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Effects of melanocortin peptides on lipopolysaccharide/interferon-gamma-induced NF-kappaB DNA binding and nitric oxide production in macrophage-like RAW 264.7 cells: evidence for dual mechanisms of action

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Abstract

The pro-opiomelanocortin-derived peptide α-melanocyte-stimulating hormone (α-MSH) mediates broad anti-inflammatory and immunomodulatory effects, which include inhibition of the production and release of proinflammatory cytokines and nitric oxide (NO) from macrophages. We investigated the effects of α-MSH, α-MSH(1–10), and α-MSH(11–13) on NO production and nuclear factor-kappaB (NF-κB) translocation in RAW 264.7 macrophages. After stimulation of the cells with bacterial lipopolysaccharide/interferon-γ (LPS/IFN-γ), all three peptides inhibited NO production with an order of potency α-MSH $\geqq$ α-MSH(11–13) $>$ α-MSH(1–10). All three MSH peptides inhibited NF-κB nuclear translocation with the maximal effect of α-MSH and α-MSH(11–13) being seen in the range 1 nM–1 μM, and that of α-MSH(1–10) at 1 μM. By use of ^{125}I-(Nle^4,D-Phe^7)α-MSH(NDP-MSH) radioligand binding, MC_1 receptor-binding sites were demonstrated on RAW 264.7 cells. α-MSH and α-MSH(1–10) competed with the ^{125}I-NDP-MSH binding at these MC_1 receptor-binding sites, but α-MSH(11–13) even in concentrations up to 1 mM did not. Moreover, α-MSH and α-MSH(1–10) caused powerful stimulation of cyclic 3′,5′-adenosine monophosphate (cAMP) in the RAW 264.7 cell, whereas α-MSH(11–13) was ineffective. Forskolin stimulated cAMP and inhibited NO production to the same extent as α-MSH and α-MSH(1–10), but did not modify the translocation of NF-κB. Whereas the protein kinase A inhibitor H89 did not modify the effect of α-MSH on NF-κB translocation, H89 caused a partial inhibition of the inhibitory effect of α-MSH, α-MSH(1–10), α-MSH(11–13), and forskolin on NO production. In addition α-MSH, α-MSH(1–10), α-MSH(11–13), and forskolin also inhibited the activity of an NF-κB-dependent luciferase reporter and these effects were partially counteracted by H89. We suggest that melanocortin peptides act via dual mechanisms of action: one cAMP-independent and causing inhibition of NF-κB translocation and the other dependent on MC_1 receptor/cAMP activation.

Keywords: Macrophages; Melanocortin peptides; Nitric oxide; NF-κB; cAMP; Inflammation

1. Introduction

The pro-opiomelanocortin-derived peptide α-MSH and its C-terminal tripeptide α-MSH(11–13) are potent antipyretic and anti-inflammatory agents [1,2]. Immunoregulatory effects of melanocortins have been demonstrated both *in vivo* [3–6] and *in vitro* [7,8]. α-MSH is known both to inhibit the production and antagonize the effect of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-1, IL-6, and IL-8 [8–10]. α-MSH has also been shown to induce the production of the anti-inflammatory cytokine IL-10 [11]. Melanocortins are known to act on five different subtypes of melanocortin receptor ($MC_{1–5}$) [12–16]. One of these, the MC_1 receptor, is thought to be

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Abbreviations: α-MSH, α-melanocyte-stimulating hormone; cAMP, cyclic 3′,5′-adenosine monophosphate; NDP-MSH, (Nle^4,D-Phe^7)α-MSH; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; LPS, lipopolysaccharide; MC, melanocortin; IFN-γ, interferon-γ, iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB; IκB, inhibitory subunit of NF-κB; NO, nitric oxide; PKA, protein kinase A; PKA_c, protein kinase A catalytic unit; IL, interleukin; and FBS, fetal bovine serum.

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involved in the anti-inflammatory activity of α-MSH, as the MC_1 receptor was found on cells of the immune system, namely neutrophils and macrophages [17–20].

Nitric oxide is a short-lived bioactive molecule that has a central role in the regulation of the immune, nervous, and cardiovascular systems [21]. A family of nitric oxide synthases (NOS) form the NO from L-arginine [22]. Among these NOSs, the inducible form (iNOS) mediates prolonged high-output production of NO after its induction by immunological and inflammatory stimuli [23]. Increases in NO production may be seen in a variety of pathophysiological conditions, including circulatory shock and inflammation [24,25]. NO plays important roles in the host cell defenses by causing damage to infectious organisms and tumor cells. Excessive NO production may also be potentially toxic for host cells, and NO is implicated to be a pathogenic mediator in autoimmune diseases.

α-MSH has been shown to inhibit NO production in different cell lines, effects which are proposed to be mediated by decreased transcription of iNOS caused by the inhibition of the activity of the NF-κB transcription factor [8,17,26,27]. NF-κB is thought to be essential for the induction of iNOS upon exposure of cells to various stimuli, such as bacterial LPS, IFN-γ, proinflammatory cytokines (e.g. TNF-α and IL-1), and viral infections [28]. NF-κB consists of homodimers and heterodimers of proteins belonging to the Rel family. The activity of NF-κB is regulated by an inhibitory protein, IκB, that forms a complex with NF-κB, thereby keeping the NF-κB in an inactive state in the cytoplasm [29]. When cells receive an activating signal, the inhibitory protein undergoes phosphorylation and ubiquitination, which subsequently leads to the degradation of IκB by proteasomes. The loss of IκB leads to release of free NF-κB units, which translocate from the cytoplasm to the nucleus, where the NF-κB units may trigger the transcription of specific target genes [29]. Moreover, the protein kinase A catalytic subunit is proposed to form a complex with IκB/NF-κB wherein it has a distinct role in the regulation of NF-κB [30].

