Obestatin improves hepatic injury induced by ischemia/reperfusion in rats: Role of nitric oxide

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Abstract. Hepatic ischemia/reperfusion (I/R) injury is a common clinical problem. The present study was conducted to evaluate the protective effect of obestatin against I/R-induced liver injury. Rats were divided into three groups (n = 10): control sham-operated group, I/R group and obestatin treatment group. Rats of I/R group and obestatin treatment group underwent partial hepatic ischemia for 60 min followed by 90 min reperfusion. At the beginning of the 90-min reperfusion period, rats of obestatin treatment group were injected with obestatin (100 μg/kg) intravenously. At the end of the experiment the animals were sacrificed and blood and liver tissue samples were obtained. Liver function enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as well as inflammatory biomarkers, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), were determined in the serum. Also, total oxidative status (TOS), total antioxidant status (TAS) and oxidative stress index (OSI) were measured in hepatic tissue. Liver tissue damage was examined by histopathology. In addition, the expression levels of nitric oxide synthase (NOS) subtypes, endothelial (eNOS) and inducible (iNOS) in liver samples were assessed by Western blotting. Obestatin significantly counteracted I/R-induced liver damage mainly through reducing oxidative stress, inhibiting the release of pro-inflammatory cytokines and modulation of nitric oxide levels.

Key words: Obestatin — Ischemia reperfusion — Liver — Nitric oxide — Reactive oxygen species — Inflammatory cytokines

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; eNOS, endothelial synthase; iNOS, inducible synthase; IL, interleukin; I/R, ischemia/reperfusion; NO, nitric oxide; NOS, NO synthase; nNOS, neuronal synthase; OSI, oxidative stress index; ROS, reactive oxygen species; TAS, total antioxidant status; TOS, total oxidant status.

Introduction

Hepatic ischemia/reperfusion (I/R) injury is a major cause of liver dysfunction and is associated with several clinical conditions and interventions including liver transplantation, hepatectomy, and shock. The restoration of the blood supply following a period of ischemia will generally cause I/R injury (Subhas et al. 2010). Liver injury development after the initiation of reperfusion is due to generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, lipid peroxides, or related species, accumulation of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukins (ILs) and the subsequent biochemical derangements in intracellular homeostasis (Montalvo-Jave et al. 2008). Nitric oxide (NO) is a key molecule, which is recognized as an important, yet controversial mediator of physiological and pathological processes inherent in I/R injury since it has been shown to have both protective and deleterious effects on cellular functions (Serracino-Inglott et al. 2008). NO is synthesized from L-arginine by three isoforms of the NO synthase (NOS), the endothelial synthase (eNOS), the inducible synthase (iNOS), and the neuronal synthase (nNOS) (Kukreja et al. 2005). eNOS is responsible for the production of basal NO, which maintains normal
vascular tone. iNOS, contrary to eNOS, is especially induced under oxidative stress conditions, with controversial results regarding its role in ischemia-reperfusion (Kukreja et al. 2005; Abu-Amara et al. 2015). Neuronal (nNOS) is involved in neural signaling with no participation in the I/R events (Zhou and Zhu 2009).

Obestatin is a circulating 23-amino-acid peptide hormone, encoded by the same gene as ghrelin (Li et al. 2011). It is predominantly produced in the stomach, and exhibits a wide range of peripheral effects including inhibition of food intake, body weight gain, gastric emptying and regulation of jejunal motility (Li et al. 2011; Trovato et al. 2014). In specific, we tested the hypothesis that obestatin exerts a protective effect on the liver, as this has been evidenced for other tissues subjected to I/R insults, with a trial to clarify some of the possible involved mechanisms. In an attempt to elaborate the mechanism of the potential hepatoprotective effects of obestatin, we investigated its effect on the redox status of the liver by assessing the levels of lipid peroxides. We also assessed its anti-inflammatory effect through estimation of inflammatory biomarkers. In addition, the expression levels of nitric oxide synthetase subtypes (eNOS and iNOS) in liver samples were assessed by Western blotting.

Material and Methods

Animals

This study was conducted on 30 adult Wistar albino male rats, 6–8 weeks old, weighing between 200 and 250 g. Animals were housed in the animal laboratory at the medical research center of Benha faculty of medicine. They were housed at room temperature (25°C) and 12 h/12 h light/dark cycle. All rats were fed a standard diet and water. The study was carried out according to the guidelines of the Ethics Committee, Faculty of Medicine, Benha University.

