0013-7227/07/$15.00/0
Printed in U.S.A.

Endocrinology 148(10):4918–4926

doi: 10.1210/en.2007-0366

Activation of Melanocortin 4 Receptors Reduces the Inflammatory Response and Prevents Apoptosis Induced by Lipopolysaccharide and Interferon-γ in Astrocytes

Carla Caruso, Daniela Durand, Helgi B. Schiöth, Rodolfo Rey, Adriana Seilicovich, and Mercedes Lasaga

Centro de Investigaciones en Reproducción (C.C., D.D., R.R., A.S., M.L.), School of Medicine, University of Buenos Aires, 1121ABG Buenos Aires, Argentina; Cedie (R.R.), Hospital de Niños R. Gutierrez, C1425 EFD Buenos Aires, Argentina; and Department of Neuroscience (H.B.S.), Uppsala University, BMC, 75124 Uppsala, Sweden

α-MSH exerts an immunomodulatory action in the brain and may play a neuroprotective role acting through melanocortin 4 receptors (MC4Rs). In the present study, we show that MC4Rs are constitutively expressed in astrocytes as determined by immunocytochemistry, RT-PCR, and Western blot analysis. α-MSH (5 μM) reduced the nitric oxide production and the expression of inducible nitric oxide synthase (iNOS) induced by bacterial lipopolysaccharide (LPS, 1 μg/ml) plus interferon-γ (IFN-γ, 50 ng/ml) in cultured astrocytes after 24 h. α-MSH also attenuated the stimulatory effect of LPS/IFN-γ on prostaglandin E_2 release and cyclooxygenase-2 (COX-2) expression. Treatment with HS024, a selective MC4R antagonist, blocked the antiinflammatory effects of α-MSH, suggesting a MC4R-mediated mechanism in the action of this melanocortin. In astrocytes, LPS/IFN-γ treatment reduced cell viability, increased the number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells and activated caspase-3. α-MSH prevented these apoptotic events, and this cytoprotective effect was abolished by HS024. LPS/IFN-γ decreased Bcl-2, whereas it increased Bax protein expression in astrocytes, thus increasing the Bax/Bcl-2 ratio. α-MSH produced a shift in Bax/Bcl-2 ratio toward astrocyte survival because it increased Bcl-2 expression and also prevented the effect of LPS/IFN-γ on Bax and Bcl-2 expression. In summary, these findings suggest that α-MSH, through MC4R activation, attenuates LPS/IFN-γ-induced inflammation by decreasing iNOS and COX-2 expression and prevents LPS/IFN-γ-induced apoptosis of astrocytes by modulating the expression of proteins of the Bcl-2 family. (*Endocrinology* 148: 4918–4926, 2007)

ASTROCYTES, THE MOST abundant glial cell type in the brain, provide metabolic and trophic support for neurons and modulate synaptic transmission and plasticity (1, 2). They have a major role in regulation of the extracellular ionic environment and protect neurons from oxidative stress and excitotoxicity (3, 4). Astrocytes are an important source of neuroactive substances such as growth factors and neurosteroids, which may subsequently influence neuronal development, survival, and neurosecretion (5). Recent data indicate a significant role of astrocytes on reproduction and neuroprotection (6).

Activation of astrocytes leads to an inflammatory response during disease, infection, trauma, and ischemia (7), producing diverse inflammatory mediators such as proinflammatory cytokines, prostaglandins (PGs) and nitric oxide (NO) (8), the latter being synthesized mainly by the inducible NO synthase (iNOS). Cytokines (9–11), bacterial lipopolysaccharide (LPS) (12), and NO (13) can induce apoptosis of astrocytes, and in turn, impairment of astrocyte functions can critically influence neuronal survival. In fact, apoptosis of astrocytes may also contribute to the pathogenesis of many acute and chronic neurodegenerative disorders, such as cerebral ischemia, Alzheimer's disease, and Parkinson's disease (14). A sustained inflammatory response present in acute and chronic brain disorders may be a first step in the development of several neurodegenerative diseases. Therefore, the regulation of astrocyte inflammatory response and apoptosis may be essential in pathological processes in the central nervous system.

α-MSH is a 13-amino-acid neuropeptide of the melanocortin family, derived from proteolytic cleavage of proopiomelanocortin (POMC). α-MSH has antiinflammatory (15), antipyretic (16), and antimicrobial (17) actions. It has been suggested that the neuroprotective effects of α-MSH are mediated through its potent antiinflammatory actions (15). α-MSH antiinflammatory activity results from the inhibition of the release and/or the action of NO and cytokines (15). Also, α-MSH reduces PG synthesis via inhibition of cyclooxygenase-2 (COX-2) expression (18). Recent reports indicate that α-MSH has an antiapoptotic role in the ischemic renal failure (19), UV-induced apoptosis of melanocytes (20), cyclosporine A-induced apoptosis of human proximal tubular cells (21), and cytokine-induced apoptosis of human dermal fibroblasts (22). Conversely, α-MSH can induce cell death in mast cells (23).

There are five known melanocortin receptors (MCRs) designated MC1R through MC5R, and they all activate adenylyl

First Published Online June 26, 2007

Abbreviations: COX-2, Cyclooxygenase 2; DAPI, 4′,6-diamido-2-phenylindole dihydrochloride; FBS, fetal bovine serum; GFAP, glial fibrillary acid protein; IFN-γ, interferon-γ; iNOS, inducible NO synthase; LPS, lipopolysaccharide; MCR, melanocortin receptor; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NF-κB, nuclear transcription factor-κB; PG, prostaglandin; POMC, proopiomelanocortin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

cyclase. MC3R and MC4R are expressed in the brain, MC4R being the predominant MCR subtype in the central nervous system (24). MC1R is involved in skin pigmentation and together with MC3R is thought to mediate systemic antiinflammatory actions of melanocortins (15). The MC4R has been a focus of interest due to its roles in controlling energy balance (25). MC4R mediates antipyretic actions of α-MSH (16) and exerts a neuroprotective action on cerebral ischemia (26).

Exposure to LPS plus interferon-γ (IFN-γ) is a strong proinflammatory stimulus for astrocytes in culture (27). LPS in combination with IFN-γ has been widely used in different *in vitro* and *in vivo* experimental approaches for the study of brain inflammatory diseases (28–29). Previous results from our laboratory indicate that *in vivo* administration of α-MSH inhibits LPS-induced hypothalamic iNOS and COX-2 expression through MC4R (30). In the present report, we show that MC4R but not MC3R is expressed in astrocytes. α-MSH attenuated the stimulatory effect of LPS/IFN-γ on NO and PGE2 production as well as on iNOS and COX-2 expression in astrocytes acting through MC4R. Also, α-MSH prevented the proapoptotic action of LPS/IFN-γ in astrocytes. This antiapoptotic action of α-MSH may be exerted by modulating the expression of Bax and Bcl-2. Besides, a selective antagonist of MC4R was also able to counteract the antiapoptotic effect of α-MSH. Thus, this study reveals antiinflammatory and antiapoptotic actions of α-MSH in astrocytes that could be relevant for the treatment of brain disorders.

Materials and Methods

Materials

LPS (*Escherichia coli*, serotype O127:B8) was purchased from Sigma-Aldrich Corp. (St. Louis, MO). α-MSH was obtained from Bachem California Inc. (Torrance, CA). IFN-γ was purchased from Boehringer Ingelheim (Buenos Aires, Argentina). HS024 was purchased from Neosystem, Strasbourg, France (31). Fetal bovine serum (FBS) was obtained from PAA laboratories GmBH (Pasching, Austria). DMEM/F-12, antibiotics, and all RT-PCR reagents were purchased from Invitrogen Life Technologies, (Carlsbad, CA), unless specified otherwise. Anti-Bcl-2 and anti-Bax antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-iNOS was purchased from BD Biosciences (CA), anti-GFAP, biotinylated donkey antimouse and antirabbit and donkey antimouse rhodamine-conjugated antibodies were obtained from Chemicon International Inc. (Temecula, CA), and anti-MC4R and anti-COX-2 antibodies were purchased from Cayman Chemical (Ann Arbor, MI). iNOS and β-actin primers were purchased from Transgenomics Inc. (Omaha, NE). COX-2 and GAPDH primers were obtained from MWG-Biotech AG (Ebersberg, Germany). MC3R and MC4R primers were from Integrated DNA Technologies, Inc. (Coralville, IA). All other media and supplements were obtained from Sigma-Aldrich, unless specified otherwise.

