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Adrenocorticotrophic hormone ameliorates acute kidney injury by steroidogenic-dependent and -independent mechanisms

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Abstract

Adrenocorticotrophic hormone (ACTH) has a renoprotective effect in chronic kidney disease; however, its effect on acute kidney injury (AKI) remains unknown. In a rat model of tumor necrosis factor (TNF)-induced AKI, we found that ACTH gel prevented kidney injury, corrected acute renal dysfunction, and improved survival. Morphologically, ACTH gel ameliorated TNF-induced acute tubular necrosis, associated with reduction in tubular apoptosis. While the steroidogenic response to ACTH gel plateaued, the kidney protective effect continued to increase at even higher doses suggesting steroid independent mechanisms. Of note, ACTH also acts as a key agonist of the melanocortin system with its cognate melanocortin 1 receptor (MC1R) abundantly expressed in renal tubules. In TNF-injured tubular epithelial cells *in vitro*, ACTH reinstated cellular viability and eliminated apoptosis. This beneficial effect was blunted in MC1R-silenced cells, suggesting this receptor mediates the anti-apoptotic signaling of ACTH. Moreover, ACTH gel protected mice against cecal ligation puncture induced septic AKI better than α -melanocyte stimulating hormone; a protein equal in biological activity to ACTH except for steroidogenesis. Thus, ACTH has additive renoprotective actions achieved by both steroid dependent mechanisms and MC1R directed anti-apoptosis. ACTH may represent a novel therapeutic strategy to prevent or treat AKI.

Keywords

adrenocorticotrophic hormone; apoptosis; melanocortin; melanocortin receptor; acute kidney injury; tumor necrosis factor; rodent

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Acute kidney injury (AKI) is a complex and heterogeneous disease entity characterized by an abrupt loss of kidney function that could be ascribed to numerous causes. In the past 30 years, while progress is indeed being made in identifying biomarkers for early diagnosis of AKI,¹⁻³ management of AKI is still largely limited to general supportive measures^{4,5}; no specific intervention that either prevents or treats the injured kidney or improves survival has been successfully developed⁶. It is imperative to develop a novel, effective and pragmatic approach to prevent kidney injury or to promote kidney repair and regeneration.⁷

Adrenocorticotrophic hormone (ACTH) is an important component of the hypothalamic-pituitary-adrenal (HPA) axis.^{8,9} In the 1950's and the early 1960's, ACTH was widely used for the treatment of lipid nephrosis.⁹⁻¹¹ Recent clinical and experimental evidence suggests that ACTH has a renoprotective effect in chronic kidney diseases.^{9,11,12} Besides governing steroidogenesis, ACTH is also an important physiological agonist of the melanocortin system.^{9,13} This system comprises multiple components, including five class A guanine nucleotide-binding protein (G protein)-coupled melanocortin receptors (MCRs) MC1R~MC5R; endogenous antagonists; and peptide agonists derived from the anterior pituitary gland including α -melanocyte stimulating hormone (MSH), β -MSH, γ -MSH and ACTH.^{9,13} Accumulating data demonstrate that melanocortins,¹⁴ in particular α -MSH¹⁵ and its synthetic mimetics,¹⁶ possess a potent protective activity in acute injuries in multiple organ systems,¹⁷⁻¹⁹ including the kidney¹⁵. Nevertheless, although α -MSH has similar MCR agonizing potency as its parent molecule ACTH,^{9,13} the effect of ACTH on AKI has been barely investigated. This study examined the effect of ACTH gel (HP Acthar Gel, Questcor Pharmaceuticals, Hayward, CA), which is an FDA approved formulation of natural porcine ACTH, on a rat model of AKI induced by tumor necrosis factor (TNF)²⁰⁻²² and a murine model of septic AKI induced by cecal ligation puncture (CLP)^{16,23,24}. We found that ACTH has a kidney protective effect on AKI that is mediated by both steroid dependent and independent mechanisms.

RESULTS

ACTH gel improves survival of rats with TNF induced systemic organ injury

TNF is a pleiotropic prodeath and proinflammatory cytokine involved in the pathogenesis of multiple organ dysfunction and systemic inflammatory response syndrome.^{20,21} A single injection of exogenous recombinant TNF immediately disturbed systemic homeostasis and resulted in 40% mortality within 8 hours (Figure 1A). ACTH pretreatment reduced mortality and markedly improved general survival in a dose dependent fashion: even at low doses (2 IU/kg), pretreatment with ACTH increased survival to 80%; at higher doses ACTH treatment resulted in 100% survival from TNF stimulation (Figure 1A), suggesting a general beneficial effect of ACTH. To determine if ACTH gel therapy has a systemic beneficial action, mean arterial pressure (MAP) was measured and the injury of the liver and lung was assessed. TNF injection induced a profound drop in MAP and caused a hypotensive shock (Figure 1B). Furthermore, TNF elicited acute liver injury as evidenced by the elevated serum levels of hepatic enzymes including aspartate aminotransferase (AST) and alanine aminotransferase (ALT). TNF also augmented lung wet to dry weight ratios, in agreement with the formation of acute pulmonary edema. ACTH gel treatment (10 IU/kg) significantly

corrected the blood pressure, lowered serum AST and ALT levels (Figure 1C) and normalized lung wet to dry weight ratios (Figure 1D), signifying a general improvement in hemodynamics and systemic organ injury.

ACTH attenuates kidney injury and dysfunction in TNF injured rats

To examine the effect of ACTH on TNF induced kidney injury and to determine the minimal dose of ACTH gel that can maximally protect the kidney, fluorescence labeled viable rat alveolar macrophages (RAM) were infused into the carotid artery of some animals 60 minutes before they were sacrificed.²² Tissues were processed for fluorescent microscopic analysis. As shown in Figure 2A, many fluorescent RAM cells were sequestered in kidneys of TNF injured animals, most prominently in the interstitium of juxtamedullary cortex. Very few were found in kidneys from rats treated with saline or ACTH gel alone. ACTH gel treatment remarkably prevented the sequestration of RAM in the kidney, denoting a kidney protective and anti-inflammatory effect. The amount of RAM infiltration was quantified by fluorometric analysis of homogenized kidney tissues, which revealed a dose dependent inhibitory effect of ACTH gel on fluorescent RAM sequestration in TNF injured kidney, consistent with the morphologic observations (Figure 2B). Because no further reduction in macrophage sequestration in the kidney was noted in animals treated with ACTH gel at higher doses than 10 IU/kg, the dose of 10 IU/kg was thus adopted as the optimal dose of ACTH gel for treatment of kidney injury in the following studies. At this dose, ACTH gel treatment also remarkably prevented the TNF induced sequestration of RAM in the liver and lung shown by fluorometric analysis of liver and lung homogenates (Supplemental Figure 1A). This was further corroborated by direct fluorescent microscopy of lung and liver specimens (Supplemental Figure 1B), consistent with a systemic protective effect of ACTH. In order to further validate the renoprotective action of ACTH gel, kidney function was estimated by the glomerular filtration rates measured by inulin clearance assay and demonstrated that ACTH gel significantly improved acute kidney dysfunction (Figure 2C). Moreover, ACTH gel treatment also prominently diminished microalbuminuria induced by TNF (Figure 2D).

