Effect of Leptin and Apelin Preconditioning on Hepatic Ischemia Reperfusion Injury in Rats

Tamer Sagioglu · Mustafa Burak Aksoy ·
Gonul Sagioglu · Hilmi Tozkir · Serhat Oguz ·
Tulin Yalta · Mehmet A. Yagci · Atakan Sezer

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Abstract Leptin and apelin are important adipocytokines involved in a variety of endocrine and paracrine functions. The aim of this study was to evaluate the effect of exogenous leptin and apelin preconditioning on hepatic ischemia reperfusion (I/R) injury in rats. Forty mice were assigned to four

groups ($n=10$): sham-operated control (sham), I/R injury, I/R + leptin (I/R + L), and I/R + apelin (I/R + A). Leptin 100 $\mu\text{g/kg/day}$ and apelin 2 $\mu\text{g/kg/day}$ were delivered intraperitoneally starting 3 days prior to surgical procedure in I/R + L and I/R + A groups, respectively. All I/R groups underwent 45 min of warm ischemia, followed by 30 min of reperfusion. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), liver malondialdehyde (MDA) and glutathione (GSH), and liver histopathology were compared between groups. MDA was elevated in I/R, but stayed similar in I/R + L and I/R + A compared to sham. I/R + A had significantly lower MDA compared to I/R. GSH levels did not differ significantly between the groups. ALT and AST were elevated in all I/R groups, but significant reduction was observed in I/R + L and I/R + A compared to I/R. Liver histopathology was mostly mild in I/R + L and I/R + A, in contrast to severe injury observed in the I/R group. Leptin and apelin preconditioning significantly reduced hepatic I/R injury in rats.

T. Sagioglu (✉) · S. Oguz · A. Sezer
Faculty of Medicine Department of General Surgery,
Trakya University,
Trakya Üniversitesi Tıp Fakültesi Genel Cerrahi, AD 22030,
Edirne, Turkey
e-mail: tamersagioglu@gmail.com

M. B. Aksoy
Faculty of Medicine Department of Emergency Service,
Trakya University,
Trakya Üniversitesi Tıp Fakültesi Acil Servis, AD 22030, Edirne,
Turkey

G. Sagioglu
Faculty of Medicine Department of Anesthesiology and
Reanimation, Trakya University,
Trakya Üniversitesi Tıp Fakültesi Genel Cerrahi, AD 22030,
Edirne, Turkey

H. Tozkir
Faculty of Medicine Department of Medical Genetics,
Trakya University,
Trakya Üniversitesi Tıp Fakültesi Tıbbi Biyoloji ve Genetik, AD
22100, Edirne, Turkey

T. Yalta
Faculty of Medicine Department of Pathology, Trakya University,
Trakya Üniversitesi Tıp Fakültesi Patoloji, AD 22030, Edirne,
Turkey

M. A. Yagci
Hakkari State Hospital,
Hakkari Devlet Hastanesi,
Hakkari, Turkey

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Introduction

Ischemia reperfusion (I/R) injury is an important complication of liver transplantation and other major liver surgeries involving vascular clamping (Pringle's maneuver) to prevent excessive blood loss. In liver transplantation, I/R injury is responsible for nearly one third of delayed graft function cases [1]. I/R injury is a complex phenomenon where hypoxia and subsequent reoxygenation of the liver lead to microcirculatory disturbances, inflammatory response, neutrophil infiltration, and accumulation of reactive oxygen species—all contributing to cellular damage in hepatocytes and endothelial cells. One way

to prevent or lessen I/R injury is to apply short periods of I/R to precondition the liver for the upcoming injury [2, 3]. Since this may not be a feasible method in many cases, several agents are being tested for pharmacological preconditioning. Pacher and Haskó [4] investigated the protective effect of cannabinoids in hepatic I/R injury and showed that cannabinoids decreased the endothelial cell inflammatory response. López-Neblina et al. [5] demonstrated the anti-inflammatory effect of dantrolene on reperfusion injury by the downregulation of TNF-alpha and interleukin-10.

