α-Melanocyte-stimulating hormone induces cell death in mast cells: involvement of NF-κB

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Abstract Mast cells play a major role in the initiation of inflammation and allergic reactions. As cell numbers are tightly controlled by the interplay of factors affecting cell proliferation, development, and death the regulation of mast cell number may be important. Melanocyte-stimulating hormone inhibits most forms of inflammation by an unknown mechanism. In the present study, we have found that the α-melanocyte-stimulating hormone (α-MSH) inhibited endotoxin-mediated nuclear transcription factor κB (NF-κB) activation in different cells correlated with the expression of α-MSH receptors. We have also found for the first time that it induces cell death alone or in endotoxin-stimulated mast cells. α-MSH-mediated apoptosis was not observed in NF-κB overexpressed cells. The inhibitory effect of α-MSH was mediated through generation of cAMP, as inhibitors of adenylate cyclase and of protein kinase A reversed its inhibitory effect. Overall, our results suggest that NF-κB is the key molecule involved in α-MSH-mediated cell death and this may help to regulate mast cell-mediated inflammation.

Key words: α-MSH; LPS; NF-κB; Mast cell apoptosis

1. Introduction

Mast cells play a central role in inflammatory and immediate allergic reactions by virtue of their ability to release preformed mediators, newly synthesized mediators, as well as cytokines upon antigen activation thereby forming the genesis of inflammation [1]. As mast cells play a key role in inflammatory and immediate allergic reactions, the regulation of mast cell activity becomes important. Mast cells reside immediately beneath the thin epithelial lining of respiratory duct and when exposed to allergens produce multi-mediators for allergic responses. Mast cells are also found in the human heart [2], and have been implicated in cardiovascular diseases [3,4] as mast cell-derived mediators cause apoptosis of cardiac myocytes and proliferation of non-myocytes [5] leading to hypertrophied and failing hearts [6]. The transcription factors thought to be involved in asthma are nuclear transcription factor κB (NF-κB), AP-1, NF-AT, CREB, STATs, and GATA-3. Most of the inflammatory genes overexpressed in asthma, such as those encoding proinflammatory cytokines, chemokines, adhesion molecules, and inflammatory enzymes, contain κB sites for NF-κB within their promoter, suggesting that these genes are controlled predominantly by NF-κB [7]. Thus, regulation of NF-κB activation in mast cells appears to be very important. NF-κB's anti-apoptotic property has an immense importance in regulating mast cells' survival in allergic responses. The size of any cell population represents a balance between cell division and programmed cell death (apoptosis). In the case of mast cells, IL-3, IL-15, and stem cell factor (SCF)-3 produced in extravascular tissues appear to promote mast cell proliferation and maturation and prevent apoptosis [8]. It is therefore important to elucidate the role of NF-κB in these processes. Most of the inflammatory genes are overexpressed in asthma. NF-κB, a transcription factor, is required for maximal transcription of many proinflammatory molecules which are overexpressed during asthma. Because these molecules are regulated at the level of transcription and are involved in the inflammatory cascade, the regulation of NF-κB is important to regulate asthma and other allergic responses. NF-κB is an ideal target for apoptosis and the mast cell death involving regulation of NF-κB may prove to be a new strategy to regulate asthma.

Lipopolysaccharide (LPS), a glycolipid, is an integral component of the outer membrane of Gram-negative bacteria. LPS mediates a number of biological manifestations which are believed to result from an uncontrolled production of proinflammatory cytokines such as TNF, IL-1, IL-6, IL-8, IL-10, IL-12 etc. produced by cells. LPS interacts with most cells through CD14, a 55 kDa glycophosphatidylinositol-anchored protein expressed on the surface of monocytes, macrophages, and neutrophils [9,10]. The binding of LPS to CD14 is enhanced by the LPS binding protein (LBP) present in the serum [10,11]. Mice lacking the CD14 gene show resistance to LPS-induced shock [12]. In this study serum-activated LPS (SA-LPS) was used to induce mast cells.

Neuropeptides have been implicated in the regulation of a number of immune responses both in human and murine systems [13]. The α-melanocyte-stimulating hormone (α-MSH), a tridecapeptide derived from pro-opiomelanocortin and found in pituitary, brain, skin, and circulation [14] has been shown to interact with various cells of the immune system and down-regulate either the production or the action of the proinflammatory cytokines IL-1, TNF-α, and IL-6 [15–18], and thus

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Abbreviations: ddAdo, dideoxyadenosine; IκBα-DN, dominant negative IκBα (inhibitory of kappa B alpha); MTT, 3-(4,5-dimethyl-2-thiozolyl)-2,5-diphenyl-2H-tetrazolium bromide; α-MSH, α-melanocyte-stimulating hormone; MDA, malondialdehyde; NF-κB, nuclear transcription factor κB; SA-LPS, serum-activated lipopolysaccharide; SEAP, secretory alkaline phosphatase

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acts as an anti-inflammatory agent. Receptors for α-MSH have been detected in different cell types [16,17]. At the molecular level, how α-MSH regulates inflammation induced by different stimuli is not understood. As NF-κB is involved both in cell proliferation and inflammation, the regulation of this transcription factor will be helpful to regulate the mast cell number, thereby mast cell-mediated inflammation. In this study, we are providing the data for the first time that α-MSH induces cell death in mast cells, which may be of immense importance to regulate mast cell-mediated inflammatory and allergic responses in different mast cell-driven diseases.

