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EFFECTS OF ULINASTATIN ON GLOBAL ISCHEMIA VIA BRAIN PRO-INFLAMMATION SIGNAL

Xiao-Ming Jiang[1],
Jing-Hai Hu[2],
Lu-Lu Wang[3],
Chi Ma[4],
Xu Wang[5]*,
Xiao-Liang Liu[1]*

[1]Department of Emergency Medicine, The First Hospital of Jilin University
Changchun, Jilin 130021, P.R. China
[2]Department of Urology, The First Hospital of Jilin University
Changchun, Jilin 130021, P.R. China
[3]Cardiovascular Center for Diagnostics and Treatment, The First Hospital of Jilin University
Changchun, Jilin 130021, P.R. China
[4]Department of Brain Tumor Surgery, The First Hospital of Jilin University
Changchun, Jilin 130021, P.R. China
[5]Department of Neurology and Neuroscience Center, The First Hospital of Jilin University
Changchun, Jilin 130021, P.R. China

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Abstract

Ulinastatin [urinary trypsin inhibitor (UTI)] plays an important role in the protection of organs against ischemic injury during severe inflammation. The purposes of this study were to examine the effects of UTI on the levels of pro-inflammatory cytokines (PICs) and protein expression of PIC receptors in the neocortex and hippocampus CA1 region of rats after transient global ischemia induced via cardiac arrest (CA). Specifically, interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were analyzed. CA was induced by asphyxia followed by cardiopulmonary resuscitation in rats. ELISA and western blot analysis were employed to determine PICs and their receptors in the neocortex and hippocampus. Our results show that IL-1β, IL-6 and TNF-α were significantly elevated in the neocortex and hippocampal CA1 field after CA. This was accompanied with an increase in PIC receptors, namely IL-1R, IL-6R and TNFR1. Systemic injection of UTI attenuated the amplification of PIC signal pathways in these brain regions. UTI also improved the modified Neurological Severity Score and brain tissue edema in CA rats. Notably, UTI resulted in an increase in survival of CA rats as compared to CA rats without treatment. In conclusion, UTI plays a beneficial role in modulating transient global ischemia induced by CA by altering PIC signal mechanisms, but further studies are needed to draw more firm conclusions.

Keywords

• Cardiac arrest • Cardiopulmonary resuscitation • Cortex • Cytokines • Hippocampus • Ulinastatin

Introduction

During cardiac arrest (CA), blood flow and oxygen delivery are abruptly halted, which leads to systemic ischemic injury in various organs including the brain [1]. Although cardiopulmonary resuscitation (CPR) is applied, inadequate blood flow and tissue oxygen delivery still persist due to myocardial dysfunction, hemodynamic instability and microvascular dysfunction. In response to ischemic and hypoxic insults, neuroprotective mechanisms are engaged in the brain by improving the permeability of the blood brain barrier, reducing brain edema formation and promoting the recovery of brain injuries [2]. Thus, it is important to study signaling pathways and determine biological agents involved in neuroprotective effects and attenuating damages evoked by global cerebral ischemia.

Pro-inflammatory cytokines (PICs) i.e., interleukins, lymphokines and cell signaling molecules are released by numerous cells including leukocytes, myocytes, microglia and astrocytes [3]. The mediators of immune and inflammatory reactions interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) play an important role in responding to cerebral ischemic insults [4, 5]. These PICs modulate the responsiveness of many cell types in a number of diseases. During diseased states, PICs can recruit cells to inflammatory sites and alter cell survival, division, proliferation and differentiation [6].

Ulinastatin, also called urinary trypsin inhibitor (UTI) is a glycoprotein that acts as a trypsin inhibitor [7]. It can be derived from urine or synthetically produced and is conventionally effective in treatment of acute and chronic pancreatitis, toxic shock and sepsis etc. [7-9]. It has also been reported that UTI suppresses neutrophil accumulation and activity. The genes and proteins regulated by UTI are implicated in the inflammatory process [10]. Therefore, UTI is not just a protease inhibitor, but can also prevent inflammation and cytokine-dependent signaling pathways. In preclinical and clinical studies, UTI has been reported to protect against acute lung injury, graft ischemia/reperfusion injury, renal failure after cardiopulmonary bypass, severe burn injury and septic shock [7].

