Inhibition of Endotoxin-Induced Uveitis and Potentiation of Cyclooxygenase-2 Protein Expression by α-Melanocyte–Stimulating Hormone

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Purpose. The efficacy of α-melanocyte–stimulating hormone (α-MSH) on endotoxin-induced uveitis (EIU) was investigated in rats. Several studies have demonstrated that there are various inflammatory reactions mediated by an α-MSH receptor in macrophages. In addition, as it is known that cyclooxygenase (COX)-2 is induced by a variety of stimuli and plays an important role in inflammation, COX-2 expression was also investigated in macrophage cells treated with α-MSH in vitro to clarify its anti-inflammatory effect.

Methods. EIU was induced in male Lewis rats by a footpad injection of lipopolysaccharide (LPS). The number of infiltrating cells and protein concentration in the aqueous humor collected 24 hours after the LPS treatment was determined. The levels of prostaglandin (PG)-E2, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and monocyte chemoattractant protein (MCP)-1 production were determined. RAW 264.7 cells were pretreated with various concentrations of α-MSH for 24 hours and subsequently incubated with 10 μg /mL LPS for 24 hours. COX-2 protein expression was analyzed by Western blot analysis.

Results. α-MSH suppressed the development of EIU in a dose-dependent fashion. The treatment with α-MSH reduced the PGE2, TNF-α, IL-6, and MCP-1 concentrations in aqueous humor. The COX-2 protein expression in the α-MSH group decreased.

Conclusions. This study suggests that α-MSH has an antiocular inflammatory effect, by suppression of PGE2, TNF-α, IL-6, and MCP-1 production and blocking of COX-2 expression. (*Invest Ophthalmol Vis Sci.* 2004;45:159–164) DOI:10.1167/iovs.03-0492

A tridecapeptide, α-melanocyte-stimulating hormone (α-MSH), known as a neuroimmunomodulator, has many effects in the pituitary gland, brain, skin, and other sites in higher organisms.[1] α-MSH also prevents local inflammation in a rat model of arthritis and increases survival in experimental endotoxemia-peritonitis.[2] Furthermore, α-MSH has a potent anti-inflammatory action in brain inflammation,[3] renal injury,[4] and liver inflammation experimental models.[5] Other reports[6,7] of studies that have used the rabbit eye inflammation model, have found that α-MSH reduces blood–aqueous barrier failure and infiltration of inflammatory cells into the aqueous humor. One of the anti-inflammatory activities of MSH is reported to be the suppression of NF-κB activation.[8] The presence of MC-1, an α-MSH receptor in macrophages, has also been confirmed. According to Ichiyama et al.,[9] the central nervous system (CNS) is not involved in the anti-inflammatory activities of MSH.

Endotoxin-induced uveitis (EIU) is an acute anterior segment intraocular inflammation that is induced by injection of lipopolysaccharide (LPS) or lipoteichoic acid.[10–14] It has been suggested that this phenomenon could serve as a model for some types of human uveitis, such as those associated with seronegative arthritis, where a Gram-negative bacteria may play a role in pathogenesis.[15] The inflammatory response in EIU is characterized by a breakdown of the blood–aqueous barrier and is cell-mediated with leakage of proteins into the anterior chamber of the eye and infiltration of a large number of inflammatory cells. Exposure to LPS stimulates cellular inflammatory responses,and releases factors such as cytokines and chemokines, including tumor necrosis factor (TNF)-α,[16] interleukin (IL)-6,[17] and monocyte chemoattractant protein (MCP)-1.[18]

Arachidonic acid metabolites such as prostaglandin (PG)-E2, thromboxane B2, and leukotriene B4, have been implicated as important inflammatory mediators. Inhibition of cyclooxygenase (COX), and therefore prostaglandin production, is the common mechanism of action of nonsteroid anti-inflammatory drugs.[19] PGE2 is a major COX product at inflammatory sites, where it contributes to local blood flow increase, edema, and pain sensitization. As it is now well appreciated, COX exists as two isoforms.[20–22] In general terms, cyclooxygenase (COX)-1 is constitutive and present in, for example, endothelium, stomach, and kidney, whereas cyclooxygenase (COX)-2 is induced by proinflammatory cytokines, by endotoxins in cells in vitro, and at inflammatory sites in vivo.[23]

In the present study, we investigated the influence of α-MSH on LPS induced uveitis in rats. Eye inflammation was assessed by measurement of cell infiltration, protein, PGE2, TNF-α, IL-6, and MCP-1 concentrations in aqueous humor. In macrophages, there are various inflammatory reactions mediated by the MC-1 receptor. We also investigated the COX-2 expression in RAW 264.7 macrophage cells treated with α-MSH in vitro to clarify the anti-inflammatory effect.