In the present study, we investigated the mechanisms involved in melanocortin peptide inhibition of induced nitric oxide production in a macrophage cell line. The data obtained are discussed with the notion that melanocortins act via dual mechanisms: one is an MC_1 receptor/cAMP-mediated inhibitory pathway and the other is cAMP-independent, acting by inhibiting NF-κB nuclear translocation.

2. Materials and methods

2.1. Reagents

LPS (from *Escherichia coli* 0111:B4) and mouse recombinant IFN-γ were from Sigma Chemical Co. [γ-^{32}P]ATP, poly (dI-dC), and [^{3}H]cAMP were from Amersham. Double-stranded oligonucleotide containing the NF-κB binding motif of the mouse macrophage iNOS promoter, 5′-CAACTGGGGACTCTCCCTTTG-3′, and a double-stranded mutated NF-κB oligonucleotide, 5′-CAACTGCTCACTCTCCCTTTG-3′, were custom-synthesized by GIBCO BRL. Peptides were synthesized in our laboratory using a Pioneer Peptide Synthesis system (PerSeptive Biosystems), and were as follows: α-MSH (*N*-Acetyl-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2), α-MSH(1–10) (*N*-Acetyl-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-NH_2), α-MSH(11–13) (Lys-Pro-Val-NH_2), and NDP-MSH. The molecular mass of peptides was verified by mass spectrometry. ^{125}I-NDP-MSH was prepared by radioiodination of NDP-MSH, using the chloramine-T method, and purified by HPLC. FBS, DMEM, and RPMI-1640 medium were from GIBCO BRL. pNF-κB-Luc reporter plasmid was from Stratagene Corp. All other chemicals were from Sigma.

2.2. Cell culture

The murine macrophage cell line RAW 264.7 was a gift from Prof. Bernhard Brune, University of Erlangen-Nurnberg, Germany. Cells were cultured in DMEM supplemented with heat-inactivated 10% FBS at 37° in humidified 5% CO_2 until experiments were performed. Cells were used when they reached 80% confluency.

2.3. Measurement of NO production

Cells were scraped off from the Petri dishes, centrifuged, and resuspended in RPMI-1640 medium without phenol red, supplemented with 2 mM L-glutamate. Cells were then seeded onto 96-well plates at a density of 2.5×10^6 cells per well and incubated in the above-mentioned medium with a combination of LPS (100 ng/mL) and IFN-γ (5 U/mL) and drugs for 16 hr at 37° in a cell incubator. Cells exposed to LPS/IFN-γ alone and unstimulated cells served as controls. After 16 hr, nitrite, a stable metabolite of NO, was determined by mixing 50 μL of culture medium with 50 μL of Griess reagent [see 31] and, after 10 min, by measuring the absorbance at 540 nm using a Novo Biolabs kinetic microplate reader (Molecular Devices). Nitrite concentrations were calculated from a sodium nitrite standard curve.

2.4. Measurement of cAMP

Cells were harvested and incubated for 20 min at 37° in 0.05 mL DMEM (without FBS) containing 0.5 mM 3-isobutyl-1-methylxanthine and appropriate concentrations of test substances. After completion of the incubation, the cAMP was extracted by adding 4.4 M perchloric acid to a final concentration of 0.4 M. After centrifugation, supernatants were neutralized with 5 M KOH/1 M Tris. Fifty microliters of the extract, or cAMP standards ranging from 0.3 to 160 nM, were then assayed by using a bovine adrenal cAMP-binding protein competitive binding assay, essentially as described [32]. In brief, the samples were incubated with

[^{3}H]cAMP (0.14 pmol, approximately 11,000 cpm, specific activity 24 Ci/mmol) and the binding protein at 4° for 150 min. The incubates were then filtered on Whatman GF/B filters using a semiautomatic Brandel cell harvester. Each filter was rinsed with 3 mL 50 mM Tris/HCl pH 7.4 punched out and placed into scintillation vials with scintillation fluid and counted. The cAMP concentrations of samples were then estimated by readings to the standard curve using a radioimmunoassay program.

2.5. Preparation of nuclear extracts

Nuclear extracts were prepared essentially according to Schreiber *et al*. [33]. In brief, 1–2 $\times$ 10^6 cells were centrifuged and washed twice with ice-cold PBS. The cell pellet was resuspended in 0.4 mL of cold lysis buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/mL of leupeptin) by gentle pipetting. The cells were allowed to swell on ice for 15 min and then lysed by addition of Nonidet P-40 to a final concentration of 0.5%. The tube was vigorously vortexed for 10 sec and then centrifuged at 13,000 $\times$ g for 30 sec. The resulting nuclear pellet was resuspended in 30 μL of cold buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2.0 μg/mL of leupeptin). After incubation for 30 min on ice with intermittent mixing, the nuclear extract was centrifuged for 5 min at 13,000 $\times$ g. The supernatants were collected and stored at -80° in aliquots until performance of the EMSA. Protein concentrations of the extracts were measured by using a bicinchoninic acid (BCA) protein assay kit (Pierce).

