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Original Article

Effects of α-MSH on ischemia/reperfusion injury in the rat sciatic nerve

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Abstract

Background: Ischemia/reperfusion (I/R) causes the production of toxic free radicals and leads to pathological changes in nerve tissue. We investigated the effect of alpha-melanocyte stimulating hormone (α-MSH) in a rat model for sciatic nerve I/R and discuss the possible cytoprotective and antioxidant mechanism of α-MSH against ischemic fiber degeneration.

Methods: Experiments were performed using 42 adult male Wistar rats. Rats were divided into six experimental groups: control group, ischemia group, I/R groups, and α-MSH treated groups. Ischemia was produced by clamping of the femoral vessels. Immediately after ischemia that lasted 3 h, 75 μg/kg of α-MSH was administered subcutaneously before reperfusion and the tissue malondialdehyde (MDA) level was evaluated as an indicator of lipid peroxidation in groups with different reperfusion periods.

Results: The reperfusion injury did not begin in the first hour of reperfusion after 3 h of ischemia, and MDA levels increased on the first day of reperfusion. During the first day, blood MDA levels were decreased in the α-MSH group compared to the control group. The tissue from animals pre-treated with α-MSH showed fewer morphological alterations. Myelin breakdown was significantly diminished after treatment with α-MSH, and the ultrastructural features of axons showed remarkable improvement. Two-way analysis of variance was used for comparing three or more groups. When a significant difference existed, the *post-hoc* multiple-comparison test was applied to demonstrate the differences.

Conclusions: The results confirm that pre-treatment with α-MSH after ischemia protected the peripheral nerves against I/R injury.

Key Words: α-MSH, ischemia/reperfusion injury, lipid peroxidation, peripheral nerve, rat sciatic nerve

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INTRODUCTION

Ischemia plays an important role in the development of pathological changes in many different neuropathies. Peripheral nerves are well-vascularized structures that are perfused by independent intrinsic and extrinsic microvascular systems. A functional peripheral nerve needs aerobic energy pathways to continuously supply local energy. Therefore, decreases in blood flow lead to the depletion of high-energy phosphates, which results in conduction failure.[23] Diabetes mellitus, vascular occlusive diseases, compression injuries, entrapments, tourniquet-induced peripheral nerve injuries, necrotizing vasculitis, and trauma are only a few of the pathological conditions that result in neuropathy associated with peripheral nerve ischemia.[17,28] The extent and type of damage to the nerve fiber depends on the duration of ischemia and blood flow amount. Furthermore, decompression and restoration of an adequate blood supply to peripheral nerves may predispose the nerves to reperfusion injury.[1-3] Generation and accumulation of reactive oxygen species (ROS) provokes lipid peroxidation, protein oxidation, and DNA damage.[5,34,36,39] Ischemia/reperfusion (I/R)-related toxic substances interrupt the protective systems of the tissue, and cause lipid peroxidation and nerve fiber degeneration.[26,27,33] The peptide alpha-melanocyte stimulating hormone (α-MSH) is an anti-inflammatory, regulatory hormone made in the hypothalamus. It inhibits the activation and production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interleukin-2 (IL-2), and gamma-interferon from neutrophils, activated macrophages, astrocytes, and microglial cells.[6,9] In this study, we aimed to investigate the effect of α-MSH in a rat model for sciatic nerve I/R and discuss the possible cytoprotective and antioxidant mechanism of α-MSH against ischemic fiber degeneration.

MATERIALS AND METHODS

Drug

α-MSH was purchased from Sigma (no. M-4135 produced by Sigma, St. Louis, MO, USA), freshly dissolved in physiological saline, and used for all experimental groups at a dose of 75 μg/kg, subcutaneously (s.c). The dosage used was similar to those previously reported.[7] Rats that were not treated with α-MSH received an equal volume of saline (0.3 ml).

Experimental groups

Experiments were performed using 42 adult male Wistar rats weighing 210–250 g each. The rats were randomly and blindly assigned to six groups of seven rats per group [Table 1]. Animals were kept under regulated environmental conditions, fed with standard pellets and chow, and allowed to have tap water. All experimental procedures employed in the study were approved by the ethical committee of Mersin University (Mersin, Turkey).