2. Materials and methods

2.1. Materials

LPS, α-MSH, 3-(4,5-dimethyl-2-thiozolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and glycine were obtained from Sigma (St. Louis, MO, USA). Penicillin, streptomycin, Dulbecco's modified Eagle's medium (DMEM), WEHI-3 conditioned medium, and fetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY, USA). Adenosine cyclic 3′,5′-phosphorothiolate triethylammonium salt (Rp-cAMPS) and H-8 [(methylamino)ethyl-5-isoquinolinesulfonamide, HCl] were obtained from Calbiochem (San Diego, CA, USA). Dideoxyadenosine (ddAdo) and dibutyryl cAMP were obtained from LC Laboratory (San Diego, CA, USA). Dihydrorhodamine was purchased from Molecular Probe, The Netherlands. Antibodies (Abs) against p50, p65, and poly ADP-ribose polymerase (PARP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell lines

MC-9, HMC-1, Jurkat, H4, HuT-78, HeLa, U-937 cells, obtained from American Type Culture Collection (Manassas, VA, USA) were cultured according to their protocol. MC-9 cells were maintained in DMEM supplemented with 0.5 μM 2-ME, 10% FBS, and 5% conditioned medium (WEHI-3) containing IL-3 (30 ng/ml). Cells were mycoplasma free, as tested by Gen-probe mycoplasma rapid detection kit (Fisher Scientific, Pittsburgh, PA, USA). To obtain bone marrow-derived mast cells, 10-week-old mice bone marrow cells were harvested and cultured at a density of 10^6 cells/ml in DMEM supplemented with 100 μg/ml penicillin/streptomycin, 0.1 μM 2-ME, 10% FBS, and 10% WEHI-3 conditioned media containing IL-3. Culture flasks were incubated in CO_2 incubator. Half of the culture media was replaced every 7 days. Mast cell purity was assessed after 7 weeks of culture and about 95% cells were mast cells as assessed by metachromatic staining of cytopreparations with acidic toluidine blue, pH 1.0 [19].

2.3. Cytotoxicity assay

The cytotoxicity was measured by the MTT assay [20]. Briefly, MTT dye (100 μg in 25 μl PBS) was added to the treated cells (10^4 cells/well of a 96-well plate). After a 2 h incubation at 37°C, 0.1 ml of the extraction buffer (20% sodium dodecyl sulfate (SDS), 50% dimethylformamide) was added. After 12 h incubation at 37°C, the absorbance was read at 570 nm.

2.4. NF-κB activation assay

NF-κB activation was assayed from nuclear extract (NE) using 4 ng of ^{32}P end-labeled 45-mer double-stranded NF-κB oligonucleotide from the HIV-LTR, 5′-TTG TTA CAA GGG ACT TTC CGC TGG GGA CTT TCC AGG GAG GCG TGG-3′ by gel retardation assay [21].

2.5. Transfection with p65 and dominant negative IκBα (IκBα-DN)

MC-9 and HuT-78 cells were transiently transfected by the calcium phosphate method with 1 ml medium containing 0.5 μg plasmid DNAs for *p65* and *IκBα* mutants (*IκBα-DN*) lacking either Ser^{32} or Ser^{36} respectively with 0.5 μg NF-κB promoter DNA linked to the heat-stable secretory alkaline phosphatase (*SEAP*) gene [22]. The total amount of DNA was maintained at 3 μg by the addition of the control plasmid *pCMVFLAG1* DNA. After 12 h, cells were treated with α-MSH for 24 h for cytotoxicity assay or NF-κB activity assay by gel retardation. The culture-conditioned medium was used for SEAP activity assay essentially as per the CLONTECH protocol (Palo Alto, CA, USA).

2.6. Western blot of PARP

Cell death was examined by proteolytic cleavage of PARP [23]. Briefly, cells were treated with α-MSH and whole cell extracts were prepared. Cell extract protein (50 μg) was analyzed for PARP by Western blot analysis and detected by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.7. Determination of lipid peroxidation and measurement of reactive oxygen intermediates (ROI)

Lipid peroxidation was determined by detection of thiobarbituric acid-reactive malondialdehyde (MDA), an end product of the peroxidation of polyunsaturated fatty acids and related esters as described [24,25]. The production of ROI was determined by flow cytometry using dihydrorhodamine 123 as fluorescent probe as described [25].

2.8. Radiolabeling of α-MSH and receptor binding assay

α-MSH was iodinated with [^{125}I]Na by the IODO-GEN method. Radiolabeled ligand was purified by G25 sepharose column. The specific activity of radiolabeled ligand was 0.5×10^7 cpm/μg protein. Cell surface receptors for α-MSH were detected following the method as described previously [26]. The specific binding was detected by subtracting the counts obtained from 50-fold excess of unlabeled ligands from labeled binding in the absence of unlabeled ligands (total).

3. Results

In this study, we examined the effect of α-MSH on the activation of transcription factor NF-κB. We used murine mast cell line (MC-9) for these studies because these cells express sufficient α-MSH receptors.