2.6. Electrophoretic mobility shift assay (EMSA)

The double-stranded NF-κB binding oligonucleotide was end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. After labeling, 5 μg of nuclear extract protein was incubated with 17.5 fmol of labeled probe in the presence of 2 μg poly(dI-dC) in binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, 50 mM NaCl). The mixture was incubated for 15 min at 37° and then applied to a 5% non-denaturing polyacrylamide gel, and electrophoresed in 0.5 $\times$ Tris-borate-EDTA buffer. The gel was finally dried under vacuum at 80° and analyzed by autoradiography. The specificity of binding was examined by specific competition with the unlabeled oligonucleotide (100-fold excess) and by non-specific competition with the double-stranded mutated NF-κB oligonucleotide.

2.7. Radioligand binding

Radioligand binding was performed essentially as described [34]. In brief, the RAW 264.7 macrophages were washed with binding buffer and distributed into 96-well microtiter plates. The cells were then incubated for 2 hr at 37° with 50 μL of binding buffer containing ^{125}I-NDP-MSH and non-labeled MSH peptides as indicated. After the incubation, cells were washed with 0.2 mL of ice-cold binding buffer and detached from the plate with 0.2 mL of 0.1 M NaOH, where after the radioactivity was counted by using a Walac Wizard automatic gamma counter.

2.8. Transient transfection assay

Transfections of RAW 264.7 cells were performed according to the manufacturer's instructions using 6 μL lipofectamine reagent (GIBCO BRL) and 1 μg pNF-κB-Luc reporter plasmid. Cells were incubated for 5 hr before the addition of 1.0 mL of DMEM/20% FBS. Twenty-four hours after the start of transfection, cells were stimulated with LPS (100 ng/mL) and IFN-γ (100 U/mL) and drugs of interest. Following 4 hr activation, cells were lysed and the luciferase activity was determined using the Promega luciferase assay system and a Turner Designs TD-20/20 luminometer. The luciferase activity was normalized to the protein concentration of the sample. H89 treatments commenced 1 hr before addition of LPS/IFN-γ, peptides, or forskolin; control cells were treated for 1 hr with vehicle.

2.9. Statistical analysis

Student's paired t-test was used to assess the statistical significance of differences.

3. Results

3.1. Inhibition of nitrite production by melanocortin peptides

Fig. 1 shows the effect of α-MSH, α-MSH(1–10), and α-MSH(11–13) on the accumulation of nitrite (a stable NO metabolite) in RAW 264.7 cells. As can be seen, 16 hr of stimulation of the cells with LPS/IFN-γ (100 ng/mL and 5 U/mL, respectively) caused a marked increase in nitrite production compared to the unstimulated macrophages, which produced only very low amounts of nitrite. All three melanocortin peptides inhibited LPS/IFN-γ-stimulated nitrite production in a dose-dependent manner, the IC_{50} values of α-MSH, α-MSH(11–13), and α-MSH(1–10) being 16 $\pm$ 5 pM, 160 $\pm$ 60 pM, and 7.7 $\pm$ 1.3 nM, respectively. The maximal inhibitory effects of α-MSH and α-MSH(11–13) were similar (70 $\pm$ 9 and 67 $\pm$ 7%), whereas the maximal inhibitory effect of α-MSH(1–10) was less (39 $\pm$ 2%). The inhibitory effects were not the result of a decreased number of macrophage cells, as the melanocortin peptides did not affect cell numbers or the viability of the stimulated macrophages.

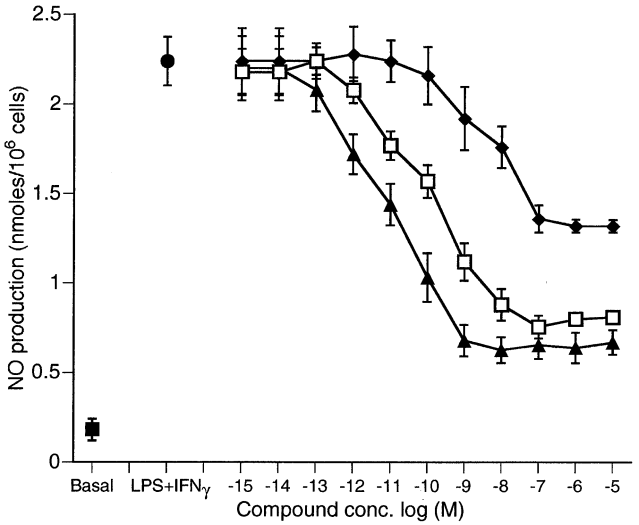

Fig. 1. Effect of melanocortin peptides on NO production. The graph shows dose–response curves for the inhibitory effect of α-MSH (▲), α-MSH(11–13) (□), and α-MSH(1–10) (◆) on NO production in RAW 264.7 macrophages. The cells were stimulated for 16 hr with LPS (100 ng/mL) and IFN-γ (5 U/mL) in the presence of various concentrations of the peptides. Control cells were incubated with or without LPS/IFN-γ. NO production was measured by determining the nitrite levels at the end of the incubation. All values represent means ± SEM of three independent experiments performed in triplicate.