Cell culture

Astrocytes were prepared from rat cerebral tissue of 1- to 2-d-old postnatal Wistar rat pups as described by McCarthy and de Vellis (32). Briefly, cerebral hemispheres were dissected, freed from meninges, and cut into small fragments. The tissue was disrupted by triturating through a needle in DMEM/F-12 medium containing 10% FBS, 40 μg/ml streptomycin, and 40 U penicillin. Then, cells were seeded in 75-cm^2 poly-L-lysine-coated culture flasks. The cultures received changes with fresh medium twice a week. After 11–14 d, astrocytes were separated from microglia and oligodendrocytes by shaking for 24 h in an orbital shaker at 200 rpm. Shaking was repeated twice after a gap of 1–3 d before subculturing to ensure the complete removal of oligodendrocytes and microglia. Cells were trypsinized, subcultured, and after 2–3 d of stabilization, incubated with the drugs for 24 h in MEM containing 2% FBS, 2 mM L-glutamine, 40 μg/ml streptomycin, and 40 U penicillin. Cultured cells were kept at 37 C in 5% CO_2. Cultures were routinely over 95% pure astrocytes, as assessed by glial fibrillary acidic protein (GFAP) immunostaining. When treated with the MC4R antagonist (HS024), astrocytes were preincubated with HS024 (0.5 μM) alone for 30 min and then treated with LPS/IFN-γ (1 μg/ml LPS and 50 ng/ml IFN-γ) in the presence of α-MSH (5 μM) and HS024 (0.5 μM) for 24 h. All the experimental procedures were carried out in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Immunofluorescent identification of astrocytes and MC4R

GFAP and MC4R were identified by indirect immunofluorescence staining. After the culture period, cells were fixed with 0.5 ml methanol: acetic acid (1:3) for 20 min and incubated with 10% normal donkey serum in PBS with 0.2% Triton X-100 and avidin blocking solution (0.2 ml/ml; Vector Laboratories, Burlingame, CA) for 60 min. The slides were then incubated with the primary antibodies mouse antirat GFAP (1:200) and rabbit antirat MC4R (1:200) in PBS with 0.2% Triton X-100, biotin blocking solution (0.2 ml/ml; Vector), and 1% normal donkey serum overnight at 4 C. After rinsing, slides were incubated for 1 h with donkey antimouse rhodamine-conjugated and biotinylated antirabbit secondary antibodies (1:200). Slides were washed and incubated with

TABLE 1. Oligonucleotide sequences of PCR primers

	Sequence (5′–3′)	No. of cycles	Size (bp)	Annealing temperature (C)
MC4R				
Forward	GGCTTCACATTAAGAGGATCGCT	33	595	65
Reverse	TTTATGGAACTCCATAGCGCCC			
MC3R				
Forward	AGCAACCGGAGTGGCAGT	30	421	63
Reverse	GGCCACGATCAAGGAGAG			
iNOS				
Forward	TAGAAACAACAGGAACCTACCA	33	907	58
Reverse	ACAGGGGTGATGCTCCCGGACA			
COX-2				
Forward	GGAGAGACGATCAAGATAGTGATC	35	860	65
Reverse	ATGGTCAGTAGACTCTTACAGCTC			
β-Actin				
Forward	ACCACAGCTGAGAGGGAAATCG	22–24	289	58–65
Reverse	AGAGGTCTTTACGGATGTCAACG			
GAPDH				
Forward	GTGAAGGTCGGTGTGAACGGATTT	24	595	65
Reverse	CACAGTCTTCTGAGTGGCAGTGAT			

fluorescein-conjugated avidin (1:400; Vector) in 10 mM HEPES buffer (pH 7.5). The slides were mounted with mounting medium for fluorescence (Vectashield; Vector) containing 4′,6-diamido-2-phenylindole dihydrochloride (DAPI) for DNA staining and visualized in a fluorescence microscope (Axiophot; Carl Zeiss, Jena, Germany). Control slides were incubated with normal donkey serum instead of primary antibody.

NO synthesis assay

Synthesis of NO was determined by assay of the culture media for nitrite, a stable product of NO with molecular oxygen. Briefly, 100 μl culture supernatants were mixed with 50 μl 1% sulfanilic acid in 5% H_3PO_4 and 50 μl 0.1% *N*-(1-naphthyl)ethylene-diamine dihydrochloride and incubated at room temperature for 15 min. The OD of the samples was read in a microplate spectrophotometer at 595 nm. Nitrite concentrations were calculated from a standard curve derived from the reaction of $NaNO_2$ in the assay.

PGE2 assay

PGE2 levels were determined in the culture media using an enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI) according to the manufacturer's protocol. The assay limit of detection was 39 pg/ml.

RT-PCR

Total RNA from cultured astrocytes, hypothalamus, and liver was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Five micrograms of total RNA were treated with 1 U DNase (Promega Corp., Madison, WI) at 37 C for 10 min and reverse transcribed as described before (30). Amplification was performed with 2 μl cDNA as template in 50 μl PCR containing 1–2 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol of each primer, and 2.5 U of *Taq* DNA polymerase in the buffer provided by the manufacturer. Temperature cycles always had an initial denaturation at 94 C for 5 min and a final extension period of 7 min at 72 C. Synthetic oligonucleotides used for PCR, annealing temperature, number of cycles, and product size are listed in Table 1. Twelve microliters of each reaction were analyzed on 2% agarose gels, stained with ethidium bromide, and visualized using UV light. RT-PCR products were quantitatively analyzed using SCION Image software. Results were normalized to the internal control β-actin. Values are expressed as relative increments of respective controls. Experiments always included non-reverse-transcribed DNase-treated RNA samples as negative controls. PCR of these RT controls never showed amplification, indicating that the RNA was genomic DNA free (data not shown).

Western blot analysis

Astrocytes (1×10^6), hypothalamus, and liver were lysed in a buffer containing 1% Igepal, 1% sodium dodecyl sulfate, 150 mM NaCl, 0.02% sodium azide, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, and 2 mM phenylmethylsulfonyl fluoride in 50 mM Tris-HCl (pH 7.4). After sonication and centrifugation at 12,000 rpm for 30 min, the supernatant was used for the immunoblot assay. The protein concentration of samples was determined by Bradford protein assay. Protein (25–30 μg) was size-fractionated in a SDS-polyacrylamide gel and then electrotransferred to polyvinylidene difluoride membrane. Blots were blocked for 2 h in 5% nonfat dry milk/TBS/0.1% Tween 20 and incubated overnight with the appropriate primary antibody in 5% milk/0.1% TBS/Tween 20 at 4 C. This was followed by 1 h incubation with the respective biotinylated secondary antibody. Proteins incubated with buffer instead of primary antibody were used as negative controls. Immunoreactivity was detected by enhanced chemiluminescence (ECL plus; Amersham Biosciences, GE Healthcare, Piscataway, NJ). Results were normalized to the internal control β-actin. Values are expressed as relative increments of respective controls.

Metabolic activity assay

The metabolic activity of viable cells was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells (5×10^4) were washed and incubated for 4 h in 100 μl Krebs buffer plus 50 μg MTT reagent dissolved in 10 μl PBS at 37 C. The developed crystals were dissolved in 100 μl 0.04 N HCl in isopropanol, and the OD was read in a microplate spectrophotometer at 595 nm.