3.1. α-MSH inhibits SA-LPS-induced NF-κB activation

To detect the role of α-MSH on endotoxin-induced NF-κB activation in murine mast cells (MC-9), cells were stimulated with SA-LPS (100 ng of LPS was incubated with 20 μl of murine serum for 1 h at 37°C and this mixture was SA-LPS) for 1 h at 37°C and then treated with different concentrations of α-MSH for 24 h at 37°C. NE was prepared and 8 μg NE proteins were analyzed in 6.6% native polyacrylamide gel electrophoresis (PAGE) to detect NF-κB by gel shift assay. The results shown in Fig. 1A indicate that α-MSH alone did not activate NF-κB, but SA-LPS-induced NF-κB activation was inhibited in a dose-dependent manner and at 10^4 pM concentration of α-MSH completely abrogated SA-LPS-induced NF-κB activation. From this results, it is clear that α-MSH alone does not alter NF-κB activation but inhibits SA-LPS-induced NF-κB activation. Various combinations of Rel/NF-κB proteins can constitute an active NF-κB heterodimer that binds to specific sequences in DNA. To show that the retarded band visualized by electrophoretic mobility shift assay (EMSA) in SA-LPS-induced cells was indeed NF-κB, we incubated the NEs from SA-LPS-activated cells with Abs p50 (NF-κBI) and p65 (Rel A) or in combination and then conducted EMSA. Abs to either subunit of NF-κB shifted the band to a higher m.w. (Fig. 1B), thus suggesting that the SA-LPS-activated complex consisted of p50 and p65 subunits. MC-9 cells were exposed to SA-LPS for 1 h, then treated with α-MSH for 0 to 36 h, and then NF-κB was assayed from NE. SA-LPS-induced NF-κB activation was inhibited maximally when the cells were treated for 24 h with α-MSH (Fig. 1C).

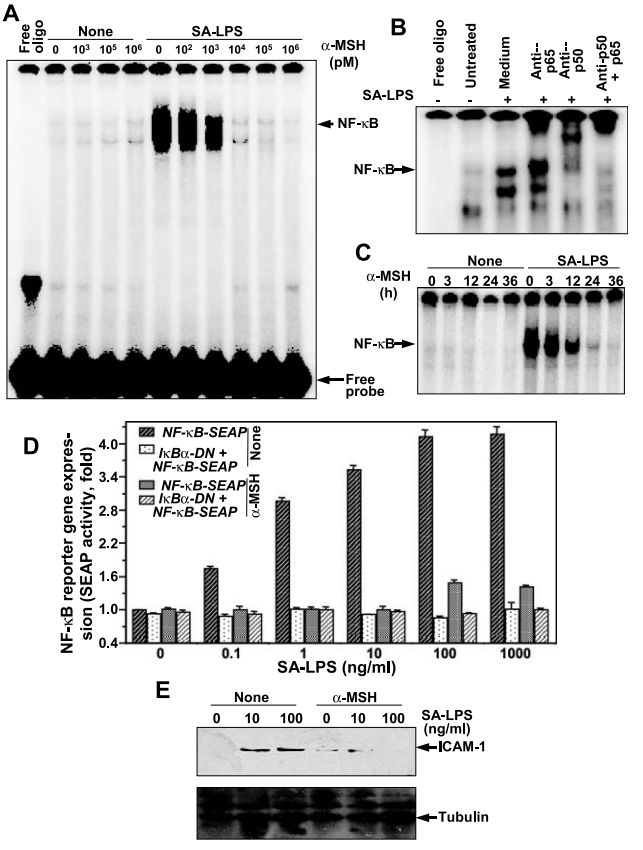

Fig. 1. A: Effect of α-MSH on SA-LPS-induced NF-κB activation. MC-9 cells (2×10^6/ml) were stimulated with SA-LPS (100 ng/ml) for 2 h and then treated with different concentrations of α-MSH for 24 h at 37°C, CO_2 incubator. NF-κB was assayed from 8 μg NE proteins B: Supershift of NF-κB band. SA-LPS-induced NE was incubated for 15 min with different Abs and then assayed for NF-κB. C: Optimum time of α-MSH treatment to downregulate SA-LPS-induced NF-κB activation. MC-9 cells, pretreated with or without SA-LPS were treated with α-MSH (10 nM) for 0–36 h and then NF-κB was assayed. D: Effect of α-MSH on SA-LPS-induced NF-κB-dependent reporter gene expression. MC-9 cells were transiently transfected with indicated plasmids along with NF-κB-containing plasmid linked to the SEAP gene. The cells were cultured for 12 h and then stimulated with different concentrations of SA-LPS for 4 h as shown in the figure. Cells were then treated with 10 nM α-MSH for another 24 h. Culture supernatant was taken and assayed for SEAP. E: Effect of α-MSH on SA-LPS-induced ICAM-1 induction. Cells were stimulated with different concentrations of SA-LPS for 2 h and then treated with α-MSH (10 nM) for 24 h at 37°C, CO_2 incubator. ICAM-1 was detected from cell extract proteins (200 μg) by Western blot analysis. Tubulin was detected by reprobing the same blot.

3.2. α-MSH inhibits SA-LPS-induced NF-κB reporter gene activation

As SA-LPS-induced NF-κB activation was blocked by α-MSH, the NF-κB-dependent gene expression also carried out. MC-9 cells were transfected with NF-κB reporter plasmid containing *SEAP* gene and/or *IκBα-DN* plasmid. Cells were stimulated with SA-LPS for 4 h and replaced with fresh medium. Cells were then treated with α-MSH for 24 h. Culture supernatant was collected and used to assay SEAP activity. The result in Fig. 1D indicated that SEAP activity was induced with increased concentrations of SA-LPS. α-MSH (10 nM) completely inhibited SEAP activity at any concentrations of SA-LPS. The IκBα-DN-transfected cells showed the basal activity of SEAP even by SA-LPS-mediated stimulation indicating the specificity of the assay.

