Vol 9 | Issue 2 | 2019 | 139-142.

ISSN 2249 - 7641
Print ISSN 2249 - 765X

International Journal of Pharmacology Research

www.ijprjournal.org

IMPACT OF ISCHEMIA/REPERFUSION ON HEPATOCYTE APOPTOSIS IN STEATOTIC LIVER: INSIGHTS FROM A MOUSE MODEL AND THERAPEUTIC POTENTIAL OF AMINOGUANIDINE

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ABSTRACT

The study investigated apoptosis of hepatocytes following ischemia and reperfusion in steatotic liver. Using a mouse model, liver histology, serum alanine aminotransferase, and peroxynitrite levels were assessed after partial hepatic ischemia. Steatotic livers exhibited increased peroxynitrite levels post-reperfusion, with upregulated caspase 9 and 3, indicating significant hepatocyte apoptosis. Aminoguanidine treatment reduced alanine aminotransferase levels and improved survival in mice with steatotic livers post-reperfusion, highlighting susceptibility to ischemia/reperfusion injury in steatotic livers.

Keywords: Steatotic liver, Ischemia/reperfusion injury, Hepatocyte apoptosis, Alanine aminotransferase, Aminoguanidine treatment.

INTRODUCTION

A growing number of people with hepatic steatosis suffer from alcohol abuse and non-alcoholic fatty liver disease (NAFLD) [1]. As a result of oxidative stress, apoptosis, and mitochondrial dysfunction, steatotic livers are susceptible to metabolic disorders. As a consequence of hepatic resection and liver transplantation, steatotis is associated with postoperative complications. Additionally, organ shortages lead to more use of steatotic liver for transplantation. [2,3] An additional clinical concern is development of steatohepatitis after neoadjuvant chemotherapy due to insufficient liver regeneration after surgery [4]. It is therefore necessary to investigate further the underlying cause of liver dysfunction after transplantation of livers with steatosis. After liver surgery for steatosis, liver dysfunction/damage is often caused by ischemia/reperfusion injury. According to experimental studies, ischemic stress, reactive oxygen species, proinflammatory mediators, neutrophil-mediated proteases, microcirculatory disruption, and apoptosis all contribute to hepatic ischemia/reperfusion injury [5-7].

NO synthase (NOS) produces nitric oxide (NO). Endothelial NOS (eNOS), neural NOS (nNOS), and inducible NOS (iNOS). NO generated by eNOS maintains microcirculation, but NO created by iNOS produces hazardous [8] peroxynitrite-producing species of reactive oxygen in the body. When hepatic ischemia/reperfusion occurs, such reactive oxygen species can cause hepatocyte apoptosis. [9] Apoptosis occurs in two ways. Fas ligands and tumor necrosis factor-alpha produce a death signal in type 1 (extrinsic) signaling. Procaspase 8 is activated by the death domains associated with tumor necrosis factor-alpha and Fas [10]. Caspase 8 activates procaspase 3, resulting in apoptosis. [11] In contrast, mitochondrial permeability transition induces type 2 signaling, which results in the release of cytochrome [12,13] In response to cytochrome c release, caspase 9 cleaves and activates caspase 3. [14] Cells switch from apoptosis to necrosis when they lack adenosine triphosphate (ATP). [15] Injury to normal livers as a result of ischemia/reperfusion is well understood, but steatotic liver ischemia/reperfusion injury remains unknown.

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Hepatocyte apoptosis is hypothesized to result from increased production of peroxynitrite in the presence of iNOS in steatotic livers. After ischemia and reperfusion of the steatotic liver, we investigated peroxinitrite production and possible apoptosis involvement.

METHODS AND MATERIALS

In the fatty liver group, the mice were genotyped as BKS. Cg-m/+Leprdb mice, with a fatty liver blood group of Leprdb/Leprdb, weighed 37 to 42 g, and the wild type group, weighed 22 to 25 g. Mice lived in a controlled environment with a 12-hour light/dark cycle.

Ischemia/reperfusion model and reagents

A partial hepatic ischemia and reperfusion model was used to anesthetize mice with sodium pentobarbital (60 mg/kg). A midline laparotomy was performed in order to interrupt the liver's arterial and portal blood supply. Vascular occlusion was not used in sham control mice. Liver tissue and blood samples were collected before surgery (baseline) and after reperfusion for 1 and 4 hours. As part of the iNOS inhibition experiment, mice were either given 0.1 ml of sterile phosphate buffered saline (PHBS) or just PHBS. According to a study, survival was evaluated after reperfusion for up to eight hours.

