C-terminal amino acids of alpha-melanocyte stimulating hormone are requisite for its antibacterial activity against *Staphylococcus aureus*

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Running title: α-MSH and Staphylocidal activity

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Keywords: alpha-MSH, *Staphylococcus aureus*, Antimicrobial peptides, Mechanisms of action, Membrane permeabilization, Electron microscopy.
ABSTRACT

Alpha-melanocyte stimulating hormone (α-MSH) is an endogenous neuropeptide, known for its anti-inflammatory and antipyretic activity. We recently demonstrated that α-MSH possesses staphylocidal activity and causes bacterial membrane damage. To understand the role of its amino acid sequences in staphylocidal mechanism, in the present study we investigated the antimicrobial activity of different fragments of α-MSH i.e., α-MSH(6-13), α-MSH(11-13) and α-MSH(1-5) and compared them with the entire peptide. Our results suggested that peptides containing the C-terminal of α-MSH, namely, α-MSH(6-13) and α-MSH(11-13) efficiently killed >90% of both methicillin sensitive and resistant *Staphylococcus aureus* cells in the micromolar range and ~50% of these cells in the nanomolar range; their efficiency was comparable to the entire α-MSH, whereas the peptide containing the N-terminal region, α-MSH(1-5), was found to be ineffective against *S. aureus*. The antimicrobial activity of α-MSH and its C-terminal fragments was not affected by the presence of NaCl or even divalent cations like Ca**+** and Mg**+**. Similar to the parent peptide, α-MSH(6-13), α-MSH(11-13) also depolarized and permeabilized *Staphylococcus* cells (~70-80% cells were depolarized and lysed after 2 h peptide exposure in micromolar concentration). Furthermore, scanning and transmission electron microscopy showed remarkable morphological and ultrastructural changes on *S. aureus* cell surface due to exposure of α-MSH based peptides. Thus, our observations indicate that C-terminal fragments of α-MSH retain the antimicrobial activity of entire peptide and their mechanism of action is similar to that of full-length peptide. These observations are important and critical in rational designing of α-MSH based therapeutics with optimal efficacy.
INTRODUCTION

The increasing drug resistance among bacterial pathogens such as *Staphylococcus aureus* (*S. aureus*) has created a need for the development of new antimicrobial agents with novel mechanisms of action. Therefore, scientists from all over the world have been trying to solve this serious problem by applying different approaches.

One such approach is the use of natural host defense antimicrobial peptides which are abundant in nature and which have been conserved across evolution as effective defense tools (45). These peptides are typically produced by immune cells, barrier cells, neutrophils and epithelial cells (17,20,41) and in the host body they act by modulating the innate and adaptive immune responses (21). Despite considerable variation in characteristics such as size, structural motifs and presence of disulphide bonds, generally host defense antimicrobial peptides are cationic and amphipathic in nature (16). Though different peptides kill pathogens by different mechanisms but their cationic and amphipathic nature allows them to interact with negatively charged microbial membranes (3,6,11,26). Microbial membrane interaction with such peptides leads to several events such as, 1) formation of multimeric pores in the lipid bilayer of the cell membrane (11,44); 2) loss of ionic balance (7); 3) interaction with DNA or RNA (4,5,30,37), all sequences eventually resulting in cell death. Their broad spectrum activity, unique mode of action and the fact that resistance is very unlikely to develop against host defense antimicrobial peptides makes them promising candidates for a new class of antibiotics (35,45).

Bacterial infections are most often accompanied by inflammation and fever and therefore intrusion of a peptide, which combines antimicrobial and anti-inflammatory properties, could be of high benefit. One such antimicrobial peptide is alpha-melanocyte stimulating hormone (*α*-MSH), an ancient linear tridecapeptide, well recognized for its
endogenous anti-inflammatory and melanogenic properties (8,15,29,36). The peptide is a cleavage product of pro-opiomelanocortic precursor (POMC), which expresses in pituitary gland and in other cells like neutrophils, monocytes, melanocytes, fibroblasts and keratinocytes (9,23,24). The sequence of this neuropeptide is: Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH₂. It controls inflammation and high temperature through immuno-modulation, down regulates several proinflammatory cytokines and stimulates production of anti-inflammatory cytokines such as Interleukin-10 (IL-10) (2,14,23). The widespread distribution of this peptide and its receptor in many barrier cells like keratinocytes and in various immune cells, suggests its potential role in host defense. α-MSH also shares a number of similarities with other natural host defense antimicrobial peptides. For example, (i) it is cationic in nature, ii) it adopts a helical structure in membrane mimetic environment (32), (iii) it causes membrane permeabilization like known antimicrobial peptides (31), (iv) it has broad spectrum of antimicrobial activity (10,12,18,31).