ACTH gel ameliorates histological AKI induced by TNF

Morphologically, TNF induced a typical pattern of acute tubular necrosis, characterized by epithelial simplification, vacuolization of proximal tubular epithelium, luminal ectasia, epithelial necrosis, sloughing of tubular cell into lumen, loss of brush border, nuclear enlargement and pleomorphism, nuclear pyknosis and karyolysis, and prominent inflammatory infiltration, concomitant with tubular apoptosis (Figure 3A). ACTH gel at 10IU/kg remarkably ameliorated these histological lesions evoked by TNF (Figure 3A). Semi-quantitative morphometric analysis confirmed and quantified the morphologic findings and revealed that ACTH gel treatment significantly reduced the tubulointerstitial injury score and the dilation sloughing score (Figure 3B&D). Computerized morphometric analysis demonstrated that the area of vacuolization as well as the number of necroapoptotic nuclei in TNF injured kidneys was remarkably diminished after ACTH gel treatment (Figure 3C&E). TNF is one of the prototypes of prodeath cytokines that could promote cell apoptosis.²¹ In support of this, TNF injection elicited massive cellular apoptosis in the kidney as indicated by TdT-mediated dUTP nick-end labeling (TUNEL) staining, mainly

located to the renal tubules in the juxtamedullary cortex (Figure 4A). ACTH gel treatment drastically abrogated the proapoptotic effect of TNF and greatly diminished the TUNEL positivity in the kidney (Figure 4A). Absolute counting of TUNEL positive cell number corroborated the morphologic findings (Figure 4C). To examine if the change in tubular cell apoptosis is associated with the change in the activity of proapoptotic signaling pathway, kidney specimens were stained for cleaved or active form of caspase-3, a pivotal transducer of the apoptotic-signaling pathway. Shown in Figure 4A, prominent expression of cleaved caspase-3 was induced predominantly in tubules in the juxtamedullary cortex of TNF injured kidneys and this could be markedly blunted by ACTH gel treatment. Moreover, immunoblot analysis of kidney homogenates indicated that ACTH gel treatment markedly attenuated the TNF induced reduction in the ratios of intact/cleaved caspase-3 and phosphorylated glycogen synthase kinase (GSK)3 β /GSK3 β (Figure 4B&D), denoting that ACTH abrogated caspase-3 directed cell death and blunts GSK3 β activation, which has been shown to be proapoptotic²⁵ and plays a detrimental role in AKI²⁶.

Steroid independent mechanism contributes to kidney protection by ACTH

ACTH possesses a canonical steroidogenic activity, through which ACTH directs the adrenal release of glucocorticoid, a typical immunomodulatory and anti-inflammatory hormone that could attenuate renal inflammation and thus might mediate the renoprotective effects of ACTH.^{9,13} Indeed, in TNF injured animals, the tubulointerstitial injury score (Figure 5B) inversely correlated with steroidogenic response to ACTH gel as determined by the plasma levels of corticosterone (Figure 5A) at low doses. However, although the steroidogenic response to ACTH gel above a dose of 5 IU/Kg reached a plateau, the renoprotective effect continued to increase with even higher doses (10 IU/Kg, 20 IU/Kg), suggesting that steroid independent mechanisms also contribute.

MC1R is the major intrarenal melanocortin receptor that is predominantly expressed on renal tubular cells

To explore the steroid independent kidney protective mechanisms, expression of all melanocortin receptors was profiled in rat kidneys by RT-PCR. The MCRs are the product of small, intronless genes; hence, genomic contamination complicates mRNA based expression analyses including RT-PCR.^{9,27} To avoid this issue, samples of mRNA extracted from kidney tissues were subjected to DNase treatment before further processes. Shown in Figure 6A, rat kidney tissues evidently expressed MC1R mRNA and to a much lesser extent MC5R mRNA. A parallel PCR amplification of an intron of the GAPDH gene was carried out simultaneously using the same cDNA samples (Table 1) to confirm the quality of mRNA: no band of the GAPDH intron was detected (Figure 6A), indicating no genomic contamination of the mRNA samples. To validate the RT-PCR results, immunoblot analysis of kidney homogenates was performed and showed that both MC1R and MC5R were expressed in the kidney, however, only MC1R is expressed in both rat kidney and cultured proximal tubular epithelial (IRPTC) cells (Figure 6B). To localize the MCR expression in the kidney, fluorescent immunohistochemistry staining was conducted and showed that MC1R was expressed intensely and predominantly by renal tubules and very weakly by glomeruli, while MC5R was weakly and sporadically by expressed the interstitial cells (Figure 6C). TNF injury enhanced the intensity of MC1R expression in the tubules but had

minor effect on MC5R expression (Figure 6C). In cultured IRPTC cells, fluorescent immunocytochemistry staining consistently revealed punctate staining of MC1R (Figure 6D), in agreement with a typical staining pattern of cytoplasmic membrane receptors. No fluorescent staining was seen when preimmune IgG was used as primary antibodies for either immunohistochemistry or immunocytochemistry staining (Figure 6C&D). Moreover, in cells transfected with MC1R specific siRNA, the immunofluorescent staining signal was drastically diminished (Figure 6D), denoting a high specificity of the MC1R immunostaining. Collectively, the above findings suggest that MC1R is the major melanocortin receptor for ACTH in the kidney that is located to renal tubular cells.

MC1R mediates the anti-apoptotic action of ACTH on renal tubular cells

In IRPTC stimulated with TNF in a culture model where the steroidogenic circuit is absent, ACTH treatment dramatically reinstated cellular viability in a dose dependent fashion with a maximal effect observed above 10^{-8} M (Figure 7A), again suggesting ACTH has a direct kidney protective activity that is independent of steroidogenesis. To examine whether this steroid independent antiapoptotic/prosurvival effect of ACTH is mediated *via* its cognate MC1R on tubular epithelial cells, RNA interference (RNAi) was applied to specifically silence the MC1R expression in cultured IRPTC cells. Shown in Figure 7B, RNAi with MC1R specific siRNA (si-MC1R) drastically knocked down MC1R expression. By contrast, RNAi with scramble siRNA (si-Control) barely affected its expression as validated by immunoblot analysis (Figure 7B) and immunocytochemistry staining (Figure 7D). TNF stimulation impaired cellular viability as estimated by tetrazolium (MTT) assay (Figure 7C) and induced apoptosis as determined by PARP activation (Figure 7B) as well as TUNEL staining (Figure 7D&E). In cells transfected with scrambled siRNA, ACTH significantly counteract TNF induced apoptotic responses (Figure 7B~E). The beneficial effects of ACTH were largely abolished in MC1R silenced cells, suggesting that MC1R is indispensable for ACTH's anti-apoptotic action in renal tubular cells.