3.3. α-MSH inhibits SA-LPS-induced ICAM-1 expression

As α-MSH inhibited SA-LPS-induced different biological responses, the adhesion molecule ICAM-1, a NF-κB-dependent gene product was detected in SA-LPS-stimulated MC-9 cells. Cells were stimulated with different concentrations of SA-LPS for 12 h and then treated with α-MSH (10 nM) for 24 h. Then 100 μg cell extract proteins were analyzed using 9% SDS–PAGE and ICAM-1 was detected by Western blot analysis. SA-LPS-induced ICAM-1 expression was shown in a dose-dependent manner but α-MSH-treated cells failed to induce ICAM-1 expression by varying concentrations of SA-LPS (Fig. 1E) suggesting α-MSH-mediated inhibition of SA-LPS-induced ICAM-1 expression in mast cells.

3.4. Inhibition of NF-κB activation by α-MSH is cell-type specific

As NF-κB activation pathways differ in many cell types

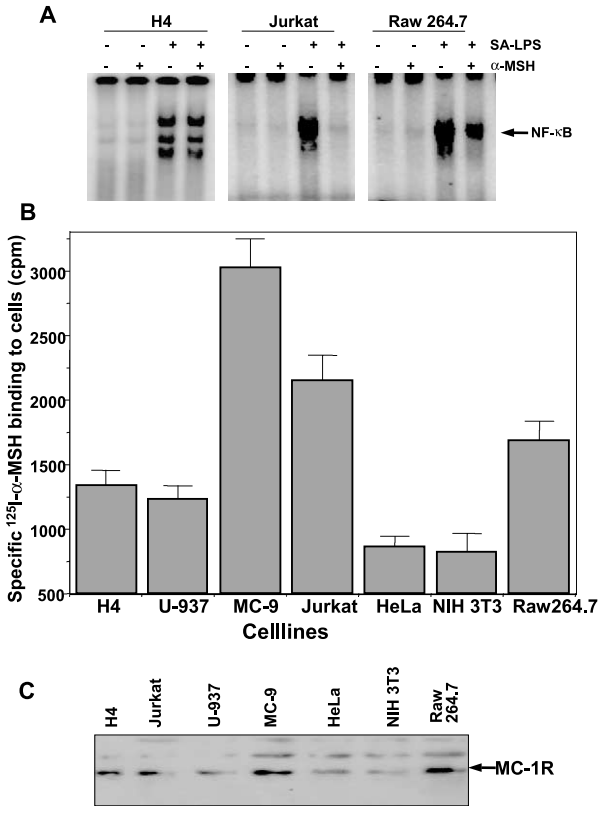

Fig. 2. A: Effect of α-MSH on SA-LPS-induced NF-κB activation in different cell lines. Human glioma (H4), T-cell (Jurkat), and mouse macrophage (Raw 264.7) cells were stimulated with SA-LPS for 2 h and then incubated with 10 nM α-MSH for 24 h, 37°C. After these treatments, NEs were prepared and then assayed for NF-κB. B: Levels of α-MSH receptors in different cells. U-937, MC-9, and Jurkat (1×10^6) cells were taken in tubes as triplicate sets. H4, HeLa, NIH 3T3, and Raw 264.7 cells were cultured in a 12-well plate as triplicate at 37°C CO_2 incubator. Cells were incubated with 4 ng iodinated α-MSH (5×10^4 cpm) per sample in presence or absence of 200 ng of unlabeled α-MSH for 2 h at 4°C. Then labeled α-MSH binding was assayed as described in Section 2. C: Level of MC-1R in different cell lines. Different cells' extract (200 μg) was analyzed in 9% SDS–PAGE and detected for MC-1R using antiMC-1R antibody.

[27–30], we therefore studied whether α-MSH affects human Jurkat (T) cells and H4 (glioma) cells and murine macrophage (Raw 264.7) cells as well. It has been demonstrated that distinct signal transduction pathways could mediate induction in epithelial, neuronal, and lymphoid cells. All the effects of α-MSH described above were conducted with MC-9 cells. We found that α-MSH blocks SA-LPS-induced NF-κB activation in Jurkat cells completely, Raw 264.7 cells partially, but not in H4 cells (Fig. 2A) suggest that this effect of α-MSH is restricted to T-cells but not glioma cells.

3.5. Levels of α-MSH receptors in different cells

To understand the specificity of α-MSH-mediated downregulation of NF-κB, we detected the level of expression of α-MSH receptors in different cells. H4, HeLa, and Raw 264.7 cells (1×10^6/well) were plated and incubated for overnight in a 12-well plate. MC-9, U937, and Jurkat cells (1×10^6/sample) were kept on ice in triplicate. Cells were incubated with 4 ng ^{125}I-labeled α-MSH (5×10^4 cpm) for 2 h at 4°C in presence or absence of 50-fold cold α-MSH and α-MSH binding was assayed. The results represented as mean specific binding in cpm ± S.D. of triplicate samples (Fig. 2B). The results indicate that the α-MSH receptors are expressed in MC-9, Jurkat, and Raw 264.7 cells which reflects the α-MSH-mediated downregulation of SA-LPS-induced NF-κB activation. α-MSH binds with its cell surface receptor specifically melanocortin-1 receptor (MC-1R). The levels of MC-1R were detected using 200 μg of different cells extract proteins by Western blot analysis (Fig. 2C).

3.6. α-MSH induces cell death in MC-9 cells

To investigate the effects of α-MSH on induction of cell death in MC-9 cells different parameters (cell viability, thymidine incorporation, lipid peroxidation, ROI generation, caspase 8, and PARP cleavage) were assayed.