3.2. Inhibition of nuclear translocation of NF-κB by melanocortin peptides

RAW 264.7 macrophages were challenged for 2 hr with LPS/IFN-γ (100 ng/mL and 100 U/mL, respectively) in the absence and presence of various concentrations of α-MSH, α-MSH(1–10), or α-MSH(11–13), and nuclear extracts were then analyzed for NF-κB binding activity. Stimulation of the RAW 264.7 cells with LPS/IFN-γ led to a marked increase in NF-κB binding compared to unstimulated cells (Fig. 2, A–C). All three MSH peptides markedly inhibited the nuclear NF-κB binding, the peak effect of α-MSH and α-MSH(11–13) being seen at 10^{-9}–10^{-6}M (Fig. 2, A and C) and that of α-MSH(1–10) at 10^{-6}M (Fig. 2B). None of the MSH peptides were effective at concentrations of 10^{-12} and 10^{-15} M (Fig. 2, A–C).

The specificity of the binding of the radiolabeled NF-κB probe to the nuclear extracts was evident, as the binding was completely prevented in the presence of a 100-fold excess of unlabeled NF-κB oligonucleotide. Moreover, a 100-fold molar excess of unlabeled mutated NF-κB oligonucleotide had no effect on the binding activity (data not shown).

3.3. Effect of MSH peptides and forskolin on cAMP

The ability of α-MSH, α-MSH(1–10), or α-MSH(11–13) to cause stimulation of cAMP production was assessed in both unchallenged and LPS/IFN-γ-challenged RAW 264.7 cells. Fig. 3A shows the effect of a 20-min treatment by MSH peptides in unchallenged cells. As seen, both α-MSH

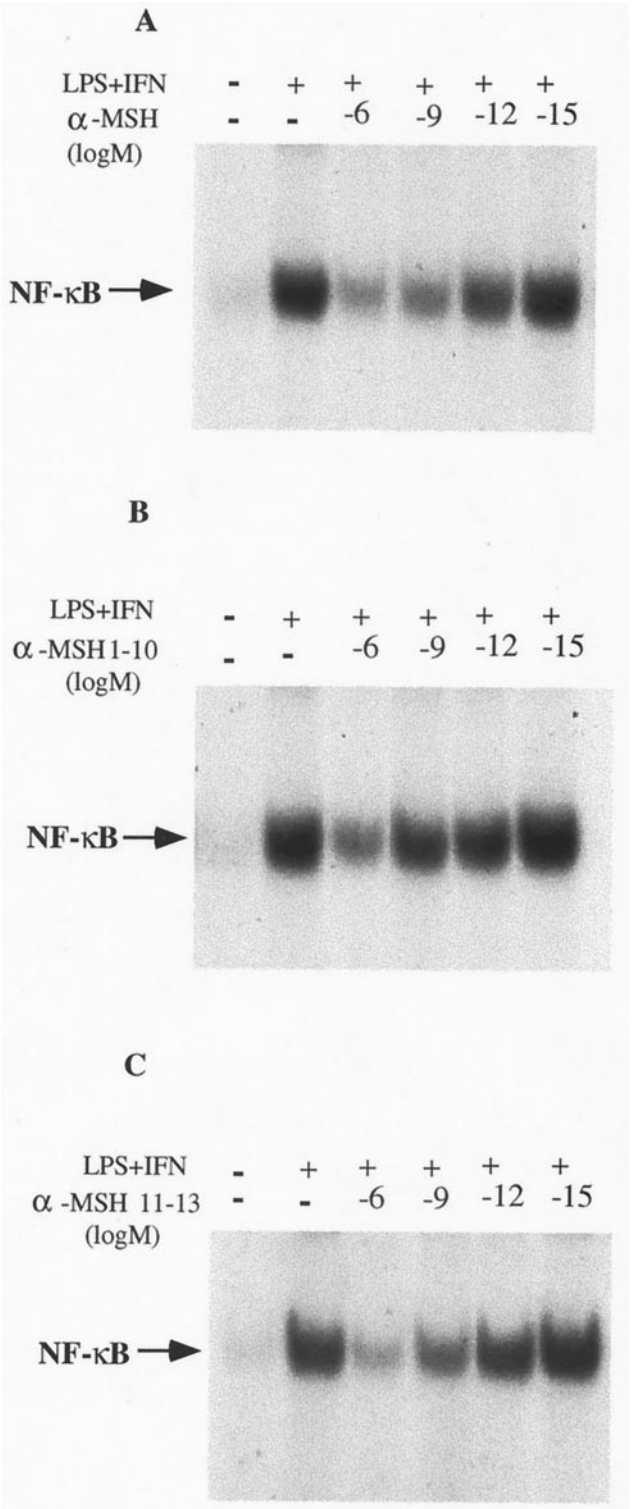

Fig. 2. Effects of melanocortin peptides on LPS/IFN-γ-induced NF-κB nuclear translocation. RAW 264.7 cells were treated for 2 hr with LPS (100 ng/mL) and IFN-γ (100 U/mL) in the presence or absence of the indicated concentrations of α-MSH (A), α-MSH(1–10) (B), and α-MSH(11–13) (C). At the end of the incubation, nuclear extracts were prepared and tested for NF-κB binding activity by EMSA as described under Materials and Methods. The graph shows one representative experiment out of three for each set of treatments with similar results.

