α-MSH and its receptors in regulation of tumor necrosis factor-α production by human monocyte/macrophages

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Departments of 1Physiology and 3Anesthesiology and Pain Management, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9040; 2Astra Hassle, 431 83 Molndal, Sweden; 3Department of Surgery, University of Michigan Medical Center, Ann Arbor, Michigan 48109-0682; and 4Third Division of Internal Medicine, IRCCS Ospedale Maggiore, Milan, Italy 20122

Taherzadeh, S., S. Sharma, V. Chhajlani, I. Gantz, N. Rajora, M. T. Demitri, L. Kelly, H. Zhao, T. Ichiyama, A. Catania, and J. M. Lipton. α-MSH and its receptors in regulation of tumor necrosis factor-α production by human monocyte/macrophages. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R1289–R1294, 1999.—Then hypothesis that macrophages contain an autocrine circuit based on melanocortin [ACTH and α-melanocyte-stimulating hormone (α-MSH)] peptides has major implications for neuroimmuno-modulation research and inflammation therapy. To test this hypothesis, cells of the THP-1 human monocyte/macrophage line were stimulated with lipopolysaccharide (LPS) in the presence and absence of α-MSH. The inflammatory cytokine tumor necrosis factor (TNF-α) was inhibited in relation to α-MSH concentration. Similar inhibitory effects on TNF-α were observed with ACTH peptides that contain the α-MSH amino acid sequence and act on melanocortin receptors. Nuclease protection assays indicated that expression of the human melanocortin-1 receptor subtype (hMC-1R) occurs in THP-1 cells; Southern blots of RT-PCR product revealed that additional subtypes, hMC-3R and hMC-5R, also occur. Incubation of resting macrophages with antibody to hMC-1R increased TNF-α concentration; the antibody also markedly reduced the inhibitory influence of α-MSH on TNF-α in macrophages treated with LPS. These results support the hypothesis that macrophages contain an endogenous regulatory circuit based on melanocortin peptides and their receptors. Targeting of this neuroimmunomodulatory circuit in inflammatory diseases in which myelomonocytic cells are prominent should be beneficial.

melanocortin peptides; melanocortin receptors; inflammation; autocrine regulation; neuroimmunomodulation; THP-1 cells; melanocortin-1 receptor antibody; α-melanocyte-stimulating hormone

THE NEUROIMMUNOMODULATORY peptide α-melanocyte-stimulating hormone (α-MSH), a tridecapeptide derived from proopiomelanocortin, has remarkable anti-inflammatory effects. The peptide is antipyretic, and it inhibits all major forms of inflammation (2, 3, 13). The mechanisms by which its anti-inflammatory influences occur are not fully known, but they involve direct actions of the peptide on its receptors in peripheral inflammatory cells; indirect actions on peripheral inflammation induced by stimulation of α-MSH receptors within the brain, receptors that give rise to descending anti-inflammatory pathways; and modulation of brain inflammation via local action of the peptide on its receptors in glial cells. One avenue to improvement of understanding of one of the actions of α-MSH in peripheral inflammation is to determine whether the peptide alters inflammatory cell activity by mimicking autocrine regulatory events that occur normally in the cells.

There is initial evidence that both murine (18) and human (17) monocyte/macrophages have autocrine circuits, based on α-MSH and its receptors, that modulate their production of inflammatory agents, thereby limiting local inflammatory processes. In murine macrophages, α-MSH inhibits inflammatory nitric oxide (NO) production by inhibiting expression of inducible NO synthase (18). These cells contain mRNA for both the melanocortin (α-MSH and ACTH-like) receptor MC-1R and for the proopiomelanocortin precursor of α-MSH, and they secrete immunoassayable α-MSH when stimulated with lipopolysaccharide (LPS) or interferon (IFN)-γ plus tumor necrosis factor (TNF)-α. Cells of the THP-1 human monocyte/macrophage line make little NO but secrete neopterin, a marker of macrophage activation (17). α-MSH inhibits neopterin production induced by coculture of THP-1 cells with IFN-γ and TNF-α. These observations raise the hypothesis that macrophages of human origin contain an endogenous autocrine anti-inflammatory circuit that depends on the neuropeptide α-MSH and specific melanocortin receptors. This hypothesis was the focus of the present research.

