# α-Melanocyte-stimulating Hormone Protects Against Renal Injury after Ischemia in Mice and Rats

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## Abstract

**Reperfusion after ischemia induces cytokines, chemoattractant chemokines, adhesion molecules, and nitric oxide (NO). The resultant neutrophil adherence and NO potentiates renal injury. α-Melanocyte-stimulating hormone (α-MSH) is a potent anti–inflammatory agent that inhibits neutrophil migration and production of neutrophil chemokines and NO. Since neutrophils and NO promote renal ischemic injury, we sought to determine if α-MSH inhibits renal injury in a model of bilateral renal ischemia. α-MSH significantly reduced ischemia-induced renal damage, measured by changes in renal histology and plasma blood urea nitrogen and creatinine in mice. α-MSH significantly decreased tubule necrosis, neutrophil plugging, and capillary congestion. Delay of α-MSH treatment for 6 h after ischemia also significantly inhibited renal damage. α-MSH also significantly inhibited ischemic damage in rats. To begin to determine the mechanism of action of α-MSH, we measured its effects on mediators of neutrophil trafficking and induction of the inducible isoform of NO synthase-II. α-MSH inhibited ischemia-induced increases in mRNA for the murine neutrophil chemokine KC/IL-8. α-MSH also inhibited induction of mRNA for the adhesion molecule ICAM-1, which is known to be critical in renal ischemic injury. α-MSH inhibited nitration of kidney proteins and induction of NO synthase-II. We conclude: (*a*) α-MSH protects against renal ischemia/reperfusion injury; and (*b*) it may act, in part, by inhibiting the maladaptive activation of genes that cause neutrophil activation and adhesion, and induction of NO synthase. (*J. Clin. Invest.* 1997. 99:1165–1172.) Key words: acute renal failure • inflammation • neutrophils • KC/IL-8 • nitric oxide • intracellular adhesion molecule-1**

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## Introduction

The mortality of acute renal failure has remained high over the last 50 yr despite advances in supportive care (1). Ischemic damage during kidney transplantation is responsible for a 20–30% incidence of delayed graft function nationwide and may increase the incidence of acute and chronic rejection (2). Dialysis, the only FDA-approved treatment for acute renal failure, can also cause renal injury that prolongs the renal failure (3). Thus, better therapeutic approaches are desperately needed, especially treatments that are effective when administered after the initial insult.

The mechanisms of renal injury were previously classified using a hemodynamic/hydraulic paradigm to explain the reduced glomerular filtration rate that is a hallmark of acute renal failure. These include vascular effects (vasospasm, reduction in glomerular filtration coefficient) and tubular events (tubular obstruction or backleak). However, more recent studies have suggested a cell injury paradigm. Whereas prolonged ischemia causes anoxic cell death, recent evidence suggests that sublethal injury is amplified by inflammatory and cytotoxic injury cascades activated during the reperfusion period (1, 4). The inflammatory component consists of the elaboration of cytokines (i.e., IL-1, TNFα), chemoattractive chemokines (IL-8), and display of adhesion molecules (ICAM-1)[1] on the vascular endothelium that cause neutrophils to accumulate in the vasa recta in the outer stripe of the outer medulla (5–7). This causes plugging of the capillaries and the resultant vascular congestion can enhance ischemic injury. Inhibition of neutrophil action (by neutrophil depletion, antibodies to ICAM-1, or ICAM-1 knock out) protects mice and rats against renal ischemic injury (6, 8–11). The cytotoxic component may involve reactive oxygen radicals or nitric oxide (NO). It has been recently shown that inhibition of NO synthesis protects isolated proximal tubules from hypoxic injury (12). Furthermore, inhibition of induction of inducible NO synthase (iNOS) protects cultured proximal tubule cells from hypoxic damage in vivo and protects against ischemic injury in vivo (4, 13). Thus, renal ischemic injury in experimental animals can be decreased by inhibiting either inflammatory or NO pathways.

α-Melanocyte-stimulating hormone is a neuropeptide with broad anti–inflammatory properties that inhibits tissue injury in a wide array of experimental models of inflammation (14). α-MSH inhibits cellular infiltration in a rat model of arthritis and a mouse model of hepatic injury from septic shock (15,

1. *Abbreviations used in this paper:* BUN, blood urea nitrogen; ICAM-1, intracellular adhesion molecule; iNOS, inducible isoform of NO synthase; MSH, melanocyte-stimulating hormone; PAS, periodic acid-Schiff; PST, proximal straight tubule.

16). α-MSH improves survival in an experimental model of endotoxemia and peritonitis (17). α-MSH inhibits the synthesis and action of cytokines and chemoattractive chemokines. For example, α-MSH inhibits the production of IFN-γ, TNFα, and the mouse KC homologue of interleukin-8 (KC/IL-8), all of which are chemoattractive for neutrophils (14, 17). α-MSH also directly inhibits neutrophil function; for example, it inhibits neutrophil migration through an IL-8 gradient (18). α-MSH is also a potent inhibitor of the induction of iNOS in cultured RAW cell macrophages, and it also inhibits induction of NO production in a sepsis model (16, 19). Since α-MSH inhibits both inflammatory and nitric oxide pathways, we hypothesized that α-MSH may be more effective in preventing renal damage after ischemia than agents that only inhibit one such pathway.

In this study, we examined the effects of α-MSH on renal damage from ischemia. Renal damage was assessed by measurements of plasma blood urea nitrogen (BUN) and creatinine and by changes in renal histology. We also sought to determine the mechanism by which α-MSH acts. Because of the prominent role of neutrophils in ischemia/reperfusion injury, we focused on a representative neutrophil chemokine (KC, the mouse isoform of IL-8) and a neutrophil adhesion molecule (ICAM-1) expressed on the surface of vascular endothelial cells. Since α-MSH inhibits NO production, we also measured the effects of α-MSH on renal NO and inducible NO synthase.