In addition, an important prior study [11] suggests that UTI can attenuate the levels of central IL-6 and TNF-α and improve brain edema, neurological function and survival in CA rats. Nevertheless, the underlying mechanisms leading to the effects of UTI on ischemic insults still need to be determined. In the present study, we examined the role played by UTI in regulating PIC mechanisms in the rats' specific brain regions, namely the neocortex

* E-mail: wangxxu2016@163.com

and hippocampus CA1 after CA induced-global ischemia. Furthermore, the specific effects of UTI on the protein expression levels of PIC receptors were examined in those brain regions of CA rats. We hypothesized that UTI plays a beneficial role in modulating the levels of IL-1β, IL-6 and TNF-α and protein expression of their respective receptors IL-1R, IL-6R and TNFR1 in those brain regions, and thereby improves neurological deficits and survival.

Materials and methods

Experimental animals

All the animal procedures were approved by the Institutional Animal Care and Use Committee of Jilin University, which were in compliance with the Guideline for the Care and Use of laboratory Animals of the U.S. National Health Institute. Male Sprague-Dawley rats (weighing 200-300 g) were used in the experiments.

Transient global ischemia induced by cardiac arrest

The ischemia produced in the CA model was induced by asphyxia. Rats were briefly anesthetized with an isoflurane-oxygen mixture (2-5% isoflurane in 100% oxygen). An endotracheal tube was first inserted and attached to a ventilator. The ventral tail artery was cannulated to monitor systemic arterial pressure. The right jugular vein was cannulated for a continuous infusion of saline (at a rate of 0.1 ml/h) to maintain baseline blood pressure and fluid balance. Body temperature was continuously monitored and maintained at 37°C with a heating pad and external heating lamps. Asphyxia was induced by stopping mechanical ventilation and clamping the tracheal tubes at the end of expiration. Resuscitation efforts began 6 min after CA was induced. For this purpose, rats were subjected to orotracheal intubation for mechanical ventilation accompanied by chest compression delivered by a mechanical compressor at a rate of 200/min for 5 min. Once a spontaneous heartbeat returned, epinephrine (2 μg) was administered to achieve a mean arterial blood pressure greater than 80 mm Hg. Ventilation was adjusted for animals to regain spontaneous respiration and achieve normoxia. The animals that survived from this procedure were used for further interventions. For pain relief those rats received analgesic buprenorphine (0.02 mg/kg) subcutaneously immediately and every 6 h for a 72 h period after the asphyxial procedures. In the control group operated animals, the same surgical procedures were performed without cardiac arrest and resuscitation.

Study interventions and animal groups

The rats were divided randomly. The group 1 rats received the same surgical procedures and endotracheal intubation that were performed, but with no asphyxia and CPR. In group 2, CA and CPR were performed and the rats were given 0.5 ml of saline (intraperitoneally, every 12 h for 3 days) after CA. In group 3, CA and CPR were carried out and 2000 units/kg body weight of UTI (intraperitoneally, every 12 h for 3 days) was injected after CA. At the end of each experiment, the rats were sacrificed and then the brains were taken out. The neocortex and CA1 region of the hippocampus were used for biochemical measurements.

Neurological examination

The modified method of the Neurological Severity Score (mNSS) was used to examine neurological functions in this study (Table 1). Note that the mNSS was generally used to assess a combination of motor, sensory, and balance functions. Neurological function was graded on a scale of 0-18 (normal score, 0; maximal deficit score, 18). If it was found that the mNSS score of rats was > 0 before CA, this indicated that the rats were abnormal and they were excluded from the experiment. It is noted that the experiments were performed in a blind manner.

