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Full Length Research Paper

Effect of ulinastatin preconditioning on the expression of hemeoxygenase-1 in limb ischemia reperfusion lung injury in rats

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To investigate the role of HO-1 in ischemic preconditioning and pharmacological preconditioning by ulinastatin in attenuating remote lung injury in rat model of lower limb ischemia reperfusion. Sixty four male SD rats were randomly divided into four groups: Sham operation group, ischemia and reperfusion group, ischemia preconditioning group (IPR), ulinastatin preconditioning group (UTI). The animals were deeply anesthetized and the lung tissue was removed at the end of the experiment. Lung W/D ratio were measured and the expression of HO-1 was determined by Immunohistochemical staining and western blotting. HO-1 was induced and expressed persistently in 2 and 4 h after ischemic preconditioning and ulinastatin preconditioning (P<0.01), and the expression in 4 h was higher than 2 h in preconditioning groups (P<0.05). The preconditioning also led to diminished lung edema (W/D ratio) (P<0.05 vs I/R), with no differences when compared to the sham group. The induction of HO-1 by ischemic preconditioning and ulinastatin preconditioning played a protective role against remote lung injury in the model of limb injury/reperfusion.

Key words: Heme oxygenase-1, preconditioning, ulinastatin, ischemia/reperfusion.

INTRODUCTION

Lower limb ischemia followed with reperfusion is an important and common clinical event. Both clinical observation and animal experiment indicate that the reperfusion can save the limbs but result in multisystem organ dysfunction, and even death in extreme cases. Although the systemic inflammation of limbs ischemia/reperfusion can damage any organ through circulation, the onset of the syndrome usually starts with the development of pulmonary dysfunction. The infiltration and activation of neutrophils and oxygen free radicals generated in the reperfused extremity are considered to be responsible for this process. In response to oxidative stress, cell often synthesize specific antioxidant proteins, such as superoxide dismutase, catalase and glutathione peroxidase. One protein that was found to be activated by oxidative stress is the heme degrading enzyme, hemeoxygenase (HO), especially its inducible form HO-1 and one of its catalytic product carbon monoxide (CO). Ischemic preconditioning (IPC) and pharmacological preconditioning suggest that brief periods of I/R or pretreatment with drugs render a tissue relatively resistant to the harmful effects of a subsequently prolonged period of ischemia. Previous studies showed the protective effect of IPC and pharmacological preconditioning in not only the ischemia tissues but also in the remote organs. Urinary trypsin inhibitor (UTI), also referred to as ulinastatin, is a protease inhibitor purified form human urine. It inhibits the functions of various enzymes simultaneously, depending on its multivalent Kunitz-type. UTI has been widely used in Japan as a drug for patient with disseminated intravascular coagulation (DIC), shock, and pancreatitis. During *in vivo* and *in vitro* animal experiments UTI has been reported to have anti-inflammatory properties apart form blocking the protease pathways; it could also inhibit the production of proinflammatory molecules such as

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thromboxane B2(TBX2), Interleukin-8(IL-8), tumor necrosis factor-a(TNF-a) induced by LPS. In the present study, we examined that whether the protection of IPC and pharmacological preconditioning correlated with upregulation of the expression of HO-1.

MATERIALS AND METHODS

Animal and surgery

Male Sprague-Dawley (SD) rats (230 to 260 g) were used for this study. One day before experiment the animals were fed on free water but no food. On the day of experiment the animals were anesthetized with an intraperitoneal injection of 4% chloral hydrate (0.6 ml/kg). Anesthesia was maintained through the experiment with additional dose of intraperitoneal chloral hydrate (0.2 to 0.3 ml/kg). The left jugular vein was cannulated (catheter 24G, Belgum) for drug administration and continuous perfusion of physiological saline (8 ml/kg/h). The animals firstly received heparin intravenously (500 IU/kg). Central temperature was maintained between 36 to 38℃ by a heating lamp placed above the animal. After the equilibration period of 30 min, the bilateral femoral aorta were dissected and clamped by noninvasive microarterial clip (HC-X018,HC-X023 Cheng-He Microsurgical instruments factory),

