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α-Melanocyte-stimulating hormone inhibits lipopolysaccharide-induced biological responses by downregulating CD14 from macrophages

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Abstract Monocytes/macrophages are the first cells involved in inflammation. α-Melanocyte-stimulating hormone (α-MSH) is known to possess an anti-inflammatory role induced by a variety of stimuli; however, the molecular mechanisms underlying these effects are not clearly defined. In this report we provide evidence that α-MSH inhibited serum-activated lipopolysaccharide (SA-LPS)-induced proteolytic enzyme release, oxidative burst response, reactive oxygen intermediate generation, nitric oxide production, and adhesion molecule expression in monocyte-derived macrophages. α-MSH also inhibited SA-LPS-induced nuclear transcription factor κB activation not only in macrophages, but also in a T-cell line and human neutrophils isolated from fresh blood. α-MSH downregulated CD14, but not interleukin-1 receptor, tumor necrosis factor receptor 1 or 2 from the surface of macrophages. Anti-CD14 antibody was unable to protect α-MSH-mediated downregulation of CD14. Overall, our results suggest that α-MSH exerts its anti-inflammatory effect by a novel mechanism in macrophages through downregulating of the endotoxin receptor CD14.

Key words: CD14; α-Melanocyte stimulating hormone; Lipopolysaccharide; Nuclear transcription factor κB; Macrophage

1. Introduction

Patients often face problems of sepsis in intensive care units as a major complication after major surgery mostly due to trauma, burn injury, pancreatitis etc. Bacterial lipopolysaccharide (LPS), a component of Gram-negative bacteria which sheds into the circulation, is the major source of sepsis. LPS finally initiates hypermetabolic shock termed endotoxic shock,collapsing the coordination of different organ leading to multiple organ dysfunction [1]. LPS mediates a number of biologic manifestations, involving activation of several kinases and transcription factors followed by an uncontrolled production and release of proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-8, IL-10, IL-12 etc., reactive oxygen species, and proteolytic enzymes [2–4] by cells. LPS interacts with most cells through CD14, a 55-kDa glycophosphatidylinositol (GPI)-anchored membrane protein expressed on the surface of monocytes, macrophages, and neutrophils [5,6]. The GPI domain of CD14 acts as a membrane anchorer and signal transducer [7]. Several proteins, which contain a GPI modification, play an important role in leukocyte and macrophage inflammatory functions [8]. GPI-anchored proteins, present in lipid rafts rich in cholesterol and sphingolipids, are needed for membrane sorting and signal transduction [9,10]. Mice lacking the CD14 gene show resistance to LPS-induced shock [11] and bone resorption [12]. LPS first binds to a 55-kDa serum protein named lipopolysaccharide binding protein (LBP) which is present in the circulation in normal conditions and this complex (LBP–LPS) triggers CD14 to activate cell signaling [6,13]. In this study serum-activated LPS (SA-LPS) was used to induce different cells (100 ng of LPS was incubated with 20 μl of human serum for 1 h at 37°C and this mixture was SA-LPS).

Neuropeptides have been implicated in the regulation of a number of immune responses both in humans and in murine systems [14]. The α-melanocyte-stimulating hormone (α-MSH), a tridecapeptide derived from pro-opiomelanocortin, is found in pituitary, brain, skin, and circulation [15]. α-MSH is essential for synthesis of melanin in melanocytes [16]. In addition, it has been shown to influence various inflammatory or immunological conditions by modulating the function of neutrophils [17], macrophages [18–21], or lymphocytes [22,23]. The interactions with various cells of the immune system have been shown to downregulate either the production or the action of the proinflammatory cytokines. This effect was correlated with a decrease in binding of DNA to nuclear transcription factor κB (NF-κB) [17–19,24–26], suggesting the downmodulation of the LPS-mediated response which reflects the inhibition of production of different inflammatory cytokines. In mouse macrophages, α-MSH has been shown to inhibit the C/EBPβ DNA binding activity and this effect is a major mechanism by which α-MSH inhibits the transcription of the NOS2 gene [27]. Neuropeptides have also been shown to possess antipyretic activity [28–30]. Receptors for α-MSH have been detected in different cell types [17,18]. Geldanamycin, an antibiotic produced by *Actinomyces* sp., has

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Abbreviations: KSCN, potassium thiocyanate; ICAM, intracellular adhesion molecule; MC-1R, melanocortin 1 receptor; α-MSH, α-melanocyte-stimulating hormone; NBT, nitroblue tetrazolium; NF-κB, nuclear transcription factor κB; SA-LPS, serum-activated lipopolysaccharide; SEAP, secretory alkaline phosphatase

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been shown to inhibit the function of heat shock proteins [31]. Interestingly, this antibiotic has been shown to downregulate CD14 by internalization followed by accumulation in the endoplasmic reticulum [32]. The mechanism underlying the accumulation of CD14 in the endoplasmic reticulum is not clearly understood.

So far, the anti-inflammatory activity of α-MSH has been shown to be mediated by inhibiting the production of inflammatory cytokines through downmodulation of NF-κB or C/EBP transcription factors. As endotoxin-mediated activation of inflammatory responses and septic shock were suppressed by α-MSH, the detailed mechanism of α-MSH-mediated responses induced by LPS was investigated. We also studied the effect of α-MSH on endotoxin receptor (CD14) regulation. In this report we provide data to establish that SA-LPS-mediated inflammatory responses were suppressed by α-MSH. For the first time we provide data on α-MSH-mediated downregulation of the endotoxin receptor CD14 from the surface of macrophages. This study will be helpful to understand the molecular mechanism of the α-MSH-mediated anti-inflammatory response in macrophages.

