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Alpha-Melanocyte-Stimulating Hormone through Melanocortin-4 Receptor Inhibits Nitric Oxide Synthase and Cyclooxygenase Expression in the Hypothalamus of Male Rats

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Key Words
Melanocortin receptors · Alpha-melanocyte-stimulating hormone · Nitric oxide · Nitric oxide synthase · Gonadotropins · Prolactin · Adrenal steroids · Lipopolysaccharide · Cyclooxygenase

Abstract
There is evidence that α-melanocyte-stimulating hormone (α-MSH) has immunomodulatory and anti-inflammatory actions within the brain. In this study, we tested whether these actions are due to inhibition of the synthesis of nitric oxide (NO) and prostaglandins induced by lipopolysaccharide (LPS). Since melanocortin subtype MC4 receptor has been detected in the hypothalamus, we investigated the effect of central administration of α-MSH and HS024 (a selective MC4 receptor antagonist) on the gene expression of inducible, neuronal and endothelial NO synthase (iNOS, nNOS and eNOS) and on cyclooxygenase (COX-1 and COX-2) expression in the mediobasal hypothalamus (MBH) of LPS-treated male Wistar rats. Peripheral administration of LPS (250 μg/rat, 3 h) induced iNOS and COX-2 gene expression in the MBH. This stimulatory effect was reduced by α-MSH (3 nmol/rat) injected 30 min before LPS. α-MSH and HS024 (1 nmol/rat) alone had no effect on iNOS and COX-2 expression. The action of α-MSH on LPS-induced iNOS and COX-2 mRNA levels was not observed in the presence of HS024, suggesting that MC4-R may be involved in the modulatory effect of α-MSH. None of these treatments produced any modifications in nNOS, eNOS and COX-1 expression in MBH. The increase in serum corticosterone levels induced by LPS was attenuated by α-MSH. Both LPS and α-MSH decreased serum LH and prolactin levels. HS024 failed to modify the inhibitory effects of LPS and α-MSH on prolactin release but reverted the effect of LPS on LH secretion, indicating that MC4-R activation may be involved in the effects of α-MSH on LH secretion in male rats. When we examined the in vitro effect of LPS (10 μg/ml) and LPS plus interferon-γ (IFN-γ, 100 ng/ml) on iNOS expression in MBH, an increase in iNOS mRNA levels was observed only in the presence of LPS + IFN-γ. This stimulatory effect was attenuated in the presence of α-MSH (5 μ*M*), which by

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itself had no effect. No changes were found in nNOS, eNOS, COX-1 or COX-2 expression. These results indicate that α-MSH reduces the induction of iNOS and COX-2 gene expression at the hypothalamic level during endotoxemia and suggest that endogenous α-MSH may exert an inhibitory tone on iNOS and COX-2 transcription via MC4 receptors acting as a local anti-inflammatory agent within the hypothalamus.

Introduction

The administration of bacterial products such as lipopolysaccharides (LPS) induces endotoxemia and causes a systemic inflammatory response affecting hypothalamic neuroendocrine functions [1]. A variety of mediators, including cytokines, nitric oxide (NO) and prostaglandins (PG), are important factors responsible for most of the manifestations leading to septic shock [1].

NO is generated by three types of NO synthases (NOS). Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed and activated by an increase in intracellular Ca^{2+}, whereas inducible iNOS is Ca^{2+}-independent and transcriptionally regulated [2]. iNOS synthesis is induced in the brain (glial and endothelial cells) during inflammation and ischemia [3, 4].

Acute systemic administration of LPS increases NOS activity in the paraventricular nucleus of the hypothalamus [5] and induces iNOS expression in hypothalamus and pituitary [6]. LPS stimulates the production and release of IL-1β, TNF-α, and NO from astrocytes and microglia [7]. Both cytokines are mediators of the stimulatory effect of LPS on NOS activity in the brain [8].

Cyclooxygenase (COX) metabolizes free arachidonic acid to PGs and thromboxanes. There are two known COX isoforms: COX-1 is constitutively expressed, whereas COX-2 has low constitutive expression but is strongly induced during inflammation [9]. Peripheral LPS injection increases PGE_2 release from rat astrocytes [10] and COX-2 mRNA levels in brain endothelial cells [11] and in microglia [12].

Melanocortins are peptide hormones derived from the precursor peptide pro-opiomelanocortin, by a series of proteolytic cleavages. Melanocortins are known to have a broad spectrum of physiological actions including immunomodulatory effects [13]. α-Melanocyte-stimulating hormone (α-MSH) is a melanocortin with potent anti-inflammatory properties [14]. Endotoxin increases plasma α-MSH in rabbits and normal human subjects [15, 16]. α-MSH inhibits the synthesis of several cytokines (i.e. TNF-α, IL-1, IL-6, IL-8) acting on immune and non-immune cells including microglia and astrocytes [17, 18]. α-MSH is a potent inhibitor of the induction of iNOS in cultured macrophages and NO production in liver and microglia [4, 17, 19]. Also, α-MSH was found to inhibit PGE_2 production in brain tissue [20, 21].

Melanocortins mediate their effects through G protein-coupled receptors by stimulating adenylate cyclase. To date, five melanocortin receptor subtypes (designated MC1-R through MC5-R) have been cloned and characterized. These receptors present different patterns of tissue expression [22], MC3-R and MC4-R being the predominant subtypes expressed in brain.

α-MSH activates both MC3-R and MC4-R. The distribution of MC4-R mRNA in the paraventricular and arcuate nuclei of the hypothalamus is consistent with the proposed roles of the central melanocortin system in neuroendocrine and autonomic regulation [23]. Central or systemic administration of α-MSH suppresses LPS activation of the hypothalamic-pituitary-adrenal (HPA) axis [24]. Melanocortins have also been involved in the regulation of the hypothalamic-pituitary-gonadal axis [25].