Several reports, including ours, have recently demonstrated that α-MSH and its C-terminal sequence (Lys-Pro-Val i.e., (α-MSH(11-13)) have strong and rapid antimicrobial activity against S. aureus including methicillin sensitive S. aureus (MSSA), methicillin resistant S. aureus (MRSA), Escherichia coli and Candida albicans (10,12,31). In our previous study we showed that α-MSH is active against staphylococcal biofilm and can retain its activity in whole blood, plasma and serum (31). Our study also demonstrated that α-MSH damages S. aureus membrane in a dose dependent manner (31). Studies by others have revealed that candidacidal effect of α-MSH might be mediated through the induction of cyclic adenosine monophosphate (cAMP) (12). Besides, an analogue of α-MSH is already under clinical trials to treat
candidal vaginitis (18). A recent study suggests that antibacterial activity of the C-terminal sequence of α-MSH is not dependent on the presence of a cationic Lys residue but on the Pro and Val residues (10). However, detailed studies have not yet been reported on the contribution of different structural regions of α-MSH towards its antimicrobial activity, particularly the role of the core sequence, α-MSH(6-9) which is common to all melanocortin peptides and responsible for binding to melanocortin receptors. In the present study, our main aim was to determine the role of the amino acid sequences, particularly the contribution of N-terminal and C-terminal regions in the staphylocidal mechanism of α-MSH. We selected four sequences of α-MSH: entire α-MSH, α-MSH(1-5), α-MSH(6-13), α-MSH(11-13) and studied their antistaphylococcal activity in detail against both MSSA and MRSA strains. The study had also been extended to understand the effect of NaCl and different ions (Mg++, Ca++) on the killing efficacy of peptides against S. aureus. In our previous study we suggested that membrane damage might be partially responsible for staphylocidal activity of α-MSH (31). In the present study we investigated how the chosen fragments of α-MSH kill S. aureus and whether their mode of antibacterial activity recapitulates with that of the entire peptide. Finally, we also examined the nature of the morphological and ultrastructural changes in S. aureus caused by exposure to these peptides in order to enhance our knowledge regarding the antimicrobial mechanism of action of α-MSH based peptides. Such analyses are important for rational designing of α-MSH based antimicrobial peptides with maximum potential.
MATERIALS AND METHODS

Antimicrobial Peptides

α-MSH, α-MSH(1-5), α-MSH(6-13), α-MSH(11-13), gramicidin D and calcein-AM were purchased/custom synthesized from Sigma-Aldrich (St. Louis, MO). DiBAC4(3) (bis-(1,3 diabarbituric acid)-trimethine oxanol) was purchased from Invitrogen (USA). Glutaraldehyde was purchased from Merck (Germany). The purity of all peptides was 97% and the concentration of α-MSH, α-MSH(6-13), α-MSH(11-13) and α-MSH(1-5) was determined spectrophotometrically (Cary 100 Bio/Varian) (31).

Bacterial strains

MSSA strain ATCC 29213 and MRSA strain ATCC 33591 (39) were used in this study. The strains were stored at -70°C in 15% (v/v) glycerol until subcultured onto Brain heart infusion (BHI, Himedia Laboratories, India) agar plate or BHI broth for further analysis. Cultured cells were collected by centrifugation, washed with PBS buffer (10 mM Na-K phosphate buffer having 150 mM NaCl, pH 7.4) and adjusted to the desired inoculum spectrophotometrically (OD_{600}=0.5 correspond to 10^8 cfu/ml). Amount of dead bacteria was estimated microscopically using LIVE/DEAD BacLight Bacterial Viability Assay™ Kit (Invitrogen, USA) (31).

Staphylocidal activities

To determine the staphylocidal activities of peptides, mid-logarithmic-phase S. aureus cells were used. After harvesting, cells were washed in PBS buffer (10 mM Na-K phosphate buffer having 150 mM NaCl, pH 7.4), and the cell density was adjusted to 10^4 -10^7 cfu/ml, as desired, in the same buffer. All studied peptides were dissolved in
PBS buffer. Cells were then exposed to peptides at various concentrations as described elsewhere (31,42). At selected time points, aliquots were plated on BHI agar and were incubated at 37°C for overnight. Survival of *S. aureus* was determined by quantitative counting of the colony and was expressed as mean percentage of survival vs. non-peptide-treated control (set at 100% survival). To determine the effect of Ca$^{++}$ and Mg$^{++}$ ions on antibacterial activity of α-MSH and its fragments α-MSH(6-13) and α-MSH(11-13), physiological concentration of CaCl$_2$ (i.e. 2mM) and MgCl$_2$ (i.e. 1mM) was included in PB (10mM Na-K phosphate buffer), pH 7.4 (28). Each experiment was performed at least three times in triplicates independently.

**Membrane permeabilization**

*S. aureus* membrane permeabilization following peptide exposure was measured via release of calcein using flow cytometry (BD FACS Calibur) as described in our previous report (31). In short, *S. aureus* cells were first loaded with calcein (2 µg/ml) and then calcein-loaded *S. aureus* cells were diluted to 10$^6$cfu/ml and exposed to peptides (each of 1µM) for 30 min, 60 min and 120 min separately. Gramicidin D, a well known membrane-targeting peptide (27) was used as positive control. A total of 10,000 cells were acquired for each flow cytometry analysis. Cells at or above a threshold of 10 fluorescence units (FL1 units) were considered to have retained calcein, indicative of an intact cytoplasmic membrane; those cells exhibiting <10 FL1 units were interpreted to have lost calcein as a result of peptide induced membrane permeabilization. Experiments were repeated at least three times independently on separate days.