Microscopic determination of DNA fragmentation by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method

Cultured cells were fixed as described above and permeabilized by microwave irradiation. DNA strand breaks were labeled with digoxigenin-deoxy-UTP using terminal deoxynucleotidyl transferase (0.18 U/μl; Roche Diagnostics, Mannheim, Germany) as described previously (33). The incorporation of nucleotides into the 3′-OH end of damaged DNA was detected with an anti-digoxigenin-fluorescein antibody (Roche). Slides were mounted as described above.

Caspase-3 activity assay

Caspase-3 activity was measured using the CaspACE kit (Promega). Astrocytes (1×10^6) were homogenized in lysis buffer and centrifuged (14,000 rpm for 20 min). The supernatant was added to the reaction mixture

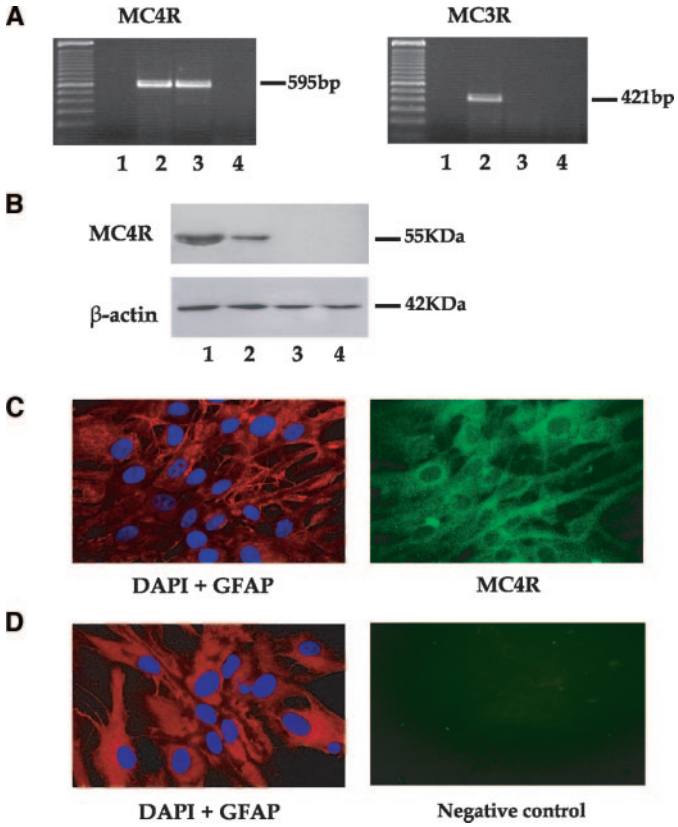

FIG. 1. Astrocytes express MC4R but not MC3R. Astrocytes were cultured in MEM with 2% FBS for 24 h. A, RNA isolated from astrocytes, hypothalamus, and liver were processed for RT-PCR as described in *Materials and Methods*. mRNA levels of MC4R (595 bp) and MC3R (421 bp) were determined. Lane 1, Blank; lane 2, hypothalamus; lane 3, astrocytes; lane 4, liver. B, Cell lysate from astrocytes, hypothalamus, and liver were fractioned by Western blot. Membranes were probed with primary antibodies against MC4R and β-actin proteins. Proteins from hypothalamus were incubated with buffer instead of MC4R antibody as a negative control. Lane 1, Hypothalamus; lane 2, astrocytes; lane 3, liver; lane 4, negative control. C, Immunofluorescent identification of MC4R in astrocytes. Cells were stained with the nuclear marker DAPI (*blue*) and with GFAP antibody (*red*) and MC4R antibody (*green*). D, The negative control shows astrocytes stained with GFAP (*red*) and the nuclear marker DAPI (*blue*), incubated with normal donkey serum instead of MC4R antibody.

containing Ac-DEVD-*p*-nitroaniline, the colorimetric substrate, and incubated at 37 C for 4 h. The OD was measured in a microplate spectrophotometer at 415 nm. The protein concentration of supernatant aliquots was determined by Bradford protein assay (Bio-Rad Laboratories, Richmond, CA) using BSA as standard. Astrocytes were incubated with 50 μM Z-VAD-FMK, a caspase-3 inhibitor, for 24 h in the presence of LPS/IFN-γ as an assay control, after which cells were treated as described before. Caspase-3 activity was expressed as OD per nanogram protein.

Statistical analysis

Data are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Dunnet or Bonferroni multiple comparisons test or two-way ANOVA with analysis of the interaction. Differences with a $P < 0.05$ were considered statistically significant. The experiments were performed at least twice. The number of apoptotic cells identified by the TUNEL method was expressed as the percentage of apoptotic cells of the total number of cells counted for each specific condition. Differences between proportions were analyzed by the χ^2 test with 95% confidence.

Results

Rat astrocytes express MC4R but not MC3R

Because MC3R and MC4R are the most abundant subtypes of MCR in the brain, we determined their expression in astrocytes. Considering that liver does not express either MC3R or MC4R (34), we used this tissue as a negative control. MC4R mRNA but not MC3R mRNA was detected in astrocytes, whereas both receptors were found in the hypothalamus and none of them in the liver (Fig. 1A). Also, MC4R was detected in astrocytes and hypothalamus by Western blot as a 55-kDa protein but not in the liver (Fig. 1B). We also observed constitutive expression of MC4R as a specific punctuate staining pattern in about 95% of astrocytes as assessed by immunocytochemistry (Fig. 1C). No staining was detected when astrocytes were incubated only with normal donkey serum (Fig. 1D).

Activation of MC4Rs is involved in the inhibitory effect of α-MSH on iNOS and COX-2 expression induced by LPS/IFN-γ in astrocytes

Previous results from our laboratory indicated that *in vivo* administration of α-MSH inhibited LPS-induced hypothalamic iNOS and COX-2 expression via MC4R (30). Now, we investigated the effect of α-MSH on NO production and