Materials and Methods

Animal Groups and EIU Induction

Eight-week-old male Lewis rats (280–220 g) were used. Inflammation was induced by injecting one footpad with 200 μg of LPS (from

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Salmonella typhimurium; Sigma-Aldrich, St. Louis, MO) diluted in 0.1 mL of sterile saline. In the α-MSH group, each rat was injected intravenously with 10, 100, or 1000 μg/kg α-MSH (Sigma-Aldrich) in 1 mL/kg PBS immediately after LPS injection. In the LPS-only group, each rat was injected intravenously with 1 mL of PBS immediately after LPS injection. In the control group, neither LPS nor α-MSH was injected into the rats.

Animals were handled and cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Number of Infiltrating Cells and Protein Concentration

Suzuma et al.[10] reported that cellular infiltration in the aqueous humor reached a maximum level 24 hours after LPS treatment in this model. The number of cells infiltrating the aqueous humor and the aqueous humor protein concentration were used as indicators of the degree of anterior inflammation.

Twenty-four hours after LPS injection, rats were killed, and the aqueous humor was collected immediately. Briefly, the aqueous humor was collected from both eyes by an anterior chamber puncture (20 μL/rat) using a 30-gauge needle under a surgical microscope. For cell counting, the aqueous humor sample was suspended in an equal amount of Türk stain solution, and the cells were counted with a hemocytometer under a light microscope. The number of cells per field (an equivalent of 0.1 mL) was manually counted, and the number of cells per microliter was obtained by averaging the results of four fields from each sample. The total protein concentration in the aqueous humor samples was measured with a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL).

The aqueous humor samples were stored in ice water until testing, and cell counts and total protein concentrations were measured on the day of sample collection.

Levels of PGE2, TNF-α, IL-6, and MCP-1

Levels of PGE2, TNF-α, IL-6, and MCP-1 in the aqueous humor were assessed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. ELISA was performed in duplicate.

Cell Culture and LPS Stimulation

RAW 264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) and 10% heat-inactivated fetal bovine serum (Invitrogen-Gibco, Grand Island, NY) and maintained at 37°C in a humidified incubator containing 5% CO_2. RAW 264.7 cells were seeded onto a 24-well plate (5×10^4 cells/well) for experiments. Cells were pretreated with 10, 100, or 1000 μM α-MSH for 24 hours and subsequently incubated with 10 μg/mL of LPS from *S. typhimurium* for 24 hours, unless otherwise stated.

Western Blot Analysis

Cells were washed with ice-cold PBS and then lysed in cold NP-40 lysis buffer (50 mM Tris-Cl [pH 7.6], 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL each of leupeptin, aprotinin, and pepstatin) for 15 minutes at 4°C. Plates were then scraped, and crude lysates were cleared by centrifugation at 14,000*g* for 10 minutes at 4°C. The total protein concentration in the lysates was measured using a BCA protein assay reagent kit (Pierce). Aliquots of the lysates were diluted in a 1:1 ratio with sample buffer (50 mM Tris-HCl [pH 6.8], 10% wt/vol SDS, 10% vol/vol glycerol, 10% vol/vol 2-mercaptoethanol, and 0.02% wt/vol bromphenol blue) and boiled for 2 minutes. Equal amounts of protein (30 μg) were loaded onto 10% SDS-polyacrylamide gels and subjected to electrophoresis for 2 hours. The separated proteins were then electrotransferred to nitrocellulose (Hybond-C Super; Amersham Pharmacia Biotech UK, Ltd., Amersham, UK) for 30 minutes After electrotransfer, the blots were incubated for 30 minutes in blocking solution (3% wt/vol dried low-fat milk and 0.1% vol/vol Tween 20 in phosphate-buffered saline) on an orbital shaker. The blots were then washed (three times, 5 minutes each) with washing buffer (Tween 20 0.1% vol/vol in phosphate-buffered saline) before being probed with anti-COX-2 antibody (rabbit anti-mouse; Alexis Biochemicals, Carlsbad, CA) diluted 1:1000 in blocking solution overnight at 4°C. After incubation with the primary antibody, the blots were washed (three times, 5 minutes) with blocking solution before being probed (1 hour at room temperature) with alkaline phosphatase-conjugate secondary antibody (anti-rabbit IgG; New England Biolabs, Ltd., Hitchin, UK) diluted 1:5000 in blocking solution. The blots were then developed using a horseradish peroxidase Western blot detection kit (Phototope; New England Biolabs, Ltd.). Images were captured on film (Hyperfilm; Amersham Pharmacia Biotech UK, Ltd.).