METHODS

Cell line. THP-1 cells were obtained from the American Type Culture Collection and maintained in stationary suspension in RPMI 1640 with 7% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 µg/ml of streptomycin at 37°C in a humidified atmosphere of 5% CO2-95% air. Cells in log phase growth were incubated in RPMI 1640-2% FBS at 5 × 105/ml in 100-mm plastic Petri dishes with 16 nM phorbol 12-myristate 13-acetate for 48 h to induce differentiation into macrophages. Nonadherent cells were aspirated, and adherent cells were released by vigorous pipetting of cells incubated in Hanks’ balanced salt solution without Ca2+ and Mg2+ at 4°C. Characteristics of monocyte differentiation were confirmed by analysis of cyto centrifuged smears using Wright-Giemsa stain and stain for endogenous peroxidase activity.

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Cell viability (>95%) was assayed by trypsin blue exclusion. The LPS used to promote TNF-α production was derived from Salmonella typhosa (Difco Laboratories, no. 0901). For RT-PCR studies, the cells were grown in RPMI 1640 with 10% FBS in 75-cm² flasks.

Peptides. α-MSH-(1—13) and ACTH-(1—24) and -(1—39) were obtained from Sigma Chemical (St. Louis, MO). The peptides, dissolved in media, were introduced into cell cultures at the times indicated.

TNF-α and α-MSH determinations. Concentrations of TNF-α in supernatants were determined from analysis of L929 fibroblast cytotoxicity with recombinant human TNF-α (R&D Systems, Minneapolis, MN) as control and for production of standard curves. Triplicate samples were incubated overnight with L929 cells in medium containing 100 µg/ml cyclohexamide (Sigma Chemical) in 96-well plates. The TNF-α concentrations were determined with reference to intensity at 550 nm in an ELISA plate reader (BT 2000, Fisher-Biotech). The L929 assay was confirmed to be specific for murine TNF-α and determined with reference to intensity at 550 nm in an ELISA plate reader (BT 2000, Fisher-Biotech).

α-MSH was measured using a commercial radioimmunoassay kit (Eurodiagnostica, Malmo, Sweden). The sensitivity of the assay is 0.5 pg/ml. There is no significant cross-reactivity with related proopiomelanocortin peptides (<0.05%).

S1 nuclease protection assay. Human melanocortin-1 receptor (hMC-1R) plasmid (Bluescript SK) contained the 951-bp gene encoding the receptor. The plasmid was linearized with Nar I, yielding a 544-bp fragment. 32P-labeled riboprobe was transcribed using the Ambion (Austin, TX) MAXIscript protocol and T3 polymerase. The labeled transcript was precipitated with 25 µg tRNA and twice with ammonium acetate-ethanol. The pellets were dried, and 50 µl of both Tris-EDTA (pH 8.0) and loading buffer were added. The sample was heated to 95°C for 3–4 min and then subjected to electrophoresis on a 5% polyacrylamide gel at 50 A for 1 h. The location of the full-length RNA transcript was determined by autoradiography; the region was excised and eluted for 1 h at 65°C in 350 µl elution buffer. β-Actin (Ambion) was included as an internal control to assess the integrity of the RNA; SP6 was used to generate a 240-bp fragment. RNA was isolated from THP-1 cells by guanidinium thiocyanate-phenol-chloroform extraction. Twenty-five micrograms of RNA were combined with 8 × 10⁵ cpm high-specific-activity hMC-1R riboprobe, and 1 × 10⁵ cpm low-specific-activity β-actin riboprobe. The samples were then precipitated with ammonium acetate-ethanol, resuspended in 10 µl hybridization buffer, heated at 95°C for 4 min, and incubated overnight at 45°C in a heating block. Two hundred microliters of digestion buffer containing 250 U/µl of RNase were added, and the samples were incubated at 37°C for 1 h. The protected fragments were precipitated, dissolved in 10 µl of loading buffer, heated to 95°C for 4 min, and fractionated on a 5% polyacrylamide gel. The protected fragments were visualized by exposure to Kodak X-OMAK film at 70°C with two intensifier screens. Human melanoma 92.1 used as a positive control for hMC-1R was provided by Dr. Jerry Niederkorn of the University of Texas Southwestern Medical Center at Dallas.