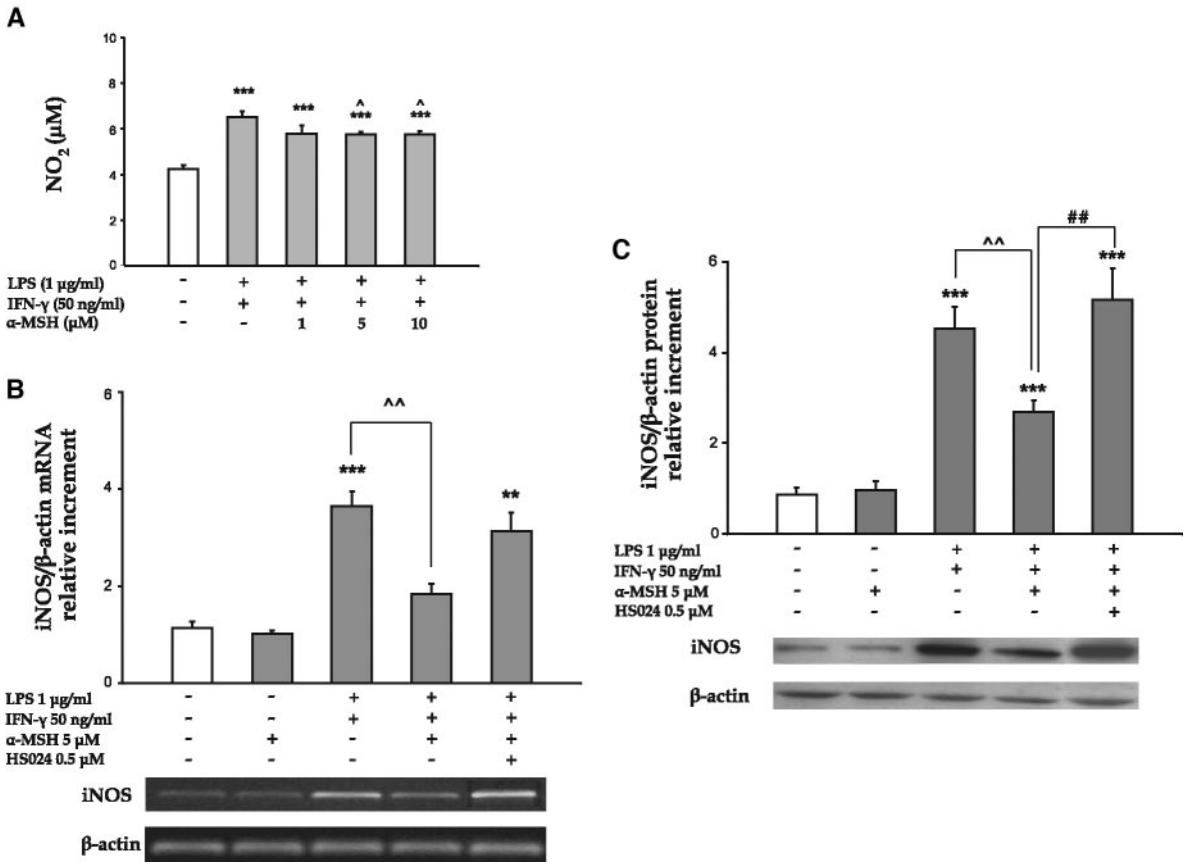

FIG. 2. α-MSH attenuates the effect of LPS/IFN-γ on NO production and iNOS expression in astrocytes. A, Nitrite was measured in supernatants of cultured astrocytes treated with LPS/IFN-γ in the presence or absence of α-MSH for 24 h. Values are the mean ± SEM of six to eight determinations per group. Data are representative of two independent experiments. ***, $P < 0.001$ *vs.* control group; ∧, $P < 0.05$ *vs.* LPS/IFN-γ. B, RNA was isolated from astrocytes treated with LPS/IFN-γ in the presence or absence of α-MSH preincubated with 0.5 μM HS024 for 24 h and processed for RT-PCR as described in *Materials and Methods*. mRNA levels of iNOS were determined and expressed as mean ± SEM of four determinations per group of relative increment of the relation iNOS/β-actin with respect to the control group of two independent experiments. **, $P < 0.01$; ***, $P < 0.001$ *vs.* control group; ∧∧, $P < 0.01$ *vs.* LPS/IFN-γ. C, Cell lysates from astrocytes treated for 24 h with LPS/IFN-γ in the presence or absence of 5 μM α-MSH with or without 0.5 μM HS024 were subjected to Western blot. Membranes were probed with antibodies against iNOS and β-actin. Values represent mean ± SEM of four determinations per group of relative increment of the relation iNOS/β-actin with respect to the control group of two independent experiments. ***, $P < 0.001$ *vs.* control group; ∧∧, $P < 0.01$ *vs.* LPS/IFN-γ; and ##, $P < 0.01$ *vs.* LPS/IFN-γ/α-MSH/HS024.

iNOS expression induced by the combination of the proinflammatory molecules LPS (1 μg/ml) plus IFN-γ (50 ng/ml) in cultured astrocytes after 24 h. The LPS/IFN-γ-stimulatory effect on NO production was significantly reduced by 5 and 10 μM α-MSH (Fig. 2A). The inhibitory action of α-MSH was not dose dependent. α-MSH (5 μM) *per se* did not modify nitrite release (data not shown), but it significantly attenuated the stimulatory effect of LPS/IFN-γ on both iNOS mRNA and protein levels (Fig. 2, B and C). α-MSH failed to reduce the effect of LPS/IFN-γ on iNOS expression when astrocytes were incubated with a selective MC4R antagonist, HS024 (Fig. 2, B and C). We also determined the effect of α-MSH on PGE2 release and COX-2 expression. Although α-MSH (5 μM) *per se* did not modify PGE2 release, it significantly reduced LPS/IFN-γ-induced PGE2 release (Fig. 3A) as well as attenuated the LPS/IFN-γ-stimulatory effect on COX-2 mRNA and protein expression (Fig. 3, B and C) in cultured astrocytes. Treatment of astrocytes with HS024 prevented the antiinflammatory effect of α-MSH on LPS/IFN-γ-induced PGE2 release (Fig. 3A) and COX-2 mRNA and protein expression (Fig. 3, B and C). Taken together, these results suggest that MC4R is involved in the antiinflammatory effect of α-MSH in astroglial cells.

α-MSH prevents the LPS/IFN-γ-induced apoptosis of astrocytes, whereas a MC4R antagonist, HS024, blocks this effect

Because inflammatory stimuli can induce apoptosis of astrocytes (9–11), we evaluated the effect of α-MSH on cell viability. LPS/IFN-γ treatment significantly decreased the viability of astrocytes. α-MSH (5 and 10 μM) prevented the LPS/IFN-γ effect (Fig. 4A), although it did not, *per se*, modify cell viability (data not shown). We further investigated apoptosis by determining DNA fragmentation with the TUNEL method. Figure 4B shows the nuclear morphology of an