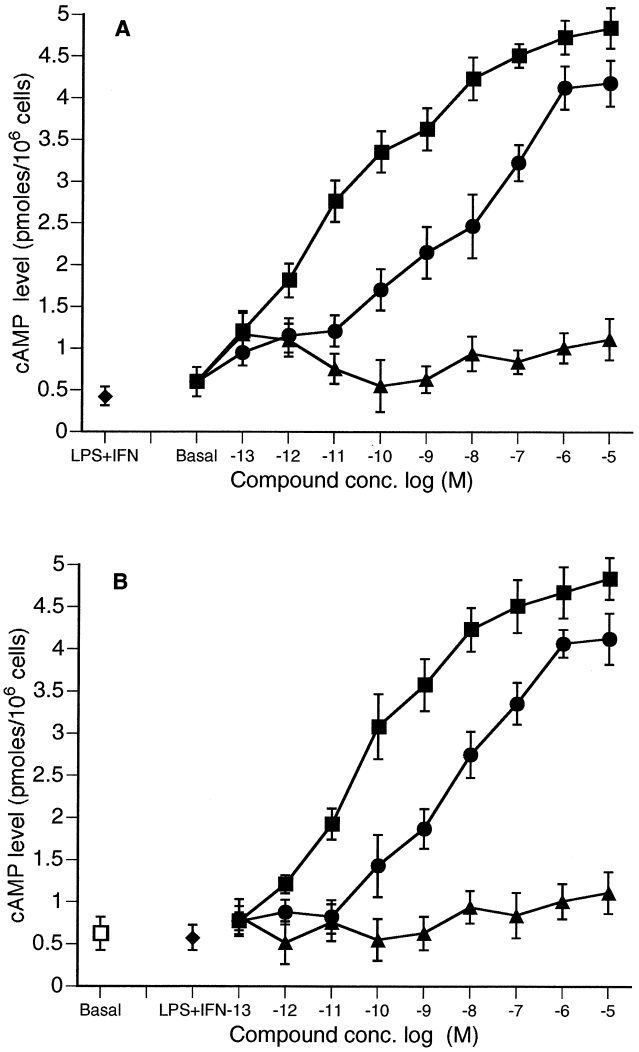

Fig. 3. Generation of cAMP in response to melanocortin peptides. RAW 264.7 cells were incubated for 20 min with various concentrations of α-MSH (■), α-MSH(1–10) (●), or α-MSH(11–13) (▲) in the absence (A) or presence (B) of LPS (100 ng/mL) and IFN-γ (5 U/mL). Each data point represents the mean ± SEM of three independent experiments, each performed with duplicate determinations.

and α-MSH(1–10) caused a dose-dependent increase in cAMP that maximally amounted to about 9-fold, the EC_{50} of α-MSH being 12 ± 1 pM and that of α-MSH(1–10) 21 ± 3 nM. By contrast, α-MSH(11–13), in the concentration range 10^{-13}–10^{-5} M, was totally devoid of effect on cAMP (Fig. 3A).

As can be seen from Fig. 3B, a 20-min treatment of the cells with LPS/IFN-γ (100 ng/mL and 5 U/mL, respectively) did not cause any effect on cAMP. Moreover, the ability of α-MSH and α-MSH(1–10) to stimulate cAMP in LPS/IFN-γ-challenged cells during the 20-min incubation (Fig. 3B) was essentially identical to that seen in LPS/IFN-γ non-challenged cells (Fig. 3A); the EC_{50} of α-MSH was 44 ± 2 pM and that of α-MSH(1–10) 5.6 ± 0.9 nM in the LPS/IFN-γ challenged cells. Moreover, also in the LPS/IFN-γ-treated cells, α-MSH(11–13) was completely devoid of effect on cAMP (Fig. 3B).

We also assessed the ability of forskolin to stimulate cAMP. A 20-min treatment with 5 or 50 μM forskolin caused about 7- and 10-fold stimulation of cAMP over the basal, respectively (data not shown).

3.4. Demonstration of MC_1 receptor binding to RAW 264.7 macrophages

In order to investigate if MSH receptor-binding sites were present on the RAW 264.7 macrophages, we applied ^{125}I-NDP-MSH radioligand binding. Saturation curves revealed that the cells possessed a high-affinity saturable ^{125}I-NDP-MSH binding site (K_d 0.17 ± 0.01 nM, B_{max} 13 ± 2 fmol/mg protein) (Fig. 4A). Competition studies using a fixed concentration of ^{125}I-NDP-MSH (0.2 nM) revealed that α-MSH and α-MSH(1–10) competed with the radioligand, with K_is of 0.87 ± 0.19 and 19 ± 1 nM, respectively. However, α-MSH(11–13), even in concentrations as high as 1 mM, was totally unable to compete for ^{125}I-NDP-MSH binding (Fig. 4B).