Four group of rats were used in this study: (I) the sham group (Sham, n=16) served as time controls, which were kept anesthetized without surgical intervention but the bilateral femoral aotra disstected; (II) the ischemia and reperfusion group (I/R,n=16) 1 ml NS was injected intravenously. Afer 30 min the animals were subjected to 2 h of ischemia followed by 2 h (n=8) or 4 h (n=8) of reperfusion. (III) The ischemia proconditioning group (IPR): the bilateral femoral artery were exposed to three cycles of ischemic proconditioning (5 min of ischemia followed by 5 min of reperfusion) then sebsequent 2 h of ischemia followed by 2 h of reperfusion (n=8) or 4 h of reperfusion (n=8). (IV) Urinary trypsin inhibitor proconditioning group (UTI) three minutes before the ischemia urinary was injected through the left jugular (50000 U/kg, dilutied in 1 ml NS) then under 2 h ischemia followed by 2 and 4 h reperfusion. The study was approved by Wenzhou medical institutional animal care and use committee and all animals were treated according to the Zhejiang province regulation for animal experimentation.

At the end of the experiments the rats were sacrificed by exsanguination. A median sternotomy was performed and the left atrium was excised allowing free pulmonary drainage. Then the pulmonary circulation was flushed with 20 ml of physiological saline at 4℃, slowly injected into the right ventricle. The left lung was harvested and stored at -80℃ for the HO-1 expression analysis with western blot; the right superior and median lung were immersed in 10% formalin for histological and morphological analyses; the inferior lobe was used for wet-dry ratio.

Lung water content

The lung water content of the obtained inferior lobe was calculated as: (wet weight/dry weight)/100.

HO-1 staining and western blot

The right superior lobe was processed for paraffin sections and then prepared for immunohistochemistry of HO-1. The sections were cooked in citrate buffer for antigen retrieval before PBS wash and in 3% H_2O_2 incubation for 15 min. Then the sections were incubated with rabbit-anti-HO-1 (Abcam, 1:2000) overnight at 4℃ in prior to secondary antibody (PV-9001 polymer HRP anti-rabbit IgG) and peroxidase-conjugated avidin based visualization. Finally the sections were counterstained with 1% Hematoxylin and mounted for microscope analysis. The left lung for western blot was homogenized in 1:10 (W/V) ice-cold homogenization buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 50 mM NaF, 1%(W/V) Triton X-100, 0.1% (W/V) SDS, 1 mM deoxycholate, 0.1 mM EDTA,1 mM sodium orthovanadate, 1 mM PMSF. The homogenates were centrifuged at 12,000 × g for 20 min at 4℃, Supernatants were collected and protein concentrations were measured with protein concentration determination kit (P0012s, Beyotime biotechno co. Ltd). Samples were then stored at -80℃ until use. An aliquot of homogenate was suspended in SDS sample buffer (P0015, Beyotime) and equal amounts of protein per lane (50 µg and 15 µl) were loaded for electrophoresis in a 10% SDS-polyacrilamide gel. After the electrophoresis, the gel was transferred onto a polyvinylidene fluoride (PVDF) membrane (FFP30, Beyotime) by clectroblotting. The membrane was blocked by incubation in Tris buffered saline with 0.1% Tween-20 (TBST) and 5% fat-free milk, and the overnight at 4℃ with primary antibodies (1:2000 Abcam,abl3243) in the same solution. Membrane was then washed and incubated with horseradish peroxidase conjugated secondary antibodies (1:10000) for 1.5 h and developed using BeyoECL Plus color substrate (Byotime, China). The intensities of the bands on the film were scanned and analysed with Quantity One 4.4.0 image analyzer (Bio-Rad Company, USA).

Statistical analysis

Data was expressed as mean± standard deviation (SD) or as median (lowest, highest). The significant differences among the four groups were determined by one-way ANOVA or Krukal-wallis H test; intergroup comparisons between groups were determined by LSD test or Dunnett T3 test; individual comparison between groups were made with the T-test or Mann-Whitney test. $P<0.05$ was considered statistically significant.