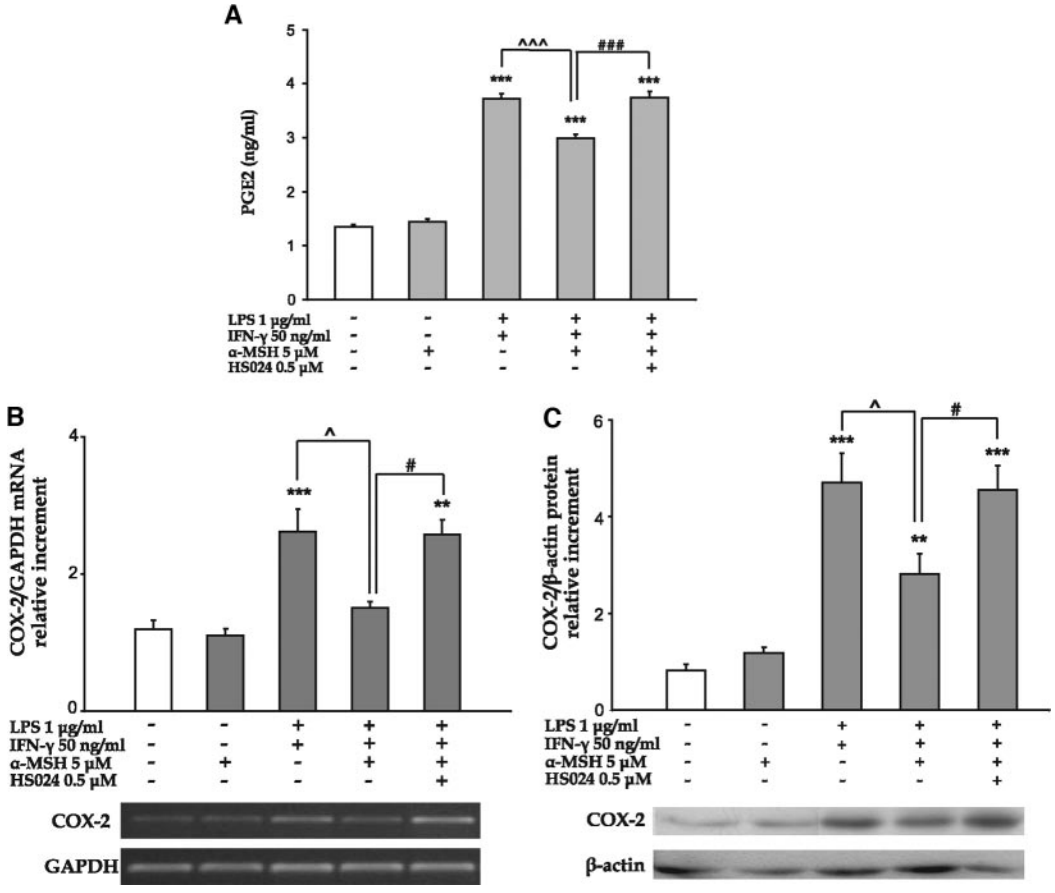

FIG. 3. α-MSH attenuates the effect of LPS/IFN-γ on PGE2 release and COX-2 expression in astrocytes. A, PGE2 was determined in supernatants of cultured astrocytes treated with LPS/IFN-γ in the presence or absence of α-MSH with or without 0.5 μM HS024 for 24 h. Values are the mean ± SEM of eight determinations per group. Data are representative of two independent experiments. ***, $P < 0.001$ *vs.* control group; ∧∧∧, $P < 0.001$ *vs.* LPS/IFN-γ; ###, $P < 0.001$ *vs.* LPS/IFN-γ/α-MSH/HS024. B, RNA was isolated from astrocytes treated with LPS/IFN-γ in the presence or absence of α-MSH preincubated with 0.5 μM HS024 for 24 h and processed for RT-PCR as described in *Materials and Methods*. mRNA levels of COX-2 were determined and expressed as mean ± SEM of four determinations per group of relative increment of the relation COX-2/GAPDH with respect to the control group of two independent experiments. **, $P < 0.01$; ***, $P < 0.001$ *vs.* control group; ∧, $P < 0.05$ *vs.* LPS/IFN-γ; and #, $P < 0.05$ *vs.* LPS/IFN-γ/α-MSH/HS024. C, Cell lysates from astrocytes treated for 24 h with LPS/IFN-γ in the presence or absence of 5 μM α-MSH with or without 0.5 μM HS024 were subjected to Western blot. Membranes were probed with antibodies against COX-2 and β-actin proteins. Values represent mean ± SEM of four to five determinations per group of relative increment of the relation COX-2/β-actin with respect to the control group of two independent experiments. **, $P < 0.01$; ***, $P < 0.001$ *vs.* control group; ∧, $P < 0.05$ *vs.* LPS/IFN-γ; #, $P < 0.05$ *vs.* LPS/IFN-γ/α-MSH/HS024.

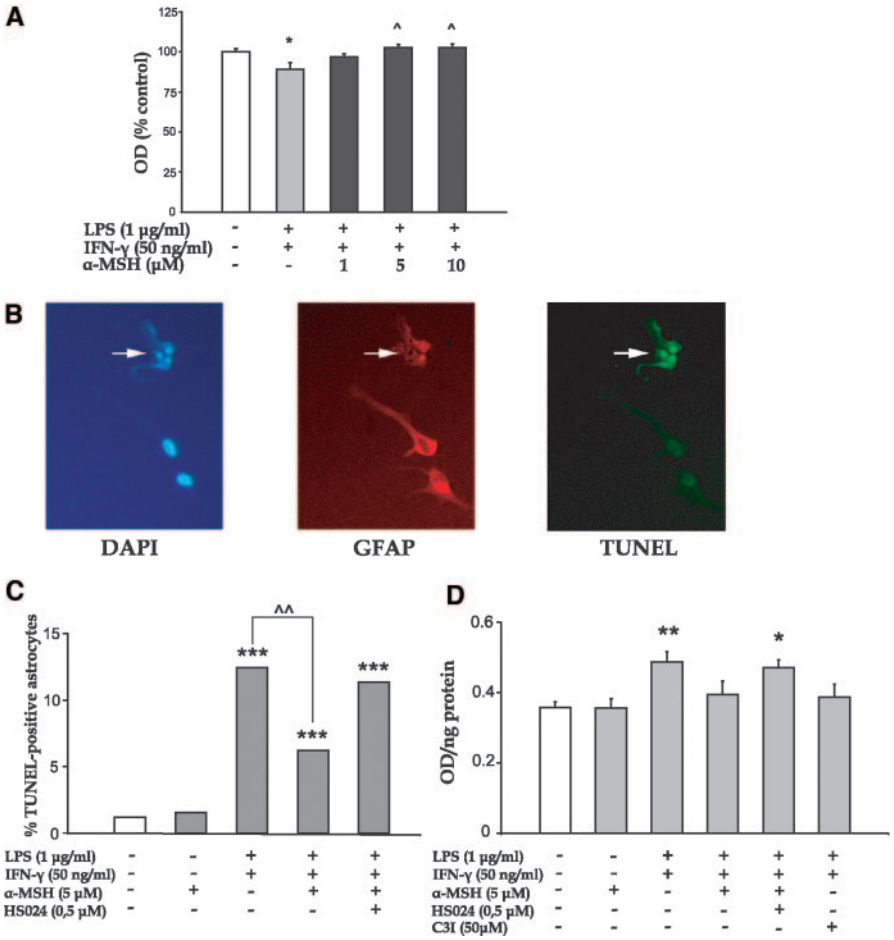

FIG. 4. α-MSH decreases LPS/IFN-γ-induced apoptosis in astrocytes. A, Cultured astrocytes were incubated with LPS/IFN-γ and α-MSH (1, 5, or 10 μM) for 24 h. The metabolic activity was assayed by MTT. Data are expressed as percentage of the control group. Values represent mean ± SEM of five to eight determinations per group of two independent experiments. *, $P < 0.05$ *vs.* control group; ∧, $P < 0.05$ *vs.* LPS/IFN-γ. B, Cells were stained with the nuclear marker DAPI (*blue*) and with GFAP antibody (*red*), and DNA fragmentation was assayed by TUNEL (*green*). The *arrow* shows a GFAP- and TUNEL-positive cell. C, Astrocytes were treated with LPS/IFN-γ with or without α-MSH in the presence or absence of 0.5 μM MC4R antagonist HS024 for 24 h. The number of TUNEL-positive astrocytes was determined and analyzed as describe in *Materials and Methods*. Values are expressed as the percentage of TUNEL-positive astrocytes of four independent experiments. ***, $P < 0.001$ *vs.* control group; ∧∧, $P < 0.01$ *vs.* LPS/IFN-γ. D, Caspase-3 activity was determined in cell lysates from astrocytes treated with LPS/IFN-γ in the presence or absence of 5 μM α-MSH with or without 0.5 μM HS024 for 16 h. The incubation of astrocytes with LPS/IFN-γ and 50 μM Z-VAD-FMK, a caspase-3 inhibitor (C3I), for 24 h is shown as an assay control. Values are expressed as OD per nanogram protein ± SEM of four to six determinations per group of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* control group.

apoptotic astrocyte characterized by nuclear condensation and fragmentation. LPS/IFN-γ treatment significantly increased the percentage of TUNEL-positive astrocytes (Fig. 4C). α-MSH (5 μM) *per se* did not modify the percentage of TUNEL-positive astrocytes but attenuated the apoptosis induced by LPS/IFN-γ (Fig. 4C). The antiapoptotic effect of α-MSH was also assessed by measuring caspase-3 activity. LPS/IFN-γ significantly increased caspase-3 activity (Fig. 4D), an effect that was not observed in the presence of α-MSH (Fig. 4D). α-MSH failed to decrease the percentage of TUNEL-positive astrocytes (Fig. 4C) and capase-3 activity (Fig. 4D) induced by LPS/IFN-γ when the cells were preincubated with the selective MC4R antagonist HS024.

α-MSH inhibits LPS/IFN-γ-induced increase of Bax/Bcl-2 ratio

To test whether the expression of pro- and antiapoptotic members of the Bcl-2 family is involved in the protective action of α-MSH, we determined the effect of α-MSH on the proapoptotic Bax and the antiapoptotic Bcl-2 protein levels in astrocytes. Although α-MSH *per se* had no effect on Bax expression, it blocked LPS/IFN-γ-induced increase of this protein (Fig. 5A). α-MSH *per se* increased Bcl-2 levels and reversed the inhibitory effect of LPS/IFN-γ on Bcl-2 expression (Fig. 5B). Because the balance between proapoptotic and antiapoptotic proteins plays a crucial role in apoptosis, we analyzed data as Bax/Bcl-2 ratio. α-MSH prevented the increase of the Bax/Bcl-2 ratio induced by LPS/IFN-γ (Fig. 5C).