Although there is evidence that melanocortins exert anti-inflammatory effects, it is not yet known whether regulation of COX plays a role in these actions of α-MSH and it is also unclear through which of the MC-R subtypes α-MSH produces the anti-inflammatory responses. In order to clarify the mechanisms involved in the local anti-inflammatory action of α-MSH in the hypothalamus, we examined the effects of α-MSH and HS024, a selective MC4-R antagonist, on the hypothalamic gene expression of NOS and COX isoforms from male rats injected with LPS. We also studied the effect of α-MSH and its antagonist on corticosterone, prolactin (PRL) and LH secretion during endotoxemia.

Material and Methods

Animals

Male Wistar rats weighing 200–250 g were obtained from the School of Pharmacy and Biochemistry, University of Buenos Aires. They were maintained in accordance with the guidelines for care and use of laboratory animals (NIH Guide). The animals were fed lab chow (dry pellet food; Cooperative Association CL, Buenos Aires, Argentina) and water ad libitum and kept under controlled conditions of light (12 h light/dark) and temperature (22–24°C).

Drugs

LPS (*Escherichia coli*, serotype O127:B8) was purchased from Sigma Chemical Company, St. Louis, Mo., USA. α-MSH was purchased from Bachem California Inc., Torrance, Calif., USA. INF-γ

was purchased from Boehringer Ingelheim, BA, Argentina. HS024 was purchased from Neosystem, Strasbourg, France. Ketamine was purchased from Holliday-Scott, BA, Argentina and Xilazine from Alfasan International BV, Woerden, The Netherlands. All RT-PCR reagents were purchased from Invitrogen Corp., Carlsbad, Calif., USA, unless specified otherwise.

In vivo Experiments

Animals were anesthetized with Xilazine (10 mg/kg) and ketamine (50 mg/kg) and implanted stereotactically with stainless steel guide cannulas in the lateral ventricle (coordinates: A-P –0.6 mm, L –2 mm, D-V –3.2 mm) and placed in individual cages, 1 week before the experiment. On the day of the experiment, rats were injected through the cannula with either α-MSH (3 nmol/rat), HS024 (1 nmol/rat) or vehicle (5 μl saline). Animals that received both α-MSH and HS024 were first injected with HS024 and 30 min later with α-MSH. After 30 min, animals were injected intraperitoneally (i.p.) with LPS dissolved in sterile saline (250 μg/rat) or vehicle and sacrificed 3 h later. All animals were killed by decapitation and trunk blood was collected for serum corticosterone, PRL and LH determination. After sacrifice, a hypothalamic fragment (MBH) that included the arcuate and periventricular nuclei and the median eminence was dissected and immediately processed for RT-PCR.

In vitro Experiments

One MBH from a non-treated rat per tube was preincubated for 15 min in a Dubnoff shaker (60 cycles/min) at 37°C in an atmosphere of 95% O_2-5% CO_2 in 0.5 ml of Krebs-Ringer bicarbonate buffer (KRB: 118.46 m*M* NaCl, 5 m*M* KCl, 2.5 m*M* $CaCl_2$, 1.18 m*M* NaH_2PO_4, 1.18 m*M* $MgSO_4$, 24.88 m*M* $NaHCO_3$, pH 7.4) containing 10 m*M* glucose, 0.1 m*M* bacitracin and 0.1% bovine serum albumin. The medium was replaced with fresh KRB containing the tested drugs for 5 h. The MBH was then immediately processed for RT-PCR.

RT-PCR

Total RNA from two MBH was extracted from tissues using Trizol (Gibco BRL, Gaithersburg, Md., USA) according to the manufacturer's protocol. 5 μg of total RNA were reverse transcribed with Superscript™ II RNAse H minus reverse transcriptase in 20 μl reaction, using 0.2 μg of oligo $(dT)_{12–18}$ as primer. cDNA amplification was performed with 1 μl of cDNA as template in 50 μl PCR reaction containing $MgCl_2$, 0.2 m*M* of each dNTP, sense and antisense primers and Taq DNA polymerase in the buffer provided by the manufacturer. Temperature cycles always had an initial denaturation at 94°C for 10 min and a final extension period of 7 min at 72°C. iNOS, nNOS, eNOS and β-actin primers were purchased from Transgenomics Inc., Omaha, Nebr., USA. Amplifications were performed with a UNO II thermocycler (Biometra, Göttingen, Germany). The number of cycles and the template input for PCR were determined empirically within the linear range of amplification.

Amplification of iNOS cDNA was performed with 1.5 m*M* $MgCl_2$, 50 pmol of each primer and 2.5 U of Taq DNA polymerase. Temperature cycles were 94°C, 58°C and 72°C steps, 30 s each. The primers for iNOS, the number of cycles and the product size were as follows: sense 5′-TAG AAA CAA CAG GAA CCT ACC A-3′, antisense 5′-ACA GGG GTG ATG CTC CCG GAC A-3′, 33 cycles, 907 bp. Amplification of nNOS was performed with 2.5 m*M* $MgCl_2$, 50 pmol of each primer and 2.5 U of Taq DNA polymerase. Temperature cycles were 94°C, 66°C and 72°C steps, 30 s each. The primers for nNOS, the number of cycles and the product size were as follows: sense 5′-GAA TAC CAG CCT GAT CCA TGG AAC ACC-3′, antisense 5′-CTC CAG GAG GGT GTC CAC CGC ATG CC-3′, 32 cycles, 599 bp. eNOS was amplified with 2 m*M* $MgCl_2$, 400 n*M* of each primer and 1.25 U of Taq DNA polymerase. Temperature cycles were 94°C (1 min), 55°C (30 s) and 72°C for 1 min. The primers for eNOS, the number of cycles and the product size were as follows: sense 5′-CTG TGT CCA ACA TGC TGC TAG AAA TTG-3′, antisense 5′-TAA AGG TCT TCT TCC TGG TGA TGC C-3′, 58 cycles, 485 bp. The primers for β-actin and the product size were as follows: sense 5′-ACC ACA GCT GAG AGG GAA ATC G-3′, antisense 5′-AGA GGT CTT TAC GGA TGT CAA CG-3′, 281 bp.

