Agouti-Related Protein Stimulates the Hypothalamic-Pituitary-Adrenal (HPA) Axis and Enhances the HPA Response to Interleukin-1 in the Primate

ENNIAI XIAO, LINNA XIA-ZHANG, NICOLAS R. VULLIÉMOZ, MICHEL FERIN, AND SHARON L. WARDLAW

Departments of Medicine and Obstetrics and Gynecology, Columbia University College of Physicians and Surgeons, New York, New York 10032

α-MSH antagonizes many of the immune and neuroendocrine effects induced by inflammatory cytokines. Studies have shown that α-MSH attenuates the stimulatory effect of IL-1 on the hypothalamic-pituitary-adrenal (HPA) axis and plays a physiological role in limiting the HPA response to IL-1. Recently an α-MSH antagonist, agouti-related protein (AGRP), has been identified in the hypothalamus, which stimulates food intake by antagonizing the effects of α-MSH at specific melanocortin receptors. It is unknown whether AGRP can also modulate neuroendocrine responses to inflammatory cytokines. We have therefore examined the effects of AGRP on the HPA axis and on prolactin (PRL) at baseline and in response to stimulation by IL-1β in nine ovarioctomized rhesus monkeys. In the first study, the effects of intracerebroventricular (icv) infusion of AGRP on the HPA axis and on prolactin (PRL) at baseline and in response to stimulation by IL-1β in nine ovarioctomized rhesus monkeys. In the first study, the effects of intracerebroventricular (icv) infusion of 20 μg (n = 6) and 50 μg (n = 4) of human AGRP (83–132)-NH₂ were compared with icv saline infusion. There was a significant stimulatory effect of 20 μg AGRP on cortisol release over time (P < 0.001). The area under the hormone response curve (AUC) for cortisol increased by 29% after 20 μg AGRP vs. saline; the AUC for ACTH increased by 166% (P = 0.028); the AUC for PRL increased by 108% (P = 0.046). There was a significant stimulatory effect of 50 μg AGRP on ACTH (P < 0.001), cortisol (P < 0.001), and PRL (P < 0.046) release over time. The AUC for ACTH after 50 μg AGRP increased by 98%; the AUC for cortisol increased by 37%; the AUC for PRL increased by 161%. The effects of AGRP on ACTH, cortisol, and PRL release were prevented by α-MSH infusion. In the second study, animals received icv either 50 ng of human IL-1β or 20 μg of AGRP followed by 50 ng IL-1β. AGRP significantly enhanced the ACTH (P < 0.05) response to IL-1β. The peak ACTH response to IL-1β alone was 124 ± 55 pg/ml vs. 430 ± 198 pg/ml after IL-1β plus AGRP; the peak cortisol response was 70 ± 8.2 μg/dl vs. 77 ± 6.2 μg/dl, but this was not significantly different. In conclusion, AGRP stimulated ACTH, cortisol, and PRL release in the monkey and enhanced the ACTH response to IL-1β. These studies suggest that, in addition to its known orexigenic effects, AGRP may play a role in neuroendocrine regulation and specifically that AGRP may interact with α-MSH to modulate neuroendocrine responses to inflammation. (Endocrinology 144: 1736–1741, 2003)

Abbreviations: AGRP, Agouti-related protein; AUC, area under the hormone response curve; CV, coefficient of variation; HPA, hypothalamic-pituitary-adrenal; icv, intracerebroventricularly; MC-R, melanocortin receptors (i.e., MC3-R and MC4-R); POMC, proopiomelanocortin; PVN, paraventricular nucleus.