Discussion

The present study shows that astrocytes express MC4R and that α-MSH through MC4R reduces the inflammatory response in these glial cells, protecting them from LPS/IFN-γ-induced apoptosis. MC4R gene expression was up-regulated in the undamaged striatum after hypoxia-ischemic brain injury (35), and MC4R-directed neuroprotective strategies have recently been proposed as a possible therapy for stroke (36). MC4R is expressed in both neuronal and nonneuronal cells. Previous studies reported that MC4R presents dense expression within specific neuron-rich areas such as the hypothalamus and the hippocampus (37, 38) as well as a widespread expression of MC4R in low levels that may suggest expression in nonneuronal cells. Very recently, Selkirk *et al.* (39) reported the selective expression of MC4R with real-time RT-PCR analysis of mRNA levels in astrocytes. Accordingly, here we report that astrocytes express MC4R because we detected MC4R mRNA and protein expression but no MC3R expression in these cells. The systemic antiinflammatory actions of melanocortins were suggested to involve MC3R (40). However, a recent study reported that the selective MC3R agonist, γ-MSH, had no protective effect in ischemic stroke (26). Therefore, our data support the idea that

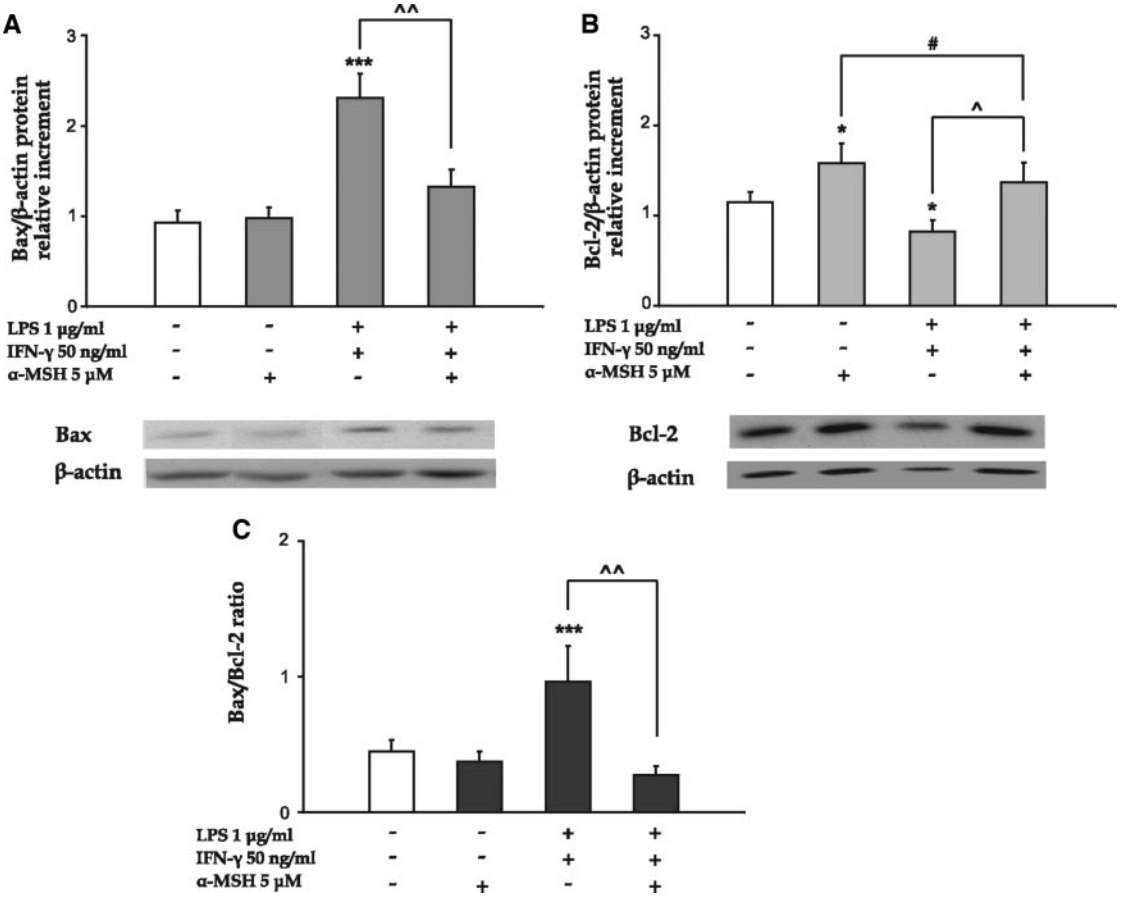

FIG. 5. α-MSH prevents LPS/IFN-γ-induced changes in Bax and Bcl-2 expression. Astrocytes were treated with LPS/IFN-γ in the presence or absence of 5 μM α-MSH for 24 h. Total cell lysates were prepared, and proteins were subjected to Western blot. Membranes were probed with antibodies against Bax, Bcl-2, and β-actin. Values represent mean ± SEM of six to eight determinations per group of relative increment of the relation Bax/β-actin (A) or Bcl-2/β-actin (B) with respect to the control group of five independent experiments. The relative increments were used to calculate Bax/Bcl-2 ratio (C). *, $P < 0.05$; ***, $P < 0.001$ *vs.* control group; ∧, $P < 0.05$; ∧∧, $P < 0.01$ *vs.* LPS/IFN-γ; #, $P < 0.05$ *vs.* α-MSH.

MC4R is the MCR subtype involved in the antiinflammatory actions of melanocortins on glial cells within the brain.

NO is one of the major inflammatory molecules produced by activated astrocytes. α-MSH has been shown to inhibit NO production in immunocompetent cells (15), through the inhibition of nuclear transcription factor-κB (NF-κB) activity leading to a decrease in iNOS transcription (41). We have previously reported that *in vivo* administration of α-MSH reduced LPS-induced increase of hypothalamic iNOS and COX-2 expression acting through MC4R (30). In this study, we show that α-MSH reduces NO production and iNOS expression induced by LPS/IFN-γ in astrocytes. This effect was not observed in the presence of HS024, a selective MC4R antagonist, suggesting that α-MSH antiinflammatory action involves MC4R activation.

Inflammatory stimuli trigger COX-2 expression, whereas COX-1 expression is unaffected. Within the brain, several cell types, including resident cells (*i.e.* neurons, microglia, astrocytes, and endothelial cells) and infiltrating blood cells, can express COX-2 (42). PGE2 synthesis elicited by LPS in rat astrocytes depends on inducible COX activity by a mechanism involving NF-κB (43). Recent studies described how the protective role of α-MSH involves a reduction in PG production via inhibition of the COX-2 pathway (18). In the present study, we provide evidence that α-MSH through MC4R attenuates the increase of PGE2 release and COX-2 expression induced by LPS/IFN-γ in astrocytes, suggesting that the antiinflammatory actions of α-MSH in brain may result, in part, from a direct action on these glial cells.

Most of the actions of α-MSH observed herein were achieved using a dose approximately 50-fold higher than the EC_{50} reported in astrocytes by Selkirk *et al.* (39). In other cell types that express MC1R, such as macrophages or neutrophils, α-MSH exerts antiinflammatory actions at lower doses (23, 44). α-MSH has higher affinity for MC1R than for MC4R (45), but MC1R activation in the central nervous system is not involved in the antiinflammatory actions of α-MSH (46). Therefore, the small changes induced by α-MSH on NO and PG release could depend on the low level of expression of MC4R in astrocytes (39).