Amplification of COX-1 and COX-2 cDNA was performed with 2 m*M* $MgCl_2$, 50 pmol of each primer (MWG-Biotech AG, Ebersberg, Germany) and 2.5 U of Taq DNA polymerase. Temperature cycles were 94°C, 46°C (30 s each) and 72°C for 1 min. The primers for each enzyme, number of cycles and product size were as follows: COX-1 sense 5′-CTG GCC GGA TTG GTG GGG GTA G-3′, antisense 5′-CAT AGG GGC AGG TCT TGG TGT TG-3′, 40 cycles, 441 bp, and COX-2 sense 5′-GGA GAG ACG ATC AAG ATA GTG ATC-3′, antisense 5′-ATG GTC AGT AGA CTC TTA CAG CTC-3′, 40 cycles, 860 bp. The primers for GADPH (MWG-Biotech AG) and the product size were as follows: sense 5′-GTG AAG GTC GGT GTG AAC GGA TTT-3′, antisense 5′-CAC AGT CTT CTG AGT GGC AGT GAT-3′, 555 bp.

10 μl of each reaction were analyzed on 1.5% agarose gels, stained with ethidium bromide and visualized using UV light. RT-PCR products were analyzed quantitatively using Scion Image densitometer. Results were normalized to the internal control β-actin or GADPH. Values were expressed as relative increments of respective controls. Experiments always included non-reverse transcribed RNA samples as negative controls.

PRL and LH Radioimmunoassay

Serum PRL and LH were measured by double-antibody radioimmunoassay with reagents provided by the National Hormone and Pituitary Program. Rat LH-RP-3 and PRL-RP-3 were used as standards, NIDDK-anti-rPRL-S-9 and NIDDK-anti-rLH-S-11 as antisera. Cross-reactivity with other pituitary hormones was negligible. The sensitivity of the assay was 0.05 ng/ml for LH and 0.1 ng/ml for PRL. The intra- and interassay coefficients of variation were less than 10%.

Corticosterone Assay

Serum corticosterone levels were determined using an enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, Mich., USA) according to the manufacturer's protocol. The assay limit of detection was 32 pg/ml. The intra- and interassay coefficients of variation were less than 8%.

Statistical Analysis

Data were expressed as mean ± SEM and were analyzed by one sample Student's t test, or two-way analysis of variance (ANOVA) with interaction terms followed by Student-Newman-Keuls multiple comparisons test. Differences with a p value of <0.05 were considered statistically significant.

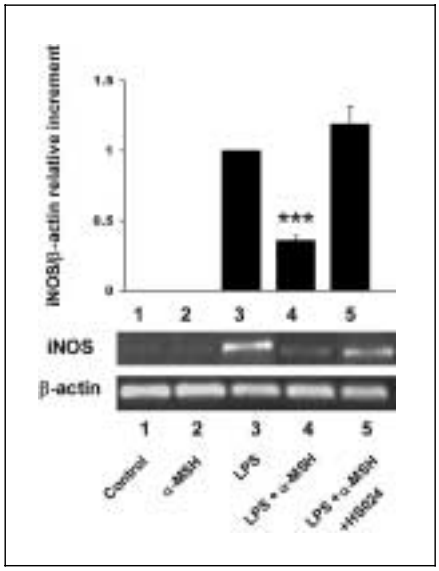

Fig. 1. Effect of α-MSH on iNOS gene expression in the mediobasal hypothalamus (MBH). Expression and semiquantification of iNOS mRNA. Control (lane 1); α-MSH i.c.v., 3 nmol/rat (lane 2); LPS i.p., 250 μg/rat (lane 3); LPS i.p., 250 μg/rat + α-MSH i.c.v., 3 nmol/rat (lane 4); LPS i.p., 250 μg/rat + α-MSH i.c.v., 3 nmol/rat + HS024 i.c.v., 1 nmol/rat (lane 5). Values are expressed as relative increment of the relation iNOS/β-actin mRNA in respect to LPS of 3 independent experiments. Data represent mean ± SEM of 4–6 determinations per group and were evaluated by one sample t test. *** $p < 0.001$ vs. LPS.

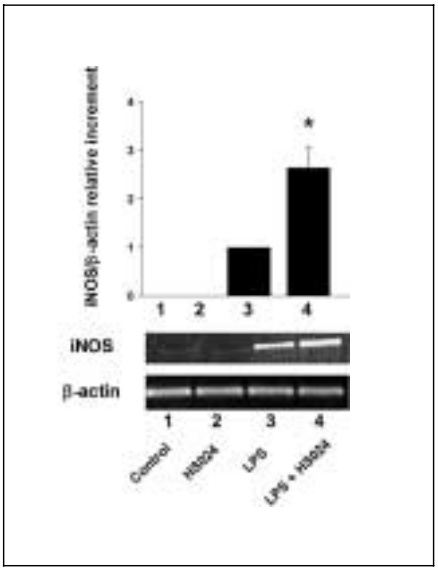

Fig. 2. Effect of HS024 on iNOS gene expression in MBH. Expression and semiquantification of iNOS mRNA. Control (lane 1); HS024 i.c.v., 1 nmol/rat (lane 2); LPS i.p., 250 μg/rat (lane 3); LPS i.p., 250 μg/rat + HS024 i.c.v., 1 nmol/rat (lane 4). Values are expressed as relative increment of the relation iNOS/β-actin mRNA in respect to LPS of 2 independent experiments. Data represent mean ± SEM of 4 determinations per group and were evaluated by one sample t test. * $p < 0.05$ vs. LPS.

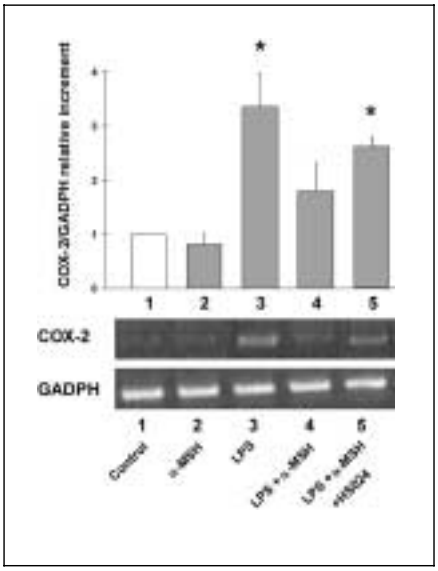

Fig. 3. Effect of α-MSH on COX-2 gene expression in MBH. Expression and semiquantification of COX-2 mRNA. Control (lane 1), α-MSH (lane 2), LPS (lane 3), LPS + α-MSH (lane 4), LPS + α-MSH + HS024 (lane 5). Values represent mean ± SEM of 4–6 determinations per group of relative increment of the relation COX-2/GADPH mRNA in respect to control group of 3 independent experiments. Data were evaluated by one sample t test. * $p < 0.05$ vs. control.