ACTH gel protects from septic AKI via an additive action

To explore if ACTH has a kidney protective efficacy comparable to α -MSH and to ascertain if the kidney protective activity of ACTH is also applicable to other forms of AKI, the effect of ACTH gel was compared with that of α -MSH and re-examined in a murine model of septic AKI induced by CLP. Mice were treated with ACTH gel (10 IU/kg) or an equal molar amount of α -MSH (71 μ g/kg), subjected to CLP and followed for 24 h. CLP induced polymicrobial sepsis disturbed systemic hemodynamics, caused hypotensive shock and resulted in a histology of acute tubular injury as well as kidney dysfunction reflected by an increase in the serum creatinine level. Consistent with previous reports¹⁶, α -MSH treatment markedly ameliorated histological AKI (Figure 8A&C) and kidney dysfunction (Figure 8B) and corrected hypotension (Figure 8D) in septic mice. Compared with α -MSH, which is equal in biological activity to ACTH except for steroidogenesis, ACTH gel exerted a much more potent protective effect, evidenced by significantly better kidney histology and more improvements in tubular injury scores, serum creatinine levels and blood pressure (Figure 8). These data suggest that the kidney protective effect exerted by ACTH might be ascribed to an additive beneficial action achieved by both steroid dependent mechanisms and MC1R-mediated α -MSH like activities.

DISCUSSION

AKI remains a therapeutic challenge despite advances in supportive care. In the present study, supplementation of exogenous ACTH attenuated acute renal histological injury, improved kidney dysfunction and promoted general survival in animal models of AKI. To the best of our knowledge, this study is the first to demonstrate a kidney protective action of ACTH on AKI.

ACTH carries vital biologic information from the brain to the adrenal cortex and to other parts of the mammalian body.^{8,9} As a crucial component of the HPA axis, ACTH stimulates the adrenal production of corticosteroids.^{8,9} The efficacy of corticosteroids on AKI remains highly controversial,^{28,29} however, its potent anti-inflammatory activity has been widely acknowledged.³⁰ Thus the beneficial effect of ACTH on AKI observed in this study might be explained partially by the steroidogenic action through which ACTH upregulates corticosterone production and subsequently suppresses TNF induced acute inflammatory injury in the kidney. In support of this, severity of the TNF-induced tubulointerstitial injury inversely correlated with steroidogenic response to ACTH as determined by the plasma levels of corticosterone at low doses. However, the steroidogenic response to ACTH above a dose of 5 IU/Kg reached a plateau, the renoprotective effect continued to increase with even higher doses, suggesting that steroid independent mechanisms also contribute.

Besides governing steroidogenesis, ACTH is also an important physiological agonist of the melanocortin system, which plays an integral role in the homeostatic control of a diverse array of physiological functions, including cellular survival, proliferation, anti-inflammation and immunomodulation.^{9,13} The anti-apoptotic and kidney protective action on AKI by α -MSH, another agonist of the melanocortin system, has been extensively examined and reproducibly demonstrated in multiple animal models of AKI, including ischemia reperfusion injury^{31–33}, renal toxicity^{34,35} and ureteral obstruction³⁶. α -MSH shares the same amino acid sequence as the N terminus of ACTH and possesses all of ACTH's biological activities with similar potency except steroidogenesis.^{9,13} Therefore, the steroid independent mechanisms accounting for the kidney protective effect of ACTH might be attributable to a non-steroidogenic melanocortin activity or an α -MSH like activity. Thus ACTH might have an additive action attributable to both corticosteroids mediated mechanisms and the non-steroidogenic α -MSH like activities. Accordingly, due its unique steroidogenic effect, ACTH might be superior to α -MSH in protecting kidney from acute injuries as revealed in our study of CLP induced septic AKI.

The detailed molecular mechanism underlying the anti-apoptotic and kidney protective effect of ACTH and α -MSH remains elusive and whether ACTH and α -MSH directly targets kidney parenchymal cells has been barely investigated. Our study found for the first time that MC1R, a cognate receptor with high affinity for both α -MSH and ACTH, is the major MCR that is abundantly expressed in the kidney and predominantly distributed on tubules, denoting renal tubules as a direct therapeutic target for both ACTH and α -MSH. Indeed, ACTH treatment of tubular epithelial cells in a culture model where the steroidogenic circuit is absent drastically obliterated TNF induced apoptosis and reinstated cellular viability. Furthermore, MC1R silencing largely abrogated ACTH's beneficial effect,

suggesting that MC1R mediated this cytoprotective effect. In agreement with our observations, Masser *et al*³⁷ demonstrated the dramatic worsening of experimental intestinal injury in mice with a nonfunctional MC1R. Moreover, the anti-apoptotic/prosurvival effect of MC1R signaling has been demonstrated previously in human melanocytes.^{38,39} Treatment with α -MSH results in reduction of ultraviolet radiation elicited apoptosis and this effect was independent of melanin synthesis, in agreement with a MC1R-mediated non-pigmentary anti-apoptotic mechanism.^{38,39} Consistently, our data suggest that ACTH intercepts the caspase directed proapoptotic signaling pathway and obliterates GSK3 β activation, which promotes apoptosis and plays dirty in AKI.

Systemic inflammatory response as well as immune dysfunction also plays an important role in the pathogenesis of AKI.^{40,41} Evidence suggests that melanocortins like α -MSH and ACTH display a potent systemic protective activity directly *via* MCRs expressed on most immune and non-immune cells.²⁷ Therefore, in addition to a direct kidney effect, ACTH might prevent AKI *via* systemic protection, immunomodulation and anti-inflammation. Indeed, our data indicate that ACTH gel treatment has a systemic beneficial action that corrected hypotension and improved histological injuries and inflammation in multiple organs including the live and lung.

In summary, ACTH gel, an FDA approved formulation of natural ACTH for the treatment of nephrotic syndrome can also ameliorate acute renal histological injury, correct acute kidney dysfunction and improve general survival in animal models of AKI. ACTH ameliorates AKI by an additive action exerted possibly *via* both steroid dependent mechanisms and MC1R directed anti-apoptotic signaling. Our findings suggest that ACTH may represent a novel and pragmatic kidney protective strategy to prevent or treat AKI.

