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Melanocortin Modulation of Inflammatory Cytokine and Neuroendocrine Responses to Endotoxin in the Monkey

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α-MSH has potent antiinflammatory properties, but little is known about the specific melanocortin receptors (MC-Rs) that mediate these effects or about the role of the melanocortin system in modulating cytokine responses to an inflammatory challenge in the primate *in vivo*. We, therefore, studied the effects of infusion of the α-MSH agonist, [Nle^4,D-Phe^7]-α-MSH (NDP-MSH); the α-MSH antagonist, SHU9119; and the selective MC3-R agonist, D-Trp8-γ-MSH, compared with saline, on proinflammatory cytokine (TNF-α, IL-1β, and IL-6), antiinflammatory cytokine [IL-10 and IL-1 receptor antagonist (IL-1ra)], and pituitary-adrenal responses to endotoxin in ovariectomized monkeys. In the first study NDP-MSH or SHU9119 was infused iv for 7 h starting at 0800 h, endotoxin was injected at 1000 h, and serial blood samples were collected (n = 6). NDP-MSH significantly attenuated proinflammatory cytokine responses to endotoxin. The area under the response curve (AUC) decreased by 61% for TNF-α ($P = 0.02$), 47% for IL-1β ($P = 0.02$), and 41% for IL-6 ($P = 0.04$); there was no effect on IL-1ra or IL-10. SHU9119 did not affect proinflammatory cytokine responses, but decreased the IL-10 response by 31% ($P = 0.03$). NDP-MSH also attenuated ACTH ($P < 0.001$) and cortisol ($P = 0.02$) responses. In a second study, the effects of D-Trp8-γ-MSH were similarly examined in seven monkeys. The AUC for IL-6 was decreased by 37% ($P = 0.04$) by D-Trp8-γ-MSH; the AUC for IL-10 was increased by 22%, but this was not significant. However, the ratio of IL-6 to IL-10 was significantly decreased by D-Trp8-γ-MSH ($P = 0.04$), consistent with a relatively more antiinflammatory cytokine environment. These results indicate that NDP-MSH can attenuate proinflammatory cytokine responses in the primate, consistent with previous studies in the rodent, and provide new evidence for a role for MC3-R in this process. Moreover, they show for the first time that SHU9119, a mixed MC3/4-R antagonist, can decrease the IL-10 response, establishing a physiological role for endogenous MSH in modulating the release of an antiinflammatory cytokine. (*Endocrinology* 147: 1878–1883, 2006)

A PEPTIDE DERIVED from the proopiomelanocortin precursor protein, α-MSH, has potent antiinflammatory properties (1–6). α-MSH can antagonize many of the biological effects of endotoxin and the proinflammatory cytokines, including effects on body temperature, immune function, endocrine function, and behavior (1, 4, 7). α-MSH can act directly on melanocortin receptors (MC-Rs) on peripheral immune cells to down-regulate the production of proinflammatory cytokines and can also act within the brain to inhibit peripheral immune responses (8, 9). α-MSH has also been shown to induce production of the antiinflammatory cytokine, IL-10, in monocytes *in vitro* (10). There is evidence that of the five known MC-Rs, MC1-R and MC3-R mediate effects on inflammation (11–13). *In vivo* infusion studies of MSH antisera or peptide antagonists in the rodent indicate that endogenous MSH participates in physiological regulation of the pyretic and hypothalamic-pituitary-adrenal (HPA) responses to inflammation (14, 15). We have previously shown in the monkey that intracerebroventricular icv infusion of α-MSH attenuates the HPA response to IL-1β, whereas the α-MSH antagonist, agouti-related protein, enhances this effect, suggesting that endogenous α-MSH plays a physiological role in this process (16). Little is known, however, about the role of the melanocortin system in modulating cytokine responses to an inflammatory challenge in a primate model *in vivo* or about the specific MC-Rs that mediate these effects. An understanding of this process in a primate model is important, because inflammatory cytokines have been implicated in the pathogenesis of a wide spectrum of human diseases. In addition, there are significant differences in the immune response and in the melanocortin system in rodent and primate models. In this study we, therefore, tested the effects of infusion of the α-MSH agonist [Nle^4,D-Phe^7]α-MSH (NDP-MSH) and the α-MSH antagonist SHU9119 on proinflammatory and antiinflammatory cytokine and HPA responses to endotoxin in female rhesus monkeys. The effects of the selective MC3-R agonist, D-Trp8-γ-MSH (17), were also examined.