With respect to endocrine function, α-MSH has been shown to antagonize the potent stimulatory effects of IL-1 on the hypothalamic-pituitary-adrenal (HPA) axis in the rodent and in the primate (15, 17, 18). In the monkey α-MSH, infused icv, blocked IL-1-stimulated cortisol release. The IL-1-induced stimulation of prolactin (PRL) release was also blocked by α-MSH in the monkey (17). In the rat, the IL-1-induced stimulation of ACTH release was significantly enhanced by icv injection of an affinity purified α-MSH antiserum, supporting a physiological role for α-MSH in limiting the HPA response to this inflammatory cytokine (19). The distribution of MC-Rs in the hypothalamus is consistent with a potential role for endogenous α-MSH in HPA regulation. Two of the five known MC-Rs (MC3-R and MC4-R) are found in the hypothalamus, including the arcuate and paraventricular nucleus (PVN), areas involved with POMC and CRH synthesis, and the HPA response to stress (20, 21). Recently, the α-MSH antagonist, AGRP, has been shown to stimulate food intake by antagonizing the effects of α-MSH at the MC4-R (8, 9). It is unknown whether AGRP can antagonize the immune or neuroendocrine effects of α-MSH and thus modulate physiological responses to inflammatory cytokines. We have therefore examined the effects of AGRP on ACTH, cortisol, and PRL release at baseline and in response to stimulation with IL-1β in female rhesus monkeys.
Materials and Methods

Animals

Nine adult female rhesus monkeys (Macaca mulatta) weighing 5–9 kg were used in these experiments. 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 Purina Monkey Chow (Purina Mills, St. Louis, MO) supplemented with fresh fruit daily. All animals were ovarectomized at least 2 months before the studies to eliminate fluctuations in estradiol levels because estradiol has been shown to attenuate the HPA response to IL-1β (22). A chronic cannula for icv peptide infusion was implanted stereotaxically into the lateral ventricle at least 1 month before experiment. Animals were sedated with ketamine (Ketaset, Henry Schein Inc., Melville, NY; 5–7 mg/kg) and intubated. Gas anesthesia (isoflurane) was then initiated. The monkey’s head was mounted on a stereotactic head holder and a Burr hole was drilled 1.5 mm laterally to the sagittal sinus. A cannula (18 g; 32–34 mm in length) was then inserted using the following coordinates: 13 mm anterior to the anterior-posterior zero reference; 1.5 mm lateral; 13–16 mm depth from dura (23). Location of the cannula tip in the lateral ventricle was confirmed by CSF reflux. The cannula was secured to the skull with dental acrylic. For protection, a plastic cap with a screw-off top was centered over the posterior zero reference; 1.5 mm lateral; 132)-NH (Phoenix Pharmaceuticals, Inc., Belmont, CA) dissolved in 30 μl of saline or 30 μl of saline was infused intracerebroventricuarily (icv) over a 30-min period. Each monkey was studied with a saline infusion and with one, two, or three doses of AGRP. Blood samples (3 ml) were collected starting at 1–1.5 h before icv infusion and continued for 5–5.5 h after icv infusion at 30-min intervals initially and then at 60 min intervals for the last 3 h of the study. Four monkeys that had already received 20 μg (n = 2) or 50 μg (n = 2) AGRP were studied on another occasion to determine whether the effects of the same dose of AGRP could be blocked by coinfusion of α-MSH. α-MSH (Peninsula Laboratories, Inc., Belmont, CA) 60 μg/h (n = 2) or [Nle4, n-Phe7]-α-MSH 20 μg/h (Phoenix Pharmaceuticals, Inc.; n = 2) was infused for 1 h before and for 4 h after AGRP infusion (n = 5). At the end of the 5-h infusion, 1 ml of saline or 30 μg AGRP was infused, and blood was collected as described above. [Nle4, n-Phe7]-α-MSH was infused with the 50 μg AGRP dose because it is a more potent and stable MSH agonist. [Nle4, n-Phe7]-α-MSH 20 μg/h was also infused alone for 5 h in three monkeys. We have previously shown that icv infusion of α-MSH at 60 μg/h did not affect baseline cortisol or PRL levels in the monkey (17). Repeated studies in the same monkey at different doses of AGRP or with α-MSH or saline were separated by at least 2 wk. In the second experiment, five monkeys were studied twice after icv infusion with recombinant human IL-1β (50 ng) plus saline alone or with IL-1β (50 ng) plus AGRP (20 μg). IL-1β was purchased from Bachem (Torrance, CA); the endotoxin level was reported to be less than 0.1 ng/μg of IL-1β. AGRP was infused icv 1 h before IL-1β, which was infused over a 30-min period. Blood samples (3 ml) were collected every 30 min for 1 h before AGRP or saline infusion and then at 30-min intervals after IL-1β infusion for the first 2.5 h and hourly for the next 3 h. Each monkey was assigned to be studied first with either IL-1β alone or with AGRP in random order. All studies in the same animal were separated by at least 3 wk. Blood samples were centrifuged and plasma was separated and stored at −20 C for hormonal assays.