MATERIALS AND METHODS

Cell culture

IRPTC cells, provided by Dr. Julie Ingelfinger, were grown in Dulbecco's modified Eagle's medium/F12 that contained 5% fetal bovine serum. Recombinant rat TNF (R&D Systems, Minneapolis, MN, USA) and porcine ACTH (Sigma, St. Louis, MO) were used to treat the culture at indicated concentrations. RAM cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in in Ham's F12K containing 15% fetal bovine serum. For fluorescent viable labeling, RAM cells (1×10^7) were incubated in media containing 5 mg/ml Calcein-AM (Molecular Probes, Eugene, OR, USA) at 37°C for 30 min.

Cellular viability assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to assess cell viability as described previously.

Fluorescent immunocytochemistry

Cells were fixed with 4% paraformaldehyde. Following serum blocking for 30 min, cells were incubated with the primary antibody to indicated MCR (Alomone Labs, Jerusalem,

Israel) or preimmune IgG and then the Alexa fluorophore 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA). Finally, cells were counterstained with 4',6-Diamidino-2-phenylindole (DAPI) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and visualized using a fluorescence microscope.²²

RNA interference

Pre-designed siRNA duplex was chemically synthesized by Ambion (Austin, TX, USA). MC1R-specific siRNA sequence (5'-GAGGUUGAAGUUCUUGAAGtt-3') was designed according to the complete coding sequence of rat MC1R gene (GenBank accession no. AB576619.2). In addition, a scrambled siRNA sequence (5'-GCGAGUAGCGCUAGGAAGUtt-3') without sequence similarity to any known gene sequences from mouse, rat, or human was designed as control for RNA interference.⁴² Efficiency of lipofectamine (Invitrogen)-mediated gene-silencing efficiency was assessed by immunocytochemistry staining or immunoblot analysis for MC1R. Near complete (~80%) suppression of MC1R protein expression was observed in cells transfected with MC1R-specific siRNA.

RNA extraction and reverse transcription-PCR

Genomic DNA (gDNA) and mRNA was extracted from kidney cortex tissues or cultured cells and the first-strand cDNA was prepared after mRNA was treated with DNase to eliminate potential genomic DNA contamination. PCR amplification of the intron or exon of GAPDH as well as indicated MCRs was carried out as previously described by using primers listed in Table 1. To confirm no contamination of mRNA by genomic DNA, cDNA samples were subject to PCR amplification of an intron and an exon of GAPDH gene; a sample of genomic DNA served as the positive control. PCR products resolved in agarose gels were photographed under ultraviolet light.

Animal experimental design

Animal experimental studies were approved by the institution's Animal Care and Use Committee and they conform to the USDA regulation and NIH guidelines for human care and use of laboratory animals.

Rat model of TNF induced AKI—Male Sprague-Dawley rats with initial weights of 200–250 g were pretreated subcutaneously with saline or ACTH gel at indicated doses 60 min before injection of recombinant rat TNF (2 mg/kg wt) (R&D system) *via* tail vein. Rats were evaluated hourly and survival recorded. To determine an optimal dose of ACTH gel for kidney protection, RAM infusion assay was performed in some rats as reported before.²² In brief, rats were anesthetized, at indicated time points after TNF injection, placed on a heated table to keep constant body temperature, and maintained euvolemic state. Fluorescent-labeled RAM cells (1×10^4) resuspended in normal saline were infused via the carotid artery. After a 15 min equilibration period, mean arterial pressure was measured and glomerular filtration rate was determined by inulin clearance assay as described before²². Rats were killed 60 min after RAM cell infusion and ureteral urine and blood were collected. Organs were harvested for further investigation. To quantify the fluorescent RAM cells sequestered

in the organs, tissue homogenates with equal amount of protein (100 mg) were subjected to fluorometric analysis.

Mouse model of septic AKI induced by CLP—Male CD-1 mice at 8 weeks old were subjected to CLP surgery to induce sepsis as previously described^{16,23,24}. In brief, under isoflurane anesthesia, 1.5 cm of the cecal tip was ligated using a 4-0 silk suture and punctured twice with a 21-gauge needle. An approximately 1-mm column of fecal material was expressed. In sham-operated mice, the cecum was isolated but neither ligated nor punctured. Following surgery all mice received 1ml of prewarmed normal saline (NS) intraperitoneally. Treatment with fluid and antibiotic was started at 12 h after surgery with subcutaneous injection of imipenem/cilastatin (14 mg/kg) in 1 mL of NS. Acthar gel (10 IU/kg) or equal molar amounts of α -MSH (71 μ g/kg, Sigma; prepared in saline containing 16% gelatin) was administered subcutaneously 60 min before surgery and at 12 h post-surgery. Vehicle treated animals received an equal volume of NS subcutaneously at the indicated times. Mouse blood pressure was measured by using the tail cuff method as described previously⁴³. Mice were killed 24 h after surgery and urine and blood were collected. Organs were harvested for further investigation.

Western immunoblot analyses

Cultured tubular epithelial cells were lysed and rat kidneys homogenized in RIPA buffer supplemented with protease inhibitors. Samples with equal amounts of total protein (50 mg/ml) were processed for immunoblot as described previously.²² The antibodies against caspase-3, PARP, p-GSK3 β , GSK3 β and actin were purchased from Cell Signaling Technology (Beverly, MA) and those against MCR were purchased from Alomone Labs.

Assessment of pulmonary edema

Lungs were removed from animals, placed in a tared microcentrifuge tube, and weighed. Lungs were then desiccated under a vacuum at 25°C and weighed again. The wet lung mass was divided by the dry lung mass to give the wet-dry ratio, which is the fraction of the wet lung weight that was water⁴⁴.

Measurement of blood and urine chemistry

Albumin levels in the ureteral urine were measured by using a rat Albumin ELISA Quantification kit (Bethyl Laboratories, Montgomery, TX, USA). Plasma corticosterone levels were measured by Corticosterone EIA Kits (Cayman Chemical, Ann Arbor, MI, USA). Serum creatinine levels were determined by a routine procedure as described previously^{25,45}. To assess hepatotoxicity, serum activity of AST and ALT were measured using spectrophotometric ELISA kits (Usen Life Science, Houston, TX, USA).

Morphological studies

Formalin-fixed kidneys were embedded in paraffin and prepared in 3- μ m-thick sections. Sections were stained with hematoxylin eosin to estimate gross histological kidney injury. One observer performed computerized as well as semi-quantitative morphometric analysis in a blinded manner. Severity of tubulointerstitial injury and tubular dilation/sloughing was

semi-quantitatively scored on a scale from 0 to 3 and reported as the mean of 20 random high-power ($\times 400$) fields per hematoxylin eosin stained section: 0, absent; 1, slight; 2, moderate; 3, marked; and 4, severe.²² Necroapoptotic nuclei were defined as nuclei dropped off into the lumen or interstitium or those with chromatin condensation, pyknosis or fragmentation. Indirect immunofluorescent staining of MCR was carried out on methanol/acetone-fixed (1:1) frozen cryostat sections using anti-MCR antibodies (Alomone Labs, Jerusalem, Israel). Then the Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) were applied. As a negative control, the primary antibodies were replaced by preimmune IgG from the same species; no staining occurred. Finally, all sections were mounted with Vectashield mounting medium (Vector Laboratories). For visualization of fluorescence RAM cells that were sequestered in the organs, cryosections were fixed with methanol/acetone and then subjected to Evans blue counterstaining.