The effect of cytokines on astrocyte survival is controversial. Both proliferative and antiproliferative actions of cytokines on astrocytes have been described depending on the state of the cells and their environment (47). Like previously reported data (12), our present results show that LPS/IFN-γ induces apoptosis of astrocytes. Few studies have focused on the ability of melanocortins to block apoptosis (19–22, 26), but the possible effect on glial cells

has been neglected. Here, we observed that α-MSH prevents LPS/IFN-γ-induced apoptosis, reducing the percentage of TUNEL-positive astrocytes and blocking the LPS/IFN-γ effect on cell viability and caspase-3 activity. This antiapoptotic action of α-MSH seems to be triggered by activation of MC4R because this melanocortin fails to protect astrocytes in the presence of a selective MC4R antagonist. The α-MSH neuroprotective effect may be associated with a suppression of the inflammatory cascade as indicated by the decrease in NO and PG production. Therefore, α-MSH may counteract the deleterious effects of NO, contributing to prevent apoptosis of astrocytes. The α-MSH neuroprotective effect could also involve downregulation of COX-2 expression, which has been associated with proinflammatory activities in neurodegenerative processes of several acute and chronic brain diseases (42). The antiapoptotic action of α-MSH could be important at early stages of the inflammatory response when preservation of astroglial function is necessary to promote neuron survival.

An extensive literature documents the capacity of Bcl-2 to block cell death in many cell types including astrocytes (48, 49). Also, increased expression of the death promoter Bax was found in LPS-induced apoptosis in the brain (50). The ratio Bax/Bcl-2 appears to be a critical determinant of a cell's apoptosis threshold (51). In our studies, LPS/IFN-γ downregulated Bcl-2 and up-regulated Bax, thereby increasing the Bax/Bcl-2 ratio. This imbalance in the Bax/Bcl-2 ratio may be important in initiating further signaling cascades leading to apoptosis. Previous studies suggested that α-MSH has a protective effect on melanocytes and tubular renal cells by modulating Bcl-2 protein levels (21). These results are in agreement with our observation that α-MSH prevents apoptosis by blocking the increase in the Bax/Bcl-2 ratio induced by LPS/IFN-γ. Also, α-MSH *per se* increased Bcl-2 levels. Thus, the control of astrocyte survival by α-MSH may depend on the expression of Bcl-2 family proteins. Ligand binding to MCRs activates adenylyl cyclase, which leads to the production of cAMP and subsequent activation of cAMP-responsive element binding protein (52). This transcription factor is responsible for cell survival during episodes of metabolic or oxidative stress (53) and modulates the expression of Bcl-2 (54). Therefore, it is possible that cAMP-responsive element binding protein activation could mediate the action of α-MSH on Bcl-2 and Bax expression. In fact, it has been recently demonstrated that activation of MC4R leads to cAMP production in cultured rat astrocytes (39).

In conclusion, our results show that α-MSH has antiapoptotic and antiinflammatory effects on astrocytes through MC4R. The antiinflammatory actions of α-MSH may result from inhibition of NO and PG synthesis. Our data also further establish α-MSH as a neuropeptide capable of preventing apoptotic events in astrocytes by modulating the expression of Bcl-2 family members.

Even small changes in the survival of astrocytes and in their capacity to release proinflammatory mediators can have important consequences in the neuronal survival, and therefore the antiinflammatory action of MC4R agonists could be potentially useful in the treatment of neurodegenerative diseases.

Acknowledgments

We are especially grateful to Dr. Juana Pasquini for her invaluable assistance.

Received March 19, 2007. Accepted June 20, 2007.

Address all correspondence and requests for reprints to: Mercedes Lasaga, Centro de Investigaciones en Reproducción, School of Medicine, University of Buenos Aires, Buenos Aires 1121ABG, Argentina. E-mail: mlasaga@fmed.uba.ar.

This work was supported by the University of Buenos Aires, the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), and the Swedish Research Council.

Disclosure Summary: The authors have nothing to declare.

References

1. **Zhao G, Flavin M** 2000 Differential sensitivity of rat hippocampal and cortical astrocytes to oxygen-glucose deprivation injury. Neurosci Lett 285:177–180
2. **Smith SJ** 1992 Do astrocytes process neural information? Prog Brain Res 94:119–136
3. **Desagher S, Glowinski J, Premont J** 1996 Astrocytes protect neurons from hydrogen peroxide toxicity. J Neurosci 16:2553–2562
4. **Dugan L, Bruno V, Amagasu S, Giffard R** 1995 Glia modulate the response of murine cortical neurons to excitotoxicity: glia exacerbate AMPA neurotoxicity. J Neurosci 15:4545–4555
5. **Garcia-Segura L, McCarthy M** 2004 Role of glia in neuroendocrine function. Endocrinology 145:1082–1086
6. **Mahesh V, Dhandapani K, Brann D** 2006 Role of astrocytes in reproduction and neuroprotection. Mol Cell Endocrinol 246:1–9
7. **Dong Y, Benveniste EN** 2001 Immune function of astrocytes. Glia 36:180–190
8. **Muñoz-Fernandez MA, Fresno M** 1998 The role of tumour necrosis factor, interleukin 6, interferon-γ and inducible nitric oxide synthase in the development and pathology of the nervous system. Prog Neurobiol 56:307–340
9. **Ehrlich L, Peterson P, Hu S** 1999 Interleukin (IL)-1β-mediated apoptosis of human astrocytes. Neuroreport 10:1849–1852
10. **Pahan K, Khan M, Singh I** 2000 Interleukin-10 and interleukin-13 inhibit proinflammatory cytokine-induced ceramide production through the activation of phosphatidylinositol 3-kinase. J Neurochem 75:576–582
11. **Saas P, Boucraut J, Quiquerez A, Schnuriger V, Perrin G, Desplat-Jego S, Bernard D, Walker P, Dietrich P** 1999 CD95 (Fas/Apo-1) as a receptor governing astrocyte apoptotic or inflammatory responses: a key role in brain inflammation? J Immunol 162:2326–2333
12. **Suk K, Lee J, Hur J, Kim YS, Lee M, Cha S, Yeou Kim S, Kim H** 2001 Activation-induced cell death of rat astrocytes. Brain Res 900:342–347
13. **Kim M, Cheong Y, So H, Lee K, Kim T, Oh J, Chung Y, Son Y, Kim B, Park R** 2001 Protective effects of morphine in peroxynitrite-induced apoptosis of primary rat neonatal astrocytes: potential involvement of G protein and phosphatidylinositol 3-kinase (PI3 kinase). Biochem Pharmacol 61:779–786
14. **Takuma K, Baba A, Matsuda T** 2004 Astrocyte apoptosis: implications for neuroprotection. Prog Neurobiol 72:111–127
15. **Catania A, Gatti S, Colombo G, Lipton JM** 2004 Targeting melanocortin receptors as a novel strategy to control inflammation. Pharmacol Rev 56:1–29
16. **Sinha P, Schiöth H, Tatro J** 2004 Roles of the melanocortin-4 receptor in antipyretic and hyperthermic actions of centrally administered α-MSH. Brain Res 1001:150–158
17. **Starowicz K, Przewlocka B** 2003 The role of melanocortins and their receptors in inflammatory processes, nerve regeneration and nociception. Life Sci 73: 823–847
18. **Liu G, Liu L, Lin C, Tseng J, Chuang M, Lam H, Lee J, Yang L, Chan J, Howng S, Tai M** 2006 Gene transfer of pro-opiomelanocortin prohormone suppressed the growth and metastasis of melanoma: involvement of α-melanocyte-stimulating hormone-mediated inhibition of the nuclear factor κB/cyclooxygenase-2 pathway. Mol Pharmacol 69:440–451
19. **Jo S, Yun S, Chang K, Cha D, Cho W, Kim H, Won N** 2001 α-MSH decreases apoptosis in ischaemic acute renal failure in rats: possible mechanism of this beneficial effect. Nephrol Dial Trans 16:1583–1591
20. **Bohm M, Wolff I, Scholzen T, Robinson S, Healy E, Luger T, Schwarz T, Schwarz A** 2005 α-Melanocyte-stimulating hormone protects from ultraviolet radiation-induced apoptosis and DNA damage. J Biol Chem 280:5795–5802
21. **Lee S, Jo S, Cho W, Kim H, Won N** 2004 The effect of α-melanocyte-stimulating hormone on renal tubular cell apoptosis and tubulointerstitial fibrosis in cyclosporine A nephrotoxicity. Transplantation 78:1756–1764
22. **Hill R, Wheeler P, Macneil S, Haycock J** 2005 α-Melanocyte stimulating hormone cytoprotective biology in human dermal fibroblast cells. Peptides 26:1150–1158
23. **Sarkar A, Sreenivasan Y, Manna S** 2003 α-Melanocyte-stimulating hormone induces cell death in mast cells: involvement of NF-κB. FEBS Lett 549:87–93