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Abbreviations: AUC, Area under the response curve; HPA, hypothalamic-pituitary-adrenal; icv, intracerebroventricular; MC-R, melanocortin receptor.

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Materials and Methods

Animals

Nine adult female rhesus monkeys (*Macaca mulatta*), weighing 5–9 kg, were used in these experiments. Five to seven animals were used in each experiment: three monkeys were used in all three experiments, three monkeys were used in two experiments, and three monkeys participated in only one experiment. Within an experiment, each monkey was studied with either saline or the various treatment peptides and thus served as its own control. Studies in the same animal within each experiment were separated by at least 3 wk. Monkeys were housed in individual cages in a temperature-controlled room (19–22 C) with a 12-h light, 12-h dark photocycle and were fed 20 Purina Monkey Chow biscuits (~6.8 g each; Ralston-Purina Co., St. Louis, MO) twice daily at 1000 and 1500 h. This was supplemented with fresh fruit or vegetables daily. All animals

were ovariectomized at least 2 months before the studies to eliminate fluctuations in estradiol levels, because estradiol has been shown to affect cytokine and neuroendocrine responses to endotoxin (18, 19). All protocols were approved by the Columbia University institutional animal care and use committee and were conducted in accordance with the National Institutes of Health guide for the care and use of laboratory animals. All animals participated in an active enrichment program provided by veterinary medicine and supervised by the institutional animal care and use committee.

Experimental protocols

Experiment 1: effects of NDP-MSH and SHU9119 on cytokine and HPA responses to endotoxin. The night before each experiment, monkeys were briefly sedated with 5–7 mg/kg ketamine, and a catheter was placed in the saphenous vein for peptide infusion and blood collection. The animals were then seated in a plastic primate chair to which they had previously been adapted, which restrained them for the period of the experiment. The next morning a blood sample was obtained at 0800 h, and then either saline or one of the following peptides was infused iv at 20 μg/h for 7 h: NDP-MSH (a stable nonselective MC-R agonist) or SHU9119, an MC3/4-R antagonist (20). Peptides were obtained from Phoenix Pharmaceuticals (Belmont, CA). Endotoxin (lipopolysaccharide *Escherichia coli* 055:B5; Sigma-Aldrich Corp., St. Louis, MO; 2.5 μg) was injected iv at 1000 h. We have shown that 2.5–5 μg endotoxin reliably stimulates cytokine and HPA responses without associated sickness behavior (21). Doses as high as 200 μg endotoxin have been used safely in the monkey, but are associated with sickness behavior (22). Blood samples were collected at 30- to 60-min intervals over the 7-h period. The animals were then returned to their housing quarters. Each monkey was studied three times in random order with saline, NDP-MSH, or SHU9119, with at least 3 wk between experiments. Six monkeys were used for this study. Blood samples were analyzed for TNF-α, IL-6, IL-1β, IL receptor antagonist (IL-1ra), IL-10, ACTH, and cortisol.

Experiment 2: effects of NDP-MSH and SHU9119 on baseline plasma cytokine, ACTH, and cortisol levels. Five monkeys were studied, as described above in experiment 1, with iv infusion of saline, NDP-MSH (20 μg/h), or SHU9119 (20 μg/h) for 7 h, but without endotoxin injection, and blood samples were analyzed for TNF-α, IL-6, IL-10, ACTH, and cortisol.

Experiment 3: effects of D-Trp8-γ-MSH on cytokine and HPA responses to endotoxin. The effects of D-Trp8-γ-MSH (Phoenix Pharmaceuticals), a selective MC3-R agonist (17), on cytokine and HPA responses to endotoxin were studied in seven monkeys. In this study, animals were briefly sedated with 5–7 mg/kg ketamine on the morning of the experiment at 0800 h, and a catheter was placed in the saphenous vein for peptide infusion and blood collection. The animals were then seated in primate chairs and infused with either saline or D-Trp8-γ-MSH at 20 μg/h from 0900–1800 h. Endotoxin (2.5 μg) was injected iv at 1300 h. Blood samples were collected at 30- to 60-min intervals from 1100–1800 h and analyzed for TNF-α, IL-6, IL-10, ACTH, and cortisol.