Tetrandrine was examined by Liu et al. [6] in rat livers during reperfusion injury and found to be protective by decreasing neutrophil infiltration. Fernández et al. [7] studied thyroid hormone preconditioning in hepatic I/R injury, and they showed that thyroid hormone preconditioning might be protective against I/R injury. Lai et al. [8] indicated that simvastatin protected the rats from liver I/R injury by hemeoxygenase-1 induction.

Adipocytokines are bioactive mediators released from adipose tissue involved in homeostasis and energy metabolism [3]. Leptin was investigated by many experimental studies in I/R injury models in various organs such as kidneys [9], bladders [10], intestines [11, 12], brains [13], and hearts [14].

Apelin is considered an adipocytokine which was determined to have endothelium-dependent vasodilator action and positive inotropic action in the heart and investigated to have cardioprotective function in rat models of I/R injury [15–21].

In this study, we aimed to determine the effect of exogenous leptin and apelin administration on hepatic I/R injury in rats.

Materials and Methods

Study Design

The study was conducted at the laboratory animals care unit in accordance with the guidelines for the care and use of laboratory animals established by the Animal Ethics Committee, which approved the study design. Forty male Sprague–Dawley rats aged 6–8 weeks and weighing 280 ± 20 g were studied. Using a computer-generated table of random numbers, rats were assigned to one of the following four groups: sham control (sham, $n=10$), I/R ($n=10$), leptin-treated I/R (I/R + L, $n=10$), and apelin-treated I/R (I/R + A, $n=10$). The animal room was maintained at a temperature of 22 ± 2 °C and a relative humidity of 55 ± 15 %, with a 12-hour light/dark cycle. Tap water and chow were easily available throughout the acclimatization and study periods.

Chemicals and Reagents

Leptin (Leptin®, Phoenix Pharmaceuticals Inc, Belmont, CA, USA) and apelin-13 (Apelin®, Phoenix Pharmaceutical,

Belmont, CA, USA) were purchased commercially. Dosages of 100 µg/kg per day leptin and 2 µg/kg per day apelin were administered intraperitoneally to the rats as described previously [11, 22]. In I/R + L and I/R + A groups, drug administration was started 3 days prior to the surgical procedure. The same amount of normal saline was administered intraperitoneally in groups I/R and C.

Surgical Procedure

After an overnight fasting period, each animal was anesthetized using 5 mg/kg of xylazine (Rompun, Bayer Ilac Sanayi, Sisli, Istanbul, Turkey) and 30 mg/kg of ketamine hydrochloride (Ketalar, Eczacibasi Ilac San, Istanbul, Turkey) via intramuscular (IM) route prior to the experiment. The abdomen of each animal was shaved and cleansed with the povidone iodine solution (Isosol, Merkez Laboratory, Ilac San, Istanbul, Turkey). Using the sterile technique, all animals underwent a laparotomy through a midline incision of about 3 cm. The hepatoduodenal ligament was exposed in the abdominal cavity. Sham control underwent hepatoduodenal ligament exposure without any clamping. In groups I/R, I/R + L, and I/R + A, an atraumatic microvascular bulldog clamp was placed across hepatoduodenal ligament for 45 min. After 30 min of reperfusion, animals were sacrificed and tissue samples were obtained from an area of liver. Liver specimens were harvested from each animal for biochemical and histopathological analysis. In addition, serum samples were obtained for measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) to assess hepatic function.

Malondialdehyde and Glutathione Assays

Liver tissue samples were washed with physiological saline and kept in deep freeze until the day of the experiment. These samples were homogenized with 150 mmol/l ice-cold KCl for the determination of malondialdehyde (MDA) and glutathione (GSH) levels. MDA concentrations in the liver tissue, an indicator of lipid peroxidation, were assayed in the form of thiobarbituric acid-reacting substances [23]. Homogenates were centrifuged at $2,600 \times g$ for 10 min at 4 °C. Then, 0.2 ml of 8.1 % sodium dodecyl sulfate, 1.5 ml of 20 % acetic acid, 1.5 ml of 0.8 % thiobarbituric acid, and 0.6 ml of distilled water were added to supernatant. This mixture was heated to 95 °C for 60 min. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of a mixture of *n*-butanol:pyridine (15:1, v/v) were added and the mixture was shaken vigorously and centrifuged at $2,600 \times g$ for 10 min at 25 °C. Absorbance of the organic layer was read at 532 nm. MDA was quantified using an extinction coefficient of 1.56×10^5 l/mol per cm and expressed as nmol MDA/mg tissue.