TUNEL staining

Apoptotic cell death in kidney sections was detected by using the TUNEL kit (Roche Molecular Biochemicals, Mannheim, Germany).²⁵ Ten randomly selected $400\times$ high-power fields of juxtamedullary cortex were counted to determine the number of apoptotic nuclei.

Statistical analyses

For immunoblot analysis, bands were scanned and the integrated pixel density was determined using a densitometer and the NIH image analysis program. All data are expressed as means \pm SD. Statistical analysis of the data from multiple groups was performed by ANOVA followed by Student-Newman-Keuls tests. Data from two groups were compared by *t*-test. Logrank test was used for Kaplan-Meier survival analysis. $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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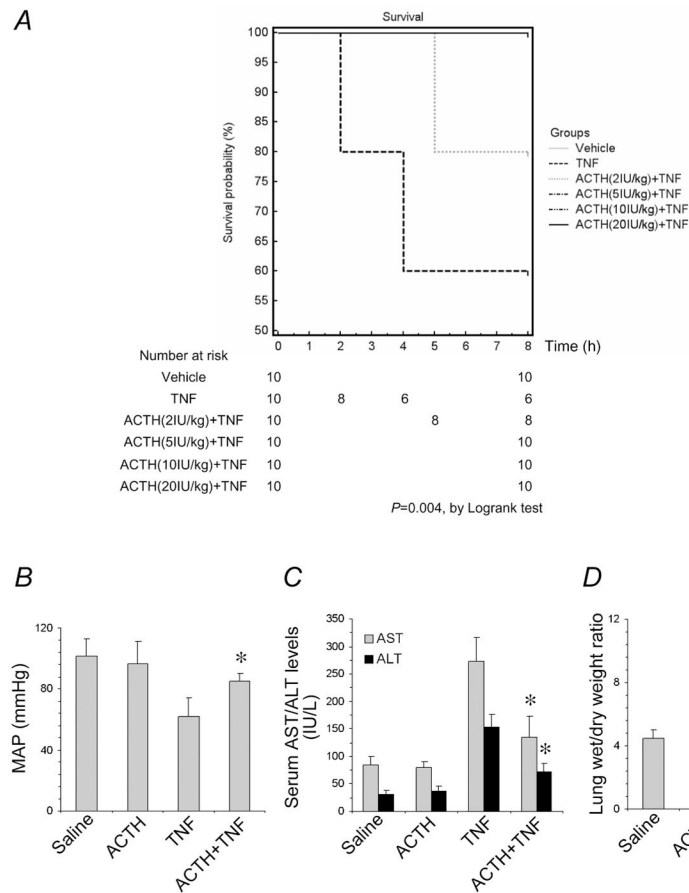


Figure 1. ACTH gel treatment significantly improves survival and ameliorates systemic organ injury in TNF injured rats

(A) Rats were pretreated subcutaneously with saline or ACTH gel at indicated doses 60 min before intravenous bolus injection of recombinant rat TNF (2 mg/kg wt). Rats were evaluated hourly and survival recorded. Survival rate was plotted against the time course. The survival probability of rats receiving different treatment was determined by Kaplan-Meier survival analysis. $P = 0.0040$ among the groups by Logrank test. (B-D) Rats were pretreated subcutaneously with saline or ACTH gel (10IU/kg) 60 min before intravenous bolus injection of recombinant rat TNF (2 mg/kg wt). Mean arterial pressure (MAP) was measured (B) and rats were sacrificed 8 hours later. Blood was collected and serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured to assess acute liver injury (C). Lung was excised and lung wet to dry weight ratio was determined to assess acute lung injury (D). * $P < 0.05$ versus TNF alone treated group ($n=5$).

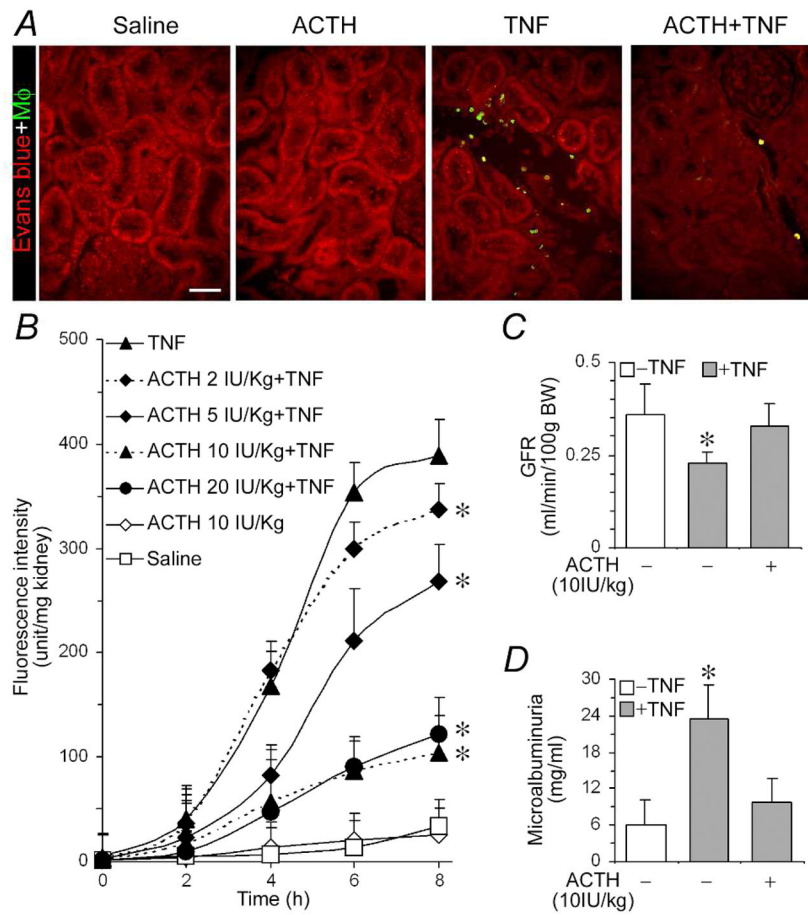


Figure 2. ACTH gel prevents acute renal injury in a dose dependent fashion and prominently improves kidney dysfunction at an optimal dose

Rats were treated with ACTH gel and TNF as described in Figure 1 and were sacrificed at indicated time. To estimate an optimal kidney protective dose of ACTH gel, fluorescence labeled RAM cells were infused into carotid artery 60 min before sacrifice. Kidney sections were counterstained with Evans blue (red) to visualize and evaluate RAM sequestration (green; A), which was further quantified by fluorometric analysis of kidney homogenates (B); * $P < 0.05$ versus TNF alone treated group ($n = 5$); scale bar = 100 μm in (A). (C) At 10IU/kg, ACTH gel significantly attenuates TNF impaired glomerular filtration rate (GFR) as estimated by inulin clearance; * $P < 0.05$ versus other groups ($n = 5$). (D) Albumin levels in the collected ureteral urine were measured by ELISA and demonstrated that ACTH gel at 10IU/kg markedly abrogates TNF induced microalbuminuria. * $P < 0.05$ versus other groups ($n = 5$).