Results

In vivo Experiments

Effect of α-MSH and HS024 on NOS Expression in MBH

iNOS mRNA was undetectable in the MBH of control and α-MSH (i.c.v., 3 nmol/rat)-treated animals. iNOS gene expression was induced 3 h after LPS (i.p., 250 μg/rat) administration (fig. 1). The administration of α-MSH significantly reduced the effect of LPS on iNOS expression (fig. 1). The modulatory effect of α-MSH on iNOS LPS-induced expression was not observed when HS024 (i.c.v., 1 nmol/rat), a specific MC4-R antagonist, was administered 30 min before α-MSH (fig. 1). HS024 significantly increased LPS induced-iNOS expression (fig. 2). LPS, α-MSH or both together modified neither nNOS nor eNOS mRNA levels in the MBH (data not shown). HS024, alone or plus LPS, had no effect on the expression of nNOS and eNOS (data not shown).

Effect of α-MSH and HS024 on COX Expression in MBH

Basal COX-2 expression was detected in MBH of control rats. LPS (i.p., 250 μg/rat) significantly increased COX-2 gene expression (fig. 3). α-MSH (i.c.v., 3 nmol/rat) per se had no effect on COX-2 mRNA levels, but reduced COX-2 expression induced by LPS. This effect was not observed in the presence of HS024 (i.c.v., 1 nmol/rat) (fig. 3). HS024 modified neither basal nor LPS-induced COX-2 expression (fig. 4). Administration of LPS, or α-MSH, or their combination failed to modify COX-1 mRNA levels in the MBH (data not shown). HS024, alone or in combination with LPS, also had no effect on COX-1 expression (data not shown).

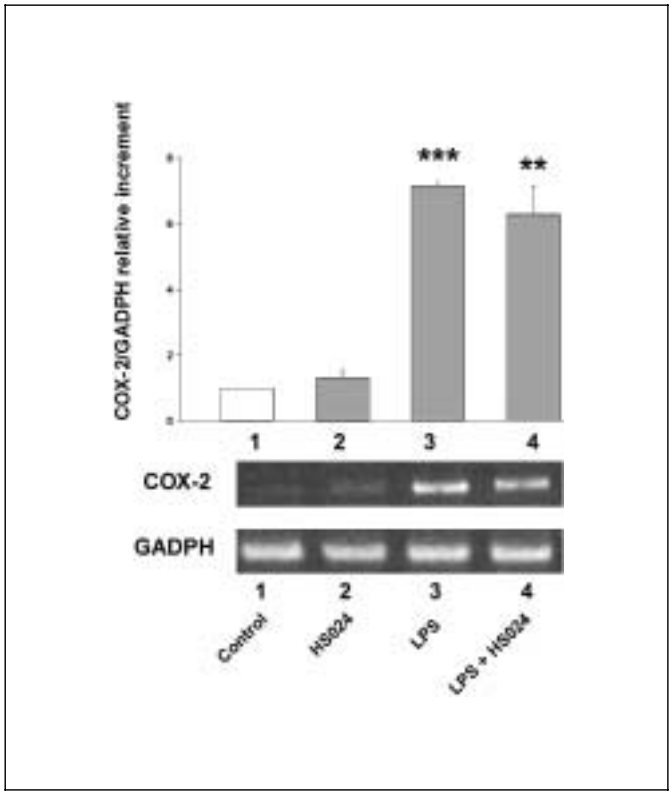

Fig. 4. Effect of HS024 on COX-2 gene expression in MBH. Expression and semiquantification of COX-2 mRNA. Control (lane 1), HS024 (lane 2), LPS (lane 3), LPS + HS024 (lane 4). Values represent mean ± SEM of 4 determinations per group of relative increment of the relation COX-2/GADPH mRNA in respect to control group of 2 independent experiments. Data were evaluated by one sample t test. ** $p < 0.01$, *** $p < 0.001$ vs. control.

Effect of α-MSH and HS024 on Serum Corticosterone Levels

Considering that LPS activates the HPA axis, we examined the effect of i.c.v. administration of α-MSH on corticosterone levels of LPS-treated rats. LPS (i.p., 250 μg/rat) increased corticosterone serum levels (fig. 5). α-MSH (i.c.v., 3 nmol/rat) significantly decreased the LPS-induced corticosterone release, but failed to modify basal corticosterone levels. HS024 had no effect on corticosterone basal or LPS-induced secretion (fig. 5).

Effect of α-MSH and HS024 on Serum PRL Levels

The administration of LPS or α-MSH significantly decreased PRL secretion and the combination of both treatments induced a further decrease (fig. 6). HS024 did not significantly modify the inhibitory effect of LPS on PRL release (fig. 6).

Effect of α-MSH and HS024 on Serum LH Levels

Administration of α-MSH or LPS significantly decreased serum LH levels (fig. 7). HS024 significantly increased LH release and blocked the inhibitory effect of LPS on LH secretion (fig. 7).