**Membrane depolarization**
Mid-log phase grown bacterial cells were diluted to $10^6$ cfu/ml and incubated with α-
MSH based peptides (each of 1µM) and gramicidin D (20 µg/ml) at 37°C for selected
time points of 30 min, 60 min and 120 min. Cells were pelleted out and washed once
with PBS, pH 7.4 and then were incubated for 5-10 min at room temperature in dark
with a membrane potential sensitive dye DiBAC$_4$(3) to a final concentration of 1µM,
whose excitation and emission wavelength are 490 nm and 520 nm respectively.
DiBAC$_4$(3) is an anionic lipophilic bis-oxonol dye, which can sense the change in
membrane potential and enters into depolarized cells. When membrane potential
decreases, oxonol fluorescence increases and hence shift in fluorescence peak is
obtained. A total of 10,000 cells were analyzed in each sample, using the cell quest
software (BD FACS Calibur) for data acquisition and analysis (37). Experiments were
repeated at least three times independently on separate days.

**Scanning Electron Microscopy**

Bacterial cells were first grown to mid-log phase and harvested by centrifugation at
4000 rpm for 10 min and resuspended in PBS, pH 7.4 to yield $10^8$ cfu/ml. As higher
density of cells was required for cell imaging, different concentrations of peptides were
used to ensure lethal activity of the studied peptides. For comparison, two different
concentrations of Gramicidin D were used. Thus, bacterial suspension was then
incubated with 12 µM and 50 µM of α-MSH based peptides and 2µg/ml and 20µg/ml of
Gramicidin D in PBS pH 7.4, for 2 h. After incubation, cells were spun down at 6000 rpm
for 10 min, washed with PBS, pH 7.4 for several times and fixed with 2.5%
glutaraldehyde in the same buffer overnight at 4 °C. After fixation, cells were washed 2-3
times in 0.1M PB, pH 7.4 and dehydrated in series of graded ethanol (30% - 100%) and
finally dried in desiccator under vacuum. An automatic sputter coater (Polaron OM-
SC7640) was used for coating the specimens with 20 nm gold particles. Then samples were viewed via SEM (Carl Zeiss EVO 40, Germany) (13).

**Transmission Electron Microscopy**

TEM was performed according to the procedure described elsewhere with little modification (1,22). In brief, *S. aureus* cells (ATCC 29231) were grown up to mid-log phase, harvested and cell density was adjusted to $10^9$ cfu/ml (OD$_{600nm}$=0.1) in PBS buffer. Cell suspension was incubated with final 50µM of each of α-MSH based peptide and 20 µg/ml of gramicidin D at 37°C for 2 h. After peptide treatment, cells were spun down and washed three times in 0.1M sodium potassium phosphate buffer pH 7.4 and fixed in 2.5%(vol/vol) glutaraldehyde in 0.1M sodium potassium phosphate buffer pH 7.4 for overnight at 4°C. The samples were then post fixed in 1.0% (wt/vol) osmium tetroxide, followed by staining with uranyl acetate, as a heavy-metal stain. Sample were then dehydrated in graded acetone and embedded in epoxy resin (Araldite CY212). Each sample was thin sectioned using microtone (Leica EM UC6), transferred on copper grid and stained by uranyl acetate (saturated solution of uranyl acetate in 50% alcohol), followed by lead citrate staining. Samples were washed three times in MQ and dried by touching whatman filter paper. Sections were viewed in electron microscope (Zeol-JEM 2001, Japan) at 120-Kev energy. For each sample three grids were prepared separately.

**Statistical analysis**

All killing experiments were performed in triplicate, repeated in three independent experiments on different days and were plotted as mean±SD. Rest of the assays were performed as three independent experiments on different days and were plotted as
mean ± SD. Statistical analysis (multiple comparison among datasets) was performed using one-way ANOVA using Minitab™ (10,31). A $P$ value $\leq 0.05$ was considered significant.
RESULTS

Antibacterial activity of α-MSH based short peptides against MSSA.

*S. aureus* ATCC 29213 (10⁴ cfu/ml) was treated with a broad range of concentration (10⁻¹¹ to 10⁻⁶M) of α-MSH, α-MSH(6-13), α-MSH(11-13) and α-MSH(1-5) for 120 min in 10mM PBS buffer, pH 7.4. Percentage of survival vs. control is presented in Fig. 1a. α-MSH and both its C-terminal containing fragments, α-MSH(6-13) and α-MSH(11-13), exhibited substantial antimicrobial activity against *S. aureus* at all concentrations tested. Staphylocidal activities of C-terminal fragments of α-MSH(6-13) and α-MSH(11-13) was comparable with the entire peptide α-MSH in micromolar range (≥95%) while at picomolar concentration the full-length peptide was found to be more active. For example, 1µM α-MSH, 1µM α-MSH(6-13), 1µM α-MSH(11-13) killed 95±0.97%, 96.2±0.46% and 97.3±0.98% cells respectively in 2 h (p<0.001), whereas 10pM α-MSH, 10pM α-MSH(6-13), 10pM α-MSH(11-13) exhibited 35±9.98%, 29±0.65% and 19±6.18% bacterial killing respectively (p<0.001). In contrast, N-terminal possessing amino acids, α-MSH(1-5) could show only 16±4.3% bacterial killing in micromolar range while 100% bacterial survival was obtained in picomolar range. The killing data of α-MSH(1-5) was significantly lower than those observed with α-MSH and its C-terminal fragments (p<0.001). In addition to killing potency, to get an idea about the time required to kill bacteria by peptides, Staphylococcus cells were incubated with α-MSH and C-terminal fragments α-MSH(6-13) and α-MSH(11-13) for indicated times (0 to120 min) and presented in Fig. 1b. The data suggests that killing activities of all the peptides were extremely rapid and ~90% of cells were killed within 15 min of incubation. Interestingly, incubation for longer period of time did not substantially increase the
staphylocidal activity. (p<0.001 compared to PBS control). There was no significant loss in viability of *S. aureus* cells in control PBS buffer over the 2 h period of incubation (Fig. 1b).