24. **Tatro J** 1996 Receptor biology of the melanocortins, a family of neuroimmunomodulatory peptides. Neuroimmunomodulation 3:259–284
25. **Chaki S, Okuyama S** 2005 Involvement of melanocortin-4 receptor in anxiety and depression. Peptides 26:1952–1964
26. **Giuliani D, Mioni C, Altavilla D, Leone S, Bazzani C, Minutoli L, Bitto A, Cainazzo M, Marini H, Zaffe D, Botticelli A, Pizzala R, Savio M, Necchi D, Schioth HB, Bertolini A, Squadrito F, Guarini S** 2006 Both early and delayed treatment with melanocortin 4 receptor-stimulating melanocortins produces neuroprotection in cerebral ischemia. Endocrinology 147:1126–1135
27. **Ramirez G, Toro R, Dobeli H, von Bernhardi R** 2005 Protection of rat primary hippocampal cultures from Aβ cytotoxicity by pro-inflammatory molecules is mediated by astrocytes. Neurobiol Dis 19:243–254
28. **Bal-Price A, Brown G** 2001 Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. J Neurosci 21:6480–6491
29. **Hemmer K, Fransen L, Vanderstichele H, Vanmechelen E, Heuschling P** 2001 An in vitro model for the study of microglia-induced neurodegeneration: involvement of nitric oxide and tumor necrosis factor-α. Neurochem Int 38: 557–565
30. **Caruso C, Mohn C, Karara A, Rettori V, Watanobe H, Schioth H, Seilicovich A, Lasaga M** 2004 α-Melanocyte-stimulating hormone through melanocortin-4 receptor inhibits nitric oxide synthase and cyclooxygenase expression in the hypothalamus of male rats. Neuroendocrinology 79:278–286
31. **Kask A, Mutulis F, Muceniece R, Pahkla R, Mutule I, Wikberg JE, Rago L, Schioth HB** 1998 Discovery of a novel superpotent and selective melanocortin-4 receptor antagonist (HS024): evaluation *in vitro* and *in vivo*. Endocrinology 139:5006–5014
32. **McCarthy K, de Vellis J** 1980 Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol 85:890–902
33. **Caruso C, Bottino MC, Pampillo M, Pisera D, Jaita G, Duvilanski B, Seilicovich A, Lasaga M** 2004 Glutamate induces apoptosis in anterior pituitary cells through group II metabotropic glutamate receptor activation. Endocrinology 145:4677–4684
34. **Gatti S, Colombo G, Turcatti F, Lonati C, Sordi A, Bonino F, Lipton JM, Catania A** 2006 Reduced expression of the melanocortin-1 receptor in human liver during brain death. Neuroimmonomodulation 13:51–55
35. **Mountjoy K, Guan J, Elia C, Sirimanne E, Williams C** 1999 Melanocortin-4 receptor messenger RNA expression is up-regulated in the non-damaged striatum following unilateral hypoxic-ischaemic brain injury. Neuroscience 89:183–190
36. **Tatro J** 2006 Melanocortins defend their territory: multifaceted neuroprotection in cerebral ischemia. Endocrinology 147:1122–1125
37. **Liu H, Kishi T, Roseberry AG, Cai X, Lee CE, Montez JM, Friedman JM, Elmquist JK** 2003 Transgenic mice expressing green fluorescent protein under the control of the melanocortin-4 receptor promoter. J Neurosci 23:7143–7154
38. **Kishi T, Aschkenasi CJ, Lee CE, Mountjoy KG, Saper CB, Elmquist JK** 2003 Expression of melanocortin 4 receptor mRNA in the central nervous system of the rat. J Comp Neurol 457:213–235
39. **Selkirk JV, Nottebaum LM, Lee J, Yang W, Foster AC, Lechner SM** 2007 Identification of differential melanocotin 4 receptor agonist profiles at natively expressed receptors in rat cortical astrocytes and recombinantly expressed receptors in human embryonic kidney cells. Neuropharmacology 52:459–466
40. **Lam C, Getting S** 2004 Melanocortin receptor type 3 as a potential target for anti-inflammatory therapy. Curr Drug Targets Inflamm Allergy 3:311–315
41. **Ichiyama T, Zhao H, Catania A, Furukawa S, Lipton JM** 1999 α-Melanocyte-stimulating hormone inhibits NF-κB activation and IκBα degradation in human glioma cells and in experimental brain inflammation. Exp Neurol 157: 359–365
42. **Minghetti L** 2004 Cyclooxygenase-2 (COX-2) in inflammatory and degenerative brain diseases. J Neuropathol Exp Neurol 63:901–910
43. **Pistritto G, Franzese O, Pozzoli G, Mancuso C, Tringali G, Preziosi P, Navarra P** 1999 Bacterial lipopolysaccharide increases prostaglandin production by rat astrocytes via inducible cyclo-oxygenase: evidence for the involvement of nuclear factor κB. Biochem Biophys Res Commun 263:570–574
44. **Yoon SW, Goh SH, Chun JS, Cho EW, Lee MK, Kim KL, Kim JJ, Kim CJ, Poo H** 2003 α-Melanocyte-stimulating hormone inhibits lipopolysaccharide-induced tumor necrosis factor-α production in leukocytes by modulating protein kinase A, p38 kinase, and nuclear factor κB signaling pathways. J Biol Chem 278:32914–32920
45. **Wikberg JES** 1999 Melanocortin receptors: perspectives for novel drugs. Eur J Pharmacol 375:295–310
46. **Ichiyama T, Sakai T, Catania A, Barsh GS, Furukawa S, Lipton JM** 1999 Systemically administered α-melanocyte-stimulating peptides inhibit NF-κB activation in experimental brain inflammation. Brain Res 836:31–37
47. **Viviani B, Bartesaghi S, Corsini E, Galli C, Marinovich M** 2004 Cytokines role in neurodegenerative events. Toxicol Lett 149:85–89
48. **Papadopoulos MC, Koumenis I, Xu L, Giffard R** 1998 Potentiation of murine astrocyte antioxidant defense by bcl-2: protection in part reflects elevated glutathione levels. Eur J Neurosci 10:1252–1260
49. **Ouyang Y, Giffard R** 2004 Changes in astrocyte mitochondrial function with stress: effects of Bcl-2 family proteins. Neurochem Int 45:371–379
50. **Semmler A, Okulla T, Sastre M, Dumitrescu-Ozimek L, Heneka M** 2005 Systemic inflammation induces apoptosis with variable vulnerability of different brain regions. J Chem Neuroanat 30:144–157
51. **Wu X, Deng Y** 2002 Bax and BH3-domain-only proteins in p53-mediated apoptosis. Front Biosci 17:151–156
52. **Montminy M** 1997 Transcriptional regulation by cyclic AMP. Annu Rev Biochem 66:807–822
53. **Persengiev S, Green M** 2003 The role of ATF/CREB family members in cell growth, survival and apoptosis. Apoptosis 8:225–228
54. **Dawson T, Ginty D** 2002 CREB family transcription factors inhibit neuronal suicide. Nat Med 8:450–451

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**