, V., Severn, A. & Liew, F. Y. (1993) *Biochem. Biophys. Res. Commun.* **195,** 1134–1138.
43. Konda, Y., Gantz, I., DelValle, J., Shimoto, Y., Miwa, H. & Yamada, T. (1994) *J. Biol. Chem.* **269,** 13162–13166.
44. Nava, E., Palmer, R. M. J. & Moncada, S. (1991) *Lancet* **338,** 1555–1557.
45. Stefanovic-Racic, M., Meyers, K., Meschter, C., Coffey, J. W., Hoffman, R. A. & Evans, C. H. (1994) *Arthritis Rheum.* **37,** 1062–1069.
46. Hutcheson, A. R. & Whittle, B. J. R. (1990) *Br. J. Pharmacol.* **101,** 815–820.
47. Shultz, P. J. & Raij, L. (1992) *J. Clin. Invest.* **90,** 1718–1725.