2. Materials and methods

2.1. Materials

LPS (*Escherichia coli* 055:B5), α-MSH, nitroblue tetrazolium (NBT), polymyxin B sulfate, formyl peptide (FMLP), phorbol myristate acetate (PMA), *p*-nitrophenyl phosphate, *p*-nitrophenyl β-D-glucuronide, Histopaque, dextran sulfate, and goat IgG (anti-rabbit) were obtained from Sigma (St. Louis, MO, USA). Penicillin, streptomycin, RPMI 1640 medium, and fetal bovine serum were obtained from Life Technologies (Grand Island, NY, USA). Dihydrorhodamine was purchased from Molecular Probes, The Netherlands. Antibodies against p50, p65, melanocortin 1 receptor (MC-1R), iNOS, IL-1R, TNFR1, TNR2, and CD14 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell lines and neutrophils

THP-1, Jurkat, U-937, HeLa, NIH 3T3, and H4 cells, obtained from American Type Culture Collection (Manassas, VA, USA), were cultured according to their protocol. THP-1 cells were stimulated with 10 ng/ml PMA for 16 h at 37°C and then adherent cells were used as macrophages [33,34]. Cells were mycoplasma-free, as tested by the Gen-probe mycoplasma rapid detection kit (Fisher Scientific, Pittsburgh, PA, USA). Polymorphonuclear neutrophils were separated from fresh human peripheral venous blood of normal healthy donors by the Dextran T-500 sedimentation method, followed by Histopaque gradient centrifugation [35]. The purity of the cells was examined by Giemsa stain and the viability of the cells was checked by the trypan blue dye exclusion test. The cell preparation contained 90–95% neutrophils, of which 95–98% were viable [35].

2.3. Enzyme release assay

Macrophages (1×10^7 cells/ml), suspended in D-phosphate-buffered saline (PBS) containing glucose (1 mg/ml) and bovine serum albumin (BSA; 5 mg/ml), were stimulated with SA-LPS at 37°C in untreated or α-MSH-pretreated cells. The supernatant was collected and used for assay of three different enzymes [35]. Myeloperoxidase, alkaline phosphatase, and β-D-glucuronidase activities were measured using orthophenylenediamine, *p*-nitrophenyl phosphate, and *p*-nitrophenyl β-D-glucuronide as substrate respectively.

2.4. Measurement of reactive oxygen intermediates

The production of reactive oxygen intermediates (ROI) was determined by flow cytometry as described [36]. Untreated or α-MSH-treated cells were exposed to dihydrorhodamine 123 and then washed with PBS. Rhodamine 123 fluorescence intensity resulting from dihydrorhodamine 123 oxidation was measured by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) with excitation at 488 nm and was detected between 515 and 550 nm.

2.5. Assay for NO synthesis

Synthesis of NO was determined by assaying nitrite, a stable reaction product of NO with molecular oxygen from culture supernatant [37]. Briefly, 100 μl of culture supernatant was allowed to react with 50 μl of Griess reagent in a 96-well plate and incubated at room temperature for 15 min. The absorbance (in OD) was measured at 570 nm using culture medium as the blank in all experiments. Nitrite concentrations were calculated from a standard curve derived from the reaction of $NaNO_2$ in the assay.

2.6. NF-κB activation assay

To determine SA-LPS-induced NF-κB activation, an electrophoretic mobility shift assay (EMSA) was conducted essentially as described previously [38]. Briefly, 8 μg nuclear extracts (NE) were incubated with ^{32}P end-labeled double-stranded NF-κB oligonucleotide from the HIV long terminal repeat, 5′-TTGTTACAAGGGACTTTC-CGCTGGGGACTTTCCAGGGAGGCGTGG-3′ (underline indicates NF-κB binding site) for 30 min at 37°C, and the DNA–protein complex formed was separated from free oligonucleotides on 6.6% native polyacrylamide gel. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. The radioactive bands from the dried gel were quantitated by PhosphorImager (Fuji, Japan) using Image Reader software.

2.7. Transfection with NF-κB-SEAP and dominant negative IκBα (IκBα-DN)

THP-1 cell-derived macrophages were transiently transfected by the calcium phosphate method with 1 ml medium containing 0.5 μg NF-κB promoter DNA linked to the heat-stable secretory alkaline phosphatase (SEAP) gene with or without 0.5 μg plasmid DNA of IκBα mutants (IκBα-DN) lacking either Ser^{32} or Ser^{36} [39]. The total amount of DNA was maintained at 3 μg by the addition of the control plasmid pCMVFLAG1 DNA. After 12 h, cells were treated with α-MSH for 24 h. The culture-conditioned medium was used for SEAP activity assay essentially as per the Clontech protocol (Palo Alto, CA, USA). For transfection control, cells were co-transfected with β-galactosidase and the activity was assayed from each transfection.

2.8. Detection of different receptors from cell surface and culture supernatant

Cells and culture supernatant was collected from macrophages, either untreated or α-MSH-treated for different times at 60% confluent culture conditions containing 1 ml medium/well of 6-well plates. Culture supernatant was concentrated. Briefly, 1 ml culture supernatant was taken in Centricon YM-3 tubes (3-kDa cut-off), purchased from Millipore (Bedford, MA, USA), and centrifuged at $3000\times g$, 4°C for 2 h and made about 100 μl. 50 μg supernatant proteins were spotted on a nitrocellulose disc. Nitrocellulose discs were blocked in 5% BSA. Fresh culture medium (500 μl) was added in each well. Both cells and discs were incubated with different antibodies (IL-1R, TNFR1, TNFR2, or CD14) for 2 h at 37°C and then incubated with [^{125}I]IgG (goat) for 1 h. Then labeled IgG binding was assayed from cells and discs by washing three times with medium. The binding of [^{125}I]IgG alone for cells or discs was subtracted from total [^{125}I]IgG binding preincubated with different antibodies to get specific binding.

2.9. Membrane preparation

Macrophage membranes were isolated from macrophages (1×10^7 cells/ml) by lysing the cells in hypotonic buffer followed by sucrose gradient centrifugation as described earlier [40].

2.10. Statistical analysis

All data were analyzed by a Student's *t*-test. Samples were run in triplicate and data represent mean ± S.D. Independent experiments were performed at least thrice.

3. Results

In this study, we examined the effect of α-MSH on SA-LPS-induced biological responses. Non-adherent THP-1 monocytic cells were converted into adherent macrophages by PMA (10 ng/ml) in 16 h and these cells were induced with SA-LPS.