Hormone and cytokine assays

Plasma ACTH was measured by immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA). Plasma cortisol was measured by solid-phase RIA (ICN Biochemicals, Inc., Costa Mesa, CA). IL-6, IL-1β, IL-10, and IL-1ra were measured by specific monoclonal sandwich immunoassays with human ELISA kits (R&D Systems, Inc., Minneapolis, MN), which we have validated for use in the rhesus monkey (23). TNF-α was assayed with a rhesus monkey ELISA kit (BioSource International, Inc., Camarillo, CA).

Data analysis

The effects of endotoxin and peptide injection on plasma hormone and cytokine levels were analyzed by ANOVA with repeated measures. Areas under the hormone and cytokine response curves (AUCs) were also calculated by trapezoid analysis, and the responses to endotoxin were compared in paired treatment groups by paired *t* test. Analyses were performed with statistical software (StatView, Abacus Concepts, Inc., Berkeley, CA).

Results

Effects of NDP-MSH and SHU9119 on cytokine and HPA responses to endotoxin

As expected, endotoxin stimulated plasma levels of TNF-α, IL-6, IL-1β, IL-1ra, and IL-10 in the saline-infused animals ($P < 0.001$). NDP-MSH infusion significantly attenuated the response of the proinflammatory cytokines, TNF-α, IL-6, and IL-1β, to endotoxin (Fig. 1A). The AUC was decreased by 61% for TNF-α ($P = 0.02$), by 47% for IL-1β ($P = 0.02$), and by 41% for IL-6 ($P = 0.04$; Fig. 1B). There was no significant effect of NDP-MSH on IL-1ra (not shown) or IL-10 (Fig. 1) responses to endotoxin. SHU9119 did not affect proinflammatory cytokine responses, but significantly decreased the IL-10 response to endotoxin by 33% ($P = 0.03$; Fig. 1). Endotoxin also stimulated plasma levels of ACTH and cortisol in the saline-infused animals ($P < 0.001$). NDP-MSH attenuated ACTH ($P < 0.001$) and cortisol ($P = 0.02$) responses to endotoxin over time (by repeated measures ANOVA), but SHU9119 had no effect on either of these responses (Fig. 2). NDP-MSH and SHU9119 had no significant effect (by repeated measures ANOVA) on plasma ACTH ($P = 0.45$) or cortisol ($P = 0.50$) levels compared with saline infusion when administered without endotoxin (data not shown). Plasma TNF-α, IL-6, and IL-10 levels were at or below the level of assay detection and did not become detectable after NDP-MSH or SHU9119 infusion in the absence of endotoxin.

Effects of D-Trp8-γ-MSH on cytokine and HPA responses to endotoxin

In a separate series of experiments, the effects of D-Trp8-γ-MSH, a selective MC3-R agonist, on TNF-α, IL-6, IL-10, ACTH, and cortisol responses to endotoxin were studied. There was a significant attenuation of the IL-6 response to endotoxin in the D-Trp8-γ-MSH-infused animals (Fig. 3). The IL-6 for AUC was decreased by 37% ($P = 0.04$). The AUC for TNF-α was decreased by 45%, but this was not significant ($P = 0.31$). The AUC for IL-10 was increased by 22%, but this was not significant ($P = 0.23$). The ratio of the AUC for IL-6, a proinflammatory cytokine, to IL-10, an antiinflammatory cytokine, was calculated for each animal and compared by paired analysis after endotoxin with either saline or D-Trp8-γ-MSH infusion. The ratio was 1.89 ± 0.43 with saline and decreased to 0.99 ± 0.13 with D-Trp8-γ-MSH infusion ($P = 0.04$). Thus, in the presence of D-Trp8-γ-MSH, there was a relatively more antiinflammatory cytokine environment. The effects of D-Trp8-γ-MSH on the HPA response are shown in Fig. 4. D-Trp8-γ-MSH had no significant effect on ACTH and cortisol responses over time, as assessed by repeated measures ANOVA. The AUC for ACTH was reduced by 23% in the D-Trp8-γ-MSH-treated monkeys compared with saline-treated monkeys ($P = 0.14$, by paired analysis). The peak cortisol level also tended to be lower in D-Trp8-γ-MSH-treated monkeys (29.7 ± 1.7 *vs.* 33.1 ± 1.6 μg/dl; $P = 0.14$, by paired analysis).