RT-PCR and Southern blots for hMC-3R and hMC-5R. Total RNA (200 µg) was isolated from THP-1 cells by guanidinium thiocyanate-phenol-chloroform extraction. Genomic DNA was digested with DNase (Promega, Madison, WI) in RT buffer for 30 min at 37°C. The DNase was inactivated by phenol-chloroform extraction. cDNA was produced using Moloney murine leukemia virus RT (BRL, Gaithersburg, MD). 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 the human MC-3R and MC-5R isoforms. The hMC-3R forward and reverse primers were 5’ to 3’ TCTCAGAGGAATGG and TATCCCAAGTTCATGCC- GTTGC, respectively. The hMC-5R forward primer was GGAAGCTTTCTTTGTAGGCTG, and the reverse primer was GGCTTAGAGCCAGAGAGAG. The primers were chosen in regions of low similarity among known MC receptors. PCR mixtures contained 1 µM primers, 1.5 mM MgCl², 200 µM dNTPs, 1× reaction buffer, 1 µl Taq DNA polymerase (Promega), and 5 µl cDNA in 20 µl. The PCR profile consisted of 35 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 75 s followed by a 5-min final extension at 72°C. The 461-bp hMC-3R and the 1,100-bp hMC-5R were size-fractionated by agarose gel electrophoresis, transferred to nylon filters, and probed with a 32P-random-primed probe corresponding to the 1,078- and 1,097-bp coding regions of the hMC-3 and hMC-5 receptors, respectively. The blot was hybridized at 68°C overnight and stringently washed in a final solution of 0.1× SSC and 0.1% SDS at 68°C for 1 h. The blot was then exposed to Kodak X-OMKR-5 film with one intensifying screen for 2 h at −70°C.

hMC-1R antibody. Polyclonal antibodies specific for hMC-1R were developed and tested in previous research (21). Antisera were raised in rabbits by immunization with peptides that had been synthesized according to the amino acid sequence predicted from the hMC-1R cDNA previously cloned by Chhajani and Wikberg (6). The peptides were selected to be substantially different from any sequences found in other melanocortin receptors. The antisera immunostained COS-7 cells that expressed hMC-1 but not control cells; malignant melanoma tissues, known to contain hMC-1R, were strongly stained with the antisera whereas melanocytes in normal skin were not. Freeze-dried antisera were reconstituted with pyrogen-free water and diluted with medium before being added to cell cultures. Control experiments were performed with rabbit IgG of the same dilution.

Statistical analysis. Numerical data were analyzed using one-way ANOVA procedures followed by Dunnett’s test for multiple comparisons of group means. Two-tail probability <0.05 was considered significant.

RESULTS

α-MSH inhibits TNF-α production by macrophages. THP-1 monocytes secreted TNF-α at rest, and this production increased when the cells were converted to macrophages. TNF-α production was increased further by incubation of the macrophages with LPS. The latter increased production was inhibited to control values by α-MSH at 10⁻¹² and 10⁻¹¹ M and to near control values by lower concentrations of the peptide (Fig. 1). Although there was little difference in the overall magnitude of the effects of concentrations of the peptide, the inhibitory effect on TNF-α production was monotonically related to α-MSH concentration. It is notable that the potency of α-MSH in inhibiting TNF-α in these experiments was substantially greater than its inhibitory effects on neopterin production by THP-1 monocyte/macrophages noted in previous research (17).

ACTH molecules that contain the α-MSH-(1—13) amino acid sequence also inhibit TNF-α production. α-MSH-(1—13) is believed to have arisen earlier in evolution than ACTH, and its amino acid sequence is...
To learn whether the larger ACTH molecule that occurs within the brain and pituitary [ACTH-(1—39)] or the amino acid sequence that is responsible for ACTH bioactivity [ACTH-(1—24)] share the anti-TNF-α effect of α-MSH, the influences of concentrations of all three peptides were tested in separate experiments (Fig. 2). Comparable basal rates of TNF-α production, and induced increases in TNF-α with LPS, allowed comparisons of the data in terms of percent inhibition by the peptides. Regarding sensitivity, an α-MSH concentration of 10^{-17} M was effective, whereas slightly greater concentrations of the ACTH molecules were required to cause substantial inhibition. With the higher concentrations of all three peptides, the maximum magnitude of the inhibitory effect was similar: a reduction of ~60% below the control TNF-α value obtained when the cells were incubated with LPS and without any peptide.

Melanocortin receptor expression in THP-1 cells. The inhibitory effects of melanocortin peptides described above must stem from activation of melanocortin receptors. There was evidence of three melanocortin receptor subtypes in these cells: hMC-1R, -3R, and -5R. S1 nuclease protection assays of RNA from THP-1 cells repeatedly revealed positive signals for hMC-1R mRNA (Fig. 3). This evidence supports our earlier observation (17) of transcripts for this receptor in THP-1 cells obtained using probes hybridized to Southern blots of RT-PCR product. Transcription of the MC-1R subtype is thus abundant enough in macrophages to be detected with nuclease protection assays. To test the idea that the α-MSH receptors MC-3R and hMC-5R, known to occur in brain tissue (9) and elsewhere, are likewise expressed in macrophages, RNA of THP-1 cells was subjected to RT-PCR and Southern analysis. Repeated analyses revealed detectable signals at 431 bp, consistent with the size of the cDNA for the hMC-3R (Fig. 4), and substantial signals at 1,100 bp, consistent with cDNA for hMC-5R.