In vitro Experiments

Effect of α-MSH on NOS Expression in MBH

When hypothalamic explants from untreated rats were incubated for 5 h, iNOS mRNA was detected in the con-

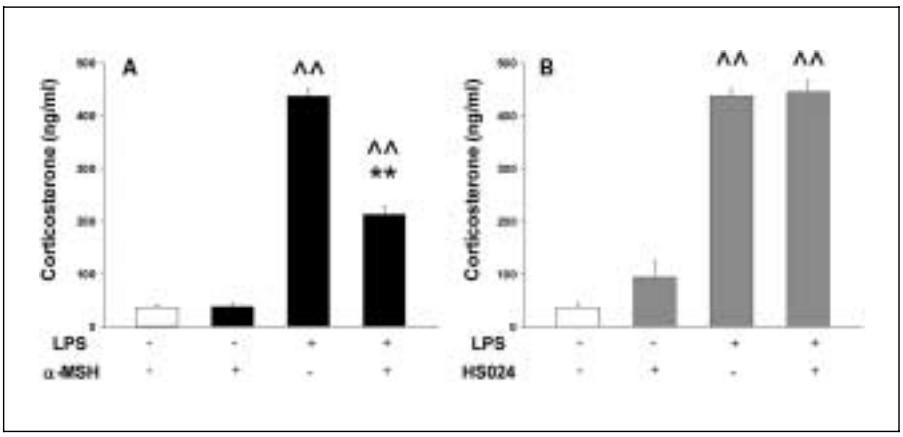

Fig. 5. Effect of α-MSH (**A**) and HS024 (**B**) on basal and LPS-stimulated serum corticosterone levels. Values represent mean ± SEM of 6–8 determinations per group. Data were evaluated by two-way ANOVA with interaction terms. ** $p < 0.01$ vs. respective control without α-MSH; ^^ $p < 0.01$ vs. respective control without LPS.

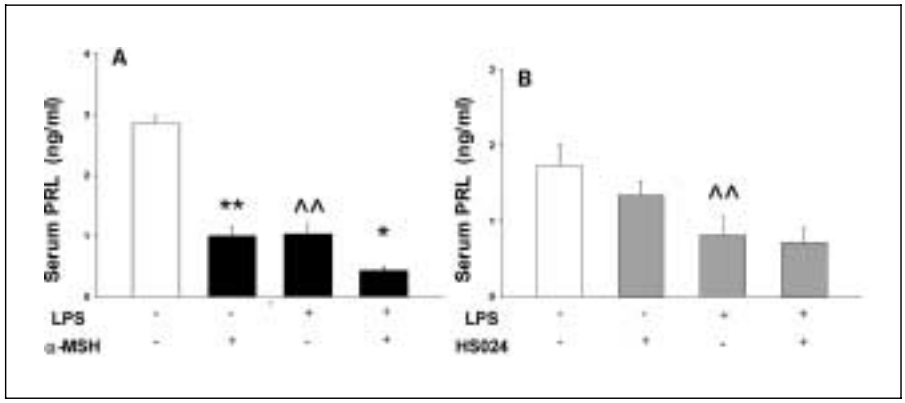

Fig. 6. Effect of α-MSH (**A**) and HS024 (**B**) on serum PRL levels. Values represent mean ± SEM of 4–6 determinations per group. Data were evaluated by two-way ANOVA with interaction terms. * $p < 0.05$, ** $p < 0.01$ vs. respective control without α-MSH; ^ $p < 0.01$ vs. respective control without LPS.

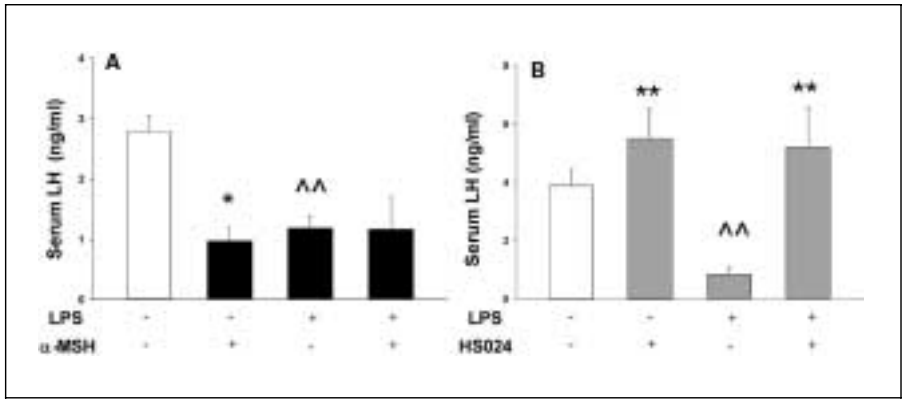

Fig. 7. Effect of α-MSH (**A**) and HS024 (**B**) on serum LH levels. Values represent mean ± SEM of 4–6 determinations per group. Data were evaluated by two-way ANOVA with interaction terms. * $p < 0.05$, ** $p < 0.01$ vs. respective control without α-MSH or HS024; ^ $p < 0.01$ vs. respective control without LPS.

trol group. LPS (10 μg/ml) had no effect on iNOS gene expression (data not shown). The incubation of the MBH explants with LPS (10 μg/ml) plus INF-γ (100 ng/ml) significantly increased iNOS expression (fig. 8). This increase was reduced in the presence of α-MSH (5 μ*M*), which per se had no effect (fig. 8). LPS + INF-γ or α-MSH or their combination did not modify nNOS, eNOS, COX-1 or COX-2 mRNA levels in the MBH (data not shown).

Discussion

Melanocortins were shown to exert anti-inflammatory actions within the brain [26]. Here, we report that central administration of α-MSH decreases LPS-induced iNOS gene expression in the MBH. The administration of HS024, a selective MC4-R antagonist, reversed the inhibitory effect of α-MSH on LPS-induced iNOS expression, suggesting that α-MSH may exert this effect, at least in part, by activating central MC4-R. Although HS024 was found to have more than a 100-fold higher affinity for MC4-R than MC3-R in the rat [27], we cannot exclude