3.5. Effect of the the PKA inhibitor H89 on MSH peptide and forskolin effects on NO production

The eventual involvement of cAMP and PKA in the effects on NO production were investigated by stimulating cells with LPS/IFN-γ in the absence and presence of the PKA inhibitor H89, and treating with MSH peptides or forskolin. Cells were first pretreated with 20 μM H89 for 1 hr, whereafter LPS/IFN-γ, forskolin, or MSH peptides were added and the incubations continued for an additional 16 hr. Cells preincubated for 1 hr with vehicle served as controls. LPS/IFN-γ (100 ng/mL and 5 U/mL, respectively) induced the expected increase in nitrite accumulation (Fig. 5). This increase in nitrite accumulation was slightly, but significantly ($P < 0.05$), inhibited by H89 (Fig. 5). α-MSH (1 nM or 1 μM) caused the expected inhibition of LPS/IFN-γ-stimulated nitrite accumulation, and this inhibition by α-MSH was significantly ($P < 0.05$) counteracted by H89 for both concentrations of α-MSH tested. In addition, α-MSH(1–10) (1 nM or 1 μM) caused the expected inhibition of nitrite accumulation, the efficacy of α-MSH(1–10) being less than that of α-MSH, again as expected (Fig. 5). Moreover, the inhibitory effect of α-MSH(1–10) was counteracted by H89, although a significant ($P < 0.05$) effect by H89 was only seen at the higher dose of α-MSH(1–10), where a substantial inhibitory effect of the peptide on nitrite accumulation had also been observed (Fig. 5). MSH(11–13) (1 nM or 1 μM) also caused the expected inhibition of nitrite accumulation, and the inhibitory effect was significantly ($P < 0.05$) counteracted by H89, at both concentrations of the MSH(11–13) peptide tested (Fig. 5). Finally, forskolin (5 and 50 μM) caused a large dose-dependent inhibition of the LPS/IFN-γ-stimulated accumulation of ni-

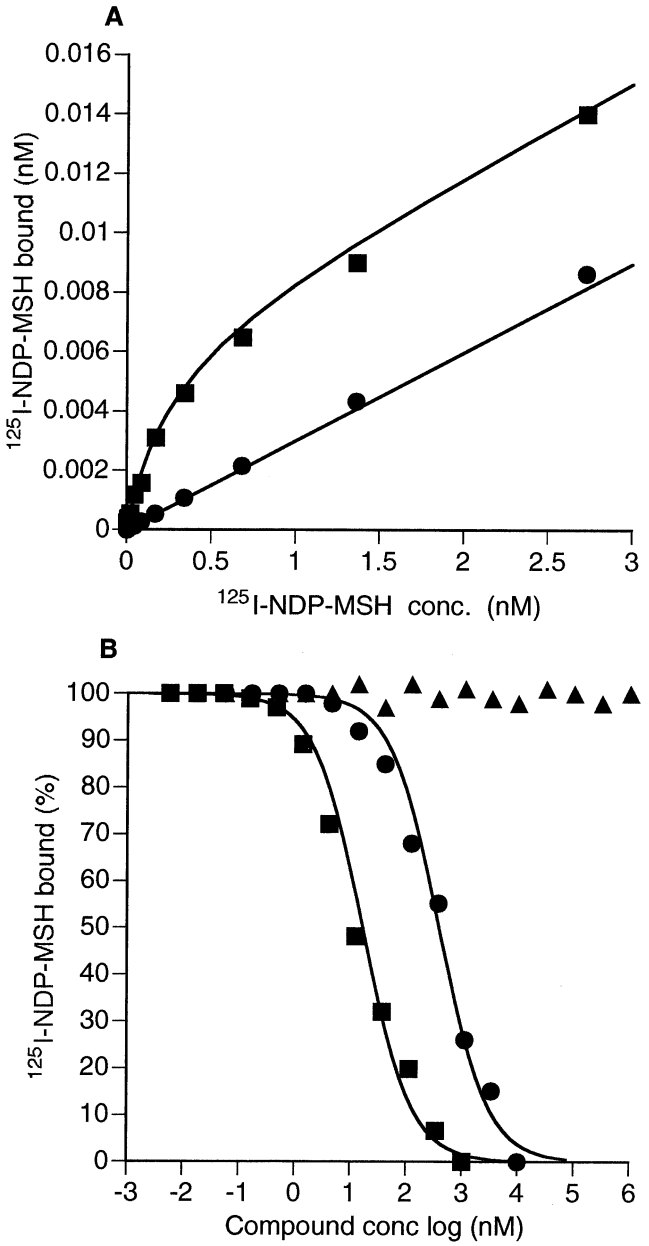

Fig. 4. ^{125}I-NDP-MSH radioligand binding in RAW 264.7 cells. Panel a shows a saturation curve for ^{125}I-NDP-MSH, the total ^{125}I-NDP-MSH binding being indicated by ■ and the non-specific binding (i.e. binding in the presence of 3 μM non-labeled NDP-MSH by ●). Panel b shows competition curves of α-MSH (■), α-MSH(1–10) (●), and α-MSH(11–13) (▲) using 0.2 nM ^{125}I-NDP-MSH. Solid lines represent the computer-drawn best fit of the data assuming that ligands bound reversibly to one site according to the law of mass action.

trite (Fig. 5). This inhibitory effect of forskolin was also counteracted by H89 (Fig. 5).

3.6. Effect of forskolin and H89 on nuclear translocation of NF-κB

The effects of forskolin and H89 on NF-κB translocation are shown in Fig. 6. Forskolin (50 μM) did not appreciably