Table 1: Experimental groups

Groups	Number of rats	Ischemia	Reperfusion	α-MSH treatment
Group 1	7	-	-	-
Group 2	7	+	-	-
Group 3	7	+	1 h	-
Group 4	7	+	1 h	+
Group 5	7	+	1 day	-
Group 6	7	+	1 day	+

+: Presence of treatment, –: Absence or no treatment

Group 1: Animals were used as sham-operated controls going through laparotomy without I/R.

Group 2: Animals used had only ischemia and were subjected to interruption of blood flow without reoxygenation.

Group 3: Animals were subjected to I/R for an hour without any drug treatment.

Group 4: Animals subjected to I/R injury, like Group 3 animals, were injected with α-MSH immediately before reperfusion.

Group 5: Animals were subjected to ischemia followed by reoxygenation period for a day.

Group 6: Animals subjected to I/R injury, like Group 5 animals, were injected with α-MSH immediately before reperfusion.

I/R injury, drug treatment, and preparation of samples

All animals were anesthetized with an intraperitoneal (i.p.) injection of ketamine-HCl (50 mg/kg, Parke Davis, Istanbul, Turkey) and xylazine (5 mg/kg, Bayer, Istanbul, Turkey) on the day of the experiment. Sciatic nerve ischemia was induced using the method described previously.[31] Right femoral vessels were exposed via an inguinal incision and were dissected free from the femoral nerve under an operating microscope. The trifurcation of the sciatic nerve into the peroneal, tibial, and sural branches was rendered almost completely ischemic via occlusion of the femoral artery and vein for 3 h with a Yaşargil microvascular clamp (Standard aneurysm clamp, FE751; Aesculap, Tuttlingen; Germany). In the reperfusion groups, the vascular clamp was removed after 3 h of ischemia and the sciatic nerve was reperfused for periods varying from 1 h to 1 day. The body temperature of all animals was maintained at 37 ± 0.5°C with a heating lamp during the experimental operation. Physiological parameters such as blood pressure and blood gases were monitored during the procedure and there was no abnormality in any group. Following reperfusion, the rats were sacrificed and sciatic nerve tissues were isolated. A sciatic nerve segment located more proximally at 2 cm length was harvested from each animal. Samples of the

sciatic nerve were stored at −40°C until the analysis for malondialdehyde (MDA) content was performed.

Biochemical measurement

MDA levels were measured using high-pressure liquid chromatography (HPLC) method. HPLC is a form of column chromatography frequently used in biochemistry and analytical chemistry to separate, identify, and quantify compounds. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvents used. MDA can then be measured by fluorescence detection in the HPLC system, which is the gold standard for assaying MDA levels. Nerve tissues devoid of surrounding connective tissue were dissected from freeze-dried samples. Plasma, standard and control solutions were pipetted into light-protected vials in 100 μl aliquots. Next, 500 μl of reactive precipitation solution was added and vortexed for 10 sec. After centrifugation at 1300 ×*g* for 5 min, 500 μl of supernatant was transferred to a new vial with a lid and mixed with 100 μl of derivatization reagent. This mixture was then incubated at 95°C for 60 min and subsequently cooled to room temperature. Finally, 500 μl of neutralization solution was added and mixed carefully.

The MDA levels of samples were assessed with the HPLC system (Chromosystems diagnostic GmbH, Munich, Germany). MDA levels were measured and expressed as nmol/mg of protein. The investigators who performed these measurements were double blinded to the experiment..

Histological assessment

The sciatic nerves were prefixed in 3% glutaraldehyde in Sorensen's phosphate buffer and then postfixed in 1% osmium tetraoxide in the same buffer. They were dehydrated in a graded alcohol series and embedded in Spurr's resin. Semi-thin sections of 0.6–1 μm thickness were stained with toluidine blue. After examination of the semi-thin sections, ultrathin sections were cut to a thickness of 200 Å. These sections were stained with uranyl acetate and lead citrate. The specimens were examined on a Carl Zeiss EM 900 electron microscope (Carl Zeiss, Oberkochen, Germany). The image analysis program Image-Pro Plus (Media Cybernetics Inc., Silver Spring, MD, USA) was used for morphological evaluation of the samples. The axonal damage or destruction, fiber degeneration, and degenerative changes in Schwann cells were assessed in a qualitative fashion. The investigators who performed these measurements were double blinded to the experimental design.

Data analysis

All data were obtained and originally analyzed using SPSS 10.0.1 for Windows (SPSS Inc., Chicago, IL, USA) by researchers who were blinded to the treatment the rats received. For comparing differences between three or more groups, two-way analysis of variance was used. When a significant difference existed in the analysis of variance, a *post-hoc* multiple-comparison test was applied to demonstrate the differences. Data are presented in the text as mean ± SD and $P < 0.05$ was accepted as statistically significant.