## Methods

*Animals.* Female BALB/c mice, age 7–8 wk (20–22 g), and male Sprague Dawley rats (200 g) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). All animals had free access to water and food (4% mouse-rat diet; Harlan Sprague Dawley Inc.). Animal care followed the criteria of the University of Texas Southwestern Medical Center for the care and use of laboratory animals in research.

*Chemicals.* α-MSH was purchased from Phoenix Pharmaceuticals, Inc. (Mountain View, CA); sterile PBS was from GIBCO BRL (Gaithersburg, MD); Naphthol AS-D Chloroacetate Esterase Stain kit (91-C; Sigma Chemical Co., St. Louis, MO); Periodic Acid-Schiff (PAS) kit (395-8; Sigma Chemical Co.), 10% formalin (Fisher Scientific Co., Pittsburgh, PA), and LPS from *Escherichia coli* 08:B (Difco Laboratories Inc., Detroit, MI). All other chemicals were purchased from Sigma Chemical Co.

*Surgery and experimental protocol.* The mice and rats were anesthetized with an injection of 100 mg/kg ketamine, 10 mg/kg xylazine, and 1 mg/kg acepromazine i.m. The abdomen was shaved and the animals were placed on a micropuncture heating table kept at 39°C to maintain constant body temperature. Blood (100 μl) was collected through a tail vein. A midline incision was made and both renal pedicles were cross-clamped for 40 min. Both kidneys were inspected for ischemia after 2 min. To help maintain thermoregulation during surgery, the abdomen contents were replaced and the abdomen was temporarily closed with several sutures. α-MSH or vehicle was given at the midpoint of the ischemic period, or after reperfusion as described below. The abdomen was reopened and the clamps were removed. The kidneys were again inspected for restoration of blood flow, and 1 ml of prewarmed (37°C) normal saline was instilled into the abdominal cavity. The abdomen was closed in two layers, and the animals were placed in a 29°C incubator overnight. Blood samples were obtained from a tail vein every 24 h. The animals were killed at 4, 24, or 72 hrs. At the time of killing, blood was collected for measurement of plasma BUN and creatinine. Both kidneys were harvested for histological study or RNA and protein analysis.

The animals were divided into three groups: sham, ischemia/PBS, and ischemia/α-MSH. Sham-treated animals went through the same surgical procedure as the other animals, including dissection of the renal pedicle; however, renal clamps were not applied. The PBS- and α-MSH–treated animals received vehicle (PBS), 25 μg (mice), or 50 μg (rats) α-MSH intravenously during the ischemic period, at 6 and 18 h postperfusion, and then every 24 h thereafter. The total volume of the injected solution was 0.3 ml. Sham mice also received PBS injections at the indicated times. In some animals, the initial dose of α-MSH was given 0.25, 1, 2, 3, or 6 h after the clamps were removed.

*BUN and creatinine.* Plasma BUN was measured by using a Jaffee colorimetric method (66-20; Sigma Chemical Co.) and read at 340 nm. Creatinine was measured by an Astra 8 autoanalyzer (Beckman Instruments, Inc., Fullerton, CA).

*RNA extraction and Northern blot hybridization.* Renal total RNA was purified using guanidinium thiocyanate-phenol-chloroform extraction (20). Samples of total RNA (20 μg) were fractionated via electrophoresis on 0.9% agarose-formaldehyde gel and transferred onto a nylon membrane. The equality of RNA samples after transfer to the membrane was substantiated by ultraviolet illumination of ethidium bromide. Membranes were fixed by baking at 80°C, and then prehybridized at 42°C in 50% formamide, 0.5% SDS, 5× Denhardts, 5× SSC, and 0.5 mg/ml salmon sperm DNA. The membranes were hybridized with [$^{32}$P]dCTP-labeled cDNA clones. 0.5–2 kB portions of mouse KC/IL-8 and ICAM-1 were generated by PCR, and the products confirmed by automated sequencing. The hybridized membranes were washed twice in 0.1× SSC and 0.1% SDS at 50°C. Loading of RNA was normalized by rehybridizing with GAPDH.

*Histological examination.* Formalin (10%)-fixed and paraffin-embedded kidney specimens were stained with hematoxylin and eosin (2 μm), naphthol AS-D chloroacetate esterase (3 μm), or PAS (2 μm) stains. Histological changes were evaluated by quantitative measurements of neutrophil infiltration, tissue necrosis, and erythrocyte congestion. Neutrophil infiltration was evaluated using naphthol AS-D chloroacetate esterase staining by counting the number of neutrophils present in glomeruli, cortex, outer stripe, inner stripe, and inner medulla. Data are expressed as neutrophils per 100 glomeruli (glomeruli) or neutrophils per square millimeter (other portions of the kidney). Tubular necrosis was measured by counting the number of necrotic and nonnecrotic cells in each zone of the kidney. Vascular congestion was evaluated by counting the number of erythrocytes seen in each zone of the kidney. Each parameter was evaluated on 10–20 (necrosis and congestion) or 50–100 (neutrophils) high power fields per section, two sections per animal from four independent animals for each treatment (sham, ischemia/PBS, ischemia/α-MSH).

*Western blot analysis.* Since the major histological damage due to ischemia is in the outer stripe of the outer medulla, the entire outer medulla was carefully dissected on ice. Kidney tissue was homogenized in ice-cold homogenization buffer (50 mM Tris, pH 7.5, 0.1% NP-40, 0.2 M NaCl, 1 mM EDTA, 50 μg/ml PMSF, 1 μg/ml pepstatin A, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). The protein content of the homogenate was determined according to the Bradford method. Protein (300 μg per lane) was fractionated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were incubated in 5% (wt/vol) nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h. The membranes were incubated with a primary antibody (in 1% dry milk), washed, and then incubated with horseradish peroxidase conjugated anti–rabbit or anti–mouse antibody (in 1% dry milk) for 1 h. The reaction products were visualized by using chemiluminescence (ECL, Amersham Corp., Arlington Heights, IL).