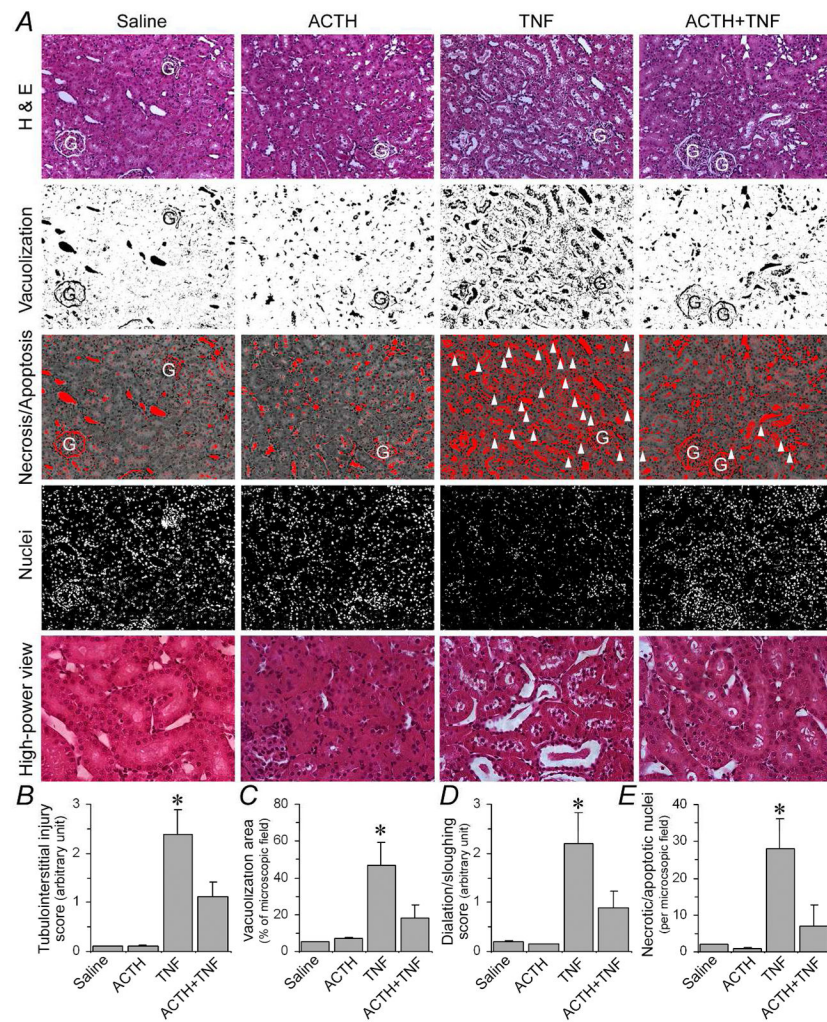


Figure 3. ACTH gel therapy ameliorates histological AKI in TNF injured rats

Rats were pretreated subcutaneously with saline or ACTH gel (10IU/kg) 60 min before intravenous bolus injection of recombinant rat TNF (2 mg/kg wt). Rats were sacrificed 8 hours later and kidney sections prepared for hematoxylin and eosin (H&E) staining to evaluate histological injury (A); Original magnification, $\times 200$ (high-power view, $\times 400$); Necroapoptotic nuclei highlighted by arrowheads; G, glomerulus. Semi-quantitative (tubulointerstitial injury score and tubular dilation/sloughing score; B and D) as well as computerized (vacuolization area and necroapoptotic nuclei; C and E) morphometric analysis was employed to estimate kidney injury. * $P < 0.05$ versus other groups ($n = 5$).

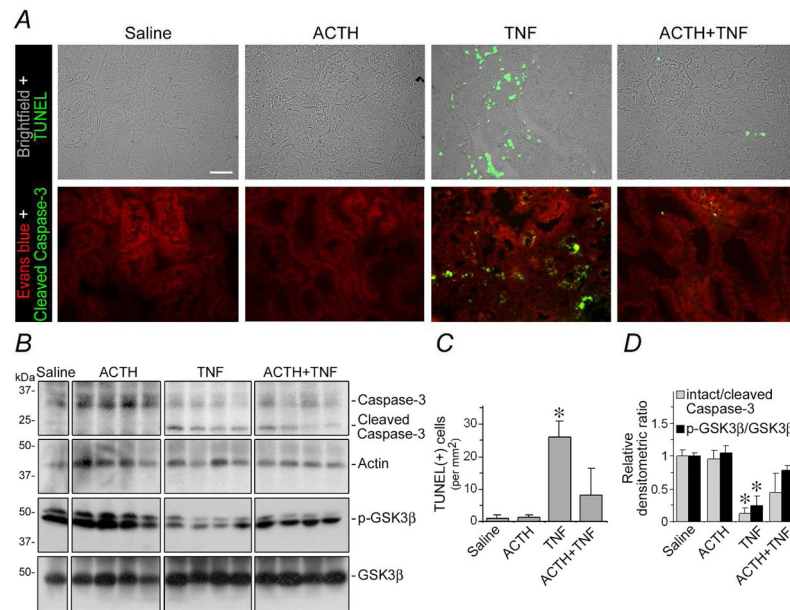


Figure 4. ACTH gel prevents renal tubular cell apoptosis in TNF injured rats

Rats were pretreated subcutaneously with saline or ACTH gel (10IU/kg) 60 min before intravenous bolus injection of recombinant rat TNF (2 mg/kg wt). Rats were sacrificed 8 hours later and kidney sections prepared for TUNEL staining (green) or immunofluorescent staining of cleaved caspase-3 (green) to evaluate renal cell apoptosis (A); scale bar=100 μ m. (B) Immunoblot analysis of kidney homogenates for intact or cleaved caspase-3, phosphorylated GSK3 β or GSK3 β and actin. (C) Absolute counting of TUNEL positive cells in the kidney sections indicates an anti-apoptotic effect by ACTH treatment. * P <0.05 versus other groups. (D) Arbitrary units of intact/cleaved caspase-3 ratios or p-GSK3 β /GSK3 β ratios expressed as immunoblot densitometric ratios of the molecules as folds of the saline treated group. * P <0.05 versus other groups (n =5).