Experimental design

Rats were randomly divided into three groups \((n = 10):\)

Group I: Control sham group \((n = 10).\) Portal structures of the left and middle liver lobe were isolated, but did not clamp, and the abdomen was subsequently closed without treatment and medication.

Group II: Hepatic I/R group \((n = 10).\) Animals underwent partial hepatic ischemia for 60 min followed by 90 min of reperfusion.

Group III: Obestatin group \((n = 10).\) Animals underwent partial hepatic ischemia for 60 min and at the beginning of the 90-min reperfusion period; the rats were injected with obestatin \((100 \mu g/kg)\) intravenously \((iv)\) (Şen et al. 2015).

Hepatic I/R injury

The overnight fasting animals were anesthetized with intraperitoneal administration of ketamine 50 mg/kg and xylazine 5 mg/kg. The abdomen was shaved and disinfected with 75% ethanol. A midline incision was performed and the hilum of the liver was exposed. All structures in the portal triad (hepatic artery, portal vein and bile duct) to the left and median liver lobes were occluded by a clamp in order to produce 70% hepatic ischemia. Sixty minutes later, the ischemic liver was reperfused by opening the clamp and reperfusion was achieved for 90 min (Zhai et al. 2004). At the end of the experimental procedure the animals were sacrificed and blood and liver tissue samples were obtained for further biochemical and histopathological investigations.

Biochemical analysis

The serum was separated by centrifugation \((5000 \text{ rpm for } 5 \text{ min})\) and used for biochemical analysis. The activities of liver enzymes such as alanine aminotransferase \((\text{ALT},\) a specific marker for hepatic parenchymal injury), and aspartate aminotransferase \((\text{AST},\) a nonspecific marker for hepatic injury) were determined by a standard automated technique using Hitachi Analyzer Model 911 and adequate kits from Roche Company \((\text{Switzerland})\). In addition inflammatory markers such as TNF-α and IL-6 were determined by ELISA technique using standard kits \((\text{Ray Biotech, Inc., USA})\).

Determination of oxidative stress biomarkers

The liver was dissected out and a part from its left lobe was washed with saline, dried and homogenized in 50 mmol/l phosphate buffer \((\text{ice cold})\) solution \((\text{pH } 7.4)\) to give 20% homogenate \((w/v)\) (Lin et al. 1998). The homogenate was centrifuged at \(3000 \times g\) for 20 min. The supernatant was separated and stored at \(-80°C\) till the colorimetric determination of total oxidant status \((\text{TOS})\) and total antioxidant status \((\text{TAS})\) using UV-160 1PC UV-visible spectrophotometer. For reading the absorbance was used Kits produced by Biodiagnostic Co., Egypt. The percent ratio TOS/TAC gave the oxidative stress index \((\text{OSI})\), an indicator of the degree of oxidative stress \((\text{Harma et al. 2003})\).

Western blotting

Proteins were extracted from liver tissues, which were subjected to ischemia and their concentrations were determined by the Bradford assay \((\text{Bio-Rad, CA, USA})\). Equivalent amounts of proteins were subjected to 10% SDS polyacrylamide gel electrophoresis. Blots were transferred onto a nitrocellulose membrane using a semidry transfer
Western blotting of iNOS and eNOS

I/R significantly decreased the expression of eNOS ($p < 0.05$) as compared to control group. Pretreatment with obestatin antagonized I/R-induced expression of eNOS as compared to ischemic group. With regard to iNOS, I/R group showed a significant increase ($p < 0.05$) in the expression of iNOS as compared to control rats. Obestatin treatment significantly decreased the expression of iNOS as compared to ischemic group (Table 3).

Table 1. Effects of obestatin on serum liver enzymes and inflammatory markers in rats subjected to I/R

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>I/R group</th>
<th>Obestatin group</th>
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<tbody>
<tr>
<td>ALT (U/l)</td>
<td>25.2 ± 6.7</td>
<td>112.6 ± 1.9*</td>
<td>78.3 ± 3.5**</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>112.6 ± 8.2</td>
<td>224.1 ± 11.3*</td>
<td>136.3 ± 4.8**</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>31.4 ± 4.2</td>
<td>129.3 ± 3.4*</td>
<td>62.6 ± 8.3**</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>51.6 ± 6.5</td>
<td>243.1 ± 4.7*</td>
<td>92.4 ± 6.6**</td>
</tr>
</tbody>
</table>

Data are mean ± SD, $n = 10$. * $p < 0.05$ significant difference compared with control group; ** $p < 0.05$ significant difference compared with I/R group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; TNF-α, tumor necrosis factor α; IL-6, interleukin-6.