We used two primary antibodies: (*a*) A rabbit polyclonal antibody to the $NH_2$-terminal peptide from mouse iNOS (gift of Dr. Kim Lau, UT-Southwestern Medical Center) at a dilution of 1:5,000. (*b*) A mouse monoclonal antibody to nitrotyrosine (gift of Dr. Joseph Beckman, University of Birmingham, Birmingham, AL) at a dilution of 1:500. To assure specificity, we determined that the binding of the antibody to antigen was blocked by preincubation of the antibody

with nitrotyrosine, or preincubation of the filter with 1 M dithionite (pH 9), which reduces nitrotyrosine to aminotyrosine (19, 21).

*Statistical analysis.* All data were described as mean±SEM. Different treatments were compared using ANOVA techniques (completely randomized design) followed by Dunnett's test for individual comparisons between group means. The null hypothesis was rejected when $P < 0.05$.

## Results

*Functional protection of renal ischemia/reperfusion injury by α-MSH.* Fig. 1 shows the effects of α-MSH on plasma BUN in the initial group of mice. 40 min of renal artery clamping caused a significant decline in renal function, as measured by BUN of 220±31 mg/dl at 24 h. The placebo group had a high death rate (four of five animals died by 24–48 h, the fifth animal died during anesthesia). The α-MSH group was treated with α-MSH (25 μg each, i.v.) immediately before release of the renal clamps and at 6, 16, 24, and 48 h postrelease of clamps. The α-MSH–treated group had a BUN of 54±8 mg/dl at 24 h, with a mortality rate of one in seven animals (one animal died during anesthesia, both $P < 0.05$). Furthermore, the BUN values remained at baseline levels for 3 d. Thus, α-MSH inhibited the decline in renal function and also decreased mortality.

The initial group of animals were allowed to recover from surgery at room temperature, which may have delayed their recovery from anesthesia and thus worsened their renal injury. We found that the animals recovered faster when kept in a 29°C incubator overnight. With this modification, the experiment was repeated in a larger group of animals (Fig. 2). We found that α-MSH significantly reduced, or inhibited, increases in plasma BUN and creatinine measured 24 h after injury. There was no change of plasma BUN or creatinine in the sham group, or in animals receiving α-MSH alone (for example, BUN before α-MSH: 39.5±3.7 mg/dl, $n = 8$; after receiving α-MSH for 24 h: 39.6±1.9 mg/dl, $n = 8$; $P$ = NS). The effect of α-MSH was evident at 4-h reperfusion (sham BUN 47±10 mg/dl, $n = 8$; ischemia 78±17 mg/dl, $n = 7$; ischemia/α-MSH 35±8 mg/dl, $n = 8$; $P < 0.05$ vs. ischemia).

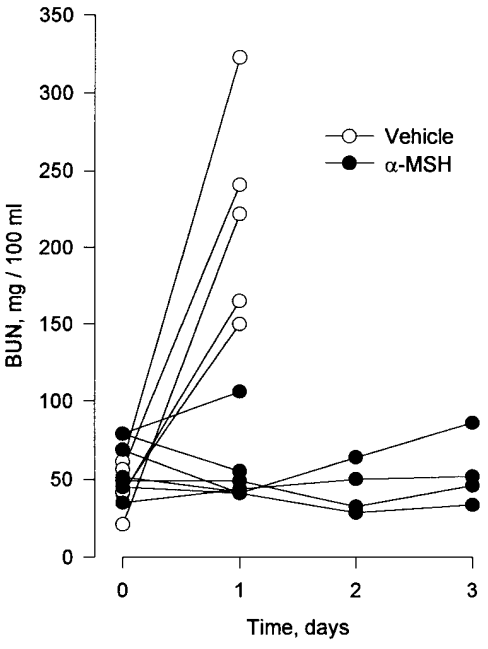


*Figure 1.* Effect of α-MSH on BUN and survival in renal ischemia. Mice were subjected to 40 min of bilateral renal ischemia, and then allowed to recover for up to 3 d. α-MSH or vehicle (PBS) was given immediately before and 6, 16, 24, and 48 h after release of renal clamps.

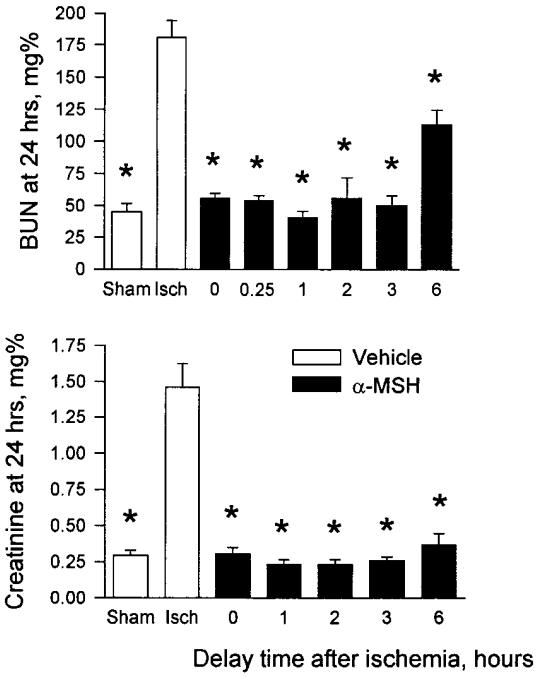


*Figure 2.* Effect of delay of α-MSH administration on plasma BUN and creatinine obtained at 24 h after ischemia. Mice were subjected to 40 min of bilateral renal ischemia (*Isch*), and then allowed to recover for 1 d. α-MSH was given at the indicated time, and at 6 and 16 h after release of renal clamps. Number of animals: sham (8), ischemia (18), 0 h (21), 0.25 (5), 1 (8), 2 (4), 3 (4), and 6 h (6). *$P < 0.05$ compared with the ischemia group. Data shown as mean±1 SEM.