The GSH level was determined according to the Ellman method [24]. The concentration of GSH was monitored

spectrophotometrically at 412 nm, and results were expressed as $\mu\text{mol/g}$ tissue.

Histopathological Examination

Tissue specimens were fixed in 10 % formalin for 24 h, and then embedded in paraffin and sliced into 5 μm sections. Slides were stained with hematoxylin and eosin (H&E) and examined under a light microscope. Each slide was evaluated by two expert investigators blinded to the experiment and data.

Statistical Analysis

MDA and GSH comparisons between groups were performed using the Kruskal–Wallis test, and pairwise comparisons were performed using the Mann–Whitney *U* test with Bonferroni correction. ALT and AST comparisons between groups were performed using one-way analysis of variance (ANOVA), and pairwise comparisons were performed using Tukey's honestly significant difference (HSD) test with Bonferroni correction. Histopathological findings were analyzed using the Monte Carlo test. *P* values of less than 0.05 were regarded as statistically significant.

Fig. 1 Malondialdehyde (MDA) (a) and glutathione (GSH) (b) levels following ischemia reperfusion injury in liver tissue samples of sham, ischemia/reperfusion (I/R), apelin-preconditioned I/R (I/R + A), and leptin-preconditioned I/R (I/R + L) groups. Liver function assessment by serum alanine aminotransferase (ALT) (c) and aspartate aminotransferase (AST) (d) levels in the same experimental groups. **P*<0.05 compared to sham; †*P*<0.05 compared to I/R

Results

Oxidative stress and antioxidant status in liver tissue were assessed using MDA and GSH (Fig. 1a and b), while liver function was assessed by ALT and AST measurements (Fig. 1c and d). The mean MDA level was significantly increased in I/R group compared to sham (*P*=0.028). In contrast, MDA levels in I/R + L and I/R + A groups stayed comparable to sham (*P*>0.05). The mean MDA level in I/R + A was significantly lower compared to I/R (*P*=0.003, Fig. 1a). There was no significant difference between the mean GSH levels of sham, I/R, I/R + L, and I/R + A (Fig. 1b).

ALT and AST levels were significantly increased in I/R compared to sham (*P*<0.001 for both). ALT was significantly elevated in I/R + L and I/R + A groups (*P*=0.037 and *P*=0.019 compared to sham), but significantly lower compared to I/R (*P*=0.003 and *P*=0.006, Fig. 1c). Similarly, AST was significantly increased in I/R + L and I/R + A groups (*P*<0.001 for both), but significantly lower compared to I/R (*P*<0.001, Fig. 1d).

A section of the liver specimen demonstrating normal histology in the sham group is shown in Fig. 2a. In the present study, the protective effects of apelin and leptin on the liver I/R

Fig. 2 **a** A section of the liver specimen demonstrating normal histology in the control group (H&E×200); **b** microscopy demonstrating severe hemorrhage and necrosis in the ischemia group (H&E×200); **c** microscopy demonstrating cytoplasmic vacuolation of the hepatocytes in the leptin group (similar to the findings in the apelin group) (H&E×200); **d** microscopy demonstrating mild injury including cytoplasmic vacuolation in the apelin group (HE×200)

were demonstrated. In the I/R group, 10 rats had severe injury (Fig. 2b), whereas 1 rat in the leptin group had mild injury (Fig. 2c) and 1 rat in the apelin group had severe injury (Fig. 2d). Liver histopathology scores were significantly different between the groups ($P<0.001$, Table 1).

Discussion

In this study, we used a rat model to determine the effect of exogenous leptin and apelin administration as pharmacological preconditioning agents in hepatic I/R injury. We showed that leptin and apelin treatments delivered for 3 days prior to induction of I/R injury decreased lipid peroxidation, improved liver function, and reduced histological tissue damage.