Antibacterial activity of α-MSH and its C-terminal fragments against MRSA

Staphylocidal activities of α-MSH and its C-terminal fragments were performed against prototype MRSA (ATCC 33591) (39) and presented in Fig. 2. As shown in figure, similar to MSSA strain, MRSA strains were also found highly susceptible to the studied peptides in the entire concentration range. For example, ~ 90% MRSA cells were killed by α-MSH and its C-terminal fragments after 2 h of incubation with 1µM of each peptide.

With lower concentration of peptide, e.g., at 10pM concentration, the percentage of survival was 64±0.06, 57±8.8 and 76±9.3 for α-MSH, α-MSH(6-13) and α-MSH(11-13) respectively. The observed killing was found significant for the entire range of concentration of each peptide (p value <0.05).

Staphylocidal activity by varying bacterial cell density

Initial antimicrobial assay was performed with lower *S. aureus* cell density (10^4 cfu/ml, Fig. 1 and Fig. 2). In order to investigate whether the antibacterial effect of α-MSH was influenced by bacterial cell number, higher no. of MSSA and MRSA cells (10^6 and 10^7 cfu/ml) were incubated for 2 h with different α-MSH peptides in PBS buffer, pH 7.4 (Fig. 3a and 3b). As can be seen from Fig. 3, bactericidal effect of α-MSH, α-MSH(6-13) and α-MSH(11-13) was decreased when higher numbers of bacterial cells were used. Thus, it was observed that there was ≥95% killing by all α-MSH based peptides (each 1µM) when 10^4 cfu/ml MSSA cells were used (Fig. 1a). However, 73±2.7%, 67±1.04% and
40±5.7% inhibition was observed when 10⁷ cfu/ml MSSA cells were exposed to same concentration of α-MSH, α-MSH(6-13) and α-MSH(11-13) respectively (Fig. 3a). Similarly, in case of MRSA strain, the killing was ~90% by all the peptides when 10⁴ cfu/ml was used (Fig. 2), while killing reduced to 75%±3, 74%±0.66 and 68%±4.9 by α-MSH, α-MSH(6-13) and α-MSH(11-13) respectively for 10⁶ cfu/ml MRSA cells and for 10⁷ cfu/ml killing was 67%±1.4, 41%±5.5 and 33%±1.9 by α-MSH, α-MSH(6-13) and α-MSH(11-13) respectively (Fig. 3b). These differences reached statistical significance for all the tested peptides (p value < 0.005 comparing data of 10⁷ cfu/ml vs. 10⁶ cfu/ml vs. 10⁴ cfu/ml for both MSSA and MRSA).

Antibacterial activity in presence of different salt

We previously showed that antibacterial activity of α-MSH was not inhibited in presence of physiological concentration of NaCl (31). In this study, the antibacterial activity of α-MSH, α-MSH(6-13) and α-MSH(11-13) was examined in 10mM Na-K phosphate buffer having no salt and in the presence of 150mM NaCl (i.e., PBS), 1mM Mg²⁺ and 2mM Ca²⁺ (Fig. 4). From the data it was observed that all the peptides were potentially active in presence of physiological concentration of salts, whereas killing was comparatively lower on the removal of salt (Fig. 4). Thus, in PBS, killing was ≥95% for all the peptides and in presence of 1mM Mg²⁺, α-MSH, α-MSH(6-13) and α-MSH(11-13) exhibited 90±1.8% (p=0.035), 81.6±5.3% (p=0.036) and 85±1.3% (p<0.001) killing respectively. Similarly in presence of 2mM Ca²⁺, α-MSH, α-MSH(6-13) and α-MSH(11-13) demonstrated 88.5±2.9% (p=0.017), 88±0.32% (p=0.008) and 84.33±0.72% (p<0.001) killing respectively. In absence of salt, killing was reduced by ~30% for all the peptides. Taken together, the data suggested that all the three peptides possess...
Membrane permeabilization by the C-terminal fragments, α-MSH(6-13) and α-MSH(11-13).