RESULTS

Administration of α-MSH before reperfusion did not result in any significant physiological disruption and none of the animals in any experimental group died during the experiment. Furthermore, hemorrhage or postoperative wound infection was not noted for any animal. According to two-way analysis of variance, the effect of α-MSH administration varied depending on the time points during the observation period for measurements of MDA. Therefore, for each time point, comparisons were performed individually between α-MSH treatment groups, ischemia, and I/R control groups.

MDA levels in sciatic nerve

The MDA levels of nerve tissue segments in different groups are presented in Figure 1. After 3 h of ischemia (Group 2), tissue MDA levels were not elevated in comparison with the control group (Group 1) ($P > 0.05$). After 3 h of ischemia and a 1-h reperfusion period, tissue MDA levels were not raised in rats either with (Group 4) or without (Group 3) α-MSH treatment ($P > 0.05$). MDA levels in Group 5 animals increased dramatically after the 1-day reperfusion period. The MDA levels treated with α-MSH (Group 6) were significantly decreased ($P < 0.05$) compared with Group 5 rats that did not receive α-MSH treatment after the 1-day reperfusion

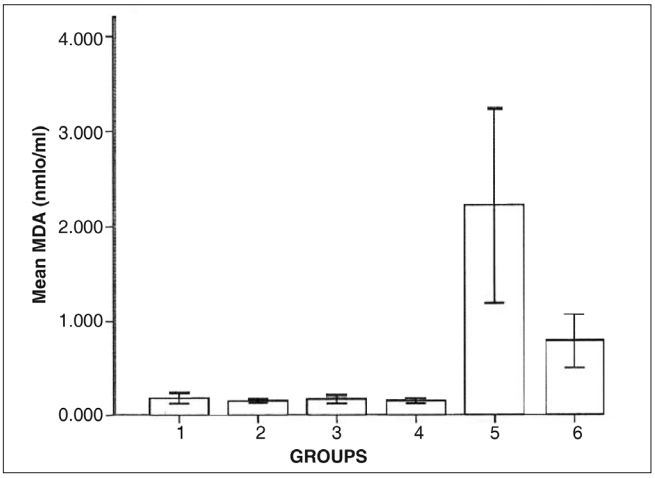

Figure 1: MDA levels for experimental groups. After 1 day of reperfusion, MDA levels in the α-MSH treated group were significantly decreased ($P < 0.005$)

period. These results clearly demonstrate that the I/R-induced elevation in tissue MDA concentrations is largely attenuated by α-MSH treatment after a 1-day reperfusion period, in contrast to those animals subjected to only I/R ($P < 0.05$). While MDA levels were not raised after 3 h of ischemia followed by reperfusion for 1 h, they were raised in Group 5 ($P < 0.005$). A visual comparison of the extent for Group 6 animals with animals of Groups 1–4 suggests that there is a difference between the latter and Group 6 animals, although α-MSH administration does reduce MDA levels compared with Group 5 animals, and there is still an increase compared with animals of Groups 1–4. This indicates that lipid peroxidation initiated at some point between 1 h and 1 day in our I/R injury model. Importantly, no statistical difference in the MDA content was observed between sham-operated control rats and α-MSH treated animals ($P > 0.05$).

Histopathologic changes

The axons, both myelinated and unmyelinated fibers, and Schwann cells all showed normal ultrastructural features in the control rats (Group 1) [Figure 2a]. However, I/R caused axonal damage in the majority of the myelinated fibers in Group 5 rats. Additionally, axonal shrinkage and swollen axons were common. The most striking morphological changes that occurred in the axonal myelins were vacuolization and lamellar separation. Total axonal destruction as well as a honeycomb appearance of the fiber was evident in some neurons. Degenerative changes were also observed in Schwann cells, including vacuolization in the cytoplasm. Fiber degeneration was associated with endoneurial edema. There was also vacuolization and degeneration of some unmyelinated fibers as well [Figure 2b]. Histologic evaluation of tissue from animals pretreated with α-MSH showed fewer morphological alterations in Group 6. Myelin breakdown was significantly diminished after treatment with α-MSH, and the ultrastructural features of axons showed remarkable improvement. Vacuolization and lamellar separation of the axonal myelin was less obvious. Furthermore, the fine structure of Schwann cells appeared normal [Figure 2c]. There were no significant changes in the ultrastructure of unmyelinated fibers.

