THE EFFECT OF α-MELANOCYTE STIMULATING HORMONE ON BURN-INDUCED OXIDANT PRODUCTION BY RAT PERITONEAL NEUTROPHILS

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ABSTRACT

Objective: Previous findings related to the anti-inflammatory effects of α-melanocyte stimulating hormone (α-MSH) indicate that the peptide might inhibit inflammation by antagonizing the effects of local cytokines or by inhibiting neutrophil functions. In addition, it is a potent inhibitor of the induction of the inducible nitric oxide synthase (iNOS) in cultured macrophages and of nitric oxide (NO) production in a sepsis model. This study was designed to investigate whether α-MSH affects reactive oxygen metabolite production by rat peritoneal neutrophils following burn injury and whether NO is involved in this effect.

Methods: The neutrophils were removed from the peritoneum of the rats 6 h after burn or sham trauma. Cells (2×10^6 /ml) were treated with the increasing concentrations of α-MSH (10^{-12} M, 10^{-10} M, 10^{-8} M or 10^{-6} M). Nitric oxide donor, sodium nitroprusside ranging from (10^{-10} - 10^{-4} M) was added to the cells in the presence of α-MSH. Reactive oxygen metabolite production was measured by chemiluminescence (CL) technique using luminol or lucigenin probes.

Results: Lucigenin CL value of cells obtained from burn animals was significantly higher than those obtained from the sham group. α-MSH caused significant reductions in high lucigenin CL values and this effect was partly inhibited by sodium nitroprusside.

Conclusion: Our results suggest that α-MSH is effective in preventing oxidant production by neutrophils activated by burn trauma, at least in part, by a mechanism involving nitric oxide.

Key Words: α-melanocyte-stimulating hormone, Burn, Reactive oxygen species, Nitric oxide, Chemiluminescence

INTRODUCTION

The neuropeptide α-melanocyte stimulating hormone (α-MSH) that occurs within the pituitary, brain, skin and other tissues has potent anti-inflammatory activity (1). Previous findings related to the anti-inflammatory effects of α-MSH indicate that the peptide might inhibit inflammation by antagonizing the effects of local cytokines or by inhibiting neutrophil functions (1, 2). It may influence neutrophil migration by direct actions on the cell or via generation of signals that alter neutrophil responses to chemotactic agents such as cytokines. Previous studies have demonstrated that the peptide given intraperitoneally blocked the accumulation of neutrophils in subcutaneously implanted sponges in response to IL-1, TNF-α and C5a (3, 4). In subsequent studies, α–MSH has been shown to inhibit the migration of neutrophils stimulated with FMLP or IL-8 (2). Recent findings showing the presence of α-MSH receptors and

mRNA for melanocortin receptor-1 (MC1) in murine and human neutrophils stimulated with interferon and lipopolysaccharide (LPS) indicate that the peptide can act directly on peripheral cells to inhibit chemotaxis (5, 6). In addition, α-MSH suppresses inflammation-related nitric oxide (NO) production by inhibiting the expression of inducible nitric oxide synthase (iNOS) in murine macrophages (6).

Based on current research findings in animals and man, a local burn insult produces oxidant-induced inflammation in various organs within hours of injury, even in the absence of shock. The local tissue trauma activates a number of systemic mediator cascades, e.g. complement activation, arachidonic acid release and cytokine production (IL-1, TNF-α), resulting in a generalized neutrophil sequestration and a "priming" of local and systemic neutrophils and macrophages. In experimental models of stress and injury, circulating endotoxins become evident probably as a result of burn wound colonization and an early gut leak (7, 8). Endotoxin and other bacterial by-products are potent activators of the primed macrophages and neutrophils. This leads to the release of massive amounts of oxidants, arachidonic acid metabolites and proteases which cause further tissue damage (9). Previous studies have shown that the increase in iNOS activity in gut mucosa after thermal trauma is well correlated with the increase in intestinal permeability which is an index for barrier failure (10). It is also known that NO which is produced in excess amounts via iNOS induction, results in peroxynitrite formation and contributes to tissue injury in many inflammatory conditions including burn injury (11).

Therefore, based on these data, the present study aimed to determine the effect of α-MSH on reactive oxygen metabolite production by rat peritoneal neutrophils following burn injury and to investigate whether NO is involved in this effect.

MATERIAL AND METHODS

Animals
Sprague-Dawley rats of both sexes (300-350 g) were made to fast for 12 h, but were allowed free access to water before burn injury. This study

was approved by Marmara University, School of Medicine, Animal Care and Use Committee.

Experimental protocol
Under brief ether anesthesia, the dorsum of the rats was shaved and exposed to a 90 °C water bath for 10 s, which resulted in a partial-thickness second degree skin burn involving 30% of the total body surface area. The degree of skin injury was verified by histopathological examination. The rats in the sham group were treated identically except that they were dipped in a 25 °C water bath for 10 s. All animals were then resuscitated with physiological saline solution (20 ml/kg; s.c.).