To analyze whether the C-terminal fragments, α-MSH(6-13) and α-MSH(11-13), killed S. aureus by membrane permeabilization like entire α-MSH, flow cytometry studies using calcein-AM were performed on both MSSA and MRSA cells (Fig. 5). As demonstrated in Fig. 5a, exposure of MSSA cells to C-terminal peptides α-MSH(6-13) and α-MSH(11-13) exhibited similar shift in peak as entire α-MSH and gramicidin D indicating release of calcein due to membrane perturbation by both the peptides. MRSA strains also exhibited leakage of calcein when exposed to α-MSH and its C-terminal peptides (Fig. 5a). Consistent with our previous observations (31), treatment of MSSA ATCC 29213 with 1 µM of α-MSH for 2 h caused 84% calcein leakage compared to untreated calcein loaded control samples. Interestingly, the time dependent profile (30 min to 120 min) of calcein- leakage from MSSA cells by all the three peptides (each at 1 µM) showed different amounts of calcein release (Fig. 5b). For instance, 2.6±0.6%, 5.8±2.2% & 9.92±0.2% calcein was released after 30 min of α-MSH, α-MSH(6-13) and α-MSH(11-13) treatment, respectively, and on increase in incubation time from 30 min to 60 min there was little increase in calcein leakage. However, a120 min exposure to all three peptides caused dramatic increase in calcein release from S. aureus cells, comparable to gramicidin D. Thus, after 120 min of peptide exposure, 84±4.1%, 85±2.1%, 90±3.3% and 95.4±0.79% calcein release was observed for α-MSH, α-MSH(6-13), α-MSH(11-13) and gramicidin D, respectively. These differences in the
percentage of membrane permeabilization over time were found to be statistically
significant for each peptide (p<0.001, comparing data of 30 min vs. 60 min vs. 120 min).
As shown in Fig. 5c, the percentage of calcein leakage from MRSA ATCC 33591 cells
on exposure to 1 µM α-MSH based peptides ranged from 50-70%.

Membrane depolarization by α-MSH based antimicrobial peptides.

Previous research reports that various membrane-targeting antimicrobial peptides such
as nisin LL-37, human neutrophil peptide-1 (HNP-1) and gramicidin D also results in
depolarization of membrane (19,34,43). In order to analyze whether α-MSH and its C-
terminal fragments cause membrane depolarization, peptide treated S. aureus cells
were incubated with membrane-potential sensitive dye DiBAC₄ (3) and compared with
untreated control. As shown in Fig. 6a, exposure of all three peptides to both MSSA and
MRSA strain for 120 min caused uptake of DiBAC₄ (3) inside the cells and resulted in
increase in fluorescence signal, indicating depolarization of the bacterial membrane. It
was also observed that all three peptides led to change in membrane potential of MSSA
cells over time (Fig. 6b). Thus, 1 µM α-MSH caused 13%, 40% and 68% depolarized
bacteria after 30, 60 and 120 min incubation, respectively. Similarly, both C-terminal
fragments caused 0-30% depolarization on 30 min incubation whereas percent of
depolarized cells was enhanced to approximately 70-83% after 120 min incubation.
Similar peptide induced membrane deplorizations were also observed for MRSA strain
as well (Fig. 6c). All three α-MSH based peptides (each of 1µM) caused 55-60%
depolarized cells after two hours of exposure. This indicated that like other membrane-
targeting peptides e.g. HNP-1 (37), α-MSH and its C-terminal fragments α-MSH(6-13)
and α-MSH(11-13) also depolarized the S. aureus cell membrane along with membrane

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permeabilization. In contrast, gramicidin D caused less depolarization at higher time points although membrane depolarization at 30 min is higher than that for α-MSH based peptides. This data reflects an immediate depolarization of staphylococcal membrane upon peptide binding followed by complete leakage of cell material which was also revealed by SEM and TEM studies as described below (Fig. 7i and Fig. 8). The difference in depolarization with time was found to be statistically significant for each peptide (p<0.05 comparing data of 30 min vs. 60 min vs. 120 min).

**Morphological changes in S. aureus on peptide exposure using Scanning Electron Microscopy.**

Morphological changes in *S. aureus* due to peptide exposure were examined by scanning electron microscopy (SEM). As cell density has to be sufficiently high (10^8 cfu/ml) for this technique, two different concentrations of α-MSH based peptides (12 µM and 50 µM) and of gramicidin D (2 µg/ml and 20 µg/ml) were used. As shown in Fig. 7a, untreated *S. aureus* cells looked round, smooth and intact. Upon exposure of 2 µg/ml of gramicidin D to the bacterial cells (Fig. 7h), there was clear indication of surface perturbation including distorted appearance, rupture lines, depression and dents, while hole formation and complete leakage of cell material was visible on incubation with 20 µg/ml gramicidin D (Fig. 7i) (22). Similar morphological changes were also observed for *S. aureus* cells exposed to α-MSH, α-MSH(6-13) and α-MSH(11-13) (Fig. 7b-7g). It is evident from Fig.7b, 7d and 7f, that exposure to 12µM of α-MSH, α-MSH(6-13) and α-MSH(11-13) caused rough and damaged surface, depression, rupture line and holes formation. On incubation of *S. aureus* cells with higher concentrations of α-MSH based peptides (each of 50µM), cell bursting and leakage were prominent (Fig. 7c, 7e and 7g).
Surface damage caused by α-MSH based peptide (used in this study) was similar but less extensive in comparison to gramicidin D, particularly at higher concentrations.