Hormone assays

ACTH was measured by a two-site immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA). Assay sensitivity is 3 pg/ml, intraassay coefficient of variation (CV) is 3.6%, and interassay CV is 7.8%. Cortisol was assayed by solid phase RIA (ICN Biomedicals, Inc., Costa Mesa, CA). Assay sensitivity is 1 μg/dl, intraassay CV is 4.4%, and interassay CV is 10.7%. PRL was measured by double antibody RIA with reagents provided by the National Hormone and Pituitary Program. Assay sensitivity is 1 ng/ml, intraassay CV is 5%, and interassay CV is 9.5%.

Statistical analysis

The effects of AGRP injection on hormone responses over time were analyzed by ANOVA with repeated measures. Areas under the hormone response curves (AUCs) were calculated by trapezoid analysis and were compared in paired treatment groups by Wilcoxon signed rank test.

Results

Effect of AGRP on ACTH, cortisol and PRL release

Icv infusion of 20 μg (n = 6) and 50 μg (n = 4) AGRP caused a significant stimulation of ACTH, cortisol and PRL release compared with icv saline infusion. No significant effects on hormone release were demonstrated with 5 μg AGRP (data not shown). The effects of 20 μg of AGRP are shown in Fig. 1. There was a significant effect of AGRP (20 μg) treatment on cortisol release over time (P < 0.001). The AUC for cortisol after AGRP was 129% of the AUC after saline. At this AGRP dose, a significant effect on ACTH and PRL release over time was not demonstrated by repeated measures ANOVA. However, a significant effect of 20 μg AGRP on ACTH was demonstrated by paired analysis of the AUC for ACTH after AGRP compared with saline infusion; the AUC after AGRP was 266% of the AUC after saline (P = 0.028). The mean peak ACTH level was 22 ± 8.8 pg/ml 3.5 h after AGRP vs. 9.4 ± 4.8 pg/ml after saline. A significant effect of 20 μg AGRP was similarly demonstrated by paired analysis of the AUC for PRL after AGRP compared with saline infusion; the AUC after AGRP was 208% of the AUC after saline (P = 0.046). The peak PRL level was 7.0 ± 3.4 ng/ml 2 h after AGRP vs. 3.0 ± 1.3 ng/ml after saline.

The effects of 50 μg of AGRP are shown in Fig. 2. There was a significant effect of 50 μg AGRP on ACTH (P < 0.001), cortisol (P < 0.001), and PRL (P < 0.001) release over time. The AUC for ACTH after AGRP was 198% of the AUC after saline; the peak ACTH level was 35 ± 6.8 pg/ml 4 h after AGRP vs. 13 ± 3.8 pg/ml after saline. The AUC for cortisol after AGRP was 137% of the AUC after saline; the peak cortisol level was 53 ± 8.9 μg/dl 4 h after AGRP vs. 37.2 ± 3.1 μg/dl 1 h after saline. The AUC for PRL after AGRP was 261% of the AUC after saline; the peak PRL level was 5.8 ± 1.1 ng/ml 3 h after AGRP vs. 1.8 ± 0.35 ng/ml.

Four monkeys were studied on another occasion to determine whether the effects of AGRP could be blocked by coinfusion of α-MSH 60 μg/h (n = 2) or [Nle4, n-Phe7]-α-MSH 20 μg/h (n = 2). As shown in Fig. 3, there was a significant effect of AGRP on ACTH (P = 0.01), cortisol (P < 0.001), and PRL (P = 0.005) release over time when compared with saline infusion. These stimulatory effects of AGRP were blocked when AGRP was coinfused with either α-MSH preparation such that these responses were no longer significantly different from the saline controls (P = 0.11 for ACTH, P =
0.23 for cortisol and \( P = 0.20 \) for PRL). The AGRP plus MSH group was also significantly different from the AGRP group (\( P = 0.04 \) for ACTH, \( P = 0.003 \) for cortisol and \( P = 0.02 \) for PRL). There was no significant effect on hormone release when [Nle\(^4\), d-Phe\(^7\)]-a-MSH 20 \( \mu \)g/h was infused alone for 5 h in three monkeys (data not shown).