Histological examination and apoptosis analysis

In this, stained liver sections with hematoxlin-eosin after formalin-fixing them and embedding them in paraffin. By using dUTP-biotin nick end-labeling with terminal deoxynucleotidyl transferase (TdT) to label double-strand breaks in DNA, we quantified the apoptotic hepatocytes. In accordance with the manufacturer's instructions, an in situ apoptosis detection kit was used to perform the assay. TUNEL-positive hepatocytes were compared to total hepatocytes in five random fields for each sample.

Western blot analysis

Liquid nitrogen was used to freeze liver tissue specimens. Homogenizing liver tissues in 150 mM NaCl, EDTA, NP-40, leupeptin, aprotinin, pepstatin, and soybean trypsin inhibitor was performed. To remove cellular debris, sonicated homogenates were centrifuged at 5,000 rpm. BCA Protein Assay kit was used to determine protein concentration. XV PANTERA was used to analyze liver lysate protein (20 g). XV PANTERA gels were electrophoresed in 7.5–15% gradients, and membranes were transferred. TBS containing 0.1% Tween 20 was used to block nonspecific binding sites for 1 h at room temperature. A rabbit polyclonal anti-mouse actin, caspase 3, and caspase 8 antibody was incubated with TBST on the membranes. We then incubated membranes with donkey anti-rabbit IgG conjugated to horseradish peroxidase. Using image analysis software, we quantitated immune-reactive proteins using enhanced chemiluminescence. An actin band-intensity ratio was normalized to the cleaved caspase 3 and caspase 8 band intensities. A total of two separate experiments were conducted.

Electrophoretic mobility shift assay

Liver extracts was prepared with bicinchoninic acid assays using precipitation using trichloroacetic acid and BSA as standards. Oligo-nucleotides labeled at the ends. In binding buffer, 20 g of protein and 35 fmols of oligonucleotide were bound for 30 min. 15 litres of reaction volume were maintained. By autoradiography, polyacrylamide gel 4% was used to separate reaction products.

Tissue protein quantification using ELISA and blood

During sacrifice, a cardiac puncture was used to obtain blood from the victim. A diagnostic kit was used to measure alanine aminotransferase (ALT) levels in serum samples. The liver's nitrotyrosine production was quantified using methods described elsewhere. Specimens were immediately frozen and homogenized in 10 volumes of homogenization buffer. The homogenate was centrifuged for 10 minutes at 12,500 g after 2 hours at 4°C. A clear lysate was obtained by centrifuging the supernatant again. Protein concentration and nitrotyrosine EIA samples were measured using a bicinchoninic assay kit.

Protein concentration per mg was calculated

Analyses based on statistics

Statistical significance was determined by the Mann-Whitney test or by a one-way analysis of variance, followed by the Student-Newman-Keuls test. The data were expressed as mean ± SEM. Kaplan-Meier survival curves were calculated and log rank tests were used to evaluate comparisons. Software such as SigmaStat 3.0 or SPSS 11.5 was used in order to analyze the data. P values of 0.05 were used to determine statistical significance.

RESULTS

Ischemia/reperfusion-induced liver apoptosis

Hepatocyte apoptosis was examined using TUNEL staining. Compared to sham mice, FL mice had significantly more TUNEL-positive apoptotic hepatocytes 1 h after reperfusion. It was not observed that the increase was present in WT mice. One and four hours after reperfusion, FL mice showed increased TUNEL-positive apoptotic cells. Caspases-3, -8, and -9 were analyzed by western blot to determine which apoptosis pathway was activated during ischemia/reperfusion. After reperfusion, FL mice were more likely to cleave caspase-3 and -9 than WT mice. The FL and WT mice expressed the same amount of cleaved caspase-8.

Peroxynitrite expression during ischemia/reperfusion

In order to examine nitrotyrosine expression by ELISA, we examined NOS-mediated NO production. FL

mice showed significantly higher nitro-tyrosine expression after reperfusion than WT mice.

NF-B is activated by ischemia and reperfusion

Since NF-κB regulates inflammation and anti-apoptosis effects [15], we assessed whether hepatic ischemia/reperfusion induced its activation. One and four hours after reperfusion, NF-κB was translocated to the nucleus of WT mice, but little activity was seen in FL mice.