3.6.1. α-MSH induces cytotoxicity and inhibits ^{3}H-thymidine incorporation. To detect α-MSH-mediated cell viability, MC-9 cells were stimulated with SA-LPS (100 ng/ml) for 2 h and then treated with different concentrations of α-MSH for 36 h. Then cell viability was assayed by MTT assay and cell proliferation was assayed by ^{3}H-thymidine incorporation by MC-9 cells. As shown in Fig. 3A, α-MSH induced cytotoxicity in a dose-dependent manner in MC-9 cells. SA-LPS stimulated cells showed about 20% increase in cell viability than unstimulated cells. SA-LPS was unable to protect α-MSH-mediated induction of cell death. Almost similar results showed for ^{3}H-thymidine incorporation by α-MSH treated cells (Fig. 3B). The results indicate that α-MSH alone causes cell death in MC-9 cells and SA-LPS has no role to protect α-MSH-mediated cell death. To show that the cell death mediated by α-MSH was not due to necrosis, the cytosolic marker enzyme lactate dehydrogenase (LDH) was assayed from the culture supernatant of α-MSH-treated cells [31]. Culture supernatant from 1 μM of α-MSH-treated for 0, 12, 24, and 36 h when incubated with substrate solution (0.23 M sodium pyruvate and 5 mM NADH in 0.1 M phosphate buffer, pH 7.5) did not decrease absorbance at 420 nm (data not shown) indicating cell death was not due to leakage of cytoplasm i.e. necrosis.

3.6.2. α-MSH induces lipid peroxidation in MC-9 cells. As lipid peroxidation is a marker of cell death, we examined the effect of α-MSH on lipid peroxidation in MC-9 cells through

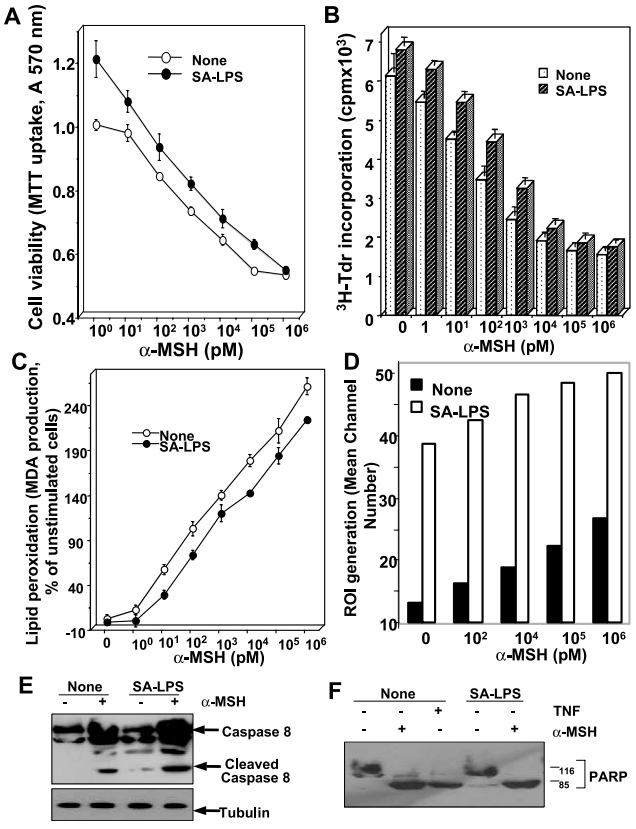

Fig. 3. A,B: Effect of α-MSH on cell viability in SA-LPS-induced MC-9 cells. MC-9 cells were stimulated with 100 ng/ml SA-LPS for 2 h and then 10^4 cells were taken in per well of a 96-well plate. Cells were then treated with different concentrations of α-MSH for 36 h and cell viability was assayed by MTT uptake (A) or cells were incubated with 0.5 μCi of ^{3}H-thymidine (Tdr) for last 18 h and then assayed for Tdr incorporation in the cells (B) as described in Section 2. C,D: Effect of α-MSH on lipid peroxidation and ROI generation in SA-LPS-induced MC-9 cells. Cell were stimulated with SA-LPS for 2 h and then treated with different concentrations of α-MSH for 24 h. Lipid peroxidation was assayed by measuring MDA as represented in percentage above unstimulated cells (C). ROI generation was assayed as the mean channel number by dihydrorhodamine in flow cytometer (D). E,F: Effect of α-MSH on caspase 8 and PARP cleavage in SA-LPS-stimulated cells. MC-9 cells were stimulated with SA-LPS for 2 h and then treated with α-MSH (10 nM) or TNF (1 nM) for 36 h. Then cell extract proteins were assayed for caspase 8 (E) and PARP (F) by Western blot.

the detection of levels of MDA production. As shown in cell viability, α-MSH-induced lipid peroxidation in a dose-dependent manner and SA-LPS did not protect α-MSH-mediated lipid peroxidation (Fig. 3C).

3.6.3. α-MSH induces ROI generation in MC-9 cells. ROI generation is an intermediate step in cell death-induced by different agents. To detect the role of α-MSH on SA-LPS-induced ROI generation, MC-9 cells were stimulated with SA-LPS for 2 h and then treated with different concentrations of α-MSH for 24 h. Then ROI generation was examined with dihydrorhodamine dye conversion to rhodamine as described in Section 2. α-MSH-mediated ROI generation was shown in a dose-dependent manner. SA-LPS-induced ROI generation was further increased with increased concentrations of α-MSH indicating its additive effect for ROI generation (Fig. 3D).

3.6.4. α-MSH induces caspase 8 and PARP cleavage in MC-9 cells. Cell death is reflected in caspases activation,

which cleave a lot of proteins including PARP. Caspase 8 activation was shown by its auto-cleavage of precursor caspase 8 into cleaved p20 caspase 8. α-MSH (10 nM) not only induced the cleaved p20 caspase 8 alone but also SA-LPS pre-stimulated cells as well (Fig. 3E). The level of precursor caspase 8 was increased by α-MSH treatment. The same blot was reprobed for tubulin protein and the intensity of bands in all these lanes were equal suggesting loading control. α-MSH (10 nM) or TNF (1 nM) at 36 h induced PARP cleavage individually as detected by Western blot (Fig. 3F). In SA-LPS pre-stimulated MC-9 cells, α-MSH induced PARP cleavage, indicating that SA-LPS was unable to protect α-MSH-mediated cell death.