RESULTS

Lung water content changes following I/R and pre-conditioning

I/R of the lower limbs was associated with an increased lung water content: 4.4±0.7 in the I/R group when compared to 3.8±0.6 in the sham group after 2 h of reperfusion; 3.2±0.1 in the sham group after 4 h of reperfusion, $P<0.05$). The water content decreased significantly in IPR groups and UTI groups ($P<0.05$), and there were no differences between sham groups and IPR/UTI groups. Moreover, after 4 h of reperfusion, the increase in the IPR and UTI group was further attenuated significantly when compared to the IPR group and UTI group after 2 h of reperfusion (Table 1).

Histological examinations of the lung tissues in different groups

In histological examinations, interstitial edema and hemorrhage were identified in rat lung tissues. Rats from I/R groups showed pulmonary lesions consisting of large areas of marked intravascular, interstitial, and

Table 1. SD rat lung tissue Wet/Dry weight ratio ($\bar{x}$ ±s, n=8).

Group	W/D	Group	W/D
S2	3.8±0.6 #	S4	3.2±0.1 # #
I/2R	4.4±0.7	I/4R	4.4±0.7
IP2R	3.5±0.6#	IP4R	2.7±0.3 # #*
U2	3.5±0.5#	U4	2.7±0.4 # #*

Compared to I/2R group, # P<0.05, compared to I/4 group, ## P<0.05, Compared to IP2R group, * P<0.05.