**Effect of AGRP on the HPA and PRL responses to IL-1\( \beta \)**

As expected, IL-1\( \beta \) stimulated ACTH (\( P = 0.006 \)) and cortisol (\( P < 0.001 \)) release. AGRP significantly enhanced the ACTH response to IL-1\( \beta \) (\( P < 0.05 \); Fig. 4). The peak ACTH response to IL-1\( \beta \) was 124 \( \pm \) 55 pg/ml vs. 430 \( \pm \) 198 pg/ml after IL-1\( \beta \) plus AGRP. The peak cortisol response was 70 \( \pm \) 8.2 \( \mu \)g/dl after IL-1\( \beta \) vs. 77 \( \pm \) 6.2 \( \mu \)g/dl after IL-1\( \beta \) plus AGRP, but this was not significantly different (Fig. 4). There was a significant stimulation of PRL release after both treatments (\( P < 0.05 \)); however, there was no difference in response between groups. The peak PRL level was 7.4 \( \pm \) 2.1 ng/ml after IL-1\( \beta \) vs. 7.1 \( \pm \) 1.7 ng/ml after IL-1\( \beta \) plus AGRP.

**Discussion**

In this study, we show that AGRP, when administered centrally, stimulates ACTH, cortisol and PRL release in the
The doses of AGRP that were used in this study are similar to those which were reported to stimulate food intake in the monkey following icv infusion (10). The stimulatory effects on ACTH, cortisol, and PRL release are blocked by infusion of α-MSH, consistent with MC-R-mediated effects. Several previous studies have documented effects of α-MSH on PRL release and on the HPA axis in the rodent (24–26). α-MSH, injected icv, has been shown to suppress basal and swimming stress-induced PRL release by stimulating the activity of the hypothalamic dopaminergic system (24). Icv injection of α-MSH has also been reported to attenuate the corticosterone response to neurogenic stress in the rat (26). In this study, we also show that AGRP augments the ACTH response to IL-1β. With respect to inflammatory stress, α-MSH has been shown to block IL-1β- and endotoxin-induced stimulation of the HPA axis in the rodent and in the primate. In the rat, icv injection of α-MSH was reported to block IL-1β-stimulated ACTH and corticosterone release in a dose-dependent manner (15). Peripheral injection of α-MSH was also shown to block IL-1β-stimulated corticosterone release in the mouse (16). We have previously shown in the monkey that icv infusion of α-MSH attenuated the HPA response to IL-1β (17). α-MSH, however, did not interfere with the stimulation of ACTH release by CRH infusion in the monkey (17). This is consistent with a hypothalamic, rather than a direct pituitary effect, of α-MSH on the HPA axis. This is further supported by two in vitro studies that have shown that α-MSH blocks IL-1β-stimulated release of CRH from rat hypothalamic explants (27, 28).