hMC-1R antibody promotes TNF-α and inhibits α-MSH activity. Production of TNF-α by resting THP-1 cells increased with increasing concentrations of hMC-1R antibody (Fig. 5). This increase is presumed to reflect blockade of an autocrine TNF-α regulatory circuit based on endogenous production of α-MSH. Production of α-MSH by THP-1 cells at rest was established previously (17). However, these observa-

Fig. 1. α-Melanocyte-stimulating hormone (α-MSH) inhibited tumor necrosis factor (TNF)-α production by THP-1 cells seeded in 96-well plates (2.5 × 10^6 cells/well) and stimulated with lipopolysaccharide (LPS; 4 ng/ml) for 3 h. Scores are means (±SE) of quadruplicate samples. Control (Con), media alone. Representative of 4 separate experiments. **P < 0.001 vs. LPS alone.

Fig. 2. In experiments separate from those of Fig. 1, ACTH sequences that contain α-MSH-(1—13) amino acid sequence inhibited TNF-α production by THP-1 cells incubated with LPS. A: α-MSH-(1—13). B: ACTH-(1—24). C: ACTH-(1—39). 0, LPS alone. Scores are means (±SE) of quadruplicate samples. Representative of 3 separate experiments.
tions were repeated in the present experiments by measuring α-MSH production by unstimulated THP-1 cells (2 \times 10^6/ml) over 24 h. Unstimulated production of α-MSH averaged 10 pg/ml over five determinations. From the data of Fig. 1, two concentrations of α-MSH, 10^{-14} and 10^{-13} M, were selected for tests with hMC-1R antibody. As in experiments above, both concentrations of peptide inhibited TNF-α production when no antibody was added. However, this inhibition was progressively reduced with increasing concentrations of hMC-1R antibody. Indeed, the greatest concentration of antibody caused not only a reversal of the inhibitory effect of α-MSH but also an enhancement of TNF-α production, presumably the result of its anti-hMC-1R activity against receptors that are required for control of TNF-α production by endogenously produced α-MSH.

**DISCUSSION**

The results are consistent with the hypothesis that monocyte/macrophages have an autocrine mechanism for modulating release of the inflammatory cytokine TNF-α. This circuit is based on the neuropeptide α-MSH, perhaps other melanocortins, and endogenous melanocortin receptors. The present data and prior evidence indicate that α-MSH is important to monocyte/macrophage regulation of TNF-α, NO (18), and neopterin (17). Support for an autocrine modulation was obtained from nuclease protection assays and Southern analyses, which indicated that human macrophages express melanocortin receptors hMC-1R, -3R, and -5R. It is likely that one or more of these receptor subtypes participate in regulating TNF-α in macrophages. The hMC-1R may be of particular importance because antibodies to this receptor promoted TNF-α in resting macrophages, decreased the inhibitory effect of α-MSH, and enhanced TNF-α production in those cells stimulated with LPS. THP-1 monocyte/macrophages produce α-MSH essential to an autocrine circuit; the peptide is produced by resting cells, and its concentration is increased when...
the cells are stimulated with LPS and/or inflammatory cytokines (17). Thus all factors required for autocrine modulation via α-MSH appear to occur in human macrophages: TNF-α is modulated by α-MSH, the cells express melanocortin receptor mRNA and produce the α-MSH neuropeptide, and anti-hMC-1R antibodies promote TNF-α in resting cells and reduce the inhibitory effect of α-MSH on TNF-α in stimulated cells. This suggested autocrine circuit, first proposed in human cells to control neopterin (17), may have a reverse counterpart in melanoma cells known to bear hMC-1R and TNF-α in resting cells and reduce the inhibitory effect of α-MSH on TNF-α in stimulated cells. This conjectured autocrine circuit, first proposed in human cells to control neopterin (17), may have a reverse counterpart in melanoma cells known to bear hMC-1R and to produce the peptide (14). In melanoma cells, melanocortins increased release of immunoreactive α-MSH. Transfection of hMC-1R DNA into IGR3 cells increased α-MSH release, which was further increased by melanocortin peptides that compete for binding with α-MSH. The importance of such a promotional or enhancing influence on α-MSH release was not established, although α-MSH is important to melanogenesis and proliferation of melanocytes and melanoma cells.

