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Asiatic acid ameliorates hepatic ischemia/reperfusion injury in rats via mitochondria-targeted protective mechanism

Yapeng Lu[a,1], Huiwen Kan[a,1], Ying Wang[a], Dan Wang[a], Xueting Wang[a], Jing Gao[b,*], Li Zhu[a,c,**]

[a] Institute of Nautical Medicine, Nantong University, Nantong 226019, China
[b] School of Medicine, Jiangsu University, Zhenjiang 212013, China
[c] Co-innovation Center of Neuroregeneration, Nantong University, Nantong 226019, China

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ABSTRACT

It has been proved that asiatic acid (AA) directly targets mitochondria and acts as a mild mitochondrial uncoupler. In this study, we aim to investigate the protective effects of AA against ischemia/reperfusion (I/R)-induced liver injury in rats and some underlying mechanisms involved were elucidated. The results showed that 50 mg/kg AA pre-treatment significantly reduced I/R-induced liver damage, characterized by the decreased release of aspartate aminotransferase (AST) and TNF-α. Furthermore, AA pre-treatment dramatically inhibited the production of MDA and increased the hepatic SOD, catalase activities and GSH levels in liver tissue of I/R rats which indicated that AA ameliorated I/R-induced liver damage by reducing oxidative stress. In isolated liver mitochondria in I/R rats, AA improved mitochondrial respiration, decreased mitochondrial MDA level, prevented I/R-induced drop of mitochondrial membrane potential (MMP) and increased ATP content, indicating the protective effect of AA against I/R-induced mitochondrial oxidative damage. In isolated liver mitochondria from normal rats, AA was found to effectively block succinate-driven H_2O_2 production no matter of the presence or absence of rotenone. In addition, AA showed a clear protective effect against anoxia/reoxygenation (A/R)-induced injury in isolated rat liver mitochondria when malate/glutamate were used as respiratory substrates. After AA treatment, mitochondrial respiratory dysfunction induced by A/R was ameliorated. Also, A/R-induced mitochondrial ROS generation was significantly inhibited by AA. In conclusion, AA can attenuate I/R-induced liver damage in rats and A/R-induced mitochondrial injury in isolated rat liver mitochondria by inhibiting oxidative stress and restoring mitochondrial function. Therefore, AA might have potential as a mitochondrial protective agent for use in clinical treatment of hepatic I/R injury.

1. Introduction

Hepatic ischemia/reperfusion (I/R) injury often occurs during liver transplantation, partial hepatic resection and trauma settings (Caldwell-Kenkel et al., 1991; Fu et al., 2014). Unfortunately, there are few effective therapies aimed at preventing or treating this devastating clinical syndrome (Hu et al., 2015). A lot of experimental data using rodent models of I/R suggest that postischemic liver tissue injury is initiated by rapid alterations in the generation of reactive oxygen species (ROS) (Kim et al., 2012; Cahova et al., 2015). A burst of ROS production from mitochondria at the beginning of reperfusion has been considered as a crucial early driver of I/R injury (Murphy and Steenbergen, 2008; Loor et al., 2011). Mitochondrial ROS not only drives acute damage but also initiates the pathology that develops over a long time following reperfusion (Eltzschig and Eckle, 2011). Firstly, ROS-induced mitochondrial oxidative damage disrupts ATP production and leads to necrotic and apoptotic cell death following reperfusion (Murphy and Steenbergen, 2008; Loor et al., 2011). Furthermore, mitochondrial disruption can also release damage-associated molecular pattern molecules such as mtDNA, which initiate the sterile inflammatory response that contributes to I/R injury and can continue for days after the initial damage (Arslan et al., 2011).