3.7. α-MSH inhibits SA-LPS-induced NF-κB activation, induces cell death and PARP cleavage in human mast cells and mouse bone marrow-derived mast cells

As shown α-MSH-mediated inhibition of endotoxin-induced NF-κB activation in murine mast cells (MC-9), this effect was also tested in human mast cell line, HMC-1 and mouse bone marrow-derived mast, BM-mast, cells. Cells, stimulated with autologus SA-LPS (100 ng/ml) for 2 h at 37°C were treated with different concentrations of α-MSH for 24 h at 37°C and NF-κB was assayed from NEs. The results indicated that α-MSH alone did not activate NF-κB, but SA-LPS-induced NF-κB activation was inhibited in a dose-dependent manner both in HMC-1 (Fig. 4A1) and BM-mast (Fig. 4A2) cells. α-MSH alone induced cell death in HMC-1 and BM-mast cells as detected by MTT assay and SA-LPS was unable to protect α-MSH-mediated cell death (Fig. 4B1,B2). However, α-MSH was unable to show the same cell death in U-937, Jurkat, and another murine mast cell line P815 (data not shown). α-MSH (10 nM) induced PARP cleavage in a time-dependent manner both in HMC-1 and BM-mast cells (Fig. 4C) indicating the similar responses of α-MSH in human mast cell line and mouse bone marrow-derived mast cell as shown in MC-9 previously.

3.8. α-MSH inhibits NF-κB activation through cAMP generation

It has been reported that α-MSH transduces its signal through cAMP. To determine the role of cAMP, we used ddAdo, a potent inhibitor of adenylate cyclase, enzyme responsible for the generation of cAMP [32]. Cells were exposed to SA-LPS (100 ng/ml) for 2 h followed by ddAdo for 1 h and then treated with α-MSH (10^6 pM) for 24 h. Then NF-κB assayed from NEs. The results shown in Fig. 4D show that ddAdo did not interfere with SA-LPS-induced NF-κB activation, but it protected against α-MSH-mediated suppression of NF-κB stimulated by SA-LPS. Treatment of cells with exogenous cAMP (dibutyryl cAMP) inhibited SA-LPS-induced NF-κB activation. (Fig. 4E). Since cAMP is known to activate PKA, we also examined the effects of two specific PKA inhibitors, Rp-cAMPS isomer and H8 [33] and PKC inhibitor, H7 on the α-MSH-induced inhibition of NF-κB activation. For this, cells were stimulated with SA-LPS for 2 h followed by treatment with either Rp-cAMPS isomer (100 μM), H-8 (2 μM), or H7 (10 nM) for 1 h at 37°C and then treated with α-MSH for 24 h. NF-κB was assayed from NE. Pretreatment with both PKA inhibitors blocked the inhibitory effects of α-MSH, while PKC inhibitor H7 had no effect on α-MSH-mediated inhibition of NF-κB activation (Fig. 4E), suggesting