**Ultrastructural changes in S.aureus on peptide exposure using Transmission Electron Microscopy**

To examine the effect of α-MSH based peptides on bacteria more accurately, transmission electron microscopy (TEM) was performed on peptide treated and untreated bacteria samples. For comparison, gramicidin D was used. As indicated in Fig. 8a, the untreated control samples had intact cell membrane and cell wall and the cells were uniformly shaped. However, huge changes were observed in the images of peptide treated cells. Fig. 8b, 8c and 8d represent the images of α-MSH, α-MSH(6-13) and α-MSH(11-13) treated S. aureus cells, respectively, and Fig. 8e shows gramicidin D treated bacterial cells. Incubation with α-MSH and its short peptides for two hours caused distortion in cell boundary; many cells were devoid of their cell walls (called ghost cells), while some cells had remnants of cell wall and cell membrane. These results were found consistent to the earlier reported peptide induced ultra structural changes (1). Gramicidin D treated cells (Fig. 8e) did not have many ghost cells but clear envelop gaps were found, through which, the cell cytoplasmic contents were leaking out.
DISCUSSION

In our previous study, we had demonstrated that α-MSH is very active against *S. aureus* in the biofilm form (31). To further our study, the structure-activity relationship of α-MSH, particularly the contribution of its amino acid sequences in staphylocidal mechanism was analyzed in the present study. We chose certain fragments of α-MSH from both C-terminal and N-terminal regions, i.e., α-MSH(6-13), α-MSH(11-13) and α-MSH(1-5) and studied their anti-staphylococcal activity and mechanism of action against MSSA and MRSA and compared them with the entire sequence of α-MSH. Since, in our previous study, α-MSH had been found to cause damage in *S. aureus* membrane (31), in the present study we investigated loss of bacterial membrane potential, if any, due to exposure to the studied peptides along with membrane permeabilization studies. Finally, membrane perturbation of *S. aureus* on peptide exposure was confirmed by investigating morphological and ultrastructural changes using SEM and TEM.

Several encouraging results were obtained from the current study. First, our activity assay clearly indicated that both the C-terminal fragments, α-MSH(6-13) and α-MSH(11-13), retained the *in vitro* antibacterial activity of entire peptide, α-MSH (Fig. 1a) in the concentration range of picomolar to micromolar and the killing was found to be very rapid for all the peptides (Fig. 1b). In contrast to the earlier observations (26, 38), presence of physiological concentrations of salts did not affect the killing potency of any of the studied peptides (Fig. 4). Of note, staphylocidal activities of C-terminal fragments of α-MSH against both MSSA and MRSA were comparable to that of the full-length peptide in micromolar range while at picomolar concentration entire peptide, α-MSH, was most effective. Our study also indicated that peptides containing the core sequence
(amino acid sequence from position 6 to 9) such as entire α-MSH and α-MSH(6-13) were more active than α-MSH(11-13), suggesting an important role of the core sequence in anti-staphylococcal activity. Further, the N-terminal region of α-MSH did not show any significant antibacterial activity at any of the concentrations tested (Fig. 1a) suggesting the importance of C-terminal fragments in the staphylocidal mechanism of α-MSH.

Host defense cationic peptides target the bacterial membrane through electrostatic interaction with negatively charged membrane components, resulting in membrane damage and eventually cell death (33,43,44,45). Our previous study suggested that the staphylocidal effect of α-MSH might be mediated through membrane disruption of S. aureus (31). Similar to entire α-MSH, both α-MSH(6-13) and α-MSH(11-13) showed substantial membrane depolarization and calcein leakage from MSSA and MRSA strains (Fig. 5 and Fig. 6) after 2 h of incubation, indicating membrane damage by all the peptides. Our data also suggests that membrane depolarization occurs prior to membrane permeabilization. As observed, 30 min and 60 min incubation of S. aureus cells with α-MSH and either of its C-terminal fragments could induce only up to 10% membrane leakage and 40% membrane depolarization, whereas >90% killing was obtained by all three peptides within 15 minutes. Comparison of the time kinetics of killing and those of membrane permeabilization and depolarization suggests membrane perturbation as a secondary event, following the lethal hit of α-MSH derived peptides rather than a major cause of staphylocidal activity. However, membrane permeabilization and depolarization assays represent results in real time, whereas killing assays (determined by colony count) depict data after overnight incubation. There is always a possibility that the entry of the peptides in S. aureus cells may require 15 min or lesser
and lethal hit probably occurs during overnight incubation period (25,37). Loss of bacterial membrane integrity was further confirmed by morphological and ultrastructural studies (Fig. 7 and 8). Our SEM images clearly indicate severe staphylococcal membrane perturbation including leakage of cell content by all three α-MSH based peptides. Further, loss of cell wall and cells without cytoplasmic content were clearly visible from TEM images of peptide-treated S. aureus cells.