*Histology.* To confirm that renal injury was present and to determine the timing of the injury, kidneys were processed for renal histology at 4 and 24 h after injury. After 24 h of reperfusion (Fig. 3), ischemic kidneys showed widespread necrosis and sloughing of the proximal straight tubule (S3 PST), with obstructing casts. There was also ischemic necrosis of medullary thick ascending limbs in the inner stripe of the outer medulla. There was neutrophil plugging of vasa recta in the outer stripe of the outer medulla (Fig. 4 *A*), and erythrocyte congestion and mild intrarenal extravasation in the inner stripe of the outer medulla and the inner medulla. In contrast, kidneys of mice treated with α-MSH had only patchy necrosis of the distal PST, dramatically decreased neutrophil plugging (Fig. 4 *B*), and no capillary congestion. Thus, α-MSH had a dramatic protective effect on renal histology. After 4 h of reperfusion, the ischemic kidneys showed widespread vascular congestion and mild tubular casts. The PST had dramatically decreased PAS staining of the brush border membrane, a few detached tubular cells, and patchy necrosis. In contrast, kidneys from α-MSH–treated animals had only a few casts and patchy congestion. The PST had moderately decreased PAS staining of the brush border membrane without detached cells or necrosis.

The histologic changes were quantified by counting the number of necrotic cells (expressed as a percentage of cell nu-

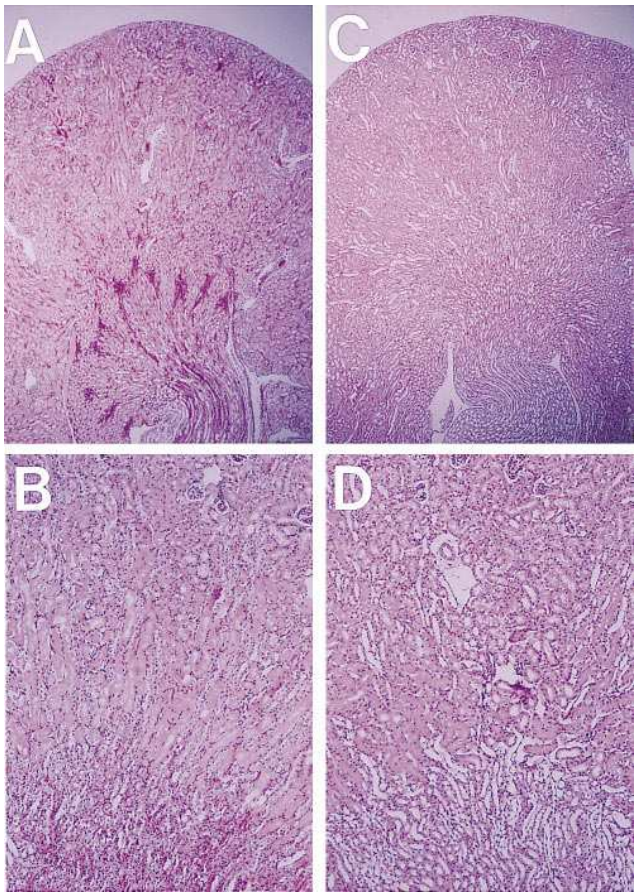


*Figure 3.* Effect of α-MSH on renal histology (hematoxylin and eosin) in mice subjected to 40 min of renal ischemia. Comparison of typical histology from animals treated with vehicle (PBS, *left*) or α-MSH (*right*). *A* and *C*: 10×, *B* and *D*: 20×. Animals were killed 24 h after treatment.

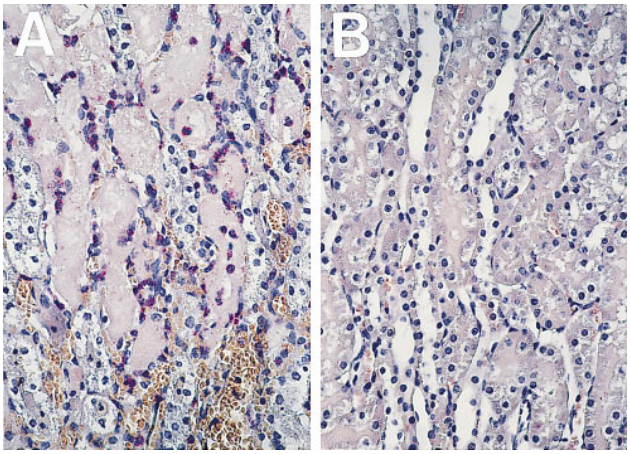


*Figure 4.* Effect of α-MSH on neutrophil plugging in mice subjected to 40 min of renal ischemia. Comparison of neutrophil (naphthol AS-D chloroacetate esterase) stain from animals treated with vehicle (PBS, *A*) or α-MSH (*B*). Animals were killed 24 h after treatment. Results shown are typical of four independent experiments.

clei), neutrophils, and erythrocytes. The data is shown in Table I and Fig. 5. α-MSH significantly reduced the cell necrosis, neutrophil infiltration, and capillary congestion at both 4 and 24 h.

*Effect of α-MSH in different experimental conditions.* To determine if α-MSH is effective at shorter ischemia times, we measured its action in mice subjected to 30 min ischemia (Table I). This protocol was chosen to replicate studies of Kelly et al. (6, 10) using anti–ICAM-1 antibody infusions, and ICAM-1 knock-out mice. We found that α-MSH was similarly protective (BUN at 24 h: Sham surgery, 46±1 mg/dl, $n = 2$; ischemia, 71±9 mg/dl, $n = 9$%; ischemia + α-MSH, 46±9 mg/dl, $n = 3$). Thus, α-MSH is also protective at this lesser amount of ischemia.

We next sought to determine if the protective effects of α-MSH are limited to mice. Therefore, rats were subjected to 40 min of renal ischemia, followed by 24 h of reperfusion. α-MSH also significantly decreased the elevation in BUN and creatinine measured 24 h after ischemia in rats (Fig. 6).