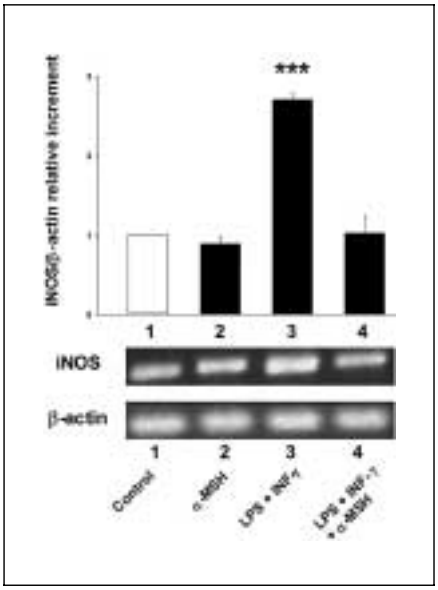

Fig. 8. In vitro effect of α-MSH on iNOS gene expression in MBH. Expression and semiquantification of iNOS mRNA. Control (lane 1), α-MSH 5 μ*M* (lane 2), LPS 10 μg/ml + INF-γ 100 ng/ml (lane 3), LPS 10 μg/ml + INF-γ 100 ng/ml + α-MSH 5 μ*M* (lane 4). Values represent mean ± SEM of 3 determinations per group of relative increment of the relation iNOS/β-actin mRNA in respect to control group of 3 different experiments. Data were evaluated by one sample t test. *** $p < 0.001$ vs. control.

the possibility that MC3-R may also be involved in α-MSH actions. Since HS024 potentiates the effect of LPS on hypothalamic iNOS mRNA levels, these observations support the idea that α-MSH may participate in the hypothalamus as an endogenous modulator of the inflammatory response.

Concordantly, it has been demonstrated that LPS upregulates gene expression of iNOS in the paraventricular nucleus of the hypothalamus [28, 29]. Also, iNOS expression was reported to be downregulated by α-MSH in microglia [17]. Preserving iNOS gene expression from its undesirable induction could be important for neuronal survival. Our findings suggest that endogenous α-MSH may prevent a potentially harmful induction of iNOS expression, acting as a local anti-inflammatory agent within the hypothalamus. In our experiments, we found no changes in nNOS and eNOS expression. Different mechanisms underlie the synthesis of nNOS, eNOS and iNOS genes [29] indicating that iNOS gene induction responds specifically to immune challenge.

The presence of IFN-γ was necessary to reveal the in vitro stimulatory effect of LPS on iNOS gene expression in hypothalamic explants. Similarly, in vitro iNOS expression in hepatocytes required the combination of LPS with IFN-γ [30], the dominant iNOS-inducing agent in macrophages [31]. In our in vitro experimental approach, α-MSH decreased iNOS expression induced by LPS plus IFN-γ.

COX-1 is a constitutively expressed enzyme responsible for producing PGs under physiological conditions. COX-2 is usually expressed at sites of inflammation following induction by various cytokines and mitogens [9, 32]. In the brain, constitutive expression of COX-2 was found in neurons of hippocampus, cerebral cortex, amygdala, and hypothalamus [11, 33]. LPS injection increases COX-2 mRNA levels in the brain endothelial cells [11, 34] and microglia [12]. PGs are involved in brain functions including fever induction, nociception, learning and memory. They are overexpressed in paradigms of excitotoxic brain injury, such as stroke and epilepsy. In previous reports, α-MSH was found to inhibit PGE_2 synthesis in brain tissue [20, 21].

In our experiments, LPS administration did not affect COX-1 gene expression but significantly increased COX-2 mRNA levels in MBH. This differential effect of LPS on COX isoforms has been reported for other tissues [35]. α-MSH attenuated the stimulatory action of LPS on COX-2 expression. This inhibitory effect of α-MSH was blocked by HS024 suggesting that it may be exerted through MC4-R. COX-2 plays a dominant role in mediating fever response to LPS [33]. The antipyretic effect of centrally administered melanocortins was shown to be exerted through MC4-R [36]. This effect could result from an inhibition of PG synthesis due to the modulatory action of α-MSH on COX-2 expression during endotoxemia. Although HS024 blocked the inhibitory effect of α-MSH on LPS-induced COX-2 expression, HS024 per se failed to modify the stimulatory effect of LPS on the expression of this enzyme, suggesting that endogenous α-MSH may not exert an inhibitory tone on COX-2 expression. Alternatively, it can be speculated that centrally administered α-MSH may gain access to different cell populations expressing COX-2 than those affected by endogenous α-MSH.

It has been reported that α-MSH antagonizes the stimulatory effect of IL-1 on the HPA (hypothalamic-pituitary-adrenal) axis in rodents and primates [37]. PGs are involved in LPS activation of the HPA axis [1].

Recently, agouti-related protein (AgRP), an α-MSH antagonist identified in the hypothalamus, has been shown to stimulate food intake by antagonizing the effects of α-MSH at MC3 and MC4 receptors [38]. Also, AgRP stimulates ACTH and cortisol in female monkeys [39]. Consistent with previous findings [40], we observed that central administration of α-MSH reduces the increase in serum corticosterone levels induced by LPS. Since α-MSH was able to reduce the stimulatory effect of LPS on both COX-2 expression and corticosterone secretion, it can be suggested that centrally administered α-MSH may decrease LPS-induced corticosterone secretion by inhibiting PGs synthesis. However, HS024 per se did not modify the stimulatory effect of LPS on serum corticosterone levels suggesting that LPS may activate HPA axis through some hypothalamic factors such as corticotrophin-releasing factor, vasopressin and catecholamines [1] that may not be affected by α-MSH.

Melanocortins have also been involved in the regulation of the hypothalamic-pituitary-gonadal axis [25]. Studies on the in vivo effect of α-MSH on LH release report contradictory results. LH response to α-MSH appears to differ according to the route of administration, time course, steroid milieu and nutritional status [25, 41–45]. In our experimental design, both LPS and α-MSH decreased serum LH levels but did not potentiate their effect. HS024 increased serum LH levels suggesting that endogenous α-MSH may inhibit LH secretion through MC4-R. Accordingly, Stanley et al. [44] demonstrated that AgRP stimulates GnRH release and LH secretion suggesting the presence of an inhibitory melanocortinergic tone on LH which may be mediated by α-MSH. More-

over, HS024 blocked the inhibitory effect of LPS on LH secretion, indicating that endogenous α-MSH may be involved in this neuroendocrine response to LPS through MC4-R.