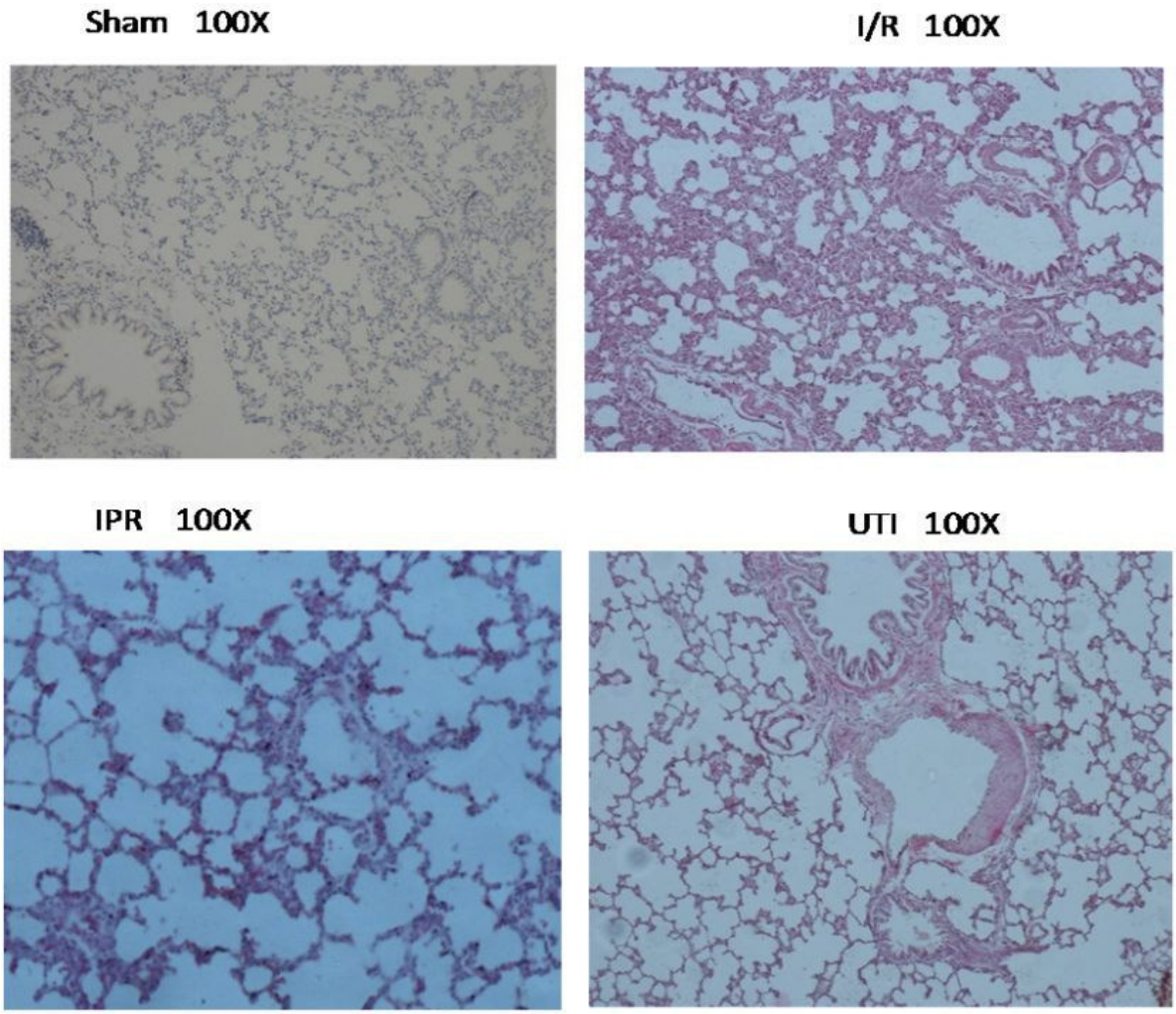

Figure 1. I/R groups, with no apparent differences in the lesions between shams, IPR and UTI groups.

intraalveolar polymorphnuclear neutrophil and macrophage infiltrations, exhibiting a multifocal distribution in the entire lung. Areas with inflammtory infiltrates were also indentified in lungs from IPR groups and UTI groups. However, these lesions were less numerous, less extended and less dense when compared to those of I/R groups, with no apparent differences in the lesions between shams, IPR and UTI groups (Figure 1).

Location and semi-quantitative assessment of HO-1 in lung

Western blot analysis revealed the expression of HO-1 was correlated with the results from immuno-

histochemistry staining. Both results indicated an increased HO-1 protein content within lungs of rats from the I/R groups with respect to Sham groups (P<0.05, Table 2; P<0.01, Figure 1), and a significant expression of HO-1 was evident in the IPR and UTI groups (when compared with I/R groups P<0.02, Table 2; P<0.01, Figure 1). Moreover, expression of HO-1 after 4 h reperfusion were more than that of 2 h reperfusion in the IPR groups and UTI groups (P<0.02, Table 3; P<0.05, Figure 2). Diffusely increased staining was found throughout the lungs, with particular abundance of HO-1 protein in the airway epithelia, endothelial cells, inflammatory cells, mild of that in the alveolar epithelial cells.

Table 2. Expression of HO-1 in lung tissue of SD rat.

Group	$\bar{x}$ ±s	Group	$\bar{x}$ ±s
S2	6.3±1.9	S4	13.2±4.2
I/2R	12.5±4.3 #	I/4R	28.2±1.1 #
IP2R	35.4±1.5 #*	IP4R	99.6±2.3 #*
U2	43.6±1.8 #*	U4	90.7±3.5 # *

Compared to S2, S4 group, # P<0.05, compared to I/2R, I/4R group, *P<0.02.

Table 3. expression of HO-1 in lung tissue of SD rat.

Group	$\bar{x}$ ±s	Group	$\bar{x}$ ±s
IP2R	35.4±1.5	IP4R	99.6±2.3 #
U2	43.6±1.8	U4	90.7±3.5 *

Compared to IP2R group, # P<0.02, Compared to U2 group, *P<0.02.