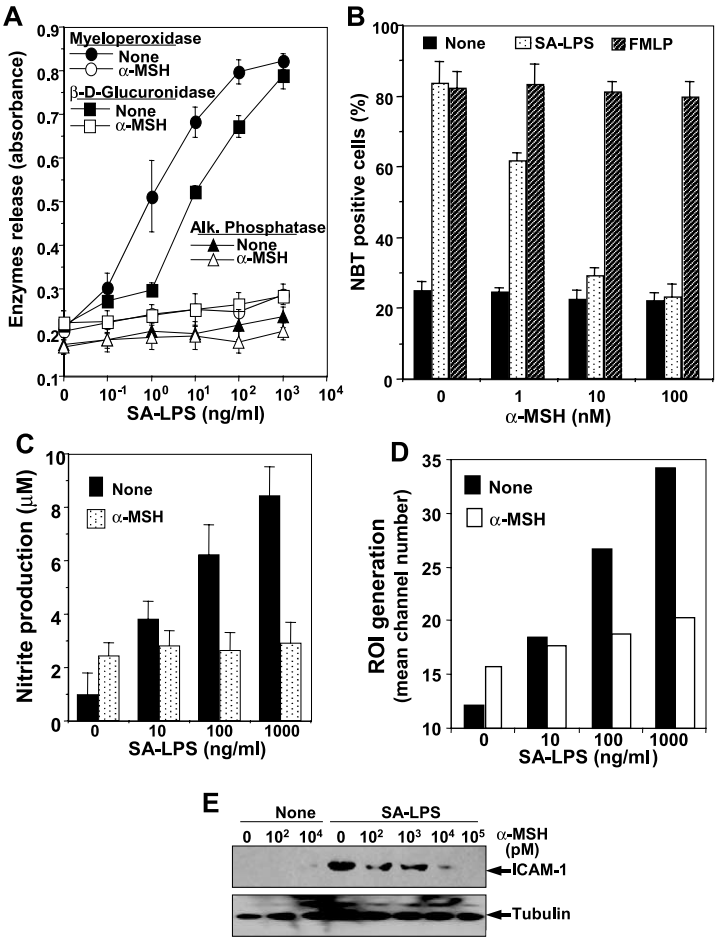

Fig. 1. A: Effect of α-MSH on SA-LPS-induced enzyme release. Macrophages were treated with 100 nM α-MSH for 24 h and then stimulated with different concentrations of SA-LPS for 2 h. The supernatant was collected and analyzed for myeloperoxidase, alkaline phosphatase, and β-D-glucuronidase. The results shown are representative of three independent experiments. B: Effect of α-MSH on SA-LPS- and FMLP-induced oxidative burst response. Macrophages were treated with different concentrations of α-MSH for 24 h and then stimulated with 100 ng/ml SA-LPS and 100 nM FMLP for 2 h. Then the NBT test was carried out. NBT-positive and -negative cells were counted under the microscope; NBT-positive cells are presented in percent. C: Effect of α-MSH on SA-LPS-induced NO production. Macrophages were treated with 100 nM α-MSH for 24 at 37°C and then stimulated with different concentrations of SA-LPS for 6 h. The nitrite concentration in the supernatants was then measured as described in Section 2. D: Effect of α-MSH on SA-LPS-induced ROI generation. Cells, either untreated or pretreated with α-MSH (100 nM) for 24 h, were stimulated with different concentrations of SA-LPS for 4 h. ROI generation was assayed as mean channel number by dihydrorhodamine in a flow cytometer. E: Effect of α-MSH on SA-LPS-induced ICAM-1 expression. Macrophages, either untreated or α-MSH (100 nM)-pretreated for 24 h, were stimulated with different concentrations of SA-LPS for 12 h. Then cell extract proteins were assayed for ICAM-1 by Western blot. The same blot was re-probed to detect tubulin by Western blot to show equal loading of extract proteins. F1: Effect of α-MSH on SA-LPS-induced NF-κB activation. Macrophages (2×10^6/ml) were treated with different concentrations of α-MSH for 24 h at 37°C in a CO_2 incubator and then stimulated with SA-LPS (100 ng/ml) for 2 h. NF-κB was assayed from 8 μg NE proteins. The result shown is representative of three independent experiments. F2: Supershift of NF-κB band. SA-LPS-induced NE was incubated for 15 min with different antibodies and cold NF-κB oligonucleotide and then assayed for NF-κB. G: Effect of polymyxin B sulfate on SA-LPS-induced NF-κB activation. 10 μg of polymyxin B sulfate was incubated with SA-LPS for 1 h at 37°C and then this mixture was added to macrophages for 2 h at 37°C. NE were prepared and assayed for NF-κB by EMSA. H: Effect of α-MSH on SA-LPS-induced NF-κB-dependent reporter gene expression. Macrophages were transiently transfected without (H1) or with IκBα-DN plasmid (H2) along with NF-κB-containing plasmid linked to the SEAP gene. The cells were cultured for 12 h, treated with 100 nM of α-MSH for different times and then stimulated with SA-LPS for 4 h. Culture supernatant was taken and assayed for SEAP. The data shown are representative of three independent experiments.

3.1. Inhibition by α-MSH of SA-LPS responses

3.1.1. α-MSH inhibits SA-LPS-induced myeloperoxidase, alkaline phosphatase, and β-D-glucuronidase activity. LPS, when incubated with autologous serum, binds with LBP of serum and forms the LPS–LBP complex. This complex then interacts with its cell surface receptor CD14 and induces biological responses. To detect the role of α-MSH in SA-LPS-mediated biological responses in macrophages, cells were incubated with α-MSH (100 nM) for 24 h at 37°C and then stimulated with different concentrations of SA-LPS for 2 h at 37°C. Culture supernatant was then used to analyze myeloperoxidase, alkaline phosphatase, and β-D-glucuronidase (Fig. 1A). The absorbance for myeloperoxidase and β-D-glucuronidase, but not for alkaline phosphatase was enhanced with increasing concentrations of SA-LPS, whereas α-MSH-pretreated macrophages showed no induction of myeloperoxidase and β-D-glucuronidase activity with different concentrations of SA-LPS. The results are mean OD of triplicate assays. From these results it is clear that the release of SA-LPS-mediated proteolytic enzymes is inhibited by α-MSH.