DISCUSSION

Brutal nerve ischemia causes low energy supply that progresses to nerve conduction failure and fiber degeneration.[7,29,33] Reperfusion creates oxidative damage in vascular endothelial cells and destroys the blood–nerve barrier located between the perineurium of peripheral nerves and the vascular endothelium of endoneurial capillaries. As a result, nerves sustain several pathological abnormalities, such as axonal degeneration and regeneration, demyelination, and diffuse or local nerve fiber edema.[27] This kind of damage is more severe in peripheral nerves than in the brain because peripheral nerves have a low metabolic rate, a high amount of intraneural–extraneural anastomoses, and reperfusion can produce energy by anaerobic pathways.[10,19,22,29] There is no standard experimental model for I/R injury in peripheral nerves. Korthals *et al.* ligated the aorta and femoral artery of cats and demonstrated ischemic changes such as axoplasmic decelerating, accumulation of organelles as well as central fasicular necrosis in the distal sciatic, tibial, and peroneal nerves. In a rabbit experimental model,[20] Hess *et al.* demonstrated that ligation of internal and external iliac arteries together or individually could constitute an ischemic lesion in the sciatic nerve or its branches.[15] Schmelzer *et al.* suggest an improved model in which they first ligated the collateral

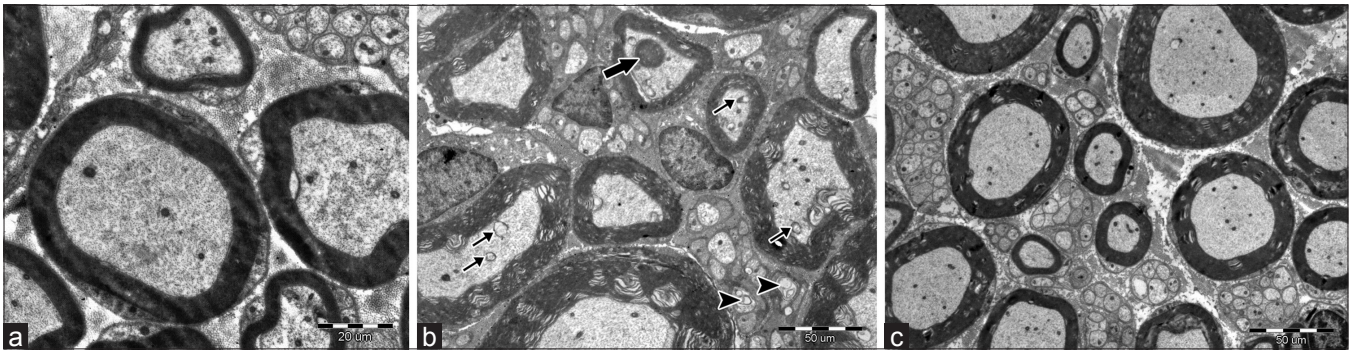

Figure 2: (a) A micrograph demonstrating fine structural features of a sciatic nerve from a control rat (Group 1). Myelinated and unmyelinated fibers show normal ultrastructural features. The cytoplasm of the axon appears unremarkable and demonstrates no morphological abnormality. Schwann cells also show normal ultrastructure (uranyl acetate and lead citrate, 2000×). (b) An electron micrograph from a rat sciatic nerve after ischemia/reperfusion (Group 5). There are many vacuoles on the myelin sheath of axons. Axonal shrinkage (arrows) is seen in some axons. Vacuolization in the cytoplasm of some Schwann cells is clear. There is vacuolization and degeneration on the unmyelinated fiber (arrow head) (uranyl acetate and lead citrate, 2000×). (c) An ultrastructural picture showing a section from the sciatic nerve of a rat that was treated with α-MSH before reperfusion (Group 6). Vacuolization on the myelin sheath decreased remarkably. Axonal shrinkage and vacuolization of Schwann cells are evident only in a few fibers. Unmyelinated fibers seem to be normal (uranyl acetate and lead citrate, 2000×)