Cell Viability

For determination of cell viability, an MTT assay was performed with 50 mg/mL of methylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide (Sigma-Aldrich) was added to 1 mL of cell suspension (6.2×10^4 cells/mL in 96-well plates) for 24 hours, and the Formosan formed was dissolved in acidic-2-propanol. Optical density was measured using a plate reader

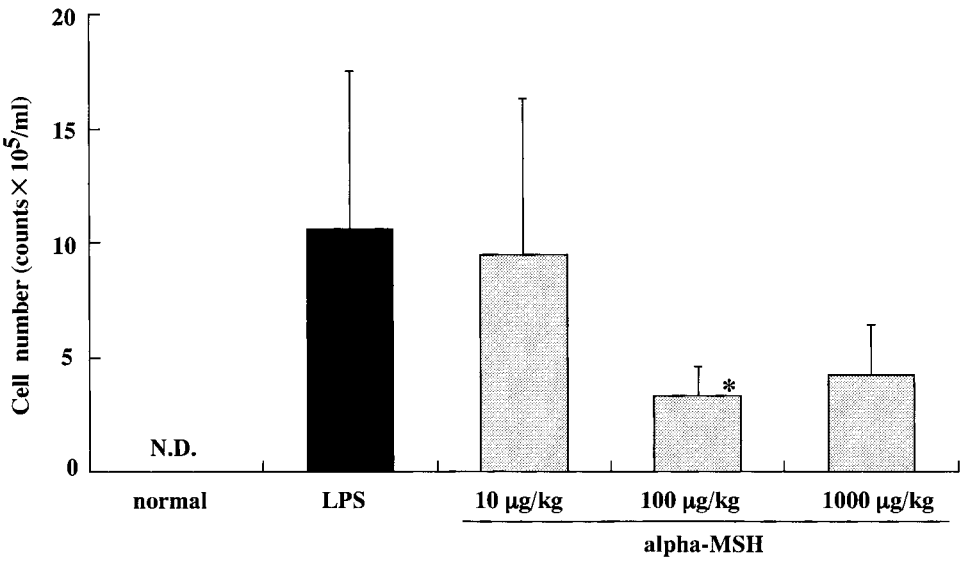

FIGURE 1. Effects of α-MSH on cellular infiltration in the aqueous humor collected 24 hours after LPS treatment and the cells counted. Data are the mean ± SD of results in five rats. *Significantly different from the LPS group at $P < 0.05$; N.D., not detected.