*Effect of delaying initial α-MSH administration.* Many agents inhibit renal injury in animals when given hours before or at the time of renal artery occlusion. From a clinical standpoint, α-MSH would be more useful if it was effective when started after ischemia. Therefore, we treated mice with PBS or α-MSH at 0.25, 1, 2, 3, and 6 h after release of the clamps. All animals also received α-MSH at 6 and 18 h postischemia. α-MSH was also effective when started 0.25, 1, 2, or 3 h into the reperfusion period (Fig. 2). Surprisingly, α-MSH was effective even when the first dose was delayed for 6 h after ischemia.

*RNA blot analysis of adhesion molecule and chemokine mRNA.* We next sought to determine how α-MSH acts in vivo. To determine if α-MSH alters signals for neutrophil activation and adhesion, we measured the changes of renal KC/IL-8 and ICAM-1 mRNA in mice by Northern blotting (Fig. 7). At 4 h after reperfusion, we found that ischemia increased renal KC/IL-8 mRNA abundance, confirming studies by Safirstein et al. (5). α-MSH inhibited the induction of KC/IL-8 at 4 h postperfusion. We found that the effect of α-MSH was still present at 24 h, although the degree of inhibition was only ∼ 50% (data not shown).

We also evaluated the effects of α-MSH on ICAM-1 induction. mRNA for renal ICAM-1 was visible at 1 h, peaked at 2 h, and remained elevated for 24 h after ischemia (data not shown), similar to that recently reported by Kelly et al. (6). We found that α-MSH inhibited the rise in renal ICAM-1 mRNA abundance in mice at 4 h postperfusion (Fig. 7).

*Effects of α-MSH on protein nitration and inducible NOS expression.* NO has been implicated as causing cytotoxicity during ischemia/reperfusion. To evaluate if α-MSH inhibits renal NO production that is stimulated by ischemia/reperfusion, we measured protein nitration of outer medullary proteins in mice. 40 min of ischemia followed by 24 h of reperfusion increased nitration of proteins extracted from the outer medulla (Fig. 8). The increased nitration was blocked by preincubation of antibody with excess nitrotyrosine, or by incubation of the filter with dithionite, which reduces nitrotyrosine to aminotyrosine (data not shown). α-MSH administration inhibited the ischemia-stimulated nitration of kidney proteins (Fig. 8).

To determine the locus of the effect of α-MSH on protein nitration, we performed Western blotting for iNOS using a rabbit polyclonal antibody to the $NH_2$-terminal portion of murine iNOS. We found that ischemia increased expression of iNOS in the outer medulla and that these changes were inhibited in animals treated with α-MSH (Fig. 9).

*Table I. Effect of α-MSH on Renal Histology*

| Treatment group | Cortex | Outer stripe | Inner stripe | Inner medulla |
|---|---|---|---|---|
| Tubular cell necrosis (%) | | | | |
| 4 h | | | | |
| Ischemia | 8.7±1.6 | 20.3±4.5 | 6.8±2.5 | 1.0±0.6 |
| Ischemia + α-MSH | 6.7±1.5 | 2.8±0.8* | 2.9±1.6* | 0.2±0.2* |
| 24 h | | | | |
| Sham | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| Ischemia | 9.9±1.3‡ | 53.3±3.0‡ | 26.5±4.8‡ | 2.9±1.6‡ |
| Ischemia + α-MSH | 3.4±1.6*‡ | 5.6±1.0*‡ | 2.0±1.2*‡ | 0.6±0.3*‡ |
| Neutrophils (per $mm^2$) | | | | |
| 4 h | | | | |
| Ischemia | 5.0±0.9 | 6.1±0.8 | 1.0±0.4 | 1.2±0.4 |
| Ischemia + α-MSH | 1.5±0.1* | 1.7±0.6* | 0.7±0.2 | 0.3±0.1* |
| 24 h | | | | |
| Sham | 0.2±0.1 | 0.1±0.0 | 0.1±0.1 | 0.0±0.0 |
| Ischemia | 18.7±5.3‡ | 382.1±99.4‡ | 116.7±57.0‡ | 27.0±18.3‡ |
| Ischemia + α-MSH | 1.1±0.1*‡ | 0.9±0.1*‡ | 0.4±0.2*‡ | 0.2±0.1*‡ |
| Erythrocytes (per hpf) | | | | |
| 4 h | | | | |
| Ischemia | 34.3±10.7 | 152.3±64.7 | 711.5±107.0 | 117.0±15.4 |
| Ischemia + α-MSH | 12.5±1.4* | 49.8±9.6* | 134.0±8.1* | 31.5±5.3* |
| 24 h | | | | |
| Sham | 14.5±4.0 | 40.0±12.2 | 104.0±35.8 | 30.0±4.1 |
| Ischemia | 81.5±28.6‡ | 169.8±65.6‡ | 1188.0±178.0‡ | 236.3±89.1‡ |
| Ischemia + α-MSH | 16.5±3.2* | 45.8±10.4* | 176.0±18.9* | 87.5±31.5* |

Renal histology was measured using quantitative scores as described in Methods. *$P < 0.05$ vs. ischemia; ‡$P < 0.05$ vs. sham by ANOVA with multiple comparisons ($n = 4$ animals per group); hpf, high power field.

## Discussion

Our data characterize the protective action of α-MSH in renal ischemia/reperfusion injury. There are three main findings: (*a*) α-MSH inhibits renal damage and improves survival from renal ischemia/reperfusion injury; (*b*) α-MSH inhibits maladaptive inflammatory and cytotoxic pathways activated during the reperfusion period; and (*c*) α-MSH is effective even when administered during the reperfusion period.