artery, followed by temporary occlusion of the abdominal and bilateral iliac arteries, which subsequently triggered an ischemic event.[33] I/R injury is known to produce reactive oxygen species (ROS) in the sciatic nerve.[27] ROS oxidize membrane lipids, proteins, and DNA, thereby resulting in cellular dysfunction and even in cell death.[39] I/R of the sciatic nerve as induced in the present study very likely led to the generation of ROS, which stimulated lipid peroxidation. ROS have been implicated in lipid peroxidation events that are critical to the axonal degeneration that occurs following I/R. The I/R injury was assessed by measurement of increased levels of MDA, a secondary product of lipid peroxidation that is widely used as an indicator of lipid peroxidation by oxygen free radicals.[3] MDA is a formed as a degradation product downstream of conjugated dienes and hydroperoxides, and is frequently employed in studies on peripheral nerves as well as other tissues.[11,18] In a previous study, ischemia was maintained for 1 h and ischemic conduction failure occurred in 30 min, followed by recovery with reperfusion. After 3 h of ischemia, however, recovery with reperfusion was evident, but insufficient, while 7 h of ischemia resulted in total necrosis.[33] Ischemic events could occur in peripheral nerves as a result of several causes including trauma, iatrogenic factors, and medical reasons. Sometimes reperfusion can allow these peripheral nerves to recover from ischemia without suffering functional or anatomical damage. When the ischemia and reperfusion periods were over a critical time (3 h), free radicals formed and targeted cell membranes, subsequently destroying intraneural microcirculation.[3,24] In the present study, lower MDA levels in α-MSH–treated nerves demonstrated the protective effect of α-MSH, which was marked within the 1-day reperfusion period. α-MSH is an ancient tridecapeptide with potent inhibitory activity in all major forms of inflammation and the capacity to precipitate regeneration after nerve injury.[16,35] It is a hormone that stimulates melanogenesis, facilitates learning and memory, and affects inflammatory and immune responses and peripheral nerve regeneration. Furthermore, it readily inhibits free radical-mediated lipid peroxidation both *in vivo* and *in vitro*, and acts as a hydroxyl radical scavenger.[13] The hydroxyl is sufficiently reactive to initiate lipid peroxidation after sciatic nerve injury.[4,12,35] *In vitro* studies have demonstrated that α-MSH stimulates axon growth by accelerating c-AMP (cylic adenosine monophospahte production in the spinal cord and dorsal root ganglion in embryonic culture.[16,37,38] Additionally, α-MSH also inhibits production of nitric oxide (NO) and TNF-α.[13] This property affords antioxidant protection during I/R by both reducing the levels of NO and lowering the generation of peroxynitrite, another reactant easily capable of initiating lipid peroxidation.[30,32] These indirect antioxidant actions of α-MSH may be significant in affording protection against neuronal injury.[13] α-MSH is known to have the ability to modulate CNS inflammation. After transient cerebral ischemia, it can reduce intracerebral proinflammatory cytokines, such as TNF and IL-6 levels in the brain. Melanocortins also have a proven neuroprotective effect that can ameliorate neurological function. Furthermore, treatment with α-MSH showed concomitant neuroprotection in the brainstem and pyramidal cell layer in the hippocampus.[7] It is well known that α-MSH provides protection against I/R injury of the kidney, myocardium, intestine, and brain.[8,9,14,21,25] Studies have repeatedly validated the protective action of α-MSH in kidney and myocardium I/R models. The results of the present study show that α-MSH has a neuroprotective effect in ischemic peripheral nerves as well.

α-MSH improves tissue functions in rats, in part, by reducing lipid peroxidation, limiting oxidative mitochondrial damage, restraining infarct volume, inhibiting NO synthesis, stimulating antioxidant enzymes, suppressing edema, and blocking excitotoxic damage.[8,9,14,21,25] Pathological alterations in ischemic nerves have been reported previously. We also know that reperfusion exacerbates the pathological condition of ischemia. We demonstrated very obvious morphological changes in myelinated fibers after the occurrence of I/R. We suggest that this might be due to fact that myelin, which is a rich source of lipids, is the main target of free radical-mediated lipid peroxidation during reperfusion. Our observations of sciatic nerves at the electron microscope level also suggest that α-MSH was effective in repairing or decreasing the morphological damage that occurs in peripheral nerves after I/R. In conclusion, α-MSH therapy promotes biochemical and morphological improvement in sciatic nerves that have been subjected to I/R. As a consequence of the therapy, lipid peroxidation was blocked, thereby protecting the peripheral nerve from I/R injury. We suggest that the neuroprotective effects of α-MSH are attributed to its direct and indirect antioxidant actions. We believe that further experimental studies are warranted to identify the mechanism of α-MSH and the optimum dosage.

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