Brain edema (brain water content) was determined 72 h after CA. The brain slices (2 mm thick) of the hemispheres and cerebellum were cut. The whole brain water content was calculated from all slices [12, 13]. The brain slices were weighed to obtain the wet weight immediately and dried in an oven at 100°C for 24 h to obtain the dry weight. The cerebellum was used as the internal control. The water content was expressed as the following formula: [(wet weight) – (dry weight)] / (wet weight) × 100%.

Table 1. Neurological Severity Scores (NSS)

Motor tests (6 points)
Raising rat by the tail (3 points) Flexion of forelimb (1) Flexion of hindlimb (1) Head moved >10° to vertical axis within 30 s (1) Placing rats on the floor (normal = 0; maximum = 3 points) Normal walk (0) Inability to walk straight (1) Circling toward the paretic side (2) Fall down to the paretic side (3)
Sensory tests (2 points)
Placing test (visual and tactile test) (1) Proprioceptive test (deep sensation, pushing the paw against the table edge to stimulate limb muscles) (2)
Beam balance tests (normal = 0; maximum = 6 points)
Balances with steady posture (0) Grasps side of beam (1) Hugs the beam and one limb falls down from the beam (2) Hugs the beam and two limbs fall down from the beam, or spins on beam (> 60 s) (3) Attempts to balance on the beam but falls off (> 40 s) (4) Attempts to balance on the beam but falls off (> 20 s) (5) Falls off: No attempt to balance or hang on to the beam (> 20 s) (6)
Reflexes absent and abnormal movements (4 points)
Pinna reflex (head shake when touching the auditory meatus) (1) Corneal reflex (eye blink when lightly touching the cornea with cotton) (1) Startle reflex (motor response to a brief noise from snapping a clipboard paper) (1) Seizures, myoclonus, myodystony (1)
Overall maximum points = 18 points

ELISA measurements

All the tissues from individual rats were sampled for the analysis. In brief, the neocortex and hippocampal CA1 region of the rats were removed under a dissection microscope. Total protein was then extracted by homogenizing the cortex and hippocampus sample in ice-cold immunoprecipitation assay buffer with protease inhibitor cocktail kit. The lysates were centrifuged and the supernatants were collected for measurements of protein concentrations using a bicinchoninic acid assay reagent kit.

The levels of PICs were examined using an ELISA assay kit (Promega Co., Madison, WI, USA) according to the provided description and modification. Briefly, polystyrene 96-well microtiter immunoplates were coated with affinity-purified rabbit anti-IL-1β, anti-IL-6 and anti-TNF-α antibodies. Parallel wells were coated with purified rabbit IgG for evaluation of nonspecific signals. After overnight incubation, plates were washed. Then, the diluted samples and these PIC standard solutions were distributed in each plate. The plates were washed and incubated with anti- IL-1β, IL-6 and TNF-α galactosidase. Then, the plates were washed and incubated with substrate solution. After incubation, the optical density was measured using an ELISA reader.

Western blot analysis

After being denatured by heating at 95°C in an SDS sample buffer, the supernatant samples were loaded onto Mini-Protean TGX Precast gels (Bio-Rad Laboratories, Hercules, CA, USA) and electrically transferred to a polyvinylidene fluoride membrane. The membrane was then incubated overnight with primary antibodies: rabbit anti-IL-1R, anti-IL-6R and anti-TNFR1 (Abcam Co., Cambridge, UK, diluted 1:200). After being fully washed, the membrane was incubated with horseradish peroxidase-linked anti-rabbit secondary antibody and visualized for immunoreactivity. The membrane was stripped and incubated with mouse anti-β-actin to show equal loading of the protein. The bands recognized by the primary antibody were visualized by exposure of the membrane onto an X-ray film. Then, the film was scanned and the optical densities of IL-1R, IL-6R, TNFR1 and β-actin bands were determined using the public domain ImageJ program (made by Wayne Rasband, Scion Corporation, Bethesda, MD, USA). Then, values for densities of immunoreactive bands/β-actin band from the same lane were determined. Each of the values was then normalized to a control sample.