In addition to demonstrating neuroendocrine effects of AGRP and the HPA Axis.
exogenous α-MSH infusion, several studies provide evidence that endogenous α-MSH participates in neuroendocrine regulation. Icv injection of an α-MSH antiserum has been shown to enhance basal and stress-induced PRL secretion in the rat, consistent with a physiological role for endogenous α-MSH in this process (29). We have shown in the rat that the IL-1β-induced stimulation of ACTH and corticosterone release was significantly enhanced by icv injection of an affinity purified α-MSH antiserum, supporting a physiological role for α-MSH in limiting the HPA response to this inflammatory cytokine (19). Our current results with AGRP further support a role for the brain melanocortin system in modulating neuroendocrine responses in the monkey. It should be noted that the effects of AGRP on ACTH and cortisol release were obtained in monkeys restrained in primate chairs. Even though the animals had been previously adapted to the chairs, this is still perceived as a form of stress as reflected by baseline cortisol measurements in the range of 40 μg/dl. Thus, it is unclear whether AGRP affects basal HPA activity or whether our results are consistent with a role for the central melanocortin system in modulating the HPA response to immobilization stress in the monkey. The enhanced ACTH response to IL-1β seen in the AGRP infused animals is consistent with a role for the central melanocortin system in modulating the HPA response to inflammatory stress. The cortisol response to IL-1β in these animals was somewhat higher, but this was not significant. It may be that the adrenal response to the high levels of ACTH released after IL-1β alone was already near maximal and was therefore not further increased by AGRP. A similar finding was reported when the effects of an α-MSH antiserum on the HPA response to different doses of IL-1β were studied in the rodent (19).

It is likely that α-MSH acts within the hypothalamus to inhibit CRH release with subsequent attenuation of the pituitary-adrenal response. Several studies in the rodent and in the primate have demonstrated that the appropriate anatomical framework exists for the regulation of CRH by the melanocortin system. Hypophysiotropic CRH neurons within the PVN receive extensive innervation from arcuate POMC and neuropeptide Y/AGRP neurons (30). MC4-Rs have also been localized to hypophysiotropic neurons in the PVN (20). The neuroanatomical distribution of AGRP immunoreactivity has been reported in the rhesus monkey and is similar to what has been reported in the rat (31). Dense fibers were found in the PVN and the median eminence in both species. AGRP could thus act within the PVN to modulate CRH synthesis and/or release or in the median eminence to modulate CRH release into hypophysial blood. The HPA axis can also interact with POMC and AGRP neurons because both express glucocorticoid receptors (32, 33), and the expression of both genes is affected by adrenalectomy and glucocorticoid replacement (34, 35).

In addition to the above neuroendocrine effects, α-MSH has also been shown to antagonize effects of IL-1 on body temperature, immune function, and sickness behavior (14). Enhanced febrile responses to IL-1 and to endotoxin have been reported when rabbits or rats were pretreated icv with either an α-MSH antiserum or an α-MSH peptide antagonist, indicating a physiological role for α-MSH in modulating these responses (36, 37). α-MSH has been shown to act both peripherally and within the brain to modulate inflammatory responses (38). α-MSH has been shown to act directly on MC-Rs on peripheral immune cells to down-regulate inflammatory cytokine production in response to endotoxin and to antagonize the proinflammatory activity of these cytokines in vitro (39, 40). At least one mechanism by which α-MSH antagonizes the effects of the inflammatory cytokines is by blocking the activation of the nuclear transcription factor κB by these cytokines (40–42). It is not known whether AGRP treatment affects IL-1β-induced IL-6 release or the release of other inflammatory mediators either peripherally or within the brain. It is likely in the current study that AGRP-injected icv is acting on MC-Rs within the brain to affect CRH release and the pituitary-adrenal response; however, a peripheral effect cannot be ruled out. It is of interest that during starvation there is increased susceptibility to endotoxin shock accompanied by increased proinflammatory cytokine levels (43). Also, during starvation, α-MSH expression is suppressed and AGRP expression is stimulated in the hypothalamus (2, 10, 44, 45). It is unknown whether the resulting decrease in melanocortin signaling could contribute to enhanced proinflammatory responses and increased susceptibility to shock in response to endotoxin.

In summary, central injection of the α-MSH antagonist, AGRP, stimulates ACTH, cortisol, and PRL release and enhances the HPA response to the inflammatory cytokine, IL-1β, in the primate. These studies suggest that, in addition to its known orexigenic effects, AGRP may play a role in neuroendocrine regulation and specifically that AGRP may interact with α-MSH to modulate neuroendocrine responses to inflammation.

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Address all correspondence and requests for reprints to: Dr. Sharon L. Wardlaw, Department of Medicine, Columbia University College of Physicians & Surgeons, 630 West 168th Street, New York, New York 10032. E-mail: sw22@columbia.edu.

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