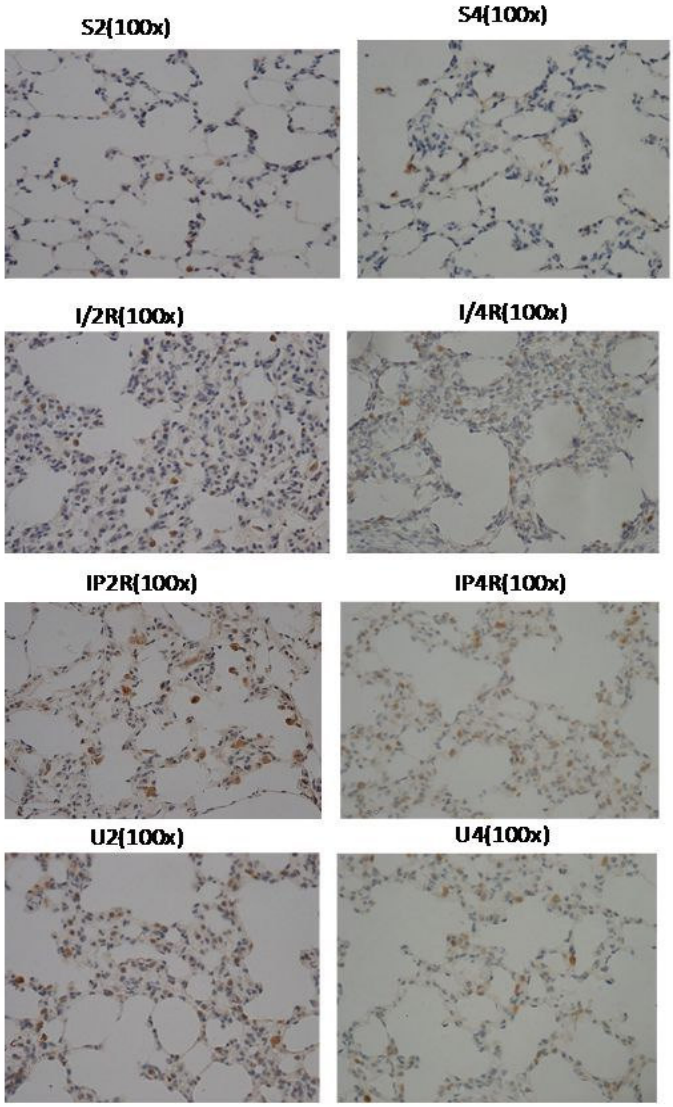

Figure 2. Immunohistochemical analysis.

Immunohistochemical analysis for HO-1 in the lung at 2 and 4 h after limb I/R

Sham group: Little almost not detectable positive HO-1 staining can be found (100x) I/R groups: Mild positive HO-1 staining is mainly found in airway epithelia, endothelial cells, inflammatory cells, mild of that in the alveolar epithelial cells IPR groups and UTI Group: intense positive HO-1 staining is found in airway epithelia, endothelial cells, inflammatory cells and alveolar epithelial cells (Figure 2, Tables 4 and 5).

DISCUSSION

In our study, lung injury can be induced by the limb ischemia only after 2 h reperfusion. HO-1 expression in the lung could be found in airway epithelia, endothelial cells, inflammatory cells, mild of that in the alveolar epithelial cells but not enough to prevent the lung injury. Ischemic preconditioning and UTI preconditioning protected the lung injury caused by edema, hemorrhage, neutrophil infiltration, correlating to an increased expression of HO-1. For example, at the end of 4 h reperfusion, the expression of HO-1 was persistently higher than that of 2 h reperfusion. The heme oxgenase (HO-1) system is the rate-limiting step in the conversion of heme into biliverdin, carbon monoxide (CO), and free iron. Three HO-1 isoform have been identified: inducible HO-1 (heat shock protein32 (HSP32)), constitutively expressed HO-2.and HO-3 that was less well characterized. Under physiological conditions, HO-2 is the major HO isoform found in mammalian tissues, particularly in brain and testis. In contrast, HO-1 expression is relatively low, with the exception of spleen

with constitutively high expression. Upregulation of HO-1 could represent one of the most critical cytoprotective

Table 4. The expression of HO-1 in the lung.

Group	Median {(5%), (95%)}	Group	$\bar{x}$ ±s
S2	0.043 (0.012, 0.232)	S4	0.33±0.05
I/2R	0.271 (0.184, 0.541)#	I/4R	0.66±0.22 #
IP2R	0.877(0.595, 1.319)#*	IP4R	1.09±0.23 #*
U2	1.019(0.829, 1.403)#*	U4	1.18±0.21 #*

Compared to S2, S4 group, # P<0.01, compared to I/2R, I/4R group, *P<0.01.