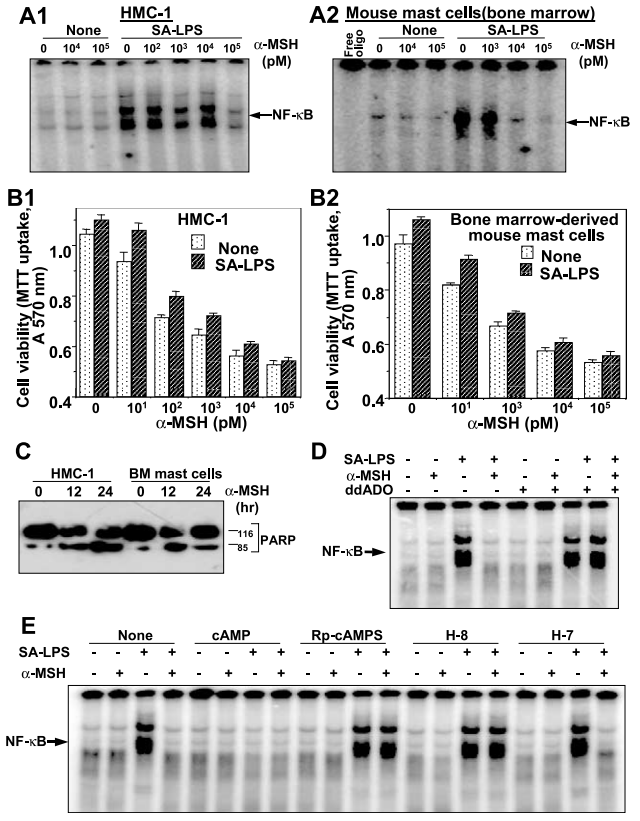

Fig. 4. A1,A2: Effect of α-MSH on SA-LPS-induced NF-κB activation in HMC-1 and bone marrow-derived primary mast cells. HMC-1 (A1) and BM-mast cells (A2) were stimulated with SA-LPS (100 ng/ml) for 2 h and then treated with different concentrations of α-MSH for 24 h at 37°C, CO_2 incubator. NF-κB was assayed from 8 μg NE proteins. B1,B2: Effect of α-MSH on cell viability in SA-LPS-induced HMC-1 and BM-mast cells. Cells (10^4/well) were stimulated with 100 ng/ml SA-LPS for 2 h and then treated with different concentrations of α-MSH for 36 h and cell viability was assayed by MTT uptake. C: Effect of α-MSH on PARP cleavage in SA-LPS-stimulated HMC-1 and BM-mast cells. Cells were stimulated with SA-LPS for 2 h and then treated with α-MSH (10 nM) for 36 h. Then cell extract proteins were assayed for PARP by Western blot. D: ddAdo protects from α-MSH-mediated inhibition of NF-κB stimulated by SA-LPS. MC-9 cells (2×10^6/ml) were incubated with SA-LPS for 2 h followed by ddAdo (250 μM) for 1 h and then treated with α-MSH for 24 h. NF-κB was assayed from NEs. E: The PKA inhibitor, cAMP-RP isomer and H8 protects α-MSH-mediated inhibition of NF-κB induced by SA-LPS. MC-9 cells, stimulated with SA-LPS for 2 h, were treated with dibutyryl cAMP (50 μM), Rp-cAMPS isomer (100 μM), H-8 (2 μM), or H7 (10 nM) for 1 h at 37°C. Cells were then treated with α-MSH (10 nM) for 24 h. NEs were prepared and analyzed by EMSA.

that the effect of the PKA to inhibit α-MSH-mediated action through generation of cAMP. As downregulation of NF-κB reflects the induction of cell death, the cAMP-mediated induction of cell death was observed in MC-9 cells and PKA inhibitors, Rp-cAMPS isomer or H-8, but not by PKC inhibitor H7 protected α-MSH-mediated cell death as detected by MTT assay (data not shown). These results suggest that α-MSH-mediated cell death occurs through cAMP production followed by downregulation of NF-κB in MC-9 cells.

3.9. Upregulated NF-κB protects α-MSH-mediated cell death in MC-9 cells

In order to detect the role of NF-κB on α-MSH-mediated cell death MC-9 cells were co-transfected with p65 plasmid

and SEAP reporter DNA. As shown in Fig. 5A1, p65-transfected MC-9 cells showed NF-κB activation and α-MSH treatment did not downregulate NF-κB. The SEAP activity was observed in p65-transfected MC-9 cells about five-fold. α-MSH did not downregulate the SEAP activity similar to NF-κB activation (Fig. 5A2). Non-transfected and p65-transfected cells were incubated with different concentrations of α-MSH for 36 h and the cytotoxicity was assayed by MTT uptake. The cell viability was decreased with increased concentrations of α-MSH in non-transfected MC-9 cells but p65-transfected cells showed about 10% decrease of cell viability (Fig. 5A3) indicating that NF-κB overexpressed cells are resistant to α-MSH-mediated apoptosis.

3.10. Downregulation of NF-κB by IκBα-DN does not induce cytotoxicity in HuT-78 cells

To confirm the role of NF-κB on α-MSH-mediated cell death, HuT-78 cells (constitutively activated with NF-κB) were transfected with IκBα-DN construct (mutation on Ser^{32} and Ser^{36} position) and then these transfected and non-transfected cells were treated with α-MSH for 24 h. NF-κB was assayed from NEs. As shown in Fig. 5B1, α-MSH-mediated downregulation of NF-κB (high activity of basal level) was not observed. Fifty-fold cold oligo suppressed the band indicating its specificity. In IκBα-DN-transfected cells, no NF-κB activity was observed. The SEAP activity from culture supernatant showed that the high basal level (four-fold) was not decreased by α-MSH. IκBα-DN-transfected cells did not show much SEAP activity (Fig. 5B2). In order to detect the role of NF-κB on α-MSH-mediated cell death, HuT-78 cells non-transfected and transfected with DN-IκBα were treated with different concentrations of α-MSH for 24 h and then cell viability was assayed by MTT uptake. HuT-78 cells showed 10–20% decrease in cell viability but DN-IκBα-transfected cells showed cell death in a dose-dependent manner by α-MSH (Fig. 5B3), indicating involvement of NF-κB in α-MSH-mediated cell death.