Statistical data analysis

Experimental data were analyzed using one-way repeated measures analysis of variance (ANOVA). As appropriate, Tukey's *post hoc* tests were used. All values were presented as mean ± standard deviation. For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed using SPSS for Windows version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Survival rate in three groups of animal

The survival rate of 72 h was 100% (16/16 rats) in sham control rats; 60% (21/35 rats) in CA rats; 89% (31/35 rats) in CA rats injected with UTI. It is noted that when CA rats received UTI treatment, the survival rate was increased during 72 h compared with CA rats with saline injection (CA rats with UTI treatment *vs.* CA rats with saline treatment). Data obtained from those survival rats were included for the analysis in this report.

Neurological function and brain edema

Figure 1A shows that CA increased the mNSS in rats ($P < 0.05$, control rats *vs.* CA rats; n = 16 in control and n = 21 in CA group). This figure further demonstrates that UTI significantly attenuated the impaired mNSS in animals 72 h following CA ($P < 0.05$, CA rats with saline treatment *vs.* CA rats with UTI; n = 31 in CA group with UTI). Likewise, fig. 1B shows that induction of CA amplified water content of brain tissues in rats and administration of UTI significantly attenuated increases of water content.

PICs and their respective receptors

Figure 2 demonstrates that IL-1β, IL-6 and TNF-α were increased in the neocortex and hippocampal CA1 region 72 h after CA ($P < 0.05$ *vs.* control, n = 16 in control and n = 21 in CA group). Administration of UTI significantly attenuated the amplified levels of IL-1β, IL-6 and TNF-α ($P < 0.05$ *vs.* saline treatment; n = 31 in CA group with application of UTI).

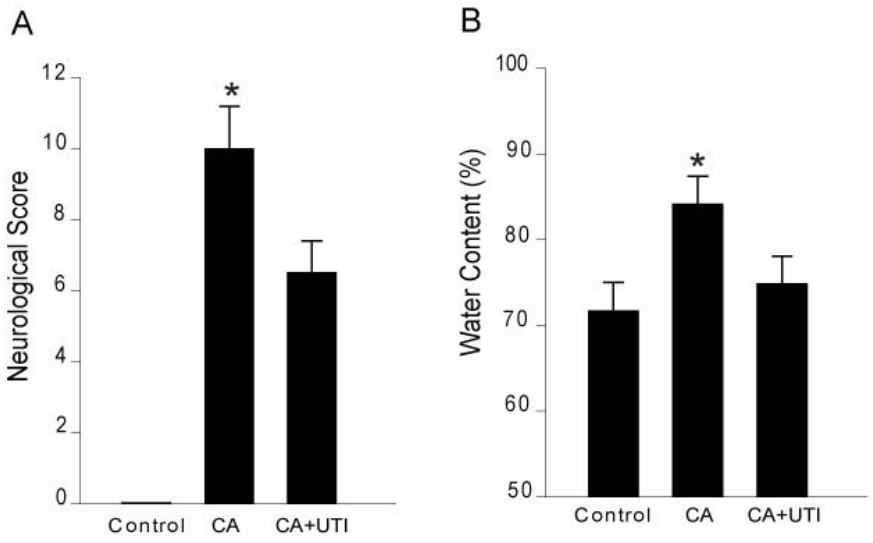

Figure 1. Panels A and B: the mNSS and water content of brain tissues were increased in rats 72 h after CA and they were improved by administration of UTI. *$P < 0.05$ *vs.* control rats and CA rats with UTI. The number of rats n = 16 in control; n = 21 in CA group and n = 31 in CA group with UTI. CA, cardiac arrest; mNSS, modified Neurological Severity Score; UTI, urinary trypsin inhibitor.

