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Melatonin attenuates hepatic ischemia through mitogen-activated protein kinase signaling

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ABSTRACT

Background: Melatonin exerts a protective effect during hepatic ischemia–reperfusion (I/R) injury through modulation of the apoptotic cell death program. Mitogen-activated protein kinases mediate various intracellular processes such as cell differentiation, survival, and death. This study investigated whether melatonin exerts a protective effect through the activation of Raf-MEK-ERK and its downstream targets, including 90 ribosomal S6 kinase (p90RSK) and Bad, during hepatic I/R damage.

Methods: Hepatic ischemia was induced in mice by occlusion of the hepatic artery, portal vein, and bile duct. Adult mice were subjected to 1 h of hepatic ischemia and 3 h of reperfusion. Vehicle or melatonin (10 mg/kg, intraperitoneal) was injected 15 min before ischemia and just before reperfusion. Serum aspartate aminotransferase and alanine aminotransferase levels were measured, and terminal deoxynucleotidyl transferase dUTP nick-end labeling histochemistry was performed. Moreover, Western blot and immunoprecipitation analyses were performed.

Results: Melatonin treatment attenuated hepatic I/R-induced increases in alanine aminotransferase and aspartate aminotransferase levels and also ameliorated hepatic injury-induced pathologic lesions and increases of positive terminal deoxynucleotidyl transferase dUTP nick-end labeling staining in hepatic tissues. Hepatic I/R injury induced decreases in the phosphorylation of Raf-1, MEK1/2, and extracellular-regulated kinase (ERK)1/2, whereas melatonin attenuated decreases in these phosphorylation levels. Moreover, melatonin prevented the injury-induced decreases in phosphorylation of downstream targets, p90RSK and Bad. Immunoprecipitation analysis showed that the interaction between phospho-Bad and 14-3-3 was decreased in vehicle-treated animals, while melatonin prevented this decrease. Melatonin also attenuated the injury-induced increase in cleaved caspase-3. In cultured hepatocytes, melatonin treatment prevented the hydrogen peroxide-induced cell death and decrease in phosphorylation of ERK1/2. Moreover, blocking MEK by PD98059 attenuated the effect of melatonin.

Conclusions: These data suggest that melatonin protects hepatic cells against hepatic I/R damage through the activation of the Raf-MEK-ERK cascade and phosphorylation of its downstream targets.

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1. Introduction

Ischemia–reperfusion (I/R) injury is a serious clinical complication in many organs including the heart, brain, kidney, and liver. Hepatic I/R injury mainly occurs during liver transplantation and hepatic resection [1]. Hepatic I/R injury causes excessive oxidative stress such as overproduction of reactive oxygen species and superoxide free radicals, leading to the destruction of hepatic cell structure and function [2]. Thus, a strong antioxidant can protect hepatic cells against hepatic I/R injury [3]. Melatonin, a product of the pineal gland, regulates seasonal and biological circadian rhythms. Melatonin and its metabolites have powerful antioxidant capacity and act as free radical scavengers [4–7]. Moreover, melatonin exerts protective effects against hepatic I/R through the reduction of mitochondrial oxidative stress and apoptotic cell death [8–11].

Mitogen-activated protein (MAP) kinases contribute to several multicellular programs including cell differentiation, proliferation, survival, and apoptosis [12–14]. Several growth factors and mitogens activate a protein kinase cascade involving Raf, MEK, and extracellular-regulated kinase (ERK)1/2, in which activation of Raf and MEK leads to the phosphorylation of ERK1/2, which subsequently phosphorylates the 90 kDa ribosomal S6 kinase (p90RSK) and Bad [12–16]. Bad, a proapoptotic protein of bcl-2 family, interacts with Bcl-x(L) and release Bax from the binding of Bax and Bcl-x(L) [17]. Bax activates the release of cytochrome C from mitochondria and promotes caspase cascade activation, ultimately inducing apoptotic cell death [17]. However, phosphorylated Bad interacts with 14-3-3 protein, which inhibits the apoptotic function of Bad [18,19]. Although melatonin has been demonstrated to protect hepatic cells against hepatic I/R injury, few data are available regarding its effect on the MAP kinase pathway in hepatic I/R injury. This study investigated the protective mechanism of melatonin through its effect on Raf-MEK-ERK and its downstream targets, p90RSK and Bad during hepatic I/R injury.