In the very long period, mitochondrial ROS generation during reperfusion has been considered a nonspecific consequence of the

Abbreviations: I/R, ischemia/reperfusion; AA, asiatic acid; A/R, anoxia/reoxygenation; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; Rh123, rhodamine 123; RCR, respiratory control ratio; RET, reverse electron transport; EGTA, ethyleneglycol-bis (β-aminoethyl)-N,N,N′,N′-tetraacetic acid; DMSO, dimethyl suifoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AST, aspartate aminotransferase

* Correspondence to: Jing Gao, School of Pharmacy, Jiangsu University, 301# Xuefu Road, Zhenjiang 212013, China.
** Correspondence to: Li Zhu, Institute of Nautical Medicine, Nantong University, 9# Seyuan Road, Nantong 226019, China.
E-mail addresses: jinggao@ujs.edu.cn (J. Gao), zhulizhou@ntu.edu.cn (L. Zhu).
[1] Yapeng Lu and Huiwen Kan contributed equally to this work.

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interaction of a dysfunctional respiratory chain with oxygen during reperfusion (Jaeschke and Woolbright, 2012). However, recent findings suggest that there is a common pathway for the initial burst of mitochondrial ROS production that underlies I/R injury (Chouchani et al., 2014). Succinate, a component of the Krebs cycle which accumulates during ischemia, fuels reverse electron transport (RET) in the first minutes following reperfusion, providing the initiating burst of superoxide that leads to I/R injury (Chouchani et al., 2016). Therefore, inhibiting RET upon reperfusion by the inhibition of complexes I and II, or by mild mitochondrial uncoupling might provide a new choice for development of therapies towards I/R injury (Chouchani et al., 2014).

Asiatic acid (AA), a pentacyclic triterpene in *Centella asiatica*, possesses a wide variety of pharmacological effects (Maquart et al., 1999; Soo Lee et al., 2003; Patil et al., 2010; Tang et al., 2012; Xu et al., 2012). For a long period of time, AA has been used as a herbal treatment for skin disorders and wounds (Maquart et al., 1999). It is detectable in human serum after oral administration (Grimaldi et al., 1990), and has no significant toxicity to mice after either subcutaneous or interperitoneal injections (Maquart et al., 1999). In recent years, AA has showed notable neuroprotective effects in preclinical experimental models of stroke (Mook-Jung et al., 1999; Lee et al., 2000; Kim et al., 2004; Krishnamurthy et al., 2009; Lee et al., 2012), highlighting its therapeutic properties (Lee et al., 2012). Recently, our group has identified that AA is a mild mitochondrial uncoupler (Lu et al., 2016). The uncoupling properties of AA with consequent effects on mitochondrial respiration might have the potential to treat I/R-induced injury. Therefore, this study was designed to tested whether AA, a mild mitochondrial uncoupler, can protect against liver injury induced by in vivo I/R and mitochondrial dysfunction induced by in vitro A/R.

2. Materials and methods

2.1. Compounds and reagents

AA, rhodamine 123 (Rh123), dimethyl suifoxide (DMSO) and commercial aspartate aminotransferase (AST) detection kit were purchased from Sigma-Aldrich Corp.(St. Louis, USA). Amplex red, horseradish peroxidase and commercial TNF-α detection kit were purchased from Invitrogen Corporation (Carlsbad, USA). The ATP assay kit and *N*-acetylcysteine (NAC) were obtained from Beyotime Biotechnology Corporation (Shanghai, China). Kits used for determination of SOD, catalase activities and GSH, MDA levels were purchased from Jiancheng Institute of Biological Engineering (Nanjing, China). Other chemicals used were of analytical reagent grade purchased locally from commercial suppliers.

2.2. Animals and surgery

The experiments were performed using 6-week-old (200–220 g) male Sprague Dawley rats purchased from the Experimental Animal Center of Nantong University (Nantong, China). All animals were maintained in a laminar-flow, specific pathogen-free atmosphere at the University of Nantong. All the studies reported here were submitted to the ethics committee on animal experimentation in Nantong University and all procedures were approved according to the Animal Care and Use Committee of Nantong University and the Jiangsu Province Animal Care Ethics Committee (Approval ID: SYXK(SU)2007-0021).