Table 5. The expression of HO-1 in the lung.

Group	Median {(5%), (95%)}	Group	$\bar{x}$ ±s
IP2R	0.877(0.595, 1.319)	IP4R	1.09±0.23 #
U2	1.019(0.829, 1.403)	U4	1.18±0.21 *

Compared to IP2R group, # P<0.05, compared to U2 group, *P<0.05.

and Alam, 1996), and it was thought to play a key role in maintaining antioxidant/oxidant homeostasis during cellular injury. It has been documented that HO-1expression in lung during the model of lung injury induced by limbs ischemia/reperfusion (Boutros et al., 2005). However the ischemia/reperfusion injury happened not only in the limb but also in small intestine tissue; in our study we manipulated to have the local I/R injury of the limb. More findings have led to a re-definition of the HO pathway as not only an anti-oxidative mechanism, but also a complex and coordinated cytoprotective system (Otterbein et al., 2003).

Ischemia and reperfusion insult, an antigen independent component of the harvesting injury, has a complex pathophysiology with a number of contributing factors. Currently there is no effective treatment available to prevent the remote organs dysfunction induced by ischemia/reperfusion; the most promising strategy explored during the last few years was the HO-1 system targeting therapy. HO-1 could amplify multiple intracellular cytoprotective mechanisms against a variety of cellular insults. The anti-oxidant function of HO-1 was more than heme degradation; it also included biliverdin/bilirubin production. Ferritin upregulation also provided antioxidant function. CO, the last byproduct of HO-1, exerted important anti-inflammatory and anti apoptotic effects via p38 MARK activation, and could also modulate the vascular tone, which leads to diminished platelet aggregation and depressed fibronigenesis.

Given the multifactorial cytoprotective properties of the HO-1 system, the recruitment of this system as a novel strategy to prevent I/R injury has been extensively studied (Katori et al., 2002; Fondecila et al., 2003). Indeed, HO-1 overexpression exerted potent cytoprotective functions in a numbers of hepatic I/R injury transplant models; HO-1 induction by pharmacological means (eg, cobalt protoporphyrin, hemin) or genetic engineering maintains tissue architecture, preserved organ function, and increased local induction of anti-apoptotic Bcl-2/Bag-1, whereas decreased iNOS/caspase-3 expression (Coito et al., 2002). Clearly, HO-1 induction exerted cytoprotection against I/R insults in association with the modulation of pro-and anti-apoptotic pathways; it has also been shown that HO-1knocked-out mice exhibited dysregulation inflammatory response to injury and more widespread apoptosis of immune cells (Tracz et al., 2007). Therefore, the elimination of oxidant or pro-oxidants formed the cell was considered to be an important mechanism of HO-1 mediated protection against oxidative stress. In the clinical applications, however, utilizing HO-1 could have some limitations because there were no known pharmacological reagents that specifically induce HO activity.

Ischemic preconditioning refers to a mechanism whereby brief periods of I/R render a tissue relatively resistant to the harmful effects of a subsequent prolonged period of ischemia (Murry et al., 1991). Olguner et al. (2006) have found that ischemic preconditioning attenuated the lipid peroxidation and remote lung injury in the rat model of unilateral lower limb ischemia reperfusion. Numerous molecules were released during preconditioning ischemia (Li et al., 2001). These included adenosine, acetylcholine, catecholamines, angiotensin II, bradykinin, endothelin, and opiods, though the end-effectors of classical preconditioning are still under debate. However, Downey and Cohen (1995) have proposed a scheme whereby the activated cell surface

mechanisms that are activated upon cellular stress, such as inflammation, ischemia, hypoxia, hyperthermia (Choi

receptors coupled through G-proteins to phospholipase-C and the formed diacylglycerol activates protein kinase C (PKC). Subsequent studies have proved the cell signaling pathways is in prevalence among various species, and

the attempts to modulate the end-effectors of IPC have spawned therapeutic research. In our study, the heme oxygenase-1 could be one of the most important end - effectors and then triggers the cascade of HO/CO. However, the protection of ischemic preconditioning used as a clinical strategy to attenuate the lung injury induced by limbs ischemic/reperfusion was limited by the inability to predict the onset of ischemia.