*α-MSH inhibits renal damage and improves survival from renal ischemia/reperfusion injury.* α-MSH protects against renal damage based on its ability to inhibit rises in plasma BUN and creatinine caused by ischemia (Figs. 1 and 2) and inhibits renal tubular cell necrosis (Figs. 3–5; Table I). The impressive effect of α-MSH was not limited to a specific experimental protocol, since α-MSH also protected against renal damage from 30 min of renal ischemia in mice and 40 min of ischemia in rats (Fig. 6). The ability of α-MSH to inhibit structural renal damage was striking. α-MSH dramatically decreased tubular injury at 4 and 24 h. In this model of ischemia, most of the tubular necrosis occurs in the proximal straight tubule in the outer stripe of the medulla. Kidneys with the most severe damage also showed necrosis of the cortical proximal straight tubule in the medullary rays and the medullary thick ascending limb in the inner stripe. In contrast, α-MSH–treated kidneys only had necrosis of proximal straight tubule in the most inner portion of the outer stripe (Fig. 3). A similar protective effect has been seen with anti–ICAM-1 antibodies and antisense oligonucleotides to iNOS (4, 10, 11). α-MSH is more effective than anti–ICAM-1 antibodies, since it largely inhibited increases in BUN or creatinine in the 40-min ischemia model, whereas anti–ICAM-1 antibodies only inhibited the rise in creatinine by 50% (10, 11). α-MSH was as effective as antisense oligonucleotides to iNOS, although the oligonucleotides had to be administered 8 h before ischemia, whereas α-MSH is effective when first administered 6 h after ischemia (4). In contrast, several growth factors, including insulin-like growth factor-1 and hepatocyte growth factor, only minimally inhibit renal damage, as assessed at 24 h, but accelerate the rate of repair of the injury (22–24). Whether α-MSH also accelerates repair is unknown, but possible since it works late in the reperfusion period, when there is already substantial renal injury.

α-MSH also had a statistically significant effect on survival in animals followed over 3 d. The untreated animals all died between 1 and 2 d, while the α-MSH–treated animals largely survived. The one death can be attributed to overzealous anesthesia very early during the study. α-MSH has been shown to increase survival in a peritonitis model of septic shock induced by cecal ligation and puncture (17); the survival advantage is similar to that of gentamicin. As will be discussed below, both ischemia and sepsis activate a systemic inflammatory response. Anti–ICAM-1 antibodies, which interrupt neutrophil adhesion to the endothelium, also improve survival under similar conditions (30- and 40-min ischemia in mice) (10). The ability of α-MSH to inhibit systemic and local inflammation may be important in improving survival in acute renal failure. If so, this could have important clinical consequences. The optimal dosing schedule for α-MSH is unknown. The plasma half-life of

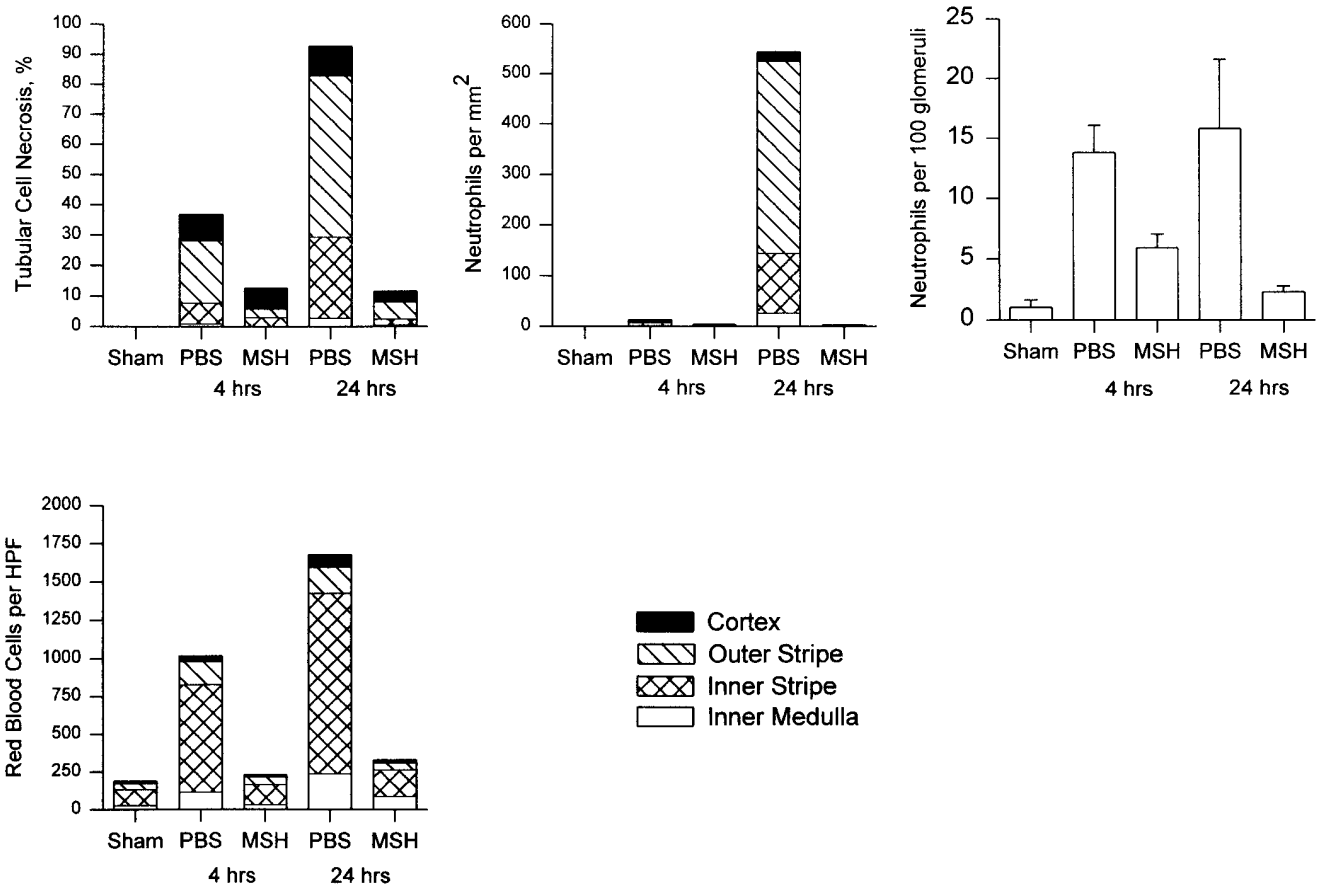


*Figure 5.* Effect of α-MSH on quantitative histological changes in renal ischemia. Kidneys ($n$ = 4 per group) were studied at 4 and 24 h after renal ischemia. α-MSH given as in Fig. 1.