To study the protective effect of AA against I/R-induced liver damage, a total of 30 rats were randomly divided into 5 groups (n = 6, 6 rats per group) as follows: SHAM group, I/R group, and 25, 50, 100 mg/kg AA-pretreated groups. To study the protective of AA against I/R-induced hepatic mitochondrial damage, a total of 18 rats were randomly divided into 3 groups (n = 6, 6 rats per group) as follows: SHAM group, I/R group, and AA-pretreated groups. Rats in AA-pretreated groups were received oral administration of AA 1 h prior to ischemia. AA was suspended in a 0.5% sodium carboxymethylcellulose solution. Rats in SHAM group and I/R group were given 0.5% sodium carboxymethylcellulose solution intragastrically 1 h prior to ischemia.

All animals were fasted for 12 h prior to surgery and subsequently anesthetized with pentobarbital (40 mg/kg) intraperitoneally. A model for partial warm I/R in the liver was established as described previously (Uchida et al., 2010). Throughout the procedure, body temperature was maintained at 37 ± 0.5 °C with a thermostatically controlled infrared lamp. 1.5 h after the induction of ischemia, the vascular clamp was slightly removed and the animals were then returned to their cages for a period of 6 h of reperfusion. The SHAM rats underwent the same surgical procedures but without vascular occlusion. After 6 h the reperfusion, the animals were euthanized and the samples of liver and blood were collected for further analysis.

2.3. Histochemical analysis

Liver tissues were taken from the median lobe after 6 h of reperfusion and stored in 10% formalin. Then formalin-fixed liver samples were embedded with paraffin and cut to 6 μm-thick sections. Tissues were stained with H&E and slides were assessed for tissue damage. The necrotic area was observed by using a microscope (DM4000B, Leica, Germany).

2.4. Plasma biochemical assays

Blood samples were centrifuged at 3000 × *g* for 10 min to collect serum, which was then stored at − 80 °C until use. To assess hepatic function and inflammation in rats following liver I/R, the serum levels of AST and TNF-α were measured. The AST levels were determined based on the catalytic activity of AST to transfer an amino group from aspartate to α-keto-glutarate results in the generation of a colorimetric (450 nm) product proportional to the AST enzymatic activity by using commercially available kits purchased from Sigma-Aldrich Corp.(St. Louis, USA). Serum levels of TNF-α were quantified with the use of specific enzyme-linked immuno-absorbent assay kits for rats, according to the manufacturer's instructions (Invitrogen Corporation). Optical density was measured by the use of a microplate reader (Synergy 2™, BioTek, US).

2.5. Isolation of mitochondria from rat liver

Mitochondria were isolated from rat liver by differential centrifugation (Lu et al., 2016). In brief, rat liver was sliced immediately after decapitation in ice-cold isolation buffer A consisting of 250 mM sucrose, 0.5 mM ethyleneglycol-bis (β-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), 0.1% defatted BSA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH (4 °C, pH 7.4) and homogenized using a Potter-Elvehjem glass homogenizer. Homogenates were then centrifuged for 5 min at 600 × *g* and the resulting supernatant further centrifuged for 10 min at 11, 000 × *g*. The pellet was resuspended in ice-cold isolation buffer B (buffer A without BSA) and the same centrifugation process was repeated. Pellets were then suspended in medium consisting of 250 mM sucrose, 0.3 mM EGTA and 10 mM HEPES-KOH (4 °C, pH 7.4) and centrifuged for 15 min at 3400 × g. The final mitochondrial pellet was suspended in storage buffer consisting of 250 mM sucrose and 10 mM HEPES-KOH (4 °C, pH 7.4) and used within 4 h. The protein content was determined by the biuret reaction.

2.6. Determination of oxidative stress-related parameters

After 6 h of reperfusion, liver samples were obtained from the median lobe, washed in cold saline and quickly placed into liquid nitrogen during sampling and maintained at − 80 °C until use. Prior to detection, the samples were first unfrozen at 4 °C and subsequently homogenized in ice cold phosphate buffer (pH 7.4). The homogenates