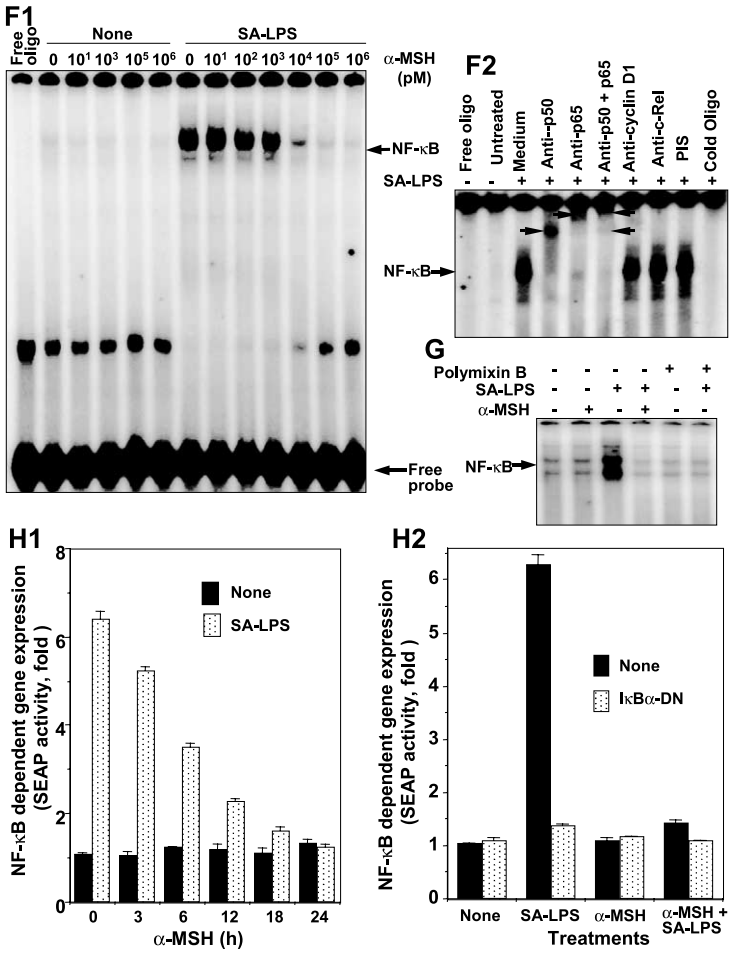

Fig. 1 (*Continued*).

3.1.2. α-MSH inhibits SA-LPS-, but not FMLP-induced oxidative burst response. That LPS and FMLP induce macrophages to generate reactive oxygen species (ROS) is well-established [35]. The generation of ROS is detected by the NBT test. To detect the role of α-MSH in SA-LPS-mediated ROS generation, macrophages were treated with different concentrations of α-MSH for 24 h and then stimulated with 100 ng/ml SA-LPS and 100 nM FMLP for 2 h at 37°C in the presence of NBT solution (0.1%). NBT-positive cells were counted under the microscope and are presented in percent above unstimulated cells (Fig. 1B). α-MSH did not increase the number of NBT-positive cells alone like in unstimulated cells. SA-LPS and FMLP induced 82% NBT-positive cells ($P < 0.001$). α-MSH inhibited SA-LPS-induced but not FMLP-induced NBT-positive cells. These data indicate that α-MSH downregulates the SA-LPS-, but not the FMLP-induced oxidative burst response suggesting specific activity of α-MSH.

3.1.3. α-MSH inhibits SA-LPS-induced NO production. To detect the role of α-MSH on SA-LPS-induced NO production, macrophages were treated with α-MSH (100 nM) for 24 h and then stimulated with different concentrations of SA-LPS for 6 h at 37°C. The culture supernatant was collected and then analyzed for NO using Griess reagent. The concentration of NO was determined from the standard curve obtained from the reaction of $NaNO_2$ ($P < 0.001$). The results in Fig. 1C indicate that SA-LPS induced NO production in a dose-dependent manner. α-MSH inhibited SA-LPS-induced NO production at all concentrations.

3.1.4. α-MSH inhibits SA-LPS-induced ROI generation. ROI generation is an intermediate step for different biological responses. To detect the role of α-MSH in SA-LPS-induced ROI generation, macrophages were treated with α-MSH (100 nM) for 24 h and then stimulated with different concentrations of SA-LPS for 4 h. Then ROI generation was examined with dihydrorhodamine dye conversion to rhodamine as described in Section 2. SA-LPS-induced ROI generation was shown in a dose-dependent manner and α-MSH inhibited ROI generation at all concentration of SA-LPS (Fig. 1D).

3.1.5. α-MSH inhibits SA-LPS-induced ICAM-1 expression. As α-MSH inhibited different SA-LPS-induced biological responses, the expression of intracellular adhesion molecule (ICAM-1) was detected. Macrophages were treated with α-MSH (100 nM) for 24 h and then stimulated with different concentrations of SA-LPS for 12 h. Then 100 μg cell extract proteins were analyzed in 9% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and ICAM-1 was detected by Western blot analysis. Fig. 1E shows that SA-LPS induced ICAM-1 expression in a dose-dependent manner but α-MSH-pretreated cells failed to induce ICAM-1 expression by varying concentrations of SA-LPS, suggesting

α-MSH-mediated inhibition of SA-LPS-induced biological responses in macrophages. Upon re-probing the gel with anti-tubulin antibody, we found that the band intensities in all lanes were uniform indicating equal loading of extracted protein in the lanes.

3.1.6. α-MSH inhibits SA-LPS-induced NF-κB activation. To detect the role of α-MSH in endotoxin-induced NF-κB activation, macrophages were treated with different concentrations of α-MSH for 24 h at 37°C and then stimulated with 100 ng/ml SA-LPS for 2 h at 37°C. NE was prepared and 8 μg NE proteins were analyzed in 6.6% native PAGE to detect NF-κB by gel shift assay. The results in Fig. 1F1 indicate that α-MSH alone did not activate NF-κB, but SA-LPS-induced NF-κB activation was inhibited in a dose-dependent manner and at 100 nM concentration α-MSH completely abrogated SA-LPS-induced NF-κB activation. From this result, it is clear that α-MSH alone does not alter NF-κB activation but inhibits SA-LPS-induced NF-κB activation. Various combinations of Rel/NF-κB proteins can constitute an active NF-κB heterodimer that binds to specific sequences in DNA. To show that the retarded band visualized by EMSA in SA-LPS-induced cells was indeed NF-κB, we incubated the NE from SA-LPS-activated cells with antibodies p50 (NF-κBI) and p65 (Rel A) or in combination and then conducted EMSA. Antibodies to either subunit of NF-κB shifted the band to a higher molecular weight (Fig. 1F2), thus suggesting that the SA-LPS-activated complex consisted of p50 and p65 subunits. Neither preimmune serum nor irrelevant antibodies such as anti-c-Rel or anti-cyclin D1 had any effect on the mobility of NF-κB. The complex completely disappeared in the presence of a 50-fold molar excess of cold NF-κB indicating its specificity for NF-κB.