In tests of the inhibitory effects of α-MSH and ACTH molecules, the similarity in effects on TNF-α was notable. It is known that macrophages produce immunoreactive ACTH-(1–24) (15); this proopiomelanocortin-derived fragment may share in autocrine regulation of macrophage activity. Phagocytosis of latex beads by peritoneal macrophages was inhibited by ACTH-(1–24) (10), which supports the functional significance of melanocortin receptors to control of macrophage activity. Because all of the melanocortin receptor subtypes identified so far bind and react to ACTH molecules (19), any one of the subtypes, all or a combination, or perhaps melanocortin receptors yet to be discovered, may be responsible for the reaction to ACTH molecules found in these experiments. With regard to those receptors for which we found evidence in the present experiments, α-MSH was previously observed to be more effective than ACTH in inducing cAMP in cells transfected with cDNA for hMC-1R (6). However, the peptides were equivalent in inducing cAMP in L cells transfected with cDNA for hMC-3R (9). In COS-7 cells transfected with hMC-5R DNA, binding of α-MSH and ACTH-(1–39) was equivalent, although affinity for melanocortin peptides was much lower than for hMC-1R in prior studies (5). From these observations, MC-1R, -3R, and -5R could all contribute to the equivalence in TNF-α inhibition caused by α-MSH-(1–13), ACTH-(1–24), and ACTH-(1–39), with perhaps a predominant contribution of hMC-3R and hMC-5R. Our observations highlight the importance of hMC-1R in macrophages but leave unexplained the precise contribution of each receptor subtype to the overall anti-inflammatory circuit in macrophages. Such an explanation can be determined with application of antibodies to specific receptor subtypes, singly and in combination.

In the history of research on peptide receptors it is common to initially find one receptor subtype in a specific cell type and to subsequently discover that other subtypes occur in the same cells. For example, in the case of opioid receptors, the µ-receptor was the first to be described and the discovery of other subtypes followed, so that now multiple opioid receptors are known in the same cells. A similar history may be in progress with regard to melanocortin receptors. So far five receptor subtypes have been described and others are likely to be discovered. The present results indicate that multiple melanocortin receptor subtypes exist in the same macrophages. It should be noted that our results differ from those of Bhardwaj et al. (1), who observed evidence for only MC-1R in human peripheral blood mononuclear cells. The reasons for this difference are not clear but likely lie in the difference between the cell types. If, as our evidence suggests, there are different receptor subtypes in macrophages, what functional significance might they have? All respond to α-MSH, so the reason is not likely to stem from differences in response to this ligand. If the receptor subtypes induce different second messengers they could mediate divergent signals, but the evidence indicates that all of the receptor subtypes induce intracellular cAMP. MC-3R, however, also signals through inositol trisphosphate (11). Other signaling pathways may also be activated by melanocortin receptor (19). Also, at this point there is no evidence that any receptor subtype has a unique role in function that is not shared by the others. To study such a possibility will require blockade of all receptor subtypes but one in a cell type.

The evidence of an autocrine circuit in host cells that is based on a neuropeptide and its receptors is a further example of the commonality of peptide signal mechanisms present in the nervous system, endocrine system, and in peripheral inflammatory cells. α-MSH is an ancient peptide, and its amino acid sequence has been highly conserved in evolution; the peptide is ubiquitous in the animal kingdom, and its amino acid sequence is observed in tissues of modern animals (hagfish, lamprey) that originated during the Paleozoic era (8). This conservation of amino acid sequence over eons and across phyla, together with the marked capacity of α-MSH to modulate inflammation, an ancient host reaction, suggests that the peptide developed very early to modulate inflammatory responses and that it has continued to be incorporated in similar functions over evolution. Support for this idea comes from the consistency of the anti-inflammatory effect of the peptide on stimulation of its receptors in different cell/tissue types. α-MSH modulates peripheral inflammation via direct actions on host cells such as macrophages (17, 18) and neutrophils (4). The peptide modulates inflammation within the brain (16), likely by acting on microglia (7) and astrocytes (20). Furthermore, through actions on neuronal receptors, α-MSH induces signals in descending anti-inflammatory pathways that modulate inflammatory processes in the periphery (12). It is likely that the peptide acts via all these mechanisms in intact organisms. Prominence of melanocortins in a macrophage autocrine circuit suggests that the peptides have come to be incorporated in local anti-inflammatory systems that represent a “layer” of melanocortin control over inflammation, one layer of a multilayered control system.
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