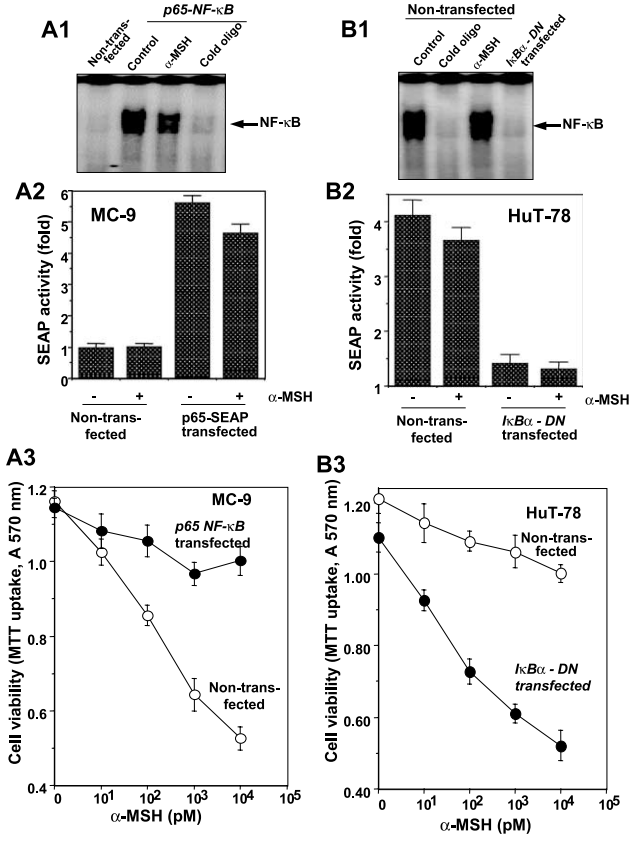

Fig. 5. A: Overexpressed NF-κB protects α-MSH-mediated inhibition of NF-κB and apoptosis in MC-9 cells. MC-9 cells were transfected with p65 linked with SEAP construct, cultured for 12 h, and then treated with α-MSH for 24 h. The NF-κB assayed from NEs using 50-fold cold oligo for specificity (A1) and SEAP assayed from cultured conditioned medium (A2). Transfected and non-transfected MC-9 cells were treated with different concentrations of α-MSH and assayed for cytotoxicity by MTT uptake (A3). B: Effect α-MSH on HuT-78 cells transfected with IκBα-DN construct. HuT-78 cells were transfected with IκBα-DN construct and culture for 12 h. Then cells were treated with α-MSH for 24 h. NF-κB was assayed from NEs (B1) and SEAP assayed from cultured conditioned medium (B2). Non-transfected and IκBα-DN-transfected HuT-78 cells were treated with different concentrations of α-MSH for 36 h and cell viability was assayed (B3). The results represent the mean absorbance ± S.D. of triplicate samples.

4. Discussion

Even though several studies indicate that certain neuropeptides, such as α-MSH, have anti-inflammatory effects, the mechanism underlying this effect is not understood. Mast cells are quick responder in allergic and inflammatory diseases. As inflammatory responses are aggravated by NF-κB activation followed by NF-κB-dependent gene activation, our strategy was to regulate this transcription factor. Surprisingly, we observed that α-MSH not only downregulates NF-κB in mast cells, but also induces cell death. In this report we observed that endotoxin induced NF-κB and NF-κB-dependent reporter gene activation was inhibited by α-MSH (Fig. 1A,D,E). α-MSH binds predominantly with its receptor MC-1R. The level of α-MSH receptors as detected by radiolabeled α-MSH binding (Fig. 2B) and Western blot (Fig. 2C) reflects the α-MSH-mediated NF-κB activation. The viable cell number as detected by MTT dye uptake (Fig. 3A) and proliferating cell number by thymidine incorporation (Fig. 3B) suggesting that the cell death occurred due to α-MSH treatment. Lipid peroxidation (Fig. 3C), ROI generation (Fig. 3D), precursor caspase 8 cleavage (Fig. 3E), and caspase-dependent PARP protein cleavage (Fig. 3F) all supported cell death. α-MSH-mediated cell death was not due to necrosis as LDH, marker for cytosol level was not observed in α-MSH-treated cells. ROI has shown to induce NF-κB and activator protein-1 activation. In combination of α-MSH and SA-LPS showed additive effect on ROI generation, though SA-LPS (100 ng/ml) alone induced about two-fold ROI generation than α-MSH (1 μM). SA-LPS has been reported to maintain NF-κB activation for a long time [20], which may be due to ROI generated by it. However, an additional generation of ROI by α-MSH may be detrimental to the cells, which needs to be studied further. α-MSH-mediated biological activities were observed not only in murine mast cell line but also in human cell line and mouse bone marrow-derived primary mast cells. However, why α-MSH did not show cell death in another murine mast cell line or human lymphoid cells is not understood. The reports so far available were that α-MSH downregulates TNF-induced NF-κB activation through generation of cAMP and activation of PKA [34]. How generated cAMP inhibits NF-κB activation was also investigated. It was

also shown that the catalytic subunit of PKA (PKAc) associates with IκBα, the inhibitory subunit of NF-κB in the cytoplasm [35]. Upon NF-κB activation by SA-LPS stimulation a lot of genes including IκBα may be upregulated. This IκBα possibly binds with PKAc forming PKAc-IκBα-p65 (NF-κB) complex thereby downregulating NF-κB. How cAMP downregulates NF-κB is interesting though the previous report confirms this effect and ddAdo, adenylate cyclase inhibitor reverses the function [34]. cAMP induces transcription factors like AP-1 and CREB, but not NF-κB [36]. NF-κB may be negatively downregulated by CREB. H7, an inhibitor of PKC, had no effect on the suppressive effect of α-MSH, indicating specificity.

Downregulation of NF-κB reflects on cell death as shown in α-MSH-treated cells. In unstimulated or SA-LPS-stimulated mast cells α-MSH induced cell death as shown by different assays. Surprisingly, α-MSH did not downregulate NF-κB in mast cells, overexpressed with p65 (NF-κB) or HuT-78, constitutively activated with NF-κB and showed 10–20% induction of cell death (Fig. 5). Mechanism of endotoxin-mediated NF-κB activation may be different than constitutively expressed NF-κB in HuT-78 cells or overexpressed with p65 (NF-κB) in MC-9 cells. From these results it is clear that α-MSH-mediated induction of cell death in mast cells depends on NF-κB.

The anti-inflammatory response of α-MSH is mediated by downregulation of key inflammatory molecule, NF-κB. The cells with overexpressed NF-κB show anti-apoptotic response [20]. In this report, we showed endotoxin induced NF-κB in mast cell MC-9 and then tested the downregulation of NF-κB by α-MSH through the production of cAMP. This downregulation of NF-κB reflected on cell death. As mast cells are key responder of allergic and inflammatory responses in asthma, gout arthritis, rheumatoid arthritis etc. and basal activation of NF-κB has been shown in these diseases, so induction of cell death by α-MSH in mast cells by downregulating NF-κB will be helpful to regulate cell number in these diseases.

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