Regarding PRL secretion, α-MSH blocks IL-1-stimulated PRL release [24] whereas AgRP stimulates PRL release in the monkey [39] supporting the idea of a physiological role for endogenous α-MSH in PRL secretion. α-MSH has been shown to suppress, whereas an α-MSH antiserum has been shown to enhance, basal and stress-induced PRL secretion [42]. We observed that both LPS and α-MSH administration decreased serum PRL levels and the combination of these treatments further decreased them. HS024 modified neither basal PRL secretion nor PRL response to LPS, suggesting that α-MSH may not be involved in the inhibitory effect of LPS on PRL secretion through MC4-R.

Previous reports indicated that HS024 diminishes proestrous and leptin-induced surges of LH and PRL [46–49]. The mechanisms involved in the control of these surges are different from those involved in maintaining basal levels of PRL and LH. Moreover, the surges in female rats have no corresponding mechanisms in male rats. Our data indicate that activation of MC4-R leads to an increase of basal serum LH levels and does not modify basal PRL levels in male rats.

In brief, our data show that α-MSH has a modulatory role through MC4-R on the hypothalamic response to endotoxin by mechanisms that involve NO and PG synthesis, thus providing further insight into the actions of α-MSH on neuroendocrine response to endotoxemia.

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References

1 Turnbull AV, Lee S, Rivier C: Mechanisms of hypothalamic-pituitary-adrenal axis stimulation by immune signals in the adult rat. Ann NY Acad Sci 1998;840:434–443.
2 Nathan C, Xie QW: Nitric oxide synthases: Roles, tolls and controls. Cell 1994;78:915–918.
3 Nelson R, Kriegsfeld L, Dawson V, Dawson T: Effects of nitric oxide on neuroendocrine function and behavior. Front Neuroendocrinol 1997;18:463–491.
4 Chiao H, Foster S, Thomas R, Lipton J, Star RA: α-Melanocyte-stimulating hormone reduces endotoxin-induced liver inflammation. J Clin Invest 1996;97:2038–2044.
5 Uribe RM, Lee S, Rivier C: Endotoxin stimulates nitric oxide production in the paraventricular nucleus of the hypothalamus through nitric oxide synthase I: Correlation with hypothalamic-pituitary-adrenal axis activation. Endocrinology 1999;140:5971–5981.
6 Satta MA, Jacobs RA, Kaltsas GA, Grossman AB: Endotoxin induces interleukin-1β and nitric oxide synthase mRNA in rat hypothalamus and pituitary. Neuroendocrinology 1998;67:109–116.
7 Lee SJ, Lee S: Toll-like receptors and inflammation in the CNS. Curr Drug Targets Inflamm Allergy 2002;1:181–191.
8 Romero LI, Tatro JB, Field JA, Reichlin S: Roles of IL-1 and TNF-α in endotoxin-induced activation of nitric oxide synthase in cultured rat brain cells. Am J Physiol 1996;270:R326–R332.
9 Needleman P, Isakson PC: The discovery and function of COX-2. J Rheumatol 1997;24 (suppl 49):6–8.
10 Pistritto G, Franzese O, Pozzoli G, Mancuso C, Tringali G, Preziosi P, Navarra P: Bacterial lipopolysaccharide increases prostaglandin production by rat astrocytes via inducible cyclo-oxygenase: Evidence for the involvement of nuclear factor κB. Biochem Biophys Res Commun 1999;263:570–574.
11 Quan N, Whiteside M, Herkenham M: Cyclooxygenase 2 mRNA expression in rat brain after peripheral injection of lipopolysaccharide. Brain Res 1998;802:189–197.
12 Schiltz JC, Sawchenko PE: Distinct brain vascular cell types manifest inducible cyclooxygenase expression as a function of the strength and nature of immune insults. J Neurosci 2002;22:5606–5618.
13 Lipton JM, Catania A, Ichiyama T: Marshaling the anti-inflammatory influence of the neuroimmunomodulator α-MSH. News Physiol Sci 2000;15:192–195.
14 Ichiyama T, Sato S, Okada K, Catania A, Lipton JM: The neuroimmunomodulatory peptide α-MSH. Ann NY Acad Sci 2000;917:221–226.
15 Martin LW, Lipton JM: Acute phase response to endotoxin: Rise in plasma α-MSH and effects of α-MSH injection. Am J Physiol 1990;259:R768–R772.
16 Catania A, Lipton JM: Peptide modulation of fever and inflammation within the brain. Ann NY Acad Sci 1998;856:62–68.
17 Delgado R, Carlin A, Airaghi L, Demitri MT, Meda L, Galimberti D, Baron P, Lipton JM, Catania A: Melanocortin peptides inhibit production of proinflammatory cytokines and nitric oxide by activated microglia. J Leukoc Biol 1998;63:740–745.
18 Ichiyama T, Zhao H, Catania A, Furukawa S, Lipton JM: α-Melanocyte-stimulating hormone inhibits NF-κB activation and IκBα degradation in human glioma cells and in experimental brain inflammation. Exp Neurol 1999;157:359–365.
19 Star RA, Rajora N, Huang J, Stock RC, Catania A, Lipton JM: Evidence of autocrine modulation of macrophage nitric oxide synthase by α-melanocyte-stimulating hormone. Proc Natl Acad Sci USA 1995;92:8016–8020.
20 Cannon JG, Tatro JB, Reichlin S, Dinarello CA: α-Melanocyte-stimulating hormone inhibits immunostimulatory and inflammatory actions of interleukin-1. J Immunol 1986;137:2232–2236.
21 Weidenfeld J, Crumeyrolle-Arias M, Haour F: Effect of bacterial endotoxin and interleukin-1 on prostaglandin biosynthesis by the hippocampus of mouse brain: Role of interleukin-1 receptors and glucocorticoids. Neuroendocrinology 1995;62:39–46.
22 Tatro JB: Receptor biology of the melanocortins, a family of neuroimmunomodulatory peptides. Neuroimmunomodulation 1996;3:259–284.