3.1.7. Polymyxin B sulfate blocks SA-LPS-induced NF-κB activation. LPS is a glycolipid, and its lipid moiety is known to be critical for its activity. Polymyxin B, a polycationic cyclic peptide, is known to bind to the lipid moiety of LPS and inactivate its activity [41]. To ascertain this exquisite specificity for NF-κB activation, we incubated 10 μg of polymyxin B sulfate with SA-LPS for 1 h at 37°C and then this mixture was added to macrophages for 2 h at 37°C. NE were prepared and assayed for NF-κB by EMSA. The results in Fig. 1G show that polymyxin B by itself had no effect on NF-κB activation, but it completely abrogated SA-LPS-induced NF-κB activation.

3.1.8. α-MSH inhibits SA-LPS-induced NF-κB reporter gene activation. As SA-LPS-induced NF-κB activation was blocked by α-MSH, NF-κB-dependent gene expression was also tested. Macrophages were transfected with NF-κB reporter plasmid containing the SEAP gene and/or IκBα-DN plasmid. Cells were treated with α-MSH (100 nM) for different times at 37°C and then stimulated with SA-LPS for 4 h. Culture supernatant was collected and used to assay SEAP activity. The results in Fig. 1H1 indicate that SEAP activity was induced by SA-LPS. α-MSH alone had no effect on SEAP activity but inhibited SA-LPS-induced SEAP activity in a time-dependent manner. The IκBα-DN-transfected cells showed the basal activity of SEAP and SA-LPS-mediated stimulation was completely abrogated by the cells transfected with IκBα-DN (Fig. 1H2) indicating the specificity of the assay. The β-galactosidase activity from cell extracts showed almost similar absorbance from different treatments (as per Promega protocol) at 420 nm (data not shown) suggesting the transfection control for each treatment.

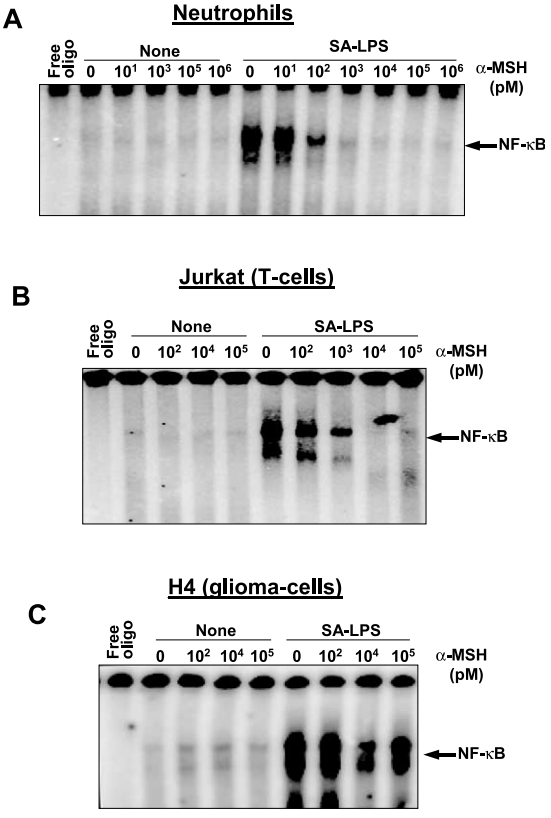

Fig. 2. Effect of α-MSH on SA-LPS-induced NF-κB activation in different cell lines. Human neutrophils isolated from fresh human blood (A), T-cell (Jurkat) (B), and glioma (H4) (C) cells were treated with different concentrations of α-MSH for 24 h and then stimulated with SA-LPS (100 ng/ml) for 2 h at 37°C. After these treatments, NE were prepared and assayed for NF-κB.

3.2. Inhibition of NF-κB activation by α-MSH is cell type-specific

As NF-κB activation pathways differ in many cell types [42–45], we studied whether α-MSH affects human Jurkat (T) cells and H4 (glioma) cells and neutrophils isolated from fresh human blood as well. It has been demonstrated that distinct signal transduction pathways could mediate induction in lymphoid, neuronal, and primary cells. All the effects of α-MSH described above were conducted with macrophages. We found that α-MSH blocks SA-LPS-induced NF-κB activation in neutrophils (Fig. 2A) and Jurkat cells (Fig. 2B) in a dose-dependent manner, but partially in H4 cells (Fig. 2C), suggesting that this effect of α-MSH is restricted to neutrophils and T-cells, but not glioma cells.

3.3. Levels of α-MSH receptors in different cells

To understand the specificity of α-MSH-mediated downregulation of NF-κB, we detected the level of expression of α-MSH receptors in different cells. H4, HeLa, and THP-1-derived macrophages (1×10^6/well) were plated and incubated overnight in 12-well plates. U937, Jurkat cells, and neutrophils (1×10^6/sample) were kept on ice in triplicate. Cells were incubated with 4 ng ^{125}I-labeled α-MSH (5×10^4 cpm) for 2 h at 4°C in the presence or absence of 50-fold cold