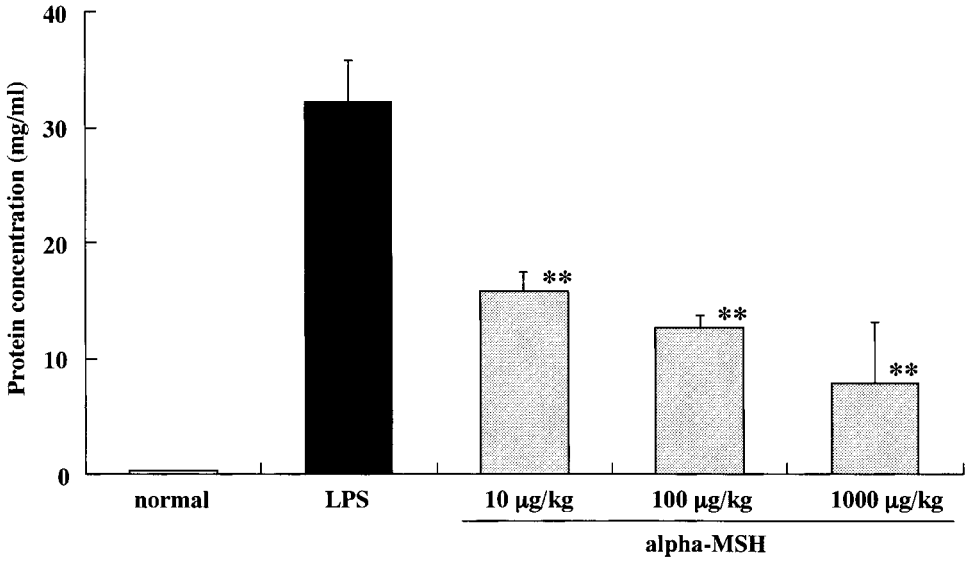

FIGURE 2. Effects of α-MSH on protein concentration in the aqueous humor collected 24 hours after LPS treatment. Data are the mean ± SD of results in five to six rats. **Significantly different from the LPS group at $P < 0.01$.

at 590 nm. The optical density of the Formosan formed by untreated cells was taken as 100%.

Statistical Analysis

Values were expressed as the mean ± SD. An unpaired Student's *t*-test was used to assess the statistical significance of differences. $P < 0.05$ was regarded as significant.

RESULTS

Effects on LPS-Induced Cellular Infiltration

Cells were not detected in aqueous humor of the control group. In the LPS group, the number of cells in the aqueous humor was $10.6 \pm 6.9 \times 10^5$ cells/mL. The group treated with 100 and 1000 μg/kg α-MSH showed reduced numbers of cells (100 μg/kg; $3.3 \pm 1.3 \times 10^5$ cells/mL, 1000 μg/kg; $4.2 \pm 2.2 \times 10^5$ cells/mL) compared with that in the LPS group. Treatment with 10 μg/kg of α-MSH produced a mild reduction in the number of cells ($9.5 \pm 6.8 \times 10^5$ cells/mL). Statistically significant differences were observed ($P < 0.05$) between the LPS and 100 μg/kg α-MSH groups (Fig. 1).

Effects on LPS-Induced Protein Infiltration

In the control group, the aqueous humor protein concentration was 0.3 ± 0.1 mg/mL. With LPS treatment, protein concentration in the aqueous humor increased dramatically by approximately 10-fold (32.3 ± 3.5 mg/mL). Treatment with 10, 100, or 1000 μg/kg of α-MSH significantly reduced protein compared with that of the LPS group (10 μg/kg; 15.8 ± 1.6 mg/mL, $P < 0.01$, 100 μg/kg; 12.6 ± 1.1 mg/mL, $P < 0.01$, 1000 μg/kg; 7.9 ± 5.2 mg/mL, $P < 0.01$, Fig. 2).

PGE2 Concentration

In the control group, PGE2 concentration in the aqueous humor was not detected, but in the LPS group it was 76.2 ± 11.9 ng/mL. The group treated with 100 and 1000 μg/kg α-MSH showed reduced PGE2 (100 μg/kg; 0.5± 0.1 ng/mL, 1000 μg/kg; 0.8 ± 0.1 ng/mL) compared with the LPS group. Treatment with 10 μg/kg of α-MSH produced a mild reduction in PGE2 (49.4 ± 15.5 ng/mL). Statistically significant differences were observed ($P < 0.01$) between LPS and the 100- or 1000-μg/kg α-MSH groups (Fig. 3).