Isolation of neutrophils
In the burn or sham groups, 2 h after the insult, oyster glycogen (1%) dissolved in 25 ml of phosphate-buffered saline (PBS; 10 mM K_2HPO_4 in 0.9 % NaCl, pH=7.4) was administered i.p. to rats under light ether anesthesia. Four hours later, that is 6 h after burn or sham trauma, the animals were exsanguinated, and cells in the peritoneum were removed by lavage with 30 ml of PBS containing 1000 U/L heparin. The suspension was centrifuged at 2000 rpm for 10 min, and erythrocytes were destroyed by lysis buffer containing 0.15 M NH_4Cl. Cells were resuspended in ice-cold PBS and centrifuged again. Finally, the pellet was suspended in 3 ml of PBS. Cells were stained with 0.01% trypan blue in PBS, and the cell counts were performed using a light microscope. None of the treatments altered the cell viability, which was >95%, as assessed by trypan blue exclusion (12).

Chemiluminescence measurement in isolated neutrophils
Cells (2×10^6/ml) were treated with increasing concentrations of α-MSH (10^{-12} M, 10^{-10} M, 10^{-8} M or 10^{-6} M). In another set of experiments, the nitric oxide donor, sodium nitroprusside ranging from 10^{-10} to 10^{-4} M was added to the cells in the presence of 10^{-6} M of α-MSH.

Chemiluminescence (CL) measurements were made at room temperature using Mini Lumat LB 9506 luminometer (EG&G Berthold, Germany) in the presence of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, 0.2 mM) or lucigenin (bis-N-methylacridinium nitrate, 0.2 mM). Counts were obtained at 15 s intervals for 10 min and the

results were given as area under the curve (AUC) / 2 X 10^6 cells / ml (13).

Statistical analysis

Values are expressed as the means ± S.E. One way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test was used for the analysis of the data. For the comparison of two different groups, the unpaired *t*-test was used. Values of *p<0.05* were regarded as significant. All calculations were done using Instat statistical analysis package (GraphPad Software, San Diego; CA).

RESULTS

The luminol CL level of the cells obtained from the burn group was not different from those of the sham group (data not shown). However, the lucigenin CL value of cells obtained from the burn animals was significantly higher than that of the sham group (3216.15 ± 385.20 AUC and 14181.54 ± 1769.10 AUC, respectively; p< 0.001) (Fig. 1). α-MSH added to the cells of the burn group, at doses of 10^{-10} M, 10^{-8} M and 10^{-6} M caused significant reductions in high lucigenin CL values (8489.73 ± 770.28 AUC, 9056.09 ± 695.26 AUC and 5871.0 ± 582.18 AUC,
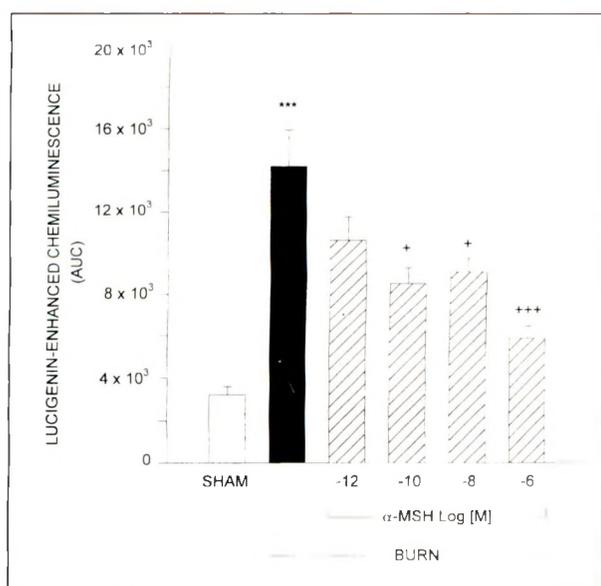
respectively; p< 0.05-0.001) (Fig. 1). In another set of experiment where the decrease in lucigenin CL value by α-MSH at a dose of 10^{-6} M was tested in the presence of different doses of sodium nitroprusside, it was observed that the NO donor at the dose of 10^{-7} M partly reversed this effect (8685.33 ± 1155.20 AUC; p< 0.05) (Fig. 2).
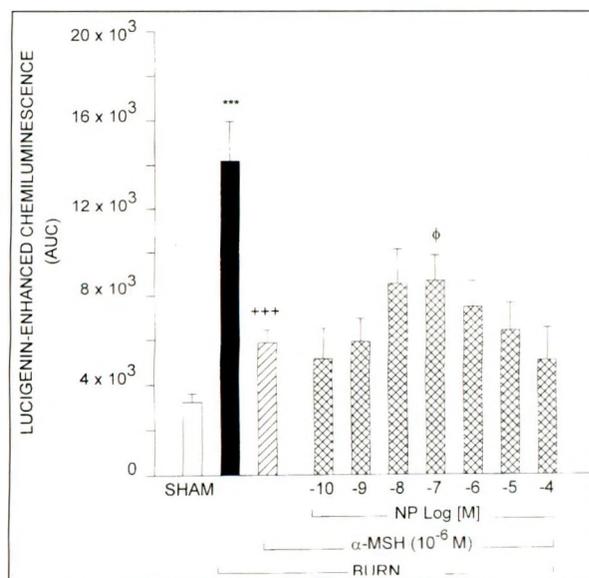