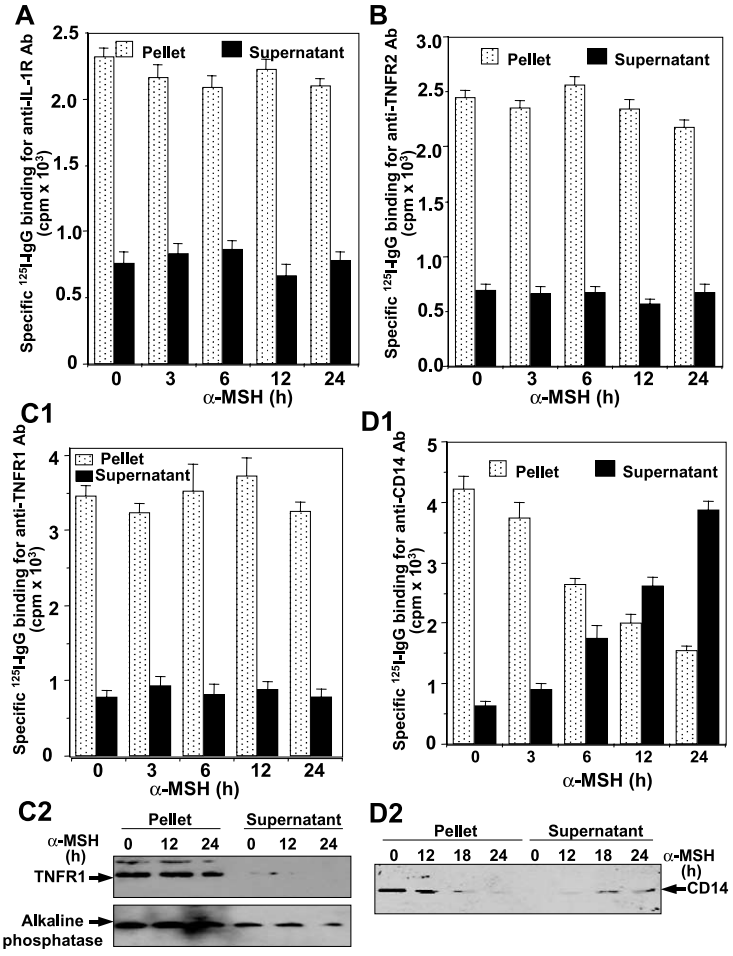

Fig. 3. Effect of α-MSH on anti-IL-1R, -TNFR2, -TNFR1, and -CD14 antibody binding. Macrophages were treated with 100 nM α-MSH for different times. After treatment, cells and 10 times concentrated culture supernatants (taken in nitrocellulose membrane discs) were incubated with anti-IL-1R (A), -TNFR2 (B), -TNFR1 (C1), or -CD14 (D1) antibodies. Then specific [^{125}I]IgG (mouse) binding was assayed as described in Section 2. The cell pellet was extracted and 100 μg of extract proteins and 50 μg of culture supernatant proteins were assayed for TNFR1 (C2) and CD14 (D2) level by Western blot analysis. The results shown are representative of three independent experiments.

α-MSH and α-MSH binding was assayed. The results are represented as mean specific binding in cpm ± S.D. of triplicate samples (Table 1). The results indicate that the α-MSH receptors are expressed in macrophages, Jurkat, and neutrophils ($P < 0.001$), which reflects the α-MSH-mediated downregulation of SA-LPS-induced NF-κB activation.

3.4. α-MSH downregulates CD14, but not IL-1R1, TNFR1, or TNFR2 in macrophages

To detect the effect of α-MSH on the level of different receptors, macrophages were treated with 100 nM α-MSH for different times. Then cells and culture supernatant (concentrated 10 times) were used to assay IL-1R1, TNFR1, TNFR2, or CD14 using [^{125}I]IgG binding assay as described in Section 2. Fig. 3 shows that specific binding for IL-1R1 (Fig. 3A), TNFR1 (Fig. 3B), or TNFR2 (Fig. 3C1) was not changed with varying time of α-MSH incubation either in cells or in culture supernatant ($P < 0.005$). The specific binding of [^{125}I]IgG for CD14 was decreased in cells with increasing time of α-MSH treatment. The level was gradually increased with increasing time of α-MSH treatment of cell supernatant (Fig. 3D1). The level of TNFR1 was unchanged in the cell extracts treated with α-MSH as assayed by Western blot (Fig. 3C2). No TNFR1 was observed in culture supernatant. The level of CD14 was decreased in the cell extract with increasing time of α-MSH treatment and the level increased gradually in the supernatant (Fig. 3D2), indicating that α-MSH induces release of CD14 from the surface of macrophages but not other receptors like IL-1, TNFR1, and TNFR2. The almost equal intensity of the alkaline phosphatase level from different lanes shows equal loading of proteins.

3.5. Anti-MC-1R antibody protects α-MSH-mediated downregulation of SA-LPS-induced NF-κB activation

To detect the specificity of α-MSH-mediated inhibition of SA-LPS-induced biological responses, macrophages (1×10^6/well of 6-well plates) were incubated with 1 μg of anti-melanocortin 1 receptor (anti-MC-1R) antibody for 2 h and then treated with α-MSH (100 ng/ml) for 24 h. Cells were then stimulated with SA-LPS (100 ng/ml) for 2 h. NE were prepared, and 8 μg nuclear proteins were analyzed to detect NF-κB by gel shift assay. Fig. 4A shows that SA-LPS-induced NF-κB activation was inhibited by α-MSH. α-MSH-treated cells did not downregulate SA-LPS-induced NF-κB activation when cells were preincubated with anti-MC-1R antibody indicating that anti-MC-1R antibody protects α-MSH-mediated downregulation of NF-κB.