23 Kishi T, Aschkenasi C, Lee C, Mountjoy K, Saper C, Elmquist J: Expression of melanocortin-4 receptor mRNA in the central nervous system of the rat. J Comp Neurol 2003;457: 213–235.
24 Shalts E, Feng YJ, Ferin M, Wardlaw SL: Alpha-melanocyte-stimulating hormone antagonizes the neuroendocrine effects of corticotropin-releasing factor and interleukin-1α in the primate. Endocrinology 1992;131:132–138.
25 Schiöth HB, Watanobe H: Melanocortins and reproduction. Brain Res Rev 2002;38:340–350.
26 Lipton JM, Catania A, Delgado R: Peptide modulation of inflammatory processes within the brain. Neuroimmunomodulation 1998;5: 178–183.
27 Schiöth H, Bouifrouri A, Rudzish R, Muceniece R, Watanobe H, Wikberg J, Larhammar D: Pharmacological comparison of rat and human melanocortin 3 and 4 receptors in vitro. Regul Pept 2002;106:7–12.
28 Lee S, Barbanel G, Rivier C: Systemic endotoxin increases steady-state gene expression of hypothalamic nitric oxide synthase: Comparison with corticotropin-releasing factor and vasopressin gene transcripts. Brain Res 1995;705: 136–148.
29 Harada S, Imaki T, Chikada N, Naruse M, Demura H: Distinct distribution and time-course changes in neuronal nitric oxide synthase and inducible NOS in the paraventricular nucleus following lipopolysaccharide injection. Brain Res 1999;821:322–332.
30 Geller DA, Nussler AK, Di Silvio M, Lowenstein CJ, Shapiro RA, Wang SC, Simmons RL, Billiar TR: Cytokines, endotoxin and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. Proc Natl Acad Sci USA 1993;90:522–526.
31 Stenger S, Thuring H, Rollinghoff M, Bogdan C: Tissue expression of inducible nitric oxide synthase is closely associated with resistance to *Leishmania major*. J Exp Med 1994;180:783–793.
32 Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, Lipsky PE: Cyclooxygenase in biology and disease. FASEB J 1998;12:1063–1073.
33 Zhang YH, Lu J, Elmquist JK, Saper CB: Specific roles of cyclooxygenase-1 and cyclooxygenase-2 in lipopolysaccharide-induced fever and Fos expression in rat brain. J Comp Neurol 2003;463:3–12.
34 Cao C, Matsumura K, Ozaki M, Watanabe Y: Lipopolysaccharide injected into the cerebral ventricle evokes fever through induction of cyclooxygenase-2 in brain endothelial cells. J Neurosci 1999;19:716–725.
35 Ichitani Y, Holmberg K, Maunsbach A, Haeggström J, Samuelsson B, De Witt D, Hökfelt T: Cyclooxygenase-1 and cyclooxygenase-2 expression in rat kidney and adrenal gland after stimulation with systemic lipopolysaccharide: In situ hybridization and immunocytochemical studies. Cell Tissue Res 2001;303:235–252.
36 Sinha PS, Schiöth HB, Tatro JB: Activation of central melanocortin-4 receptor suppresses lipopolysaccharide-induced fever in rats. Am J Physiol Regul Integr Comp Physiol 2003;284: R1595–R1603.
37 Papadopoulos AD, Wardlaw SL: Endogenous α-MSH modulates the hypothalamic-pituitary-adrenal response to the cytokine interleukin-1β. J Neuroendocrinol 1999;11:315–319.
38 Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS: Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science 1997; 278:135–138.
39 Xiao E, Xia-Zhang L, Vulliemoz NR, Ferin M, Wardlaw SL: Agouti-related protein stimulates the hypothalamic-pituitary-adrenal (HPA) axis and enhances the HPA response to interleukin-1 in the primate. Endocrinology 2003;144: 1736–1741.
40 Huang QH, Hruby VJ, Tatro JB: Systemic α-MSH suppresses LPS fever via central melanocortin receptors independently of its suppression of corticosterone and IL-6 release. Am J Physiol 1998;275:R524–R530.
41 Newman CB, Wardlaw SL, Frantz AG: Suppression of basal and stress-induced prolactin release and stimulation of luteinizing hormone secretion by α-melanocyte-stimulating hormone. Life Sci 1985;36:1661–1668.
42 Khorram O, Bedran de Castro JC, McCann SM: Stress-induced secretion of α-melanocyte-stimulating hormone and its physiological role in modulating the secretion of prolactin and luteinizing hormone in the female rat. Endocrinology 1985;117:2483–2489.
43 Scimonelli T, Celis ME: A central action of α-melanocyte-stimulating hormone on serum levels of LH and prolactin in rats. J Endocrinol 1990;124:127–132.
44 Stanley S, Small C, Kim M, Heath M, Seal L, Russell S, Ghatei M, Bloom S: Agouti-related peptide stimulates the hypothalamus pituitary gonadal axis in vivo and in vitro in male rats. Endocrinology 1999;140:5459–5462.
45 Schiöth HB, Kakizaki Y, Kohsaka A, Suda T, Watanobe H: Agouti-related peptide prevents steroid-induced luteinizing hormone and prolactin surges in female rats. Neuroreport 2001; 12:687–690.
46 Watanobe H, Schiöth HB, Wikberg JE, Suda T: The melanocortin-4 receptor mediates leptin stimulation of luteinizing hormone and prolactin surges in steroid-primed ovariectomized rats. Biochem Biophys Res Commun 1999; 257:860–864.
47 Watanobe H, Yoneda M, Kakizaki Y, Kohsaka A, Suda T, Schiöth HB: Further evidence for a significant participation of the melanocortin-4 receptor in the preovulatory prolactin surge in the rat. Brain Res Bull 2001;54:521–525.
48 Watanobe H, Schiöth HB, Izumi J: Pivotal roles of α-melanocyte-stimulating hormone and the melanocortin-4 receptor in leptin stimulation of prolactin secretion in rats. J Neurochem 2003;85:338–347.
49 Watanobe H, Yoneda M: Evaluation of the role of melanocortin 3 and 4 receptors in leptin-stimulated and spontaneous growth hormone secretion in rats. Neuroendocrinology 2003;78: 331–338.