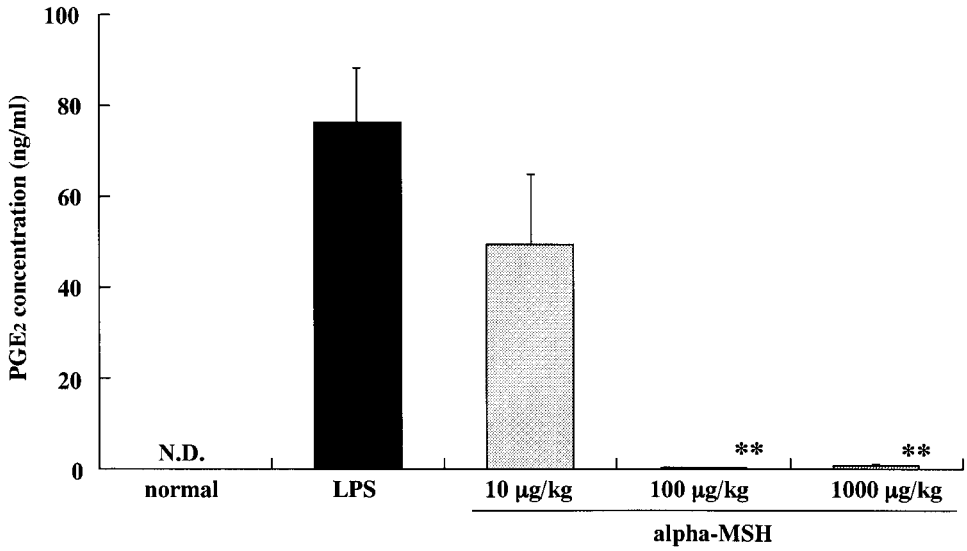

FIGURE 3. Effects of α-MSH on PGE2 concentration in the aqueous humor collected 24 hours after LPS treatment. Data are the mean ± SD of results in five rats. **Significantly different from the LPS group at $P < 0.01$. N.D., Not detected.

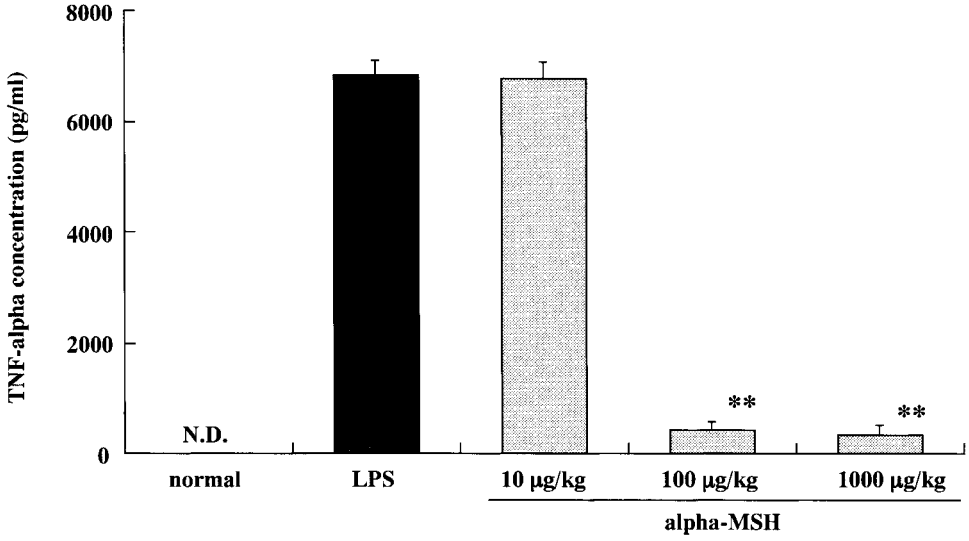

FIGURE 4. Effects of α-MSH on TNF-α concentration in the aqueous humor collected 24 hours after LPS treatment. Data are the mean ± SD of results in five rats. **Significantly different from the LPS group at $P < 0.01$. N.D., not detected.

Levels of TNF-α

In the control group, TNF-α was not detected in the aqueous humor, but in the LPS group the concentration was 6839.6 ± 259.6 pg/mL. The group treated with 100 and 1000 μg/kg α-MSH exhibited reduced TNF-α (100 μg/kg; 427.3 ± 138.3 pg/mL, 1000 μg/kg; 321.2 ± 184.1 pg/mL) compared with the LPS group. Treatment with 10 μg/kg of α-MSH produced no reduction in TNF-α (6781.4 ± 297.2 pg/mL). Statistically significant differences were observed ($P < 0.01$) between LPS and the 100- and 1000-μg/kg α-MSH groups (Fig. 4).

IL-6 Concentration

In the control group, the IL-6 concentration in the aqueous humor was 31.4 ± 54.5 pg/mL, but with LPS treatment, it increased markedly (to 13,767.9 ± 3,006.2 pg/mL). The group treated with 100 and 1000 μg/kg α-MSH exhibited reduced IL-6 (100 μg/kg; 8895.2 ± 2257.2 pg/mL, 1000 μg/kg; 5497.1 ± 2842.0 pg/mL) compared with the LPS group. Treatment with 10 μg/kg of α-MSH produced no reduction in IL-6 (11,419.4 ± 5,509.3 pg/mL). Statistically significant differences were observed ($P < 0.01$) between LPS and the 100 or 1000 μg/kg α-MSH groups (Fig. 5).