α-MSH is 20–25 min in humans (25, 26). The biological half-life is unknown, although α-MSH was effective when given twice a day for 21 d in an arthritis model (15).

*α-MSH inhibits maladaptive inflammatory pathways activated during reperfusion.* The histologic data obtained in this study indicates that α-MSH inhibited neutrophil adherence/plugging of the vasa recta in the outer stripe of the outer medulla, and subsequent erythrocyte congestion in the inner stripe of the outer medulla and inner medulla. These effects were demonstrated at both 4 and 24 h after injury. Previous studies have shown that α-MSH inhibits neutrophil infiltration in intradermal and air-pouch models of acute inflammation. α-MSH also inhibits acute inflammation in a solid organ, the liver, after systemic LPS injection (16).

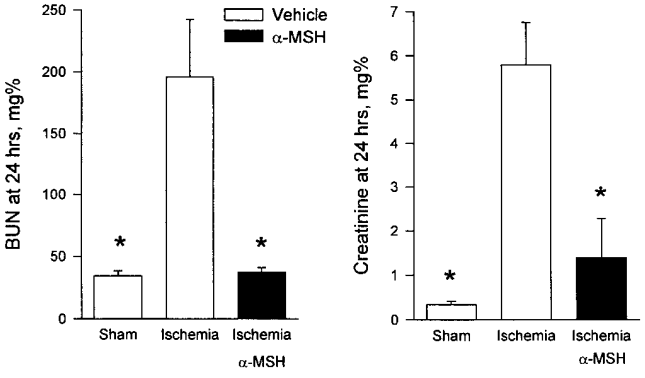


*Figure 6.* Effect of α-MSH administration on plasma BUN and creatinine obtained at 24 h after ischemia in rats. Rats were subjected to 40 min of bilateral renal ischemia, and then allowed to recover for 1 d. α-MSH or vehicle (PBS) was given immediately before and at 6 and 16 h after release of renal clamps. *$P < 0.05$ compared with the ischemia group (four to six animals per group).

We then focused on factors that cause neutrophil infiltration early in inflammation. Neutrophils are attracted to inflammatory sites by chemoattractant α-chemokines. IL-8 (or mouse KC) is the most potent neutrophil chemokine. Neutrophil adherence to endothelial cells is promoted by ICAM-1 expressed on endothelial cells. Indeed, both IL-8 and ICAM-1 are upregulated within 1–2 h by renal ischemia (5, 6). We confirmed that ischemia upregulates both KC/IL-8 and ICAM-1, and extended these findings to show that α-MSH inhibits induction of mRNA for both KC/IL-8 and ICAM-1. This was demonstrated at 4 h, before there are many neutrophils in the kidney (Fig. 5), suggesting that these events may be important to attract neutrophils to the kidney. We previously found that α-MSH inhibits KC/IL-8 induction before cellular infiltration in the liver during sepsis (16). In the current study, we also found that α-MSH inhibited induction of mRNA for ICAM-1. ICAM-1 is a neutrophil adhesion molecule that promotes neutrophil adhesion in the kidney after ischemia. Disruption of ICAM-1 activity (either by anti–ICAM-1 antibody or ICAM-1

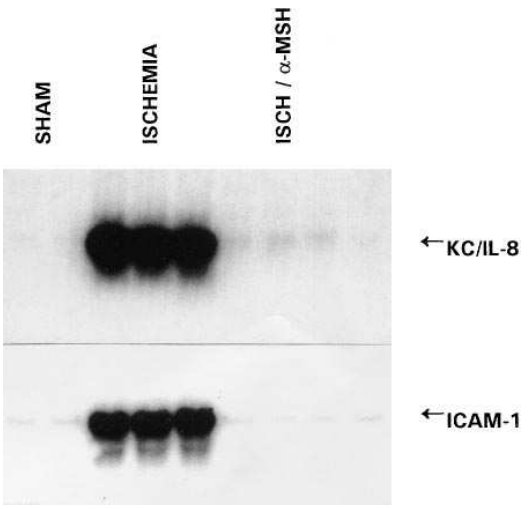


*Figure 7.* RNA blots of neutrophil chemokine and adhesion molecule mRNA abundance. Mice were subjected to sham surgery ($n = 2$), ischemia ($n = 3$), or ischemia + α-MSH ($n = 4$). Animals were killed 4 h after ischemia. Whole kidney total RNA was isolated and electrophoresed (10 μg/lane). These blots are representative of two independent experiments.

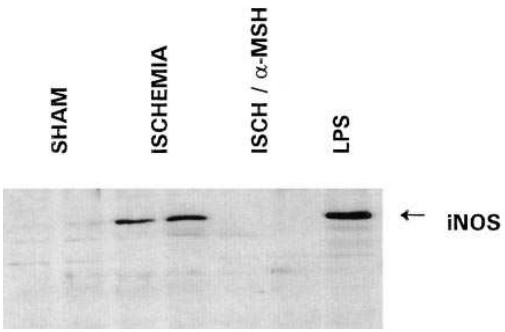


*Figure 9.* Western blot of inducible NOS in the outer stripe. Samples were prepared as in Fig. 8, except that one mouse was treated with LPS 24 h before killing. Proteins were extracted from the outer medulla and electrophoresed (300 μg/lane). The blot is representative of two independent experiments.

knock-out animals) reduces neutrophil infiltration and ischemic damage in rats and mice (6, 10, 11). Thus, α-MSH inhibits the activation of genes that promote neutrophil activation and adhesion.