Similarly, fig. 3A and B illustrate that induction of CA increased the protein expression of IL-1R, IL-6R and TNFR1 in the cortex and hippocampus CA1 region ($P < 0.05$, control rats *vs.* CA rats; n = 6-10 in each group). When UTI was injected, increases in protein expression of these PIC receptors were significantly attenuated in CA rats.

Discussion

A rat model of asphyxia induced CA followed by CPR has widely been used to study transient global ischemia. Prior studies have demonstrated that hypoxia inducible factor-1 (HIF-1) subtype HIF-1α is expressed in the brain tissues including the neocortex and hippocampus and engaged in neuronal apoptosis after induction of global ischemia [14-17]. In particular, a recent study using this model has further demonstrated that CA increases IL-1β, IL-6 and TNF-α and upregulates their receptors IL-1R, IL-6R and TNFR1 in the hippocampus [18]. Systemic activation of HIF-1α attenuates these exaggerated PIC signal pathways [18], and as a result neuronal apoptosis and neurological deficits induced by CA are attenuated [19]. In the present study, we administered UTI and then examined its effects on neurological deficits observed in CA rats. Our data suggests that PIC mechanisms are engaged in the protective role played by UTI in the pathophysiological process of transient global ischemia.

In an important prior study [11], UTI has been shown to play a protective role in CA induced-cerebral ischemia by attenuating brain edema and inflammatory responses (i.e., central IL-6 and TNF-α) and thereby improving survival and neurological function. In contrast, in the present study, we further studied the effects of UTI on the protein expression levels of PIC receptors in CA rats. It should also be noted that we determined the levels of IL-1β, IL-6 and TNF-α as well as their respective receptors in the specific brain regions, cortex and hippocampus CA1 region. Those specific brain regions were selected in our study because expression of PICs and their receptors is upregulated in those regions following CA in rats and in general they are also more related to neurological deficits after cerebral ischemia than other brain regions [18, 20]. Our results showed that CA amplifies the levels of IL-6 and TNF-α in addition to IL-1β, which is consistent with the previous report [11]. Notably, we further found that the protein expression of PIC receptors such as IL-1R, IL-6R and TNFR1 were also increased in the cortex and hippocampus CA1 regions. In addition, administration of UTI attenuated increases in those PICs and their receptors in CA rats. Taken together, UTI plays a beneficial role in regulating cerebral ischemia.

PICs including IL-1β, IL-6 and TNF-α are responsive to global ischemic stress [5] and during this response PICs recruit cells to inflammatory sites and modulate cell survival, division, proliferation and differentiation [6]. This process is likely to modulate the responsiveness of many cell types under diseased conditions. Using a rat model it has previously been shown that CA increases IL-1β, IL-6 and TNF-α in the hippocampus CA1 region [18]. CA also augments a representative nucleus transcription factor indicating cell apoptosis, caspase-3, which is linked to upregulation of PIC signal pathways. In the current study, we found that systemic injection of UTI attenuates amplification of PIC levels and their respective