And although these stimuli are powerful means of protecting the lung injury elicited limbs ischemia/ reperfusion from irreversible injury, their clinical applicability may be limited since: 1) the mechanical intervention may require precise, timed pulsations of ischemia and reperfusion, different among different species; 2) A reservation of physicians to purposely create an ischemic tissues; 3) the requirement of training of emergency medical professionals in this technique to provide timely intervention. Therefore, an alternative means of harnessing this protection by the use of specific clinical drug could provide a feasible means of effectively producing protection clinically.

UTI protects against SIRS pathophysiology and subsequent organ damage induced by LPS in mice, partly through the modulation of the proinflammatory cytokine IL-1, as well as chemokines such as MIP-2, MCP-1.and KC. These *in vivo* results provided direct and novel molecular evidence for the "rescue" therapeutic potential of UTI against systemic inflammatory responses syndrome such as DIC, acute lung injury, and multiple organ dysfunction syndrome (Inoue et al., 2008). UTI not only plays an important role in protease inhibition, but also has a beneficial effect on inflammatory diseases. Intravenous administration of UTI reduces ischemia-reperfusion injury in the rat brain (Yano et al., 2003), improves impaired cardiac function during hemorrhagic shock in rats (Masuda et al., 2003), and ameliorates clinical parameters including survival rate, cardiac index, as well as blood pressure during septic shock in canines (Tani et al., 1993). However all these studies had a critical limit in that the animals were treated with human-derived UTI as a foreign protein, leaving the direct effect of UTI on inflammatory disease including systemic inflammatory response syndrome unexamined in knock-out mice.

Inoue et al. (2005) have demonstrated the protective role of UTI in acute lung inflammation induced by LPS in a UTI-null mouse model. With UTI there is no significant increase in the lung water content and in the neutrophil numbers in BAL fluid in the presence of LPS, suggesting that UTI inhibited proteases and subsequent cascades of inflammatory events. On the other hand, it also can be speculated that in the absence of UTI, the lung damage can be more severe, can be longer lasting, or perhaps can result in even more elevated level of cytokines.

In normal human subjects, the physiological concentration of UTI in serum ranges form 6 to 50 U/ml (Fries and Blom, 2000), and the concentration increased to 150 U/ml after administration of 5000 U/kg of UTI (Sato et al., 2000). Because 100 U/ml of UTI inhibited TNF-a production in LPS-stimulated monocytes *in vitro*, as shown in the present study (Molor-Erdene et al., 2005), it is possible that the therapeutic dose (5000 U/kg) of UTI inhibits monocytic TNF-a production in patient with sepsis. In the present study, 50000 U/kg of UTI, 10 times higher than the therapeutic dose of UTI in the clinical setting, was required to inhibit inflammatory responses and hypotension in rats given with LPS. Yamaguchi et al. (2000) also reported that ischemia-reperfusion induced proinflammatory cytokines production in rats was inhibited by 50000 U/kg of UTI but not by 5000 U/kg. The need of such a large dose of UTI to reduce LPS-induced pathological events in rats is unclear at present. Possibly, the differences between human and rat UTI explains the lower sensitivity of rats to human UTI. There lacks a competitive enzymatic inhibitor of HO activities. ZnPP not only inhibit the activities of HO-1, but also interrupt the production of the normal level of HO-2 and NO, which led to protection for the cells; though ZnPP has also been reported to have adverse effects including bone marrow suppression (Lutton et al., 1997).

In summary, our results showed that the induction of HO-1 by ischemic preconditioning and ulinastatin preconditioning played a protective role against remote lung injury in the model of limb injury/reperfusion. The more powerful pharmacological agents targeting HO-1 system would bring more clinical benefits.

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