MCP-1 Concentration

The MCP-1 concentration in the aqueous humor from rats without LPS (control group) was 71.0 ± 11.3 pg/mL, and in the LPS group was 1027.4 ± 28.1 pg/mL. The MCP-1 concentration in the groups treated with 100 μg/kg and 1000 μg/kg α-MSH were significantly lower than in the LPS group (100 μg/kg; 627.1 ± 125.3 pg/mL, $P < 0.05$ and 1000 μg/kg; 377.6 ± 203.7 pg/mL, $P < 0.01$). Treatment with 10 μg/kg α-MSH produced only a mild reduction in protein concentration (816.5 ± 74.3 pg/mL), and there was no significant difference from that of the LPS group. (Fig. 6).

Expression of COX-2 Protein

No expression of the COX-2 protein as a 72-kDa protein was detected in normal cells (Fig. 7, lane 1), but there was strong expression in LPS-stimulated cells (Fig. 7, lane 2). Expression of COX-2 protein decreased in the 10 to 1000 μM α-MSH group in a dose-dependent fashion (Fig. 7, lanes 3– 5). α-MSH did not decrease cell viability in RAW 264.7 cells when these cells were incubated with 1000 μM α-MSH alone for 24 hours (data not shown).

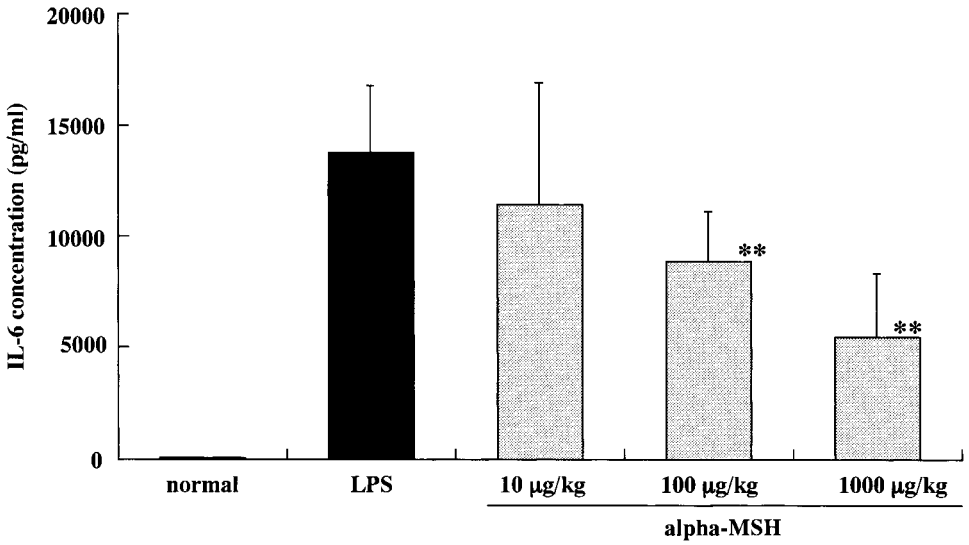

FIGURE 5. Effects of α-MSH on the IL-6 concentration in aqueous humor collected 24 hours after LPS treatment. Data are the mean ± SD of results in five rats. **Significantly different from the LPS group at $P < 0.01$.