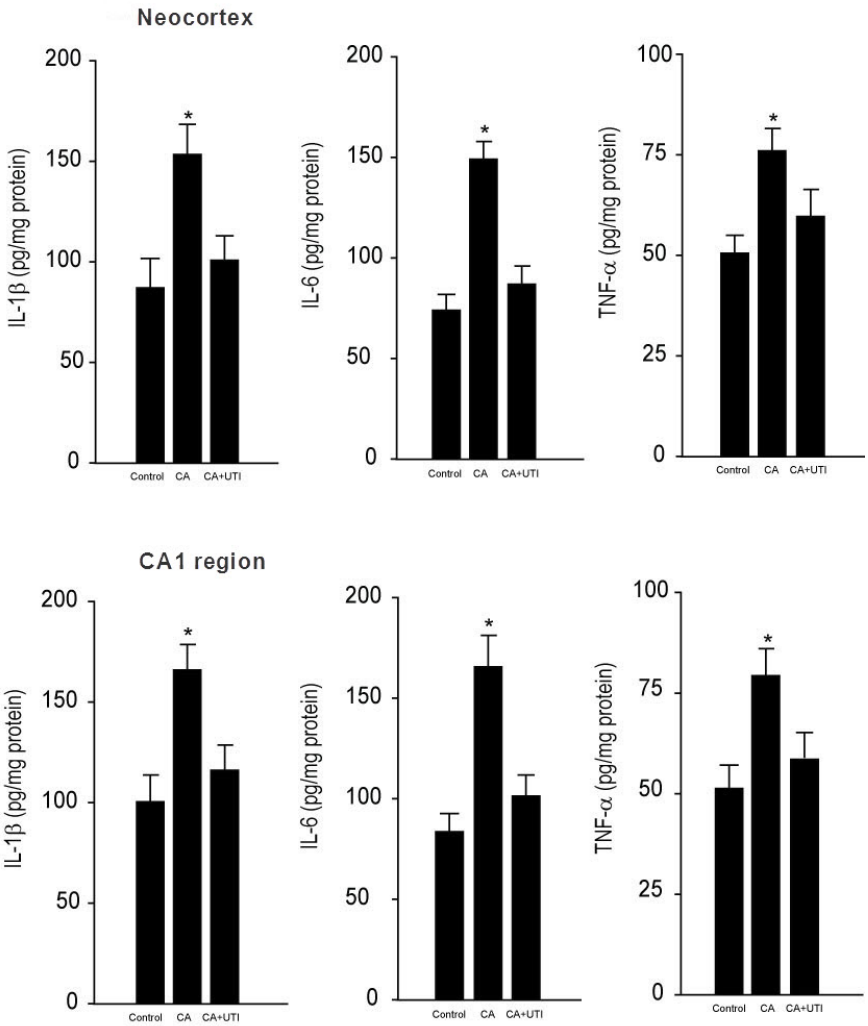

Figure 2. Induction of CA amplified the levels of PICs in the cortex (top panel) and hippocampus (bottom panel). Systemic administration of UTI attenuated increases of PICs in rats 72 h after CA. *$P < 0.05$ *vs.* control rats and CA rats injected with UTI. The number of rats n = 16 in control; n = 21 in CA group and n = 31 in CA group with UTI. CA, cardiac arrest; PICs, pro-inflammatory cytokines; UTI, urinary trypsin inhibitor.

receptor expression in the cerebral cortex and CA1 region of ischemic rats. Importantly, UTI can improve the neurological Severity Score, brain tissue edema and survival in CA rats. Data of the current study specifically suggests a beneficial role played by UTI in modulating transient global ischemia induced by CA and attenuating PIC signaling mechanisms.

Blood flow and oxygen delivery to various organs such as the brain are largely decreased during CA, thereby leading to ischemic injury in those organs [1]. In response to hypoxic stress, HIF-1α is synthesized and the expression of its downstream products such as vascular endothelial growth factor (VEGF) is upregulated and remains elevated [14, 21]. This contributes to neuroprotection against brain ischemic conditions by improving the permeability of the blood brain barrier, reducing brain edema formation and promoting the recovery of brain injuries [2]. A recent study has shown that stabilizing HIF-1α significantly attenuates increases in caspase-3 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, labeling of apoptotic cells) evoked by induction of CA [19]. As a result, this process improves neurological deficits and neuronal edema observed in rats after CA. Also, activation and maintenance of HIF-1α attenuates the upregulation of central nervous PIC pathways in rats with transient global ischemia [18]. Data of our current study further provides evidence suggesting that inhibition of PIC signal pathways is likely involved in neuroprotective effects and attenuating cerebral damages evoked by global ischemia.