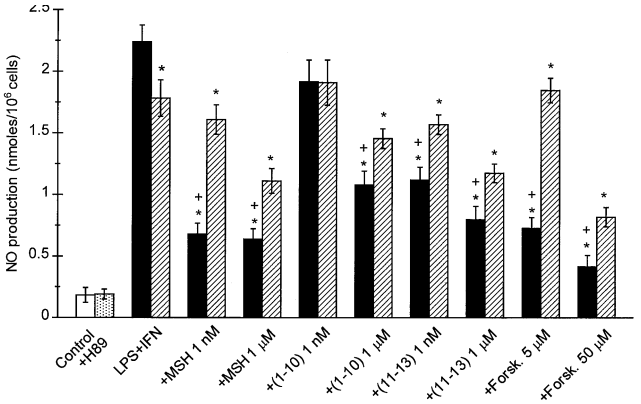

Fig. 5. Effect of the PKA inhibitor H89 on LPS/IFN-γ-induced NO production and on the effects of melanocortin peptides and forskolin. RAW 264.7 cells were pretreated with or without H89 (20 μM) for 1 hr and then activated with LPS (100 ng/mL) and IFN-γ (5 U/mL), with or without α-MSH, α-MSH(1–10), α-MSH(11–13), or forskolin for 16 hr, after which the accumulation of nitrite was assessed. Cells treated with LPS/IFN-γ with or without the indicated addition of peptide or forskolin are indicated by black bars. Cells treated with LPS/IFN-γ and H89, with or without the indicated addition of peptide or forskolin, are indicated by obliquely striped bars. Controls represent cells incubated in the absence of LPS/IFN-γ, with or without H89 as indicated. All values represent means ± SEM of three independent experiments performed in triplicate. Key: * $P < 0.05$ compared with LPS/IFN-γ treatment; $^{+}$ $P < 0.05$ compared with 1-hr pretreatment with H89.

modify LPS/IFN-γ-stimulated NF-κB translocation. Moreover, 20 μM H89 did not appear to affect the inhibition of NF-κB nuclear translocation caused by 1 nM α-MSH (Fig. 6).

3.7. Effect of MSH peptides and H89 on NF-κB-dependent reporter activity

After transient transfection of the RAW 264.7 cells with an NF-κB-dependent luciferase reporter plasmid, LPS/

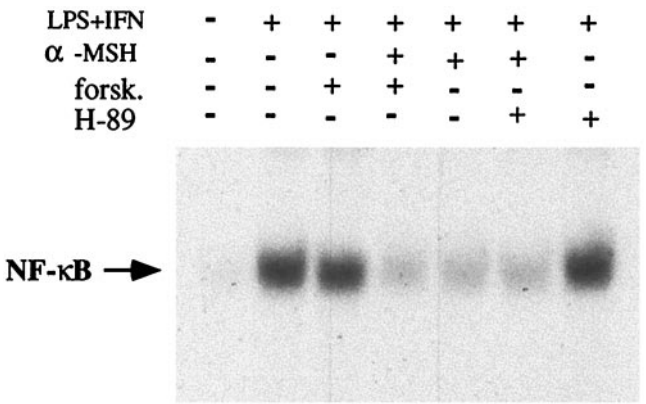

Fig. 6. Effect of H89 and forskolin on the α-MSH inhibition of NF-κB nuclear translocation. Shown is the effect of 2-hr stimulation with LPS/IFN-γ (100 ng/mL; 100 U/mL) with or without α-MSH (1 nM), H89 (20 μM), and forskolin (50 μM). H89 treatments commenced 1 hr before addition of LPS/IFN-γ and α-MSH. The picture shows one representative experiment out of three with similar results.

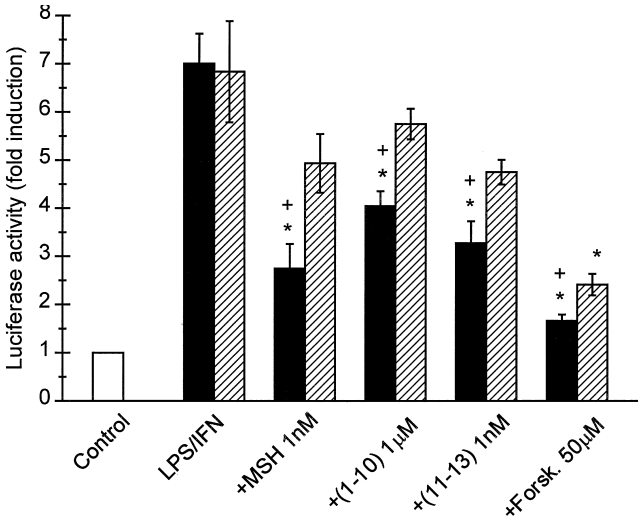

Fig. 7. Effects of MSH peptides on NF-κB-dependent luciferase reporter activity. RAW 264.7 cells were transfected with the reporter and incubated for 1 hr with or without 20 μM H89, and then activated with LPS (100 ng/mL) and IFN-γ (100 U/mL) in the absence or presence of α-MSH, α-MSH(1–10), α-MSH(11–13), or forskolin for 4 hr, after which the luciferase activity was determined. Black bars represent cells treated with LPS/IFN-γ with or without the indicated addition of peptide or forskolin. Obliquely striped bars represent the corresponding cells pretreated for 1 hr with H89. The white bar indicates non-treated control cells. All values represent means ± SEM of three independent experiments performed in triplicate. Fold induction is relative to luciferase activity/mg protein of transfected unstimulated cells. Key: *, $P < 0.05$ compared with LPS/IFN-γ treatment; $^{+}$ $P < 0.05$ compared with 1-hr pretreatment with H89.

IFN-γ (100 ng/mL and 100 U/mL, respectively) induced an almost 6-fold stimulation of the luciferase activity over the baseline (Fig. 7). As seen in the figure, α-MSH (1 nM), α-MSH(1–10) (1 μM), α-MSH(11–13) (1 nM), and forskolin (50 μM) all significantly inhibited LPS/IFN-γ-induced luciferase activity. Moreover, 1-hr preincubation with H89 (20 μM) partially reversed the inhibitory effects of all three peptides and forskolin (Fig. 7).