Discussion

α-MSH has potent antiinflammatory properties that have been demonstrated in many animal models of inflammation

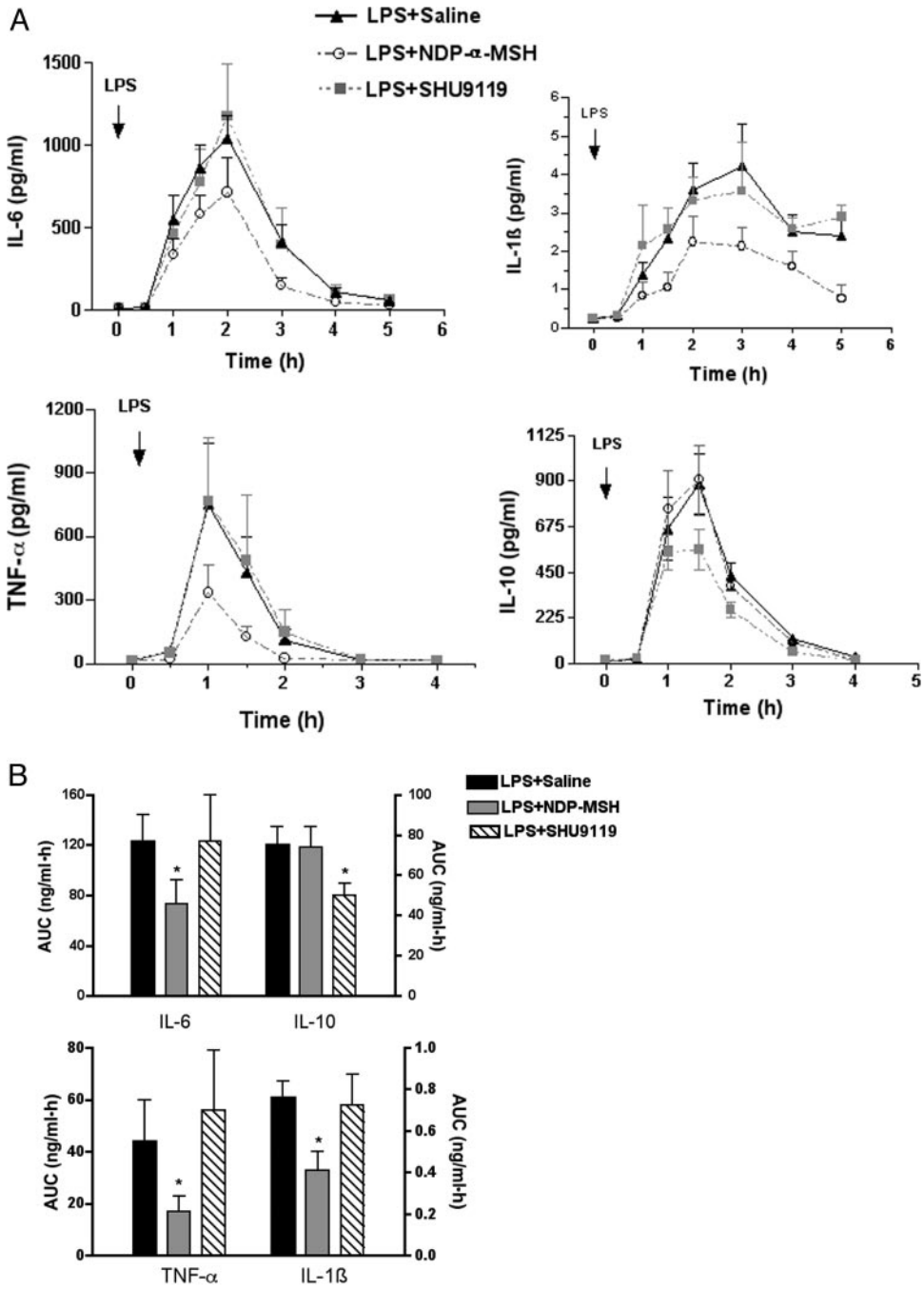

FIG. 1. A, Mean (±SEM) plasma IL-6, IL-1β, TNF-α, and IL-10 responses to endotoxin (LPS) injection in six monkeys studied three times after saline infusion (▲), NDP-MSH infusion (○), or SH9119 infusion (▨). NDP-MSH or SHU9119 was infused for 2 h before LPS injection at time zero and for 5 h afterward. Endotoxin stimulated plasma levels of IL-6, IL-1β, TNF-α, and IL-10 in the saline-infused animals ($P < 0.001$). NDP-MSH infusion significantly attenuated the response of the proinflammatory cytokines, IL-6, IL-1β, and TNF-α, to endotoxin ($P < 0.05$). There was no significant effect of NDP-MSH on IL-10. SHU9119 did not affect proinflammatory cytokine responses, but significantly decreased the IL-10 response to endotoxin ($P < 0.05$). B, Graphs of the cytokine AUCs for plasma IL-6, IL-10, TNF-α, and IL-1β after endotoxin (LPS) in the six monkeys depicted in A with saline (■), NDP-MSH (▨), or SHU9119 (▧) infusion. Data are expressed as the mean AUC (nanograms per milliliter per hour) ± SEM. The AUCs for the proinflammatory cytokines, IL-6, TNF-α, and IL-1β, were all attenuated by NDP-MSH ($P < 0.05$), but were not affected by SHU9119. In contrast, the AUC for the antiinflammatory cytokine, IL-10, was attenuated by SHU9119 ($P < 0.05$), but was not affected by NDP-MSH.

(6). Administration of α-MSH can antagonize the effects of both endotoxin and proinflammatory cytokine injection and can also reduce inflammation in experimental models of arthritis and skin inflammation (5, 24). Exogenous α-MSH injected either iv or icv suppresses endotoxin- or IL-1-induced fever and activation of the HPA axis (2, 4). In contrast, enhanced febrile and HPA responses to IL-1 have been reported after pretreatment with either an α-MSH antiserum or an α-MSH peptide antagonist, indicating that endogenous α-MSH plays a physiological role in modulating these responses (14, 15, 25). Most *in vivo* studies examining the role of the melanocortin system in modulating cytokine responses to an inflammatory challenge have been performed in rodent models. We have previously shown in the monkey that icv infusion of α-MSH attenuated the HPA response to IL-1β, whereas the α-MSH antagonist, agouti-related protein, enhanced this effect, suggesting that endogenous α-MSH plays a physiological role in this process (16). There are no data, however, on the role of the melanocortin system in modulating cytokine responses to an inflammatory challenge in a primate model. In addition, little is known in any species about the role of selective MC-R activation or antagonism on pro- and antiinflammatory cytokine responses *in vivo*. The current study demonstrates that iv infusion of NDP-