One of the study limitations is that the cerebellum was used as an internal control to assess the brain water content, but one should note that the cerebellum was likely altered in a rat model of global cerebral ischemia. Nonetheless, consistent with the prior report [11] our data indicates that UTI can attenuate amplified brain water content induced by CA. Second, it is known that CA-induced cerebral ischemia is associated with inflammation and release of numerous and various inflammatory mediators [4, 5]. In the present study, we studied only IL-1β, IL-6 and TNF-α signaling pathways. Thus, inhibition of those specific cytokines is likely a part of the protective roles played by UTI and additional studies need to clarify the precise mechanisms of UTI. Other limitations included the possible imperfections in isolation of the hippocampal CA1 region and the fact that specific tests for assessment of cognitive abilities, particularly in relation to hippocampal dysfunction, were not utilized.

In summary, transient global ischemia induced by CA increases the levels of PICs and their receptors in the cortex and hippocampus CA1 region. Systemic administration of UTI can attenuate amplified expression of PIC signal pathways in ischemic animals and thereby improve the modified Neurological Severity Score, brain tissue edema and survival. Our data indicate the beneficial role played by UTI in alleviating cerebral ischemia reperfusion injury partly via PIC mechanisms, but larger more detailed studies on these lines should be made in order to reach firm conclusions.

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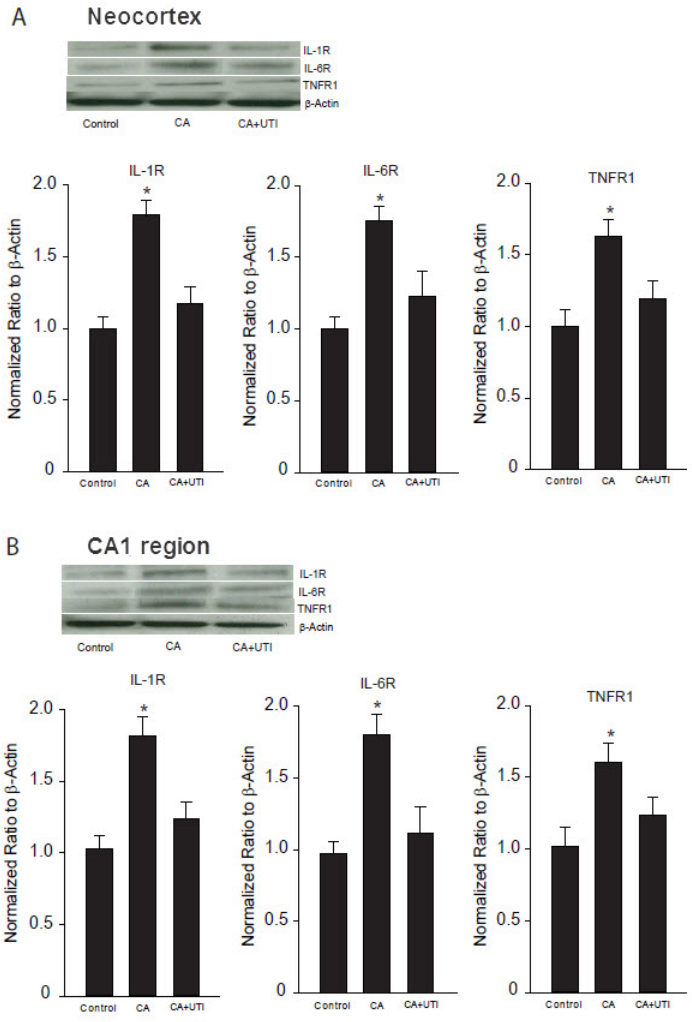

Figure 3. The protein expression of IL-1R, IL-6R and TNFR1 in the neocortex (panel A) and hippocampus (panel B) tissues was increased in rats 72 h after CA. UTI attenuated the upregulation of PIC receptors evoked by CA. Panels A and B: top panels indicate representative bands and bottom panels indicate averaged data. *$P < 0.05$ *vs.* control and CA rats with administration of UTI. The number of rats n = 6-10 in each group. CA, cardiac arrest; IL-1R, interleukin-1 receptor; IL-6R, interleukin-6 receptor; PICs, pro-inflammatory cytokines; TNFR1, tumor necrosis factor receptor 1; UTI, urinary trypsin inhibitor.

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