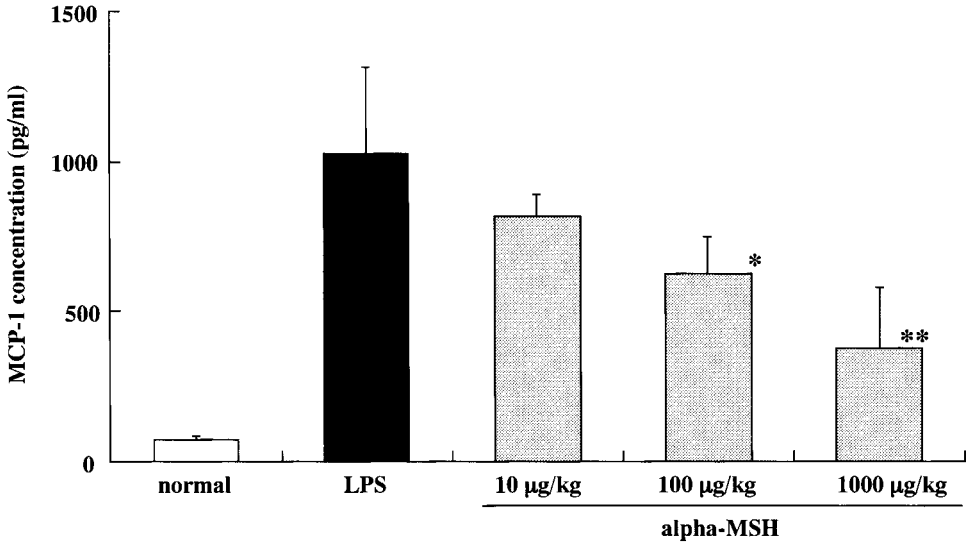

FIGURE 6. Effects of α-MSH on MCP-1 concentration in the aqueous humor collected 24 hours after LPS treatment. Data are the mean ± SD of results in five rats. *, **Significantly different from the LPS group at $P < 0.05$ and $P < 0.01$, respectively.

DISCUSSION

In the present study we investigated the efficacy of α-MSH on EIU in rats. The results of this study indicate that α-MSH suppressed the development of EIU in a dose-dependent fashion. Ohgami et al.[24] reported that steroids and certain carotenoids have anti-inflammatory effects in the rat EIU model. The anti-inflammatory activity of 10 mg/kg of prednisolone was comparable to that of 1000 μg /kg of α-MSH in the present study. The anti-inflammatory activity of 100 mg/kg of astaxanthin, a carotenoid, was also comparable to that of 1000 μg/kg of α-MSH.

TNF-α is a pleiotropic cytokine, produced principally by activated macrophages and monocytes, and it has an important role in the nonspecific resistance against various infectious agents.[25,26] IL-6 is also a multifunctional cytokine that plays important roles in acute phase reactions and immune responses. TNF-α has been postulated to serve as an inducer of IL-6 in several models.[27–29] de Vos et al.[30] has reported on the kinetics of systemic and intraocular TNF-α and IL-6, and found that the interactions between TNF-α and IL-6 during eye inflammation are more complex than originally thought. The results of the present study indicate that α-MSH can decrease the TNF-α and IL-6 concentrations in a dose-dependent manner. Although the mechanism of α-MSH action on TNF-α and IL-6 production cannot be clarified based solely on the results of the present study, α-MSH may somehow affect monocytes and macrophages that produce TNF-α, thus revealing its anti-inflammatory action.

MCP-1 is uniquely essential for monocyte recruitment during inflammation.[31] MCP-1 is expressed in humans during acute anterior uveitis,[32] and its expression has also been described in the rat EIU model.[33] Data from its study have shown that α-MSH can decrease the MCP-1 concentration in aqueous humor in a dose-dependent manner. These findings suggest that systemic administration of α-MSH affects monocyte and macrophage recruitment and thus the expression of anti-inflammatory action. Therefore, monocytes and macrophages may play an important role in the anti-inflammatory action of α-MSH in the EIU model.

The results of the present study indicate that α-MSH can decrease the concentration of PGE2. To examine the α-MSH mechanism of anti-inflammation, we investigated the effect of α-MSH on LPS-induced COX-2 in the RAW 264.7 macrophage cell line. We found that there was a dose-dependent decrease in LPS-induced COX-2 in α-MSH–treated cells. Thus, the α-MSH–induced suppression of PGE2 that was observed occurred due to the direct inhibition of COX-2 expression. Our study also found that even at a concentration of 1000 μM α-MSH, the cell viability did not change. Therefore, inhibition of LPS induced COX-2 expression by α-MSH was not the result of cell cytotoxicity. According to Chiang et al.,[34] NF-κB was involved in the induction of COX-2. The results of the present study suggest that the suppressive effects of MSH on COX-2 induction are attributable to its ability to inhibit NF-κB activation.

In summary, this study indicates that α-MSH shows a dose-dependent antiocular inflammatory effect on EIU. A possible mechanism for the antiocular inflammatory effect of α-MSH, is the suppression of the production of PGE2, TNF-α, IL-6, and MCP-1 and blocking of the expression of COX-2.

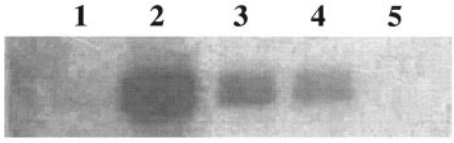

FIGURE 7. Western blot analysis demonstrating the expression of COX-2 protein in the mouse macrophage cell line RAW264.7. *Lane 1*: control; *lane 2*: LPS; *lane 3*: 10 μM α-MSH; *lane 4*: 100 μM α-MSH; and *lane 5*: 1000 μM α-MSH.

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