At least some of the protective effects of α-MSH are likely to be via its direct effect on neutrophils. α-MSH is known to inhibit acute inflammation caused by intradermal injection of IL-8, and α-MSH inhibits in vitro neutrophil migration in an IL-8 gradient (18). Neutrophils express mRNA for the melanocortin-1 MSH receptor (18); this MSH receptor is found on macrophages and keratinocytes and is expressed in the liver (19, 27, 28). Taken together, the above findings indicate that α-MSH inhibits an acute inflammatory response that occurs after renal ischemia.

*α-MSH inhibits maladaptive cytotoxic pathways activated during reperfusion.* Since α-MSH inhibits induction of iNOS both in vitro and in vivo, we also focused on its effects of renal NO and NOS during ischemia/reperfusion. We used nitration of tyrosine on intrinsic kidney proteins as a "footprint" of NO, since nitrated proteins have been demonstrated in the places of high NO production (21), and we previously showed that protein nitration is modulated in concert with NO production (19). In the current study, we found that protein nitration is increased in renal ischemia and that α-MSH inhibited nitration of kidney proteins. Others have shown that NO production is increased in hypoxic cortical tubules ex vivo and cortical tubules obtained from ischemic kidneys (4, 12). Our studies extend these findings to suggest that there are elevated levels of peroxynitrite, a powerful cellular oxidant produced when high levels of NO react with superoxide. Peroxynitrite reacts with tyrosine to form nitrotyrosine, which is then detected by the antinitrotyrosine antibody (21). This is important since peroxynitrite is the NO metabolite that attacks proteins and kills brain cells stimulated with *N*-methyl-D-aspartic acid (29). Thus, we conclude that α-MSH inhibits NO production activated by renal ischemia.

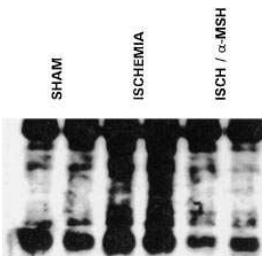


*Figure 8.* Western blot of protein nitration in proteins from the outer stripe. Mice were subjected to sham surgery ($n = 2$), ischemia ($n = 3$), or ischemia + α-MSH ($n = 4$). Animals were killed 24 h after ischemia. Each lane has 40 μg protein. The filters were blotted with an antinitrotyrosine antibody. The blot is representative of two independent experiments.

Since iNOS is the only NO synthase that produces high concentrations of NO, we next evaluated whether α-MSH inhibits iNOS induction. We found that iNOS is increased in ischemia, as shown by Noiri et al. (4). While we could not detect iNOS in protein extracts from whole kidney, we could easily detect iNOS in extracts from outer medulla. This indicates that the outer medulla is enriched in iNOS during ischemia. Furthermore, we found that α-MSH also inhibited the ischemic induction of iNOS. Since we previously found that α-MSH inhibits induction of iNOS in cultured mouse RAW cell macrophages and systemic production of NO during sepsis (16, 19), this suggests that these stresses induce iNOS via a common pathway that is inhibited by α-MSH. α-MSH is likely to inhibit the transcription of iNOS since iNOS is primarily regulated by transcription and α-MSH also inhibits induction of iNOS mRNA in RAW cells.

The finding that antisense oligonucleotides directed to iNOS inhibit tubular nitrate production and renal injury in a rat model of renal ischemia suggests that much, if not all, of the excess NO is produced by iNOS (4). That α-MSH also inhibits iNOS induction and NO production suggests that part of α-MSH's actions occur via inhibition of iNOS induction by ischemia. Whether the induction occurs by ischemia per se or by events initiated during the reperfusion period is unknown.

*α-MSH is effective when administered during the reperfusion period.* Because the morbidity and mortality of acute renal failure are so high, many agents have been tried as a prophylaxis to protect against acute renal failure to prevent further damage to existing renal failure, and to enhance repair mechanisms. The current clinical recommendations try to prevent renal injury: optimize volume status, avoid nephrotoxins, etc. In contrast, we found that α-MSH was protective even when administered 6 h after ischemia sufficient to cause irreversible

uremia and death in most of the animals. Administration of α-MSH during the reperfusion period significantly inhibited further damage that occurs during the reperfusion period. Thus, the renal injury after ischemia/reperfusion does not occur because of ischemia per se, but is a consequence of the maladaptive activation of genes such as IL-8, ICAM-1, and iNOS during reperfusion. If these genes are not activated, as occurs with α-MSH, then renal injury does not ensue. That α-MSH was partially protective at 6 h was impressive, since significant tubular necrosis was already present at 4 h postreperfusion (Fig. 5). Alternatively, α-MSH may promote recovery of cellular function, perhaps by preventing destruction of renal architecture that provides a scaffolding for cells to repair the damage during the recovery phase of acute renal failure. In previous studies, we found that α-MSH inhibited liver damage when given 30 min after LPS injection, although the results are more impressive in renal ischemia. While the reasons for the temporal differences are unknown, the liver model chosen was an accelerated liver injury model by which LPS was given to animals pretreated with *Corynebacterium parvum* to induce large aggregates of neutrophils and macrophages in the liver. This accelerated model therefore produced rapid liver injury, and α-MSH was effective after LPS injection, but only for a short while.

*Clinical significance.* The ability of α-MSH to work even when given after the ischemic period is clinically important, since physicians are not often present at the time of injury. Few agents work after injury has occurred. For example, calcium channel blockers work only if given during ischemia (30). IGF-1 modestly decreases renal damage, although it does increase renal regeneration (22, 23). Anti–ICAM-1 antibodies work when given 2 h after injury, but are not effective when given 8 h after injury (10). The broad mechanism of action on both inflammatory and cytotoxic pathways, and the extent of protection afforded even late in the reperfusion period, distinguishes α-MSH from other agents that protect against ischemia/reperfusion injury. This suggests that α-MSH may have important therapeutic effects in patients.

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