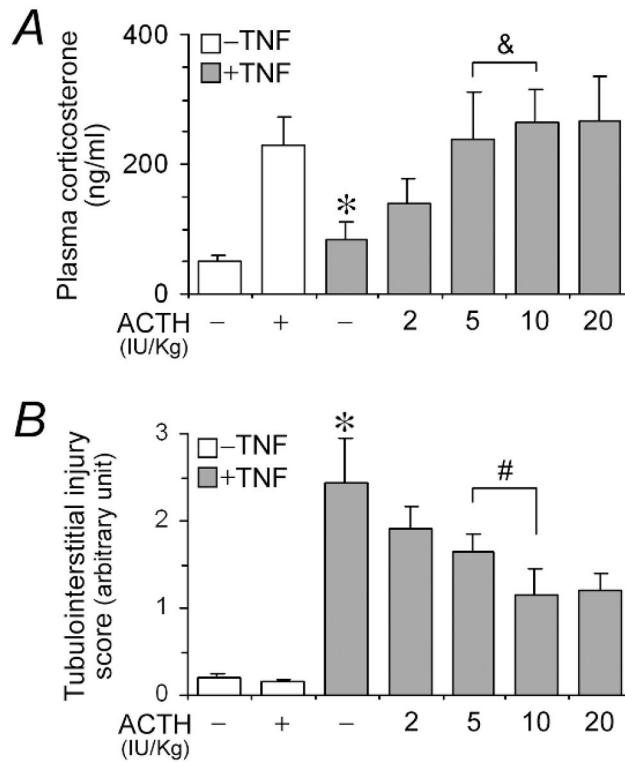


Figure 5. ACTH gel therapy attenuates AKI in TNF injured rats VIA, at least in part, a steroid independent mechanism

Rats were pretreated with saline or ACTH gel subcutaneously at indicated doses 60 min before intravenous bolus injection of recombinant rat TNF (2 mg/kg wt). Rats were sacrificed 8 hours later and plasma and kidney specimens collected. (A) Plasma corticosterone levels were determined by ELISA assay and suggested that the steroidogenic response to ACTH above a dose of 5 IU/kg reaches a plateau; * $P < 0.05$ versus other groups ($n = 5$); & Not significant. (B) Severity of tubulointerstitial injury was scored as stated in Figure 3 and revealed that the renoprotective effect of ACTH continues to increase with even higher doses than 5 IU/kg, suggesting that steroid independent mechanisms also contribute to kidney protection; * $P < 0.05$ versus other groups ($n = 5$); # $P < 0.05$.

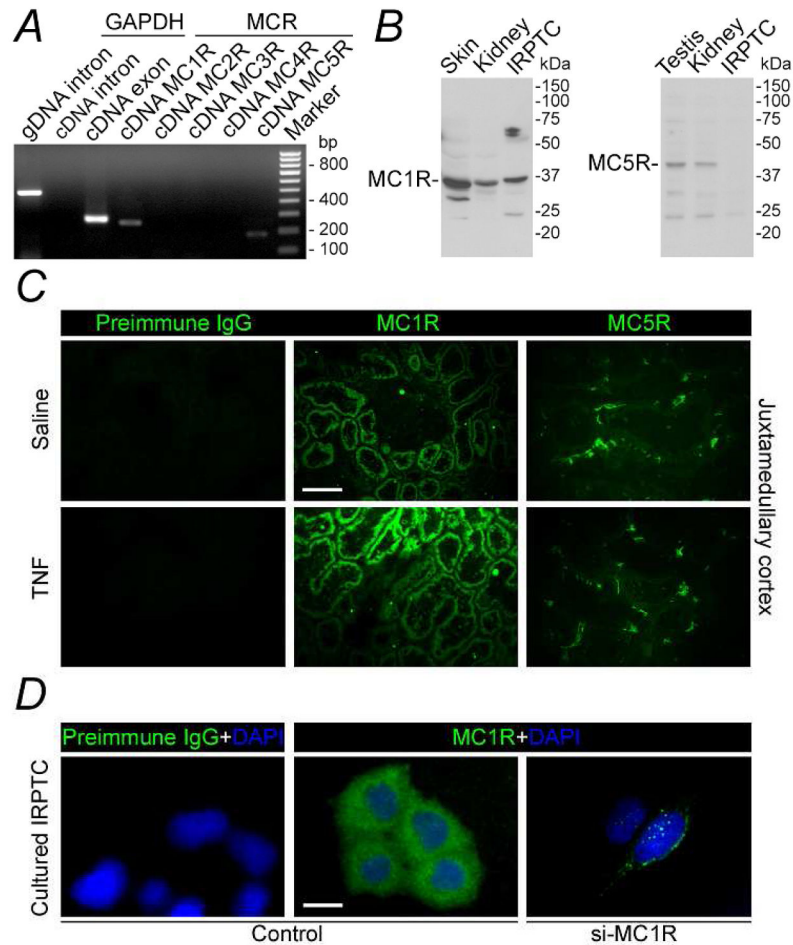


Figure 6. MC1R is a major intrarenal melanocortin receptor predominantly expressed by renal tubular epithelial cells

(A) Genomic DNA (gDNA) and mRNA were extracted from rat kidney and cDNA was prepared from mRNA following DNase treatment of mRNA samples. Both gDNA and cDNA samples were subjected to PCR amplification for indicated intron or exon of GAPDH as well as indicated melanocortin receptor (MCR) subtypes. PCR results were resolved in agarose gels and visualized under ultraviolet light and indicated that rat kidney expresses mainly MC1R and, to a much lesser extent, MC5R. (B) To validate the RT-PCR data, protein was prepared from rat kidneys as well as cultured rat proximal tubular epithelial cells (IRPTC) cells, and subjected to immunoblot analysis for MC1R and MC5R. Samples prepared from skin and testis served as positive controls. Rat kidney expresses both MC1R and MC5R, however, only MC1R is expressed in both rat kidney and cultured IRPTC cells. (C) Fluorescent immunohistochemistry staining in control (saline) or TNF treated kidney indicates that MC1R (green) is abundantly expressed by renal tubules and induced after TNF injury, while MC5R (green) is expressed not by the tubules but weakly and sporadically by interstitial cells in rat kidneys; scale bar=100 μ m. (D) Cultured IRPTC cells (control) or those transfected with MC1R specific (si-MC1R) siRNA for RNA interference were subjected to fluorescent immunocytochemistry of MC1R, which reveals punctate staining

(green) consistent with a typical staining pattern of cytoplasmic membrane receptors; counterstained with DAPI (blue); scale bar=20 μ m.