It has been reported that a given host defense antimicrobial peptide may use more than one mechanism for its microbicidal activity (40). It appears from our present study as well as our previous study that the bacterial membrane is the major target for staphylocidal activity of α-MSH and its C-terminal fragments. However, other targets can not be ruled out, particularly due to the fact that the killing by α-MSH based peptides was very rapid while substantial membrane disruption occurred at later time points. These suggest either membrane damage is a secondary effect or α-MSH based peptides target bacterial components other than membranes such as nucleic acids or important enzymatic process leading to cell death and, thereafter, membrane damage. Occurrence of all the above said processes simultaneously may be another possibility of antibacterial mechanism of action of α-MSH based peptides. To elucidate the mode of staphylocidal action of α-MSH and its C-terminal fragments, a detailed study on inhibition of macromolecular synthesis needs to be performed and targets in the bacterial envelope need to be identified.

In conclusion, C-terminal fragments of α-MSH including α-MSH(6-13) and tripeptide α-MSH(11-13) exhibited rapid and potent antimicrobial activities against both MSSA and MRSA strain, demonstrating their importance in α-MSH mediated staphylocidal effect. Like other host defense antimicrobial peptides, these peptides caused membrane
depolarization followed by membrane permeabilization and the mechanism of action of
full-length α-MSH and its C-terminal fragments were equivalent. Electron microscopic
images of S. aureus demonstrated the remarkable morphological and ultrastructural
changes as a result of peptide exposure, including leakage of cell material and loss of
cell wall. Importantly, N-terminal fragments of α-MSH, i.e., α-MSH(1-5) did not show any
antimicrobial activity. It is also important to mention that the minimum sequence
required for anti inflammatory activity of α-MSH is the C-terminal tripeptide i.e., α-
MSH(11-13). Therefore, the C-terminal fragments of α-MSH having both anti-
inflammatory and antibacterial properties could emerge as excellent antibacterial agents
in the treatment of staphylococcal infection.
ACKNOWLEDGEMENTS

This research was supported by grant from the Indian Council of Medical Research and Department of Biotechnology; India to K. M. Madhuri Singh acknowledges the fellowship from the University Grant Commission. We thank Dr. G. Sainy, Mr. S. C. P. Sharma and Dr. R. Pal of AIRF, JNU for helping during acquisition of SEM and TEM images. We are grateful to Dr. B. Dhawan (AIIMS, New Delhi, India) for providing MSSA strain ATCC 29213. We thank Dr. S. S. Komath (SLS, JNU, India) and Dr. N. Gupta (ICMR, India) for their input on the manuscript. Finally, we thank editor and the reviewers for their insightful comments which were very helpful in improving the manuscript.

This study was presented in part at the International Conference on Physics Biology interface, December 13-16, 2009, Saha Institute of Nuclear Physics, Kolkata, India".
REFERENCES


FIGURE LEGENDS

Figure 1. a) Killing activity of α-MSH and its fragments at different concentrations against logarithmic phase MSSA ATCC 29213 cells after 120 min of peptide administration. These data represent the means (±SD) of three independent experiments. Symbols: α-MSH (open), α-MSH(6-13) (filled) and α-MSH(11-13) (striped); α-MSH(1-5) (dotted). Multiple comparison among data sets indicate significant changes (*p <0.001, **p<0.01, ***p<0.05). b) Killing activity of α-MSH and its C-terminal fragments (each of 1µM) at different time points starting from 0 min to 120 min against MSSA ATCC 29213. Symbols: No peptide (diamond), α-MSH (circle); α-MSH(6-13) (triangle); α-MSH(11-13) (square). These data represent the means (±SD) of three independent experiments. *p<0.001 compared to PBS control.

Figure 2. Killing activity of α-MSH and its fragments at different concentrations against logarithmic phase MRSA ATCC 33591 cells after 120 min of peptide administration. These data represent the means (±SD) of three independent experiments. Symbols: α-MSH (open), α-MSH(6-13) (filled) and α-MSH(11-13) (striped). Multiple comparison among data sets indicate significant changes (*p <0.001, **p<0.01, ***p<0.05).

Figure 3. Killing activity of α-MSH, α-MSH(6-13) and α-MSH(11-13) against logarithmic phase S. aureus (a) MSSA ATCC 29213 and (b) MRSA ATCC 33591 cells having bacterial density $10^6$ and $10^7$ cfu/ml. These data represent the means (±SD) of three independent experiments. *p<0.001, **p<0.01, ***p<0.05, comparing data of bacterial density of $10^7$cfu/ml vs. $10^6$ cfu/ml vs. $10^4$ cfu/ml.
Figure 4. Killing efficacy of α-MSH and its C-terminal fragments in different micro-environmental condition, against MSSA cells after 2 h of 1µM peptide incubation. Killing activity of α-MSH (open), α-MSH(6-13) (filled) and α-MSH (11-13) (striped) in phosphate buffer without salt (-NaCl), with 150mM NaCl (i.e., PBS) (+NaCl), with 1mM Mg++ (+MgCl₂) and with 2mM Ca++ (+CaCl₂). Each experiment was done thrice on independent day and represent here as means (±SD). *p<0.001, **p<0.01, ***p<0.05, compared data of different salt with phosphate buffer.