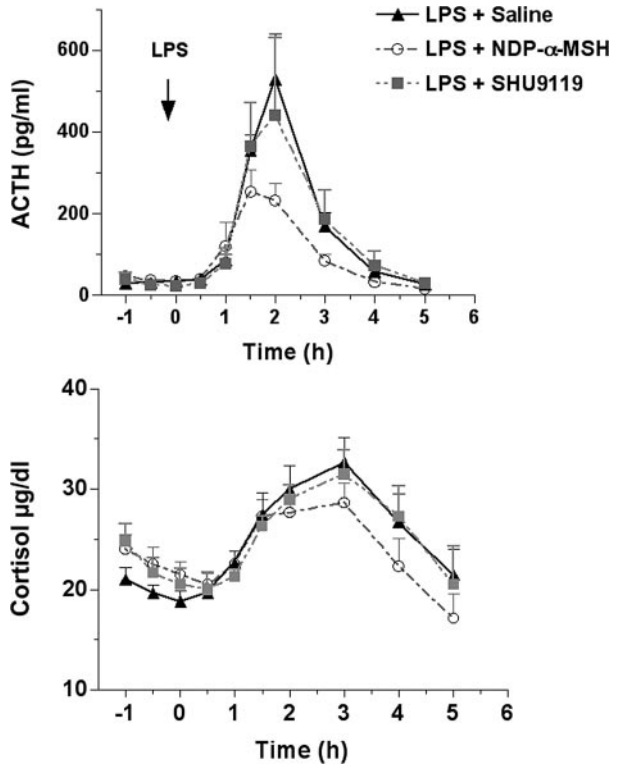

FIG. 2. Mean (±SEM) plasma ACTH and cortisol responses to endotoxin (LPS) injection in six monkeys studied three times after saline infusion (▲), NDP-MSH infusion (○), or SH9119 infusion (▨). NDP-MSH or SHU9119 was infused for 2 h before LPS injection at time zero and for 5 h afterward. Endotoxin stimulated plasma levels of ACTH and cortisol in the saline-infused animals ($P < 0.001$). NDP-MSH attenuated ACTH ($P < 0.001$) and cortisol ($P = 0.02$) responses to endotoxin over time (by repeated measures ANOVA) compared with saline, but SHU9119 had no effect on either of these responses.

MSH, a nonselective α-MSH agonist, attenuated the proinflammatory cytokine (TNF-α, IL-1β, and IL-6) and HPA responses to endotoxin in the monkey. These studies are consistent with what has been reported in the rodent *in vivo*. Infusion of SHU9119, an MC-3R and MC4-R α-MSH antagonist, had no effect on proinflammatory cytokine levels in response to endotoxin, but caused a significant decrease in the plasma level of IL-10, establishing for the first time a physiological role for α-MSH in modulating the release of an antiinflammatory cytokine. This is consistent with an *in vitro* study showing that α-MSH induced IL-10 production in human monocytes (10). It might be expected that MC-R antagonism would cause an enhanced proinflammatory cytokine response to endotoxin. However, there is evidence that although SHU9119 has antagonist activity at the human MC-3R and MC-4R, it has agonist activity at the human MC1-R (20). Such mixed activity at different human MC-Rs could be a potential explanation for the failure to detect an enhanced proinflammatory cytokine response with SHU9119. Infusion of NDP-MSH or SHU9119 in the absence of endotoxin had no significant effect on plasma cytokine, ACTH, or cortisol levels. However, in the absence of endotoxin, plasma cytokine levels were at or below the level of assay detection. These were not highly sensitive assays and could thus have missed small changes in circulating cytokine levels. Unfortunately, the commercially available, highly sensitive human assays that we evaluated did not cross-react sufficiently for use in the monkey.

Infusion of D-Trp8-γ-MSH, which has been shown to be selective for the human MC3-R (17), also significantly attenuated the IL-6 response to endotoxin. D-Trp8-γ-MSH tended to cause a decrease in the TNF-α response and an increase in the IL-10 response, but these were not significant. However, the ratio of IL-6 to IL-10 released after endotoxin was significantly decreased by 48% in the D-Trp8-γ-MSH- *vs.* saline-treated animals. Thus, in the presence of D-Trp8-γ-MSH, the cytokine environment was relatively less proinflammatory and relatively more antiinflammatory. In contrast to NDP-MSH, the effects of D-Trp8-γ-MSH on the TNF-α and HPA responses to endotoxin were not significant, although there was a tendency toward lower TNF-α, ACTH, and cortisol responses in D-Trp8-γ-MSH-treated animals. This may be related in part to some differences in the experimental groups (four of the nine animals were common to both studies) or to possible differences in the *in vivo* stability of both compounds. It may also indicate that other MC-Rs in addition to the MC3-R are important in mediating some of the antiinflammatory and HPA effects of melanocortins.