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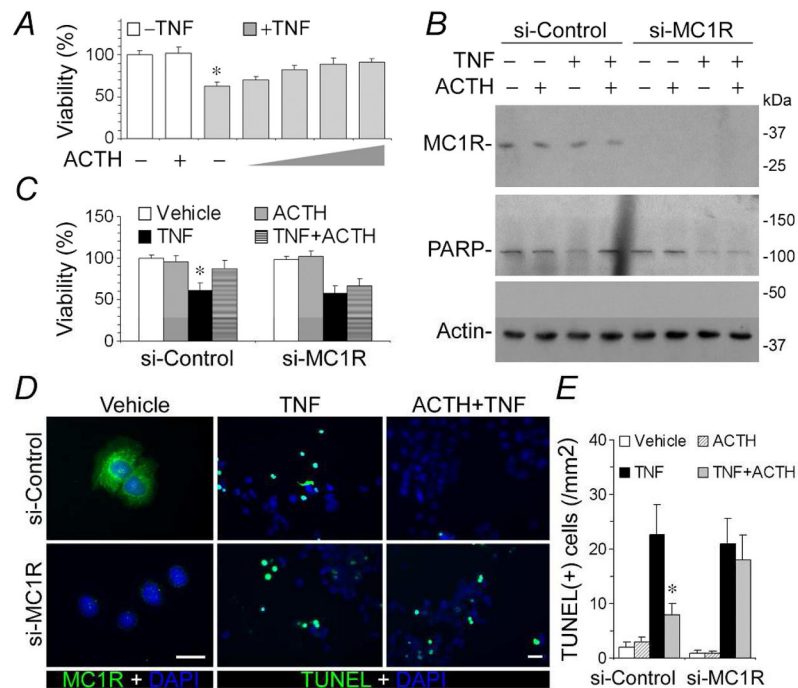


Figure 7. MC1R mediates the anti-apoptotic effect of ACTH in renal tubular epithelial cells (A) ACTH reinstates the viability of TNF injured IRPTC cells in a dose dependent fashion. IRPTC cells were treated with vehicle, TNF (10ng/ml), ACTH (10⁻⁸M), or TNF in combination with ACTH (10⁻¹⁰M, 10⁻⁹M, 10⁻⁸M, 10⁻⁷M) for 6 h before cellular viability was estimated by MTT assay; **P*<0.05 versus other groups. (B) After MC1R silencing, ACTH fails to prevent TNF induced PARP cleavage. IRPTC cells were transfected with scrambled (si-Control) or MC1R specific (si-MC1R) siRNA for RNA interference. Cells were then treated with vehicle, TNF (10ng/ml) and/or ACTH (10⁻⁸M) for 6 h. Cell lysates were subjected to immunoblot analysis for MC1R, PARP and actin (B); cellular viability assessed by MTT assay (C); **P*<0.05 versus other groups in si-Control. (D) Fixed cells were prepared for TUNEL staining (green), fluorescent immunocytochemistry staining for MC1R (green) and counterstaining of DAPI (blue); scale bar=30μm. (E) Absolute counting of TUNEL positive cells in cell cultures; **P*<0.05 versus other groups in si-Control.

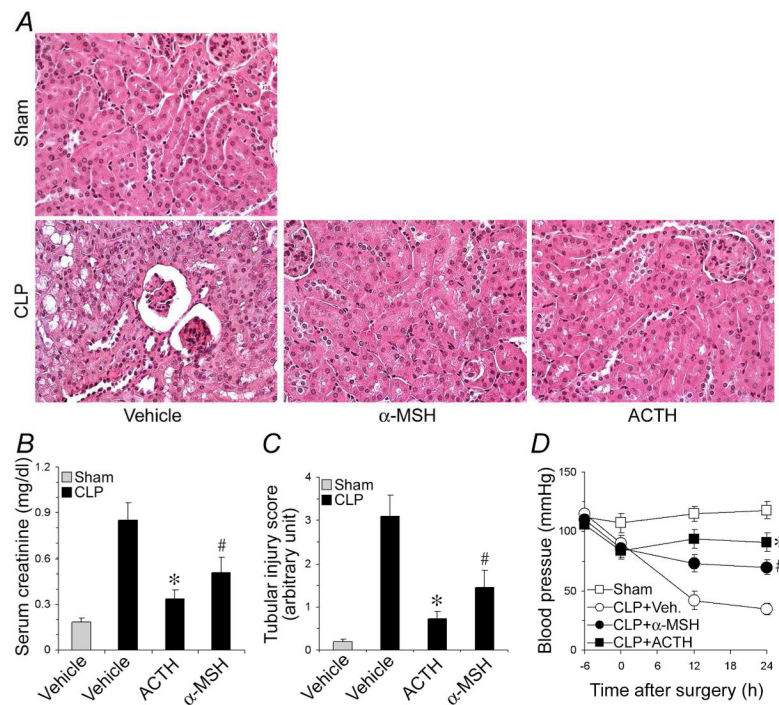


Figure 8. ACTH gel protects from cecal ligation puncture (CLP) induced septic AKI better than α -melanocyte stimulating hormone (MSH)

Mice were pretreated with ACTH gel (10IU/kg) or an equal molar amount of α -MSH (71 μ g/kg) or an equal volume of vehicle (normal saline) and subjected to CLP or sham surgery. Mice were followed for 24 h and sacrificed. (A) Kidney sections prepared for hematoxylin and eosin staining to evaluate histological injury; Original magnification, $\times 400$. (B) Blood was collected and serum creatinine levels were determined; *, # $P < 0.01$ versus vehicle treated CLP group ($n = 8$); * $P < 0.05$ versus α -MSH treated CLP group ($n = 8$). (C) Tubular injury score was estimated; *, # $P < 0.01$ versus vehicle (Veh.) treated CLP group ($n = 8$); * $P < 0.05$ versus α -MSH treated CLP group ($n = 8$). (D) Systolic blood pressure was measured at indicated time points; *, # $P < 0.01$ versus vehicle (Veh.) treated CLP group ($n = 8$); * $P < 0.05$ versus α -MSH treated CLP group ($n = 8$).

Table 1

Nucleotide sequences of the primers used for PCR.

Molecules	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplimer Size (bp)
MC1R	TGTGGTAGCCATCACAAAA	CCAGGAAGCAGAGACTGGAC	230
MC2R	ATCTGCAGTTTGGCCATTTT	GCAATCACAGACAGGCTGAA	188
MC3R	AGGACCAATTCATCCAGCAC	AGGGCTTTCCTAACCATCAT	160
MC4R	CTTTACGCGCTCCAGTACC	GTAATTGCGCCCTTCATGTT	285
MC5R	CCTCGGAGGACAACATCCTA	CTAGGCTGCCACAAAGAAG	189
GAPDH exon	GGTGATGCTGGTGCTGAGTA	ACTGTGGTCATGAGCCCTTC	272
GAPDH intron	AGCTGTTGCTCCCCTTGTA	ATCCATCACCTGGCCTACAG	417

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