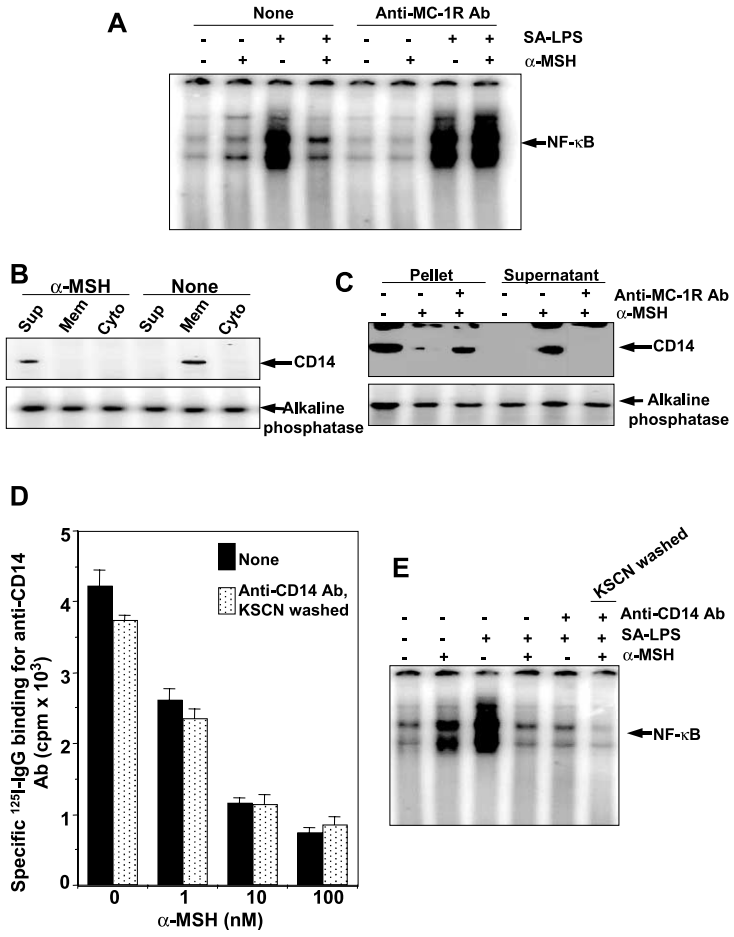

Fig. 4. A: Effect of anti-MC-1R antibody on α-MSH-mediated downregulation of SA-LPS-induced NF-κB activation. Macrophages, preincubated with anti-MC-1R antibody (1 μg/ml) for 2 h, were treated with 100 nM α-MSH for 24 h. Cells were then stimulated with 100 ng/ml SA-LPS for 2 h. NF-κB was assayed from NE. The result is representative of three independent experiments. B: Effect of α-MSH on downregulation of CD14. Macrophages were stimulated with α-MSH (100 nM) for 24 h and then culture supernatant (Sup) was concentrated 10-fold and 100 μg protein was taken in a tube. The plasma membrane (Mem) and cytoplasm (Cyto) were fractionated from untreated and α-MSH-treated cells (1×10^6 cells). The supernatant, membrane and cytosol were boiled in SDS–PAGE sample buffer. CD14 was detected by Western blot. The same blot was re-probed for alkaline phosphatase by Western blot analysis and bands were detected by chemiluminescence by PhosphorImager. C: Effect of anti-MC-1R antibody on α-MSH-mediated downregulation of CD14. Macrophages, pre-incubated with anti-MC-1R antibody (1 μg/ml) for 2 h, were treated with 100 nM of α-MSH for 24 h. The cell extract proteins (100 μg) and concentrated culture supernatant proteins (50 μg) were analyzed in 10% SDS–PAGE to detect CD14 by Western blot analysis. The same blot was re-probed with alkaline phosphatase. The result shown is representative of three independent experiments. D,E: Role of anti-CD14 antibody to protect α-MSH-mediated downregulation of CD14 and NF-κB. Macrophages were incubated without or with 1 μg/ml anti-CD14 antibody for 2 h and then treated with different concentrations of α-MSH for 24 h. Cells, then washed with 0.5 M KSCN for 10 s, were incubated with 1 μg/ml anti-CD14 antibody for 2 h followed by [^{125}I]IgG for 1 h at 37°C and specific binding was assayed (D). After KSCN washing cells were stimulated with SA-LPS (100 ng/ml) for 2 h, NE were prepared, and assayed for NF-κB by EMSA (E).

3.6. α-MSH downregulates CD14 by secretion from macrophage cell surface

To detect the mode of CD14 downregulation by α-MSH-treated macrophages, cells were treated with α-MSH (100 nM) for 24 h and then cell supernatant, plasma membrane and cytoplasm (including cell organelle) were prepared. Cell supernatant (100 μg proteins, after 10 times concentration), plasma membrane and cytosol (from 1×10^6 cells) were solubilized in SDS–PAGE sample buffer and then analyzed for CD14 by Western blot analysis. The bands were detected by ECL^{Plus} reagent (Amersham, IL, USA) and detected in PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). The level of CD14 was observed in the membrane fraction of unstimulated cells and in the supernatant in α-MSH-treated cells (Fig. 4B). CD14 was not observed in the cytosolic

Table 1
Specific [^{125}I]α-MSH binding to cells

Cells	Specific binding ± S.D. (cpm)
H4	1340 ± 120
U-937	1230 ± 102
THP-1	1672 ± 214
Macrophages	3020 ± 230
Jurkat	2140 ± 210
HeLa	858 ± 92
NIH 3T3	812 ± 152
Neutrophils	2678 ± 156

Different cells were incubated with [^{125}I]α-MSH for 2 h at 4°C in the presence or absence of 50-fold unlabeled α-MSH. The specific α-MSH binding was assayed after subtracting the non-specific binding (binding in the presence of 50-fold cold α-MSH) from total binding (binding in the absence of cold α-MSH). The results indicate mean specific binding (in cpm) of triplicate assays.

fraction. The equal intensities of alkaline phosphatase bands indicated the loading control.

3.7. Anti-MC-1R antibody protects α-MSH-mediated downregulation of SA-LPS-induced NF-κB activation

To detect the role of the melanocortin receptor on α-MSH-mediated downregulation of CD14, macrophages were incubated with anti-MC-1R antibody (1 μg/ml) for 2 h and then treated with α-MSH (100 nM) for 24 h. Then cell supernatant was collected and concentrated (10 times). The cell pellet was extracted and 100 μg extract proteins and 50 μg cell supernatant proteins were analyzed in 10% SDS–PAGE and detected for CD14 by Western blot analysis. The results in Fig. 4C indicate that pretreatment with anti-MC-1R antibody protected the α-MSH-mediated CD14 downregulation. Reprobing the blot with anti-alkaline phosphatase antibody showed the equal loading of proteins.