Figure 5. Membrane permeabilization of S. aureus ATCC 29213 by α-MSH(6-13) and α-MSH(11-13). a) Calcein leakage assay: Logarithmic MSSA ATCC 29213 and MRSA ATCC 33591 were labeled with calcein and analyzed for membrane permeabilization after incubation with α-MSH based peptides and gramicidin D. A total of 10,000 cells were acquired for each flow cytometry analysis. Cells at or above a threshold of 10 fluorescence units (FL1 units) were considered to have retained calcein, indicative of an intact cytoplasmic membrane; those cells exhibiting <10 FL1 units were interpreted to have lost calcein as a result of α-MSH induced membrane permeabilization. First row histograms (left to right) are showing calcein loaded MSSA ATCC 29213 cells untreated (control) and treated with α-MSH(6-13), α-MSH(11-13) and gramicidin D. Second row (left to right) is showing histograms of calcein loaded MRSA ATCC 33591 cells untreated (control) and treated with α-MSH, α-MSH(6-13) and α-MSH(11-13). b) Time dependent changes in membrane permeabilization of MSSA ATCC 29213 by α-MSH (open), α-MSH(6-13) (filled), α-MSH(11-13) (striped) and gramicidin D(dotted) as quantified by calcein leakage using flow cytometry. Data represents that 30 min and 60 min treatment of all the tested peptide could lead only ~10% calcein leakage which was followed by sharp increase in calcein release after 120 min peptide treatment. These
data represent the means (±SD) of three independent experiments. (*p<0.001 comparing data of 30 min vs. 60 min vs. 120 min). c) Percentage of calcein leakage from MRSA ATCC 33591 on exposure of 1µM of α-MSH, α-MSH(6-13) and α-MSH(11-13). These data represent the means (±SD) of three independent experiments.

Figure 6. Membrane depolarization of S. aureus by α-MSH, α-MSH(6-13) and α-MSH(11-13). a) Depolarization of bacterial membrane leads to uptake of anionic dye DiBAC₄(3), resulting in increase in fluorescence signal. MSSA and MRSA incubated without (control) and with 1µM of each peptide for 2 h and then incubated with DiBAC₄(3) and analysed by flow cytometry, with a total of 10000 cells were acquired for analysis. Cells below FL-1 10 were considered as unloaded and above FL-1 10 were considered as loaded. Histograms are showing uptake of DiBAC₄(3) by both MSSA and MRSA cells treated α-MSH, α-MSH(6-13) and α-MSH(11-13) compared to untreated control and demonstrate the shift of fluorescence peak in case of peptide treated loaded cells. b) Time dependent changes in MSSA bacterial membrane potential expressed as % of depolarized cells after 30 min, 60 min and 120 min of peptide treatment compared to untreated control S. aureus. α-MSH (open), α-MSH(6-13) (filled), α-MSH(11-13) (striped) and gramicidin D (dotted); these data represent the means (±SD) of three independent experiments. (**p≤0.01 comparing data of 30 min vs. 60 min vs. 120 min).

c) Percentage of depolarization occurring in MRSA ATCC 33591 on exposure of 1 µM of each of α-MSH, α-MSH(6-13) and α-MSH(11-13) for 2h. These data represent the means (±SD) of three independent experiments.

Figure 7. Scanning electron microscopic images of S. aureus of (a) untreated control cells and after 2 h exposure with (b) 12 µM α-MSH, (c) 50 µM α-MSH, (d) 12 µM α-MSH(6-13), (e) 50 µM α-MSH(6-13), (f) 12 µM α-MSH(11-13) , (g) 50 µM α-MSH(11-
(h) 2 µg/ml gramicidin D (i) 20 µg/ml gramicidin D. *S. aureus* cells exposed to lower concentration of all peptide were showing morphological changes including surface roughness, depression and dents formation whereas higher dose incubation caused leakage of cells material which was absent in untreated control cells. Similar appearances were found in separate experiments on different days.

**Figure 8.** Transmission electron microscopic images of *S.aureus* cells treated with (a) no peptide (Control), (b) 50µM α-MSH (c) 50µM α-MSH(6-13), (d) 50µM α-MSH(11-13) and (e) 20 µg/ml gramicidin D for 2 h. Left and right panels are images of same sample with different magnification. Arrows pointed to the peptide treated cells without cell walls, cell leakage and cells without cytoplasmic content.
Fig. 1

(a) Concentration-response curve for α-MSH, α-MSH (6-13), α-MSH (11-13), and α-MSH (1-5) on the percentage of survival versus control. The x-axis represents the concentration in molar, while the y-axis shows the percentage of survival. The bars indicate the mean ± standard error of the mean (SEM).

(b) Time-response curve for the same compounds. The x-axis represents time in minutes, and the y-axis shows the percentage of survival. The graph shows a significant decrease in survival over time for all compounds compared to the control.
Fig. 3
Fig. 4
Fig 6

(a) Counts of MSSA and MRSA

(b) Time course of % of depolarized cells

(c) Comparison of % of depolarized cells

** and *** indicate statistical significance.
Fig. 7
Fig. 8