Fig.2 : The lucigenin-enhanced chemiluminescence values of peritoneal neutrophils (2 X 10^{-6} cells/ml) obtained from rats in sham (n=13) and burn (n=13) groups and the effect of the NO donor, sodium nitroprusside to α-MSH (10^{-6} M) treated cells. *** p< 0.001, compared to sham group. +++ p< 0.001, compared to burn group. φ p< 0.05, compared to α-MSH treated group.

DISCUSSION

The exudate obtained from the peritoneal cavity of rats 4 h after glycogen injection has been confirmed to contain >98 % neutrophils by us and others (12). It is well-known that stimulated neutrophils produce superoxide and other highly reactive oxygen species (ROS) capable of inducing cellular injury. Although it is difficult to quantitate reactive oxygen metabolites because of their reactive nature and short half lives, chemiluminescence (CL) used for the quantitation of ROS production in tissues and inflammatory cells is a simple but reproducible technique. The two CL probes, luminol and lucigenin, differ in selectivity. Luminol detects H_2O_2, OH^-, hypochlorite, peroxynitrite, and lipid peroxyl radicals, whereas lucigenin is particularly

Fig.1 : The lucigenin-enhanced chemiluminescence values of peritoneal neutrophils (2 X 10^{-6} cells/ml) obtained from rats in sham (n=13) and burn (n=13) groups and the effect of α-MSH added to the cells of the burn animals. *** p< 0.001, compared to sham group. + p< 0.05 and +++ p< 0.001, compared to untreated burn group.

sensitive to superoxide radical (14, 15). In our study, the marked increase in the lucigenin CL values of rat peritoneal neutrophils following burn trauma indicates that local burn insult activates neutrophils to produce reactive oxygen species, mainly the superoxide radicals.

Intraperitoneal injection of the neuropeptide α-MSH inhibits the synthesis and actions of various cytokines (i.e. TNF-α, IL-1, IL-6, IL-8), NO production by murine macrophages via suppression of iNOS (1, 6) and blocks in vivo and in vitro migration of neutrophils (3-5). These are the prominent effects of the peptide as an anti-inflammatory agent. The inhibition of neutrophil chemotaxis by α-MSH is believed to be the result of stimulation of MC1 receptors expressed on neutrophils.

In our study, α-MSH caused significant reductions in lucigenin CL values of peritoneal neutrophils which were activated by burn insult. These data demonstrate that α-MSH is effective in the inhibition of superoxide radical production by activated neutrophils due to burn insult and are in accordance with the previous findings showing the inhibition of neutrophil chemotaxis by the peptide.

The reduction in lucigenin CL values by α-MSH raised the possibility that α-MSH could have a direct antioxidant effect. In an attempt to clarify this possibility, we compared the lucigenin CL values of xanthine-xanthine oxidase in the absence and presence of α-MSH (10^{-10} – 10^{-5} M) in another experimental setup. Our results revealed that the peptide (10^{-9} – 10^{-6} M) decreased the lucigenin CL values significantly (author's unpublished observation). Although this may suggest that α-MSH shows superoxide scavanging activity per se, it does not exclude the possibility that the peptide might decrease reactive metabolite production via inhibiting the actions of proinflammatory cytokines (i.e. TNF-α, IL-1, IL-6, IL-8).

It has been widely proposed that NO produced by cNOS has cytoprotective actions, while excessive NO produced by iNOS exerts cytotoxic actions. Clancy et al, have demonstrated that NO can inhibit superoxide production from neutrophils by directly inhibiting NADPH oxidase, the enzyme system responsible for the oxidative

burst (16). Previous studies have shown that increased iNOS expression and peroxynitrite production play an important role in multiple organ damage following thermal injury (10, 11). Chen et al, have shown that the activity of iNOS increased 24 h after the injury and up to a maximum of twofold on postburn Day 2 and decreased thereafter (10).

In our study, high lucigenin CL values of cells from burn animals may suggest that the amount of superoxide generated by neutrophils probably exceeds the capacity of NO to scavenge them. α-MSH added to these activated cells might cause significant reductions in superoxide production by either its own scavenging effect or inhibiting the actions of proinflammatory cytokines. However, this effect is partially reversed by the NO donor, sodium nitroprusside. Although our data cannot reveal how this agent interferes with the actions of α-MSH, it is possible that the inappropriate release of NO from the NO donor can lead to cytotoxicity which results from direct cytotoxicity of the NO radical or a subsequent metabolite such as peroxynitrite radical.

In summary, this study shows that the neuropeptide α-MSH suppresses superoxide production by activated rat neutrophils following burn insult by a mechanism which is attenuated in the presence of nitric oxide.

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