Ischemia/reperfusion-induced hepatocellular injury

Serum ALT levels were measured to assess hepatic injury during ischemia/reperfusion. One and four hours following reperfusion, it was found that FL mice had higher serum levels of ALT than WT mice. FL mice showed focal hepatic necrosis 4 hours after reperfusion.

Ischemia-reperfusion-induced apoptosis and liver damage caused by iNOS

Using TUNEL staining, we determined that iNOS is involved in the process of liver apoptosis. The apoptotic index, calculated after 4 hours of reperfusion in both WT and FL mice, was significantly higher than in sham-operated controls. As a result of AG administration as an iNOS specific inhibitor, the apoptotic index of WT mice significantly increased. When compared with PBS, AG significantly reduced FL mice's apoptotic cells. In order to determine whether ischemia/reperfusion injury resulted from iNOS, the levels of alanine aminotransferase (ALT) were measured. Controls treated with sham-operated PBS or AG did not differ from WT mice in serum ALT levels. In WT mice, AG significantly decreased serum ALT levels after reperfusion. AG administration also attenuated FL mice's serum ALT levels 1 and 4 h after reperfusion.

Ischemia/reperfusion survival

Apoptosis and hepatic reperfusion/ischemia may be related to death in mice after AG administration. Both WT and FL mice did not die after being treated with PBS or AG. Eight hours after reperfusion, none of the WT mice died. After reperfusion, FL mice with ischemia for 90 minutes had a lower survival rate. FL mice treated with AG survived significantly longer following reperfusion.

DISCUSSION

As a result of steatotic liver surgery, there is a risk of postoperative morbidity and mortality, as well as nonfunctioning primary grafts. [16] Ischemia/reperfusion injury could be a cause of hepatic ischemia/reperfusion injury. Steatosis in the liver can be caused by ischemia and reperfusion.

Additionally, we have found a connection between this type of injury and reactive oxygen species. iNOS produces hazardous reactive oxygen species, which makes NO harmful. In the current study, FL mice had significantly higher peroxynitrite concentrations than WT mice, indicating that iNOS may promote hepatocyte apoptosis by upregulating reactive oxygen species. [18] Induction of apoptosis by AG reduced hepatocyte apoptosis, indicating that upregulation of iNOS contributes to the induction of apoptosis.

Apoptosis was not augmented in the steatotic liver by death signaling, suggesting death signaling may not be responsible for enhancing apoptosis in the steatotic liver. Therefore, cleaved caspase 9 and caspase 3 were activated during ischemia/reperfusion injury-induced apoptosis, suggesting mitochondrial permeability is important in steatotic liver injury. [19] There is evidence that type 2 iNOS pathway occurs more rapidly in hepatocytes. Hepatocytes expressing iNOS were immunohistochemically identified. Further, in periportal hepatocytes, Kupffer cells engulf apoptotic cells, releasing reactive oxygen. As a result of reperfusion in steatotic livers, apoptosis of hepatocytes was significant. [20]

An important role for NF-κB has been considered proinflammatory during hepatic ischemia/reperfusion injuries. Inflammatory injury after ischemia/reperfusion can also be reduced by therapeutic modalities that reduce NF-κB activation. [21] Other studies have indicated that activation of NF-κB has anti-apoptotic effects. During the present study, we observed that NF-κB activation in whole livers decreased after hepatic ischemia/reperfusion in livers with steatosis. Steatosis after reperfusion caused hepatocyte apoptosis, possibly due to NF-B-dependent anti-apoptotic functions. [22]

When the liver is ischemic/reperfused, it has low ATP levels. We have previously shown that apoptosis consumes nicotinamide adenine dinucleotide, lowering ATP levels. Cellular responses to secondary oncotic necrosis are altered by apoptosis and ATP depletion.(31) Hepato-cyte apoptosis, coupled with low ATP levels, leads to secondary oncotic necrosis. Steatosis caused significant liver damage and lowered survival rates in the late phase of reperfusion, where ALT levels were highest. Ischemia/reperfusion injury in the liver is induced by iNOS-mediated hepatocyte apoptosis in FL mice treated with AG. [23] It is suggested that other mechanisms may be involved in reducing liver injury as measured by serum ALT levels when iNOS is blocked.

An early increase in iNOS-related peroxinitrite in the steatotic liver causes hepatocyte apoptosis and and subsequent oncotic necrosis following ischemia/reperfusion injury. If ischemia/reperfusion injuries occur during surgery for steatosis of the liver, these data may be helpful in expanding our knowledge of steatosis.

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