MC-Rs have been detected in the rodent on peritoneal macrophages and splenic lymphocytes and on circulating human monocytes and macrophages (11, 26–28). The expression of MC-R1, -3, and -5 has been reported by immune cells, and there are data to support a functional role for both MC1-R and MC3-R in modulating inflammatory responses (11, 26). There is growing evidence in the rodent that the MC3-R is particularly important in mediating the antiinflammatory effects of MSH peptides. MSH peptides can still exert antiinflammatory effects in recessive yellow (e/e) mice that lack functional MC1-Rs (12). The mixed MC3/4-R antagonist, SHU9119, was shown to block the antiinflammatory effects of the MSH agonist, MTII, in the rodent *in vivo* and *in vitro*; however, the selective MC4-R antagonist, HS024, was without effect (26). Our results in the monkey are consistent with a role for the MC-3R in modulating pro- and antiinflammatory responses. However, additional study is necessary to better define the role of specific MC-Rs in modulating the inflammatory response in the primate. It will be important to test the effects of MC-1R antagonism in our model. SHU9119 is selective for MC3/4-R and would not be expected to lead to enhanced inflammatory cytokine production in our model if MC-1R was the relevant receptor. In addition, SHU9119 has some agonist activity at the human MC1-R (20).

α-MSH is normally produced in the pituitary, brain, and several peripheral tissues, including immune cells, by posttranslational processing of the proopiomelanocortin precursor protein (29–31) and has been shown to act both peripherally and within the brain to modulate the inflammatory response (9). α-MSH has been shown to act directly on MC-Rs on peripheral immune cells to down-regulate proinflammatory cytokine production in response to endotoxin *in vitro* (11). The icv injection of α-MSH has also been shown to inhibit peripheral inflammation in the skin (32). This appears to be mediated by descending antiinflammatory neural pathways induced by the stimulation of MC-Rs within the brain (9). Central MC-Rs have also been shown to modulate tem-

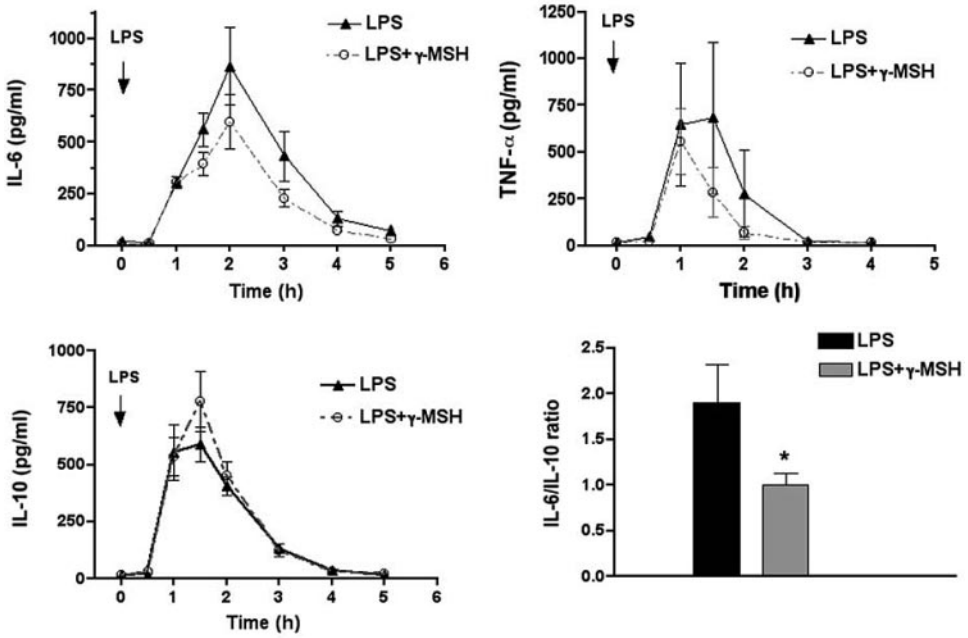

FIG. 3. Mean (±SEM) plasma IL-6, TNF-α, and IL-10 responses to endotoxin (LPS) injection in seven monkeys studied twice after either saline infusion (▲) or D-Trp8-γ-MSH infusion (○). Endotoxin stimulated plasma levels of IL-6, TNF-α, and IL-10 in the saline-infused animals ($P < 0.001$). There was a significant attenuation of the IL-6 response to endotoxin in the D-Trp8-γ-MSH-infused animals; the AUC for IL-6 was decreased by 37% ($P = 0.04$). The AUC for TNF-α was decreased, and the AUC for IL-10 was increased, but neither was significant. The ratio of the AUC for IL-6, a proinflammatory cytokine, to IL-10, an antiinflammatory cytokine, was calculated for each animal (*lower right*); the IL-6/IL-10 ratio was significantly decreased by D-Trp8-γ-MSH infusion ($P = 0.04$).