2. Materials and methods

2.1. Experimental animals and melatonin treatment

Adult ICR mice (male, 25–30 g, $n = 40$) were purchased from Samtako Co (Animal Breeding Center, Osan, Korea) and were maintained under controlled temperature (25°C) and lighting (12/12 light–dark cycle). All procedures for animal use were approved by Institutional Animal Care and Use Committee at Gyeongsang National University (GNU-LA-14). The animals were fasted for 12 h before surgery. The animals were divided into four groups as follows: vehicle + sham group, melatonin + sham group, vehicle + hepatic I/R group, and melatonin + hepatic I/R group. Vehicle or melatonin administration was carried out as a previously described method [10]. Melatonin (Sigma, St. Louis, MO) was dissolved in 5% ethanol in saline as the vehicle. Vehicle or melatonin (10 mg/kg) was injected intraperitoneally at 15 min before ischemic induction and before the onset of reperfusion [10,11].

2.2. Hepatic I/R

Before surgical operation, the animals were anesthetized with pentobarbital sodium (50 mg/kg). Partial hepatic ischemia was induced as a previously described surgical operation [20]. Briefly, the abdomen was opened through midline incision, and the portal vein and associated structures were exposed. The animals were subjected to 1 h of hepatic ischemia by cross-clamping the left branches of the portal vein, hepatic artery, and bile duct with a single microvascular clamp. At the end of the ischemia period, reperfusion was established by removal of the clamp. Reperfusion was kept up for 3 h, and blood samples were collected by cardiac puncture, and liver tissues were isolated. The sham-operated animals received laparotomy by the same surgical procedure as the other operated groups without vascular clamping. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using Vet test 8008 (IDEXX-GmbH; Worrstadt, Germany) as indicators of hepatic injury.

2.3. Histologic analysis

The hepatic tissues were fixed in 4% neutral-buffered paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. Sections were deparaffinized and stained with hematoxylin and eosin as the general method. After dehydration in gradual alcohol series and xylene, sections were mounted with Permount (Fisher Scientific, Fair Lawn, NJ) and observed using light microscopy.

2.4. Terminal deoxynucleotidyl transferase dUTP nick-end labeling histochemistry

DNA Fragmentation Detection Kit (Oncogene Research Products, Cambridge, MA) was used according to the manufacturer's protocol. The deparaffinized sections were incubated in equilibration buffer for 1 h and terminal deoxynucleotidyl transferase enzyme at 37°C for 1 h. The reaction was terminated by stop buffer for 5 min. Sections were labeled with digoxigenin peroxidase and visualized with diaminobenzidine tetrahydrochloride (Sigma) substrate. Sections were counterstained with hematoxylin and observed using light microscopy. Five histologic fields were chosen for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cells counting. The total cell number and TUNEL-positive cell number were counted in each field. Apoptotic index was described as the percentage of the number of apoptotic cells to the total number of cells.

2.5. Western blot analysis

Western blot analysis was performed as a previously described method [21]. The hepatic tissues (median lobe) were rapidly isolated and lysed in buffer (1% Triton X-100 [Sigma], 1 mM EDTA in 1 × phosphate-buffered saline [pH 7.4]) containing 10 μM of leupeptin and 200 μM of phenylmethylsulfonyl fluoride. The lysates were centrifuged at 15,000*g* for 20 min at 4°C. The supernatants were collected, and the protein concentration of each lysate was determined using the bicinchoninic acid kit (Pierce, Rockford, IL) according to the manufacturer's protocol.