4. Discussion

The present study provides evidence that melanocortins cause their actions in a mouse macrophage cell line via at least two different pathways. All three tested melanocortin peptides, α-MSH, α-MSH(1–10), and α-MSH(11–13), inhibited NO production and translocation of NF-κB into the nucleus. However, only α-MSH and α-MSH(1–10) stimulated cAMP in the macrophage cells, while α-MSH(11–13) was devoid of cAMP stimulatory effect. Since forskolin was also able to cause an inhibition of NO production without affecting the translocation of NF-κB to the nucleus, it is conceivable that elevation of cAMP may cause inhibition of NO independently of NF-κB. The finding that the α-MSH-mediated inhibition of NF-κB translocation was not affected by the PKA inhibitor H89 supports the idea that translocation of NF-κB is a cAMP-independent process. Moreover, the finding that α-MSH(11–13) inhibited NF-κB translocation without affecting cAMP supports this idea.

The present ^{125}I-NDP-MSH binding results are fully compatible with the notion that the mouse macrophage cell line contains MC_1 receptors. Thus, the high affinities of ^{125}I-NDP-MSH and α-MSH for the RAW 264.7 cells correspond exactly to those expected for an MC_1 receptor. Substantially lower binding affinities would have been seen if this binding site had been any one of the MC_{3-5} receptors [35]. Moreover, Star *et al*. [17] have shown by use of polymerase chain reaction that MC_1 receptor mRNA is present in RAW 264.7 cells.

It is well known that the core sequence His-Phe-Arg-Trp in the MSH is essential for binding to MC receptors [36]. The combined results of the present study, showing that α-MSH and α-MSH(1–10) (both of which share the core sequence) bind to a high-affinity binding site and elevate cAMP, are fully compatible with the idea that both these peptides stimulate MC_1 receptors in the mouse macrophages. Moreover, the observation that α-MSH(11–13) (which lacks the core sequence) neither binds to the ^{125}I-NDP-MSH binding sites nor elevates cAMP in these cells indicates that α-MSH(11–13) is not capable of activating MC_1 receptors in macrophage cells. These findings are supported by similar observations in a melanoma cell line, where α-MSH(11–13) was found not to compete for ^{125}I-NDP-MSH binding to the MC_1 receptors on these cells [37]. Moreover, we have shown that α-MSH(11–13) is totally unable to compete for ^{125}I-NDP-MSH binding to human MC_1, MC_3, MC_4, and MC_5 receptors.[1] Ichiyama *et al*. [38] also recently showed by use of EMSA that intracerebroventricular administration of α-MSH(11–13) was as effective in inhibiting NF-κB binding to brain extracts in a mouse strain with genetically defective MC_1 receptors as it was in normal mice. Thus, these data lend further support for an action of α-MSH(11–13) being mediated by a mechanism distinct from the MC_1 receptor.

The mechanism for NF-κB activation of nuclear transcription is a complex process. Strong evidence suggests that PKA_c forms a complex with IκB/NF-κB [30]. During the degradation of the IκB, the PKA_c unit becomes disinhibited and causes phosphorylation of the NF-κB. While this phosphorylation is suggested not to influence the translocation of NF-κB into the nucleus, it does seem to enhance the ability of NF-κB to promote nuclear gene transcription [30]. However, a direct negative control by cAMP on the transcriptional activity of NF-κB is also indicated, as cAMP/PKA activation is well known to decrease the expression of various NF-κB-dependent genes, such as those for tissue factor, endothelial leukocyte adhesion molecule-1, and tumor necrosis factor-α [39]. Moreover, cAMP elevation was found to decrease the transcriptional activity of an NF-κB-dependent reporter system when transfected into

[1] Muceniece R and Wikberg JE, unpublished data.

RAW 264.7 macrophages (present study). Whatever the exact mechanism involved in NO inhibition, the ability of the PKA inhibitor H89 to inhibit the effect of α-MSH, α-MSH(1–10), and forskolin would support the idea that a cAMP/PKA-mediated mechanism is involved. However, it is notable that the effect of α-MSH(11–13) on NO production was also partially reversed by H89. The reason why the PKA inhibitor had an effect even in the absence of elevated cAMP is not known. Perhaps PKA_c released from the NF-κB/IκB complex could play a role, but further studies are needed to settle the issue.

In the interpretation of the present data, it should also be borne in mind that activated macrophages produce a variety of cytokines such as IL-1, IL-6, and tumor necrosis factor-α, and that melanocortin peptides have been shown to suppress this production of cytokines [40,41]. Moreover, it has been shown that α-MSH, as well as α-MSH(11–13), induces the release of IL-10 in human monocytes [11]. IL-10 has been shown to inhibit LPS-stimulated production of inflammatory cytokines (IL-1, IL-6, TNF-α) by inhibiting NF-κB activity [42]. It has also been shown that IL-10 may inhibit IFN-γ-stimulated NO production [43]. It remains elusive whether or not the MSH peptides cause their actions indirectly by altering the production of cytokines.

In summary, we have here provided evidence that MSH peptides mediate their actions in macrophages via at least two different pathways. One pathway may be cAMP-independent and cause inhibition of nuclear translocation of the NF-κB; the other may be related to the activation of the MC_1 receptor and result in elevation of cellular cAMP.

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