MDA and GSH are one of the end products of decomposition of lipid peroxidation and are commonly used as a biomarker of oxidative damage. In the current study, the

MDA level in I/R+A was found significantly lower compared to I/R ($P=0.003$), but there was no significant difference between the mean GSH levels of sham, I/R, I/R + L, and I/R + A. Further investigations should be done to confirm the accuracy of GSH levels in hepatic I/R injury in which adipocytokines treatment was applied. Reperfusion is associated with microvascular dysfunction where endothelium-dependent vasodilation is impaired; fluid filtration is increased and leukocyte infiltration is enhanced. Endothelial production of superoxide exceeds nitric oxide production, and this imbalance leads to the production and release of inflammatory mediators [25].

Adipocytokines are bioactive mediators released from adipose tissue involved in homeostasis and energy metabolism [3]. Leptin is a 167-amino acid peptide encoded by the *ob* (*obese*) gene, bearing structural similarities to IL-6 family of cytokines. In addition to its role in control of appetite and energy homeostasis through the central nervous system, leptin was determined to have important peripheral roles such as promoting hemopoiesis and angiogenesis; modulating immune response and inflammation; affecting fertility and the onset of puberty—all in connection with food intake and energy expenditure [3]. Recent experimental studies suggest that exogenous leptin administration may alleviate the symptoms of I/R injury in various organs such as kidneys [9], bladders [10], intestines [11, 12], brains [13], and hearts [14].

Apelin is a small peptide which was first identified as the ligand of an orphan receptor APJ [15]. Apelin has various isoforms, all sharing a conserved C-terminal domain, but apelin-13 (13-amino acid peptide) shows the highest biological activity. Apelin is considered an adipocytokine as it is expressed in white adipose tissue [16]. In addition, apelin and APJ are both present in the central nervous system, and a variety of peripheral tissues with highest levels seen in the lung, heart, and mammary glands [17]. Apelin was determined to have endothelium-dependent vasodilator action and positive inotropic action in the heart [18, 19]. Recently, apelin was reported to have cardioprotective function in rat models of I/R injury [20, 21]. Leptin was shown to induce nitric oxide production, leading to vasodilation on rat arteries [26, 27]. Accordingly, some studies suggested that the nitric oxide signaling pathway may be responsible for the protective action of leptin against I/R injury [11, 12]. Recently, endogenous leptin levels were shown to fluctuate in response to partial hepatic I/R injury, suggesting that leptin is involved in the physiological response to I/R [28]. This finding is also corroborated by increased serum leptin in organ transplant patients [29].

Apelin is known mostly for its role in the cardiovascular system, lowering mean arterial blood pressure and potentiating cardiac function [18, 19]. Some studies have demonstrated that apelin has a protective function against I/R injury in the heart. Apelin/APJ expression is upregulated in cardiomyocytes in

Table 1 Liver histopathology of Sham, I/R, I/R + L and I/R + A groups

	Sham	I/R	I/R + L	I/R + A	<i>p</i> value
Normal	10 (100)	0 (0)	2 (20)	0 (0)	<0.001
Mild	0 (0)	0 (0)	7 (70)	9 (90)	
Moderate	0 (0)	0 (0)	1 (10)	0 (0)	
Severe	0 (0)	10 (100)	0 (0)	1 (10)	

I/R: ischemia/reperfusion; I/R + L: I/R + leptin; I/R + A: I/R + apelin

Values are presented as number (percentage)

Pearson Chi-Square (Monte Carlo)

response to I/R injury [30, 31]. Apelin appears to inhibit apoptosis in cardiomyocytes through activation of reperfusion injury salvage kinase pathway [21, 31–33]. However, nitric oxide or other unknown pathways may also be involved in apelin-mediated cardioprotection [30, 34]. As far as we know, our study is the first to show a protective role of apelin against I/R injury outside the heart. Interestingly, the only published study regarding apelin function in the liver showed increased apelin expression in rats and humans with cirrhosis and determined that delivery of APJ antagonist improved liver pathology, cardiovascular performance, and renal function in rats with cirrhosis [35]. The mechanism through which apelin exerts its protective function in liver I/R injury remains to be elucidated.

Conclusions

In conclusion, our results suggest that adipocytokines leptin and apelin have similar protective effects against hepatic I/R injury in the experimental rat model. Further studies are required to elucidate the mechanism of action and to determine whether these agents could be used in humans undergoing liver surgery.

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