perature, neuroendocrine, and behavioral responses to inflammatory stimuli (7, 16, 33). In the current monkey study, MSH was administered iv and probably acted on peripheral MC-Rs. The attenuation of the ACTH and cortisol responses to endotoxin by NDP-MSH is most likely in part secondary to the attenuated release of IL-1β, TNF-α, and IL-6, all of which are known to stimulate the HPA axis (34). There may, however, be some central effects of peripherally administered peptide. We have previously shown that icv infusion of α-MSH attenuated the HPA response to icv IL-1β in the monkey, consistent with an effect on central MC-Rs (35). α-MSH has been shown to block IL-1-induced stimulation of the HPA axis in the rodent by inhibiting CRH release within the hypothalamus (36, 37). Thus, α-MSH may act both centrally and peripherally to modulate the HPA response to inflammatory stimuli.

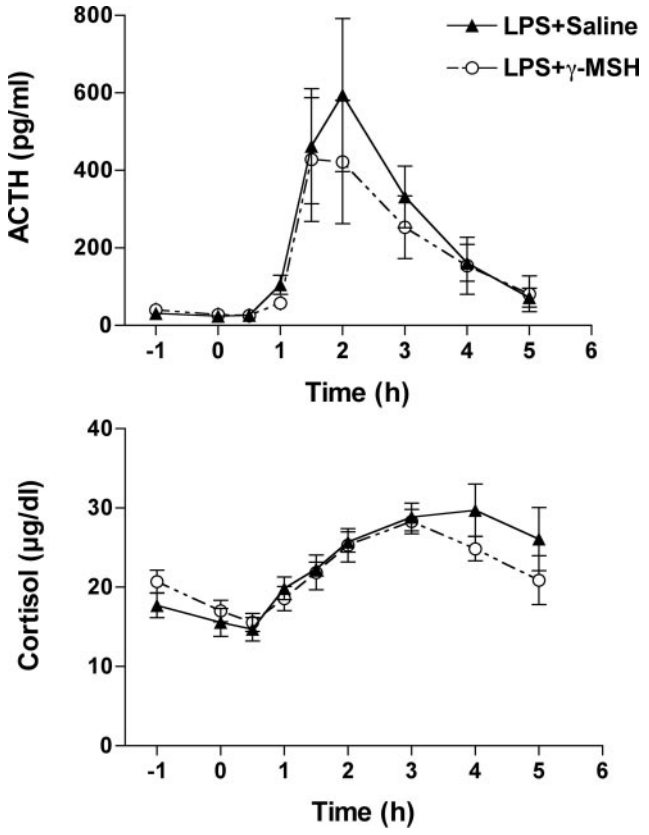

FIG. 4. Mean (±SEM) plasma ACTH and cortisol responses to endotoxin (LPS) injection in seven monkeys studied twice after either saline infusion (▲) or D-Trp8-γ-MSH infusion (○). Although there was a tendency toward lower ACTH and cortisol responses in the D-Trp8-γ-MSH-treated animals, these differences were not significant.

Several studies have addressed the molecular mechanisms by which α-MSH exerts its antiinflammatory effects. α-MSH has been shown to block the activation of NF-κB, an important transcription factor for the inflammatory cytokine genes, by various inflammatory agents (8, 38, 39). α-MSH has also been shown to block Toll-like receptor signaling on macrophages and thus attenuate endotoxin stimulation of macrophages (40). In addition, α-MSH has been reported to inhibit the production of chemoattractant chemokines, endothelial cell adhesion molecules, and nitric oxide, which all contribute to the inflammatory process (39, 41). Thus, α-MSH can affect the inflammatory response at multiple levels and by multiple mechanisms. An understanding of these mechanisms is potentially quite important given that inflammatory cytokines have been implicated in the pathogenesis of a wide spectrum of human diseases. Although our study provides evidence that α-MSH can modulate pro- and antiinflammatory cytokine responses in the primate *in vivo*, there is still much to learn about the physiological role of the melanocortin system in this process. Additional study is necessary to define the role of specific MC-Rs and the mechanisms by which MSH peptides modulate cytokine and neuroendocrine responses to inflammation.

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All of the authors have nothing to declare.

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