3.8. Anti-CD14 antibody does not protect α-MSH-mediated downregulation of CD14 and NF-κB activation

To detect the role of anti-CD14 antibody on α-MSH-mediated CD14 downregulation, macrophages were incubated with anti-CD14 antibody (1 μg/ml) for 2 h and then treated with different concentrations of α-MSH for 24 h. Cells were then washed with 0.5 M potassium thiocyanate (KSCN) for 10 s. The KSCN wash was given to remove anti-CD14 antibody bound to its receptor [35]. After washing, cells were incubated with anti-CD14 antibody for 2 h followed by [^{125}I]IgG binding at 37°C for 1 h. The specific binding was calculated. The results in Fig. 4D indicate that α-MSH decreased the CD14 level (by decreasing [^{125}I]IgG binding) at increasing concentrations ($P < 0.001$). A similar pattern of CD14 binding was shown in cells preincubated with anti-CD14 antibody followed by α-MSH treatment and washing with KSCN. From this result it is clear that anti-CD14 antibody does not protect α-MSH-mediated downregulation of CD14. Anti-CD14 antibody-preincubated macrophages did not show protection towards α-MSH-mediated inhibition of SA-LPS-induced NF-κB activation (Fig. 4E). These results suggest that anti-CD14 antibody is unable to protect the α-MSH-mediated action.

4. Discussion

Even though several studies indicate that certain neuropeptides, such as α-MSH, have anti-inflammatory effects, the mechanism underlying this effect is not understood. Primarily, the cells in the brain and pituitary are responsible for production of α-MSH. α-MSH interacts through its receptors (MC-R) [46,47] in different cells and it also stimulates melanocytes and other cell types. It was reported that both human and murine macrophage cell lines and human neutrophils express MC-R [18,19]. Our data also confirm the expression of α-MSH receptors in different cell types (Table 1). Thus, it is likely that the effects of α-MSH described here in macrophages and other cells are mediated through activation of the MC-R. The inflammatory responses are aggravated by NF-κB activation due to the NF-κB-dependent gene activation. It has been reported that α-MSH inhibits NF-κB activation in the monocytic cell line U-937 [48]. As macrophages play a central role in responding to a microbial pathogen and triggering the inflammation due to bacterial secretion of LPS, a major component of the outer membrane via the activation of NF-κB, the regulation of this transcription factor is important in macrophages. As LPS first binds to the serum glycoprotein LBP and stimulates cells through CD14, we incubated LPS with autologous serum and this mixture, SA-LPS, which serves as an endotoxin, was used as an inducer of inflammation in vitro. LPS is a glycolipid, and its lipid moiety is known to be critical for its activity. Polymyxin B, a polycationic cyclic peptide, is known to bind to the lipid moiety of LPS and inactivate its activity [41]. Polymyxin B sulfate inhibited SA-LPS-induced NF-κB activation (Fig. 2B), indicating specific endotoxin-mediated activities in macrophages.

Upon infection, macrophages release several proteolytic enzymes to remove microbes in vivo. Stimulation of macrophages with SA-LPS led to release of myeloperoxidase and β-D-glucuronidase and α-MSH inhibited SA-LPS-induced release of those enzymes but not alkaline phosphatase as detected by enzyme activity assay (Fig. 1A). Myeloperoxidase and β-D-glucuronidase are released from azurophilic granules but alkaline phosphatase is released from secretory vesicles [49]. SA-LPS might activate the macrophages by releasing the azurophilic contents but not the secretory vesicles. Activated cells show the oxidative burst response and generate different oxygen species. NBT dye converted into formazan granules reacted with those oxygen species and was deposited inside the cells. α-MSH inhibited the SA-LPS-induced oxidative burst response as detected by NBT-positive cell count (Fig. 1B). The oxidative burst response causes generation of different reactive oxygen and nitrogen species. α-MSH inhibited SA-LPS-induced nitrite and ROI generation (Fig. 1C,D). In this report we observed that endotoxin-induced NF-κB activation was inhibited by α-MSH (Fig. 1F1). α-MSH also downregulated SA-LPS-induced ICAM-1 expression (Fig. 1E). Expression of adhesion molecules is believed to be mediated by activation of NF-κB which is correlated with the downregulation of NF-κB (induced by SA-LPS) by α-MSH. This downregulation of NF-κB was further confirmed by NF-κB-dependent reporter gene (SEAP activity) assay (Fig. 1H). Several genes such as cytokines, cyclooxygenase-2, metalloproteases, urinary plasminogen activator, and cell surface adhesion molecules are involved in inflammation and tumor promotion is regulated by NF-κB [50–54]. As α-MSH blocked NF-κB activation and SEAP expression, so it may play a critical role in inflammation exhibiting anti-inflammatory effects.

α-MSH-mediated downregulation of NF-κB was observed not only in macrophages but also in a human T-cell line and human neutrophils isolated from fresh human blood. However, why α-MSH did not show downregulation of NF-κB in human glioma cells is not understood. The level of α-MSH receptors on the glioma cell surface is lower than on macrophages, neutrophils, or T-cells (Table 1). The low level of α-MSH receptor may be the cause of α-MSH insensitivity in glioma cells. However, there is no report that Jurkat cells have CD14 so the downregulation of NF-κB in these cells by α-MSH might follow some other mechanism like an increase in cAMP level [48,55] which needs to be further studied. α-MSH downregulated CD14, an endotoxin receptor from macrophages, but not TNFR1 and TNFR2 or IL-1 receptors (Fig. 3). The level of CD14 decreased from macrophages and it appeared in culture supernatant. CD14 is secreted from the surface of macrophages by α-MSH treatment. Recently Vega

and Maio [32] reported that geldanamycin downregulated CD14 from macrophages and it internalized and localized in a cytoplasmic organelle, the endoplasmic reticulum. Our results suggest that α-MSH downregulates CD14 from macrophages by secreting from the surface but not by residing in the cytoplasmic organelles. The anti-CD14 antibody did not protect α-MSH-mediated downregulation of CD14. The mechanism underlying the α-MSH-mediated secretion of CD14 from the macrophage surface needs to be studied. A possible role of different proteases induced by α-MSH cannot be ruled out. However, in this report we provide a novel mechanism by which endotoxin-mediated inflammatory responses are ameliorated by α-MSH indicating its anti-inflammatory activity in macrophage-mediated inflammation. Our present study indicates that α-MSH may prove to be a boon in the treatment of sepsis.

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