Table 2. Effects of obestatin on liver oxidative stress markers in rats subjected to I/R

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>I/R group</th>
<th>Obestatin group</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOS (nmol/mg protein)</td>
<td>9.8 ± 1.2</td>
<td>16.4 ± 1.5*</td>
<td>12.6 ± 2.2**</td>
</tr>
<tr>
<td>TAS (nmol/mg protein)</td>
<td>2.5 ± 4.7</td>
<td>1.2 ± 2.8*</td>
<td>2.7 ± 3.1**</td>
</tr>
<tr>
<td>OSI (%)</td>
<td>3.2 ± 0.7</td>
<td>7.6 ± 0.9*</td>
<td>3.9 ± 0.9**</td>
</tr>
</tbody>
</table>

Data are mean ± SD, $n = 10$. * $p < 0.05$ significant difference compared with control group; ** $p < 0.05$ significant difference compared with I/R group. TOS, total oxidant status; TAS, total antioxidant status; OSI, oxidative stress index.

Table 3. Effects of obestatin on I/R-induced iNOS and eNOS expression in rat hepatic tissues measured by Western blotting

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>I/R group</th>
<th>Obestatin group</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS (µg/gm wet tissue)</td>
<td>1.5 ± 0.02</td>
<td>0.4 ± 0.07*</td>
<td>0.9 ± 0.06**</td>
</tr>
<tr>
<td>iNOS (µg/gm wet tissue)</td>
<td>0.1 ± 0.05</td>
<td>0.7 ± 0.08*</td>
<td>0.3 ± 0.04**</td>
</tr>
</tbody>
</table>

Data are mean ± SD, $n = 10$. * $p < 0.05$ significant difference compared with control group; ** $p < 0.05$ significant difference compared with I/R group. eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase.
Histopathological examination

Histopathological findings in the control group showed normal hepatic architecture with central vein and radiating hepatic cords. In I/R group, dilated and congested portal venule with perivenular necrosis and inflammatory cells infiltration were observed. In contrast, obestatin group showed restoration of hepatocytes and less inflammatory cells infiltration (Figure 1).

Discussion

Hepatic I/R injury is observed following major liver surgery, transplantation, trauma and sepsis and may cause metabolic and structural hepatic damage (Van Gulik et al. 2007; Shin et al. 2008). This remains a significant problem in surgical procedures, and is a limiting factor in liver transplantation (He et al. 2005). Because of the complicated pathophysiology of I/R-induced liver injury, many researches have been done to find the optimal measures and treatments to protect against the post ischemic hepatocyte damage.

Obestatin is a newly discovered peptide, which derives from the ghrelin peptide precursor pre-pro-ghrelin (Zhang et al. 2005; Pemberton and Richards 2008; Ren et al. 2009). Although it has been suggested that obestatin participate in a complex regulatory system (Soares et al. 2008), the role of obestatin in pathophysiological conditions are largely unknown. Recent studies have shown that obestatin exhibits protective and regenerative effects in some organs including the stomach, kidney, brain and the pancreas. However, no studies investigated the effect of obestatin administration on the liver. To elucidate the potential hepatoprotective effects of obestatin, hepatic I/R injury was induced in male rats. Previous studies have shown that inflammation, apoptosis, and altered microcirculation are evidently found even in the early stage of hepatic I/R injury.

In the present study, ALT and AST levels significantly elevated after I/R most likely due to cell membrane damage. These findings are in agreement with those of Sepodes et al. (2006). ALT and AST levels are generally measured as indirect biochemical indices of liver injury (Niemelä and Alatalo 2010). Obestatin treatment markedly attenuated ALT, AST levels, suggesting a possible protective effect of obestatin treatment in the hepatic I/R condition.

In the current study, it was preferred to measure oxidants and antioxidant capacity simultaneously to assess oxidative stress more exactly. TOS was evaluated to reflect the oxidative status, while TAS was evaluated to reflect the antioxidative status. Also OSI was taken as an indicator of the degree of oxidative stress. The data of the present study confirm that liver I/R increases oxidative stress, an effect that not only produces direct tissue damage, but also modulates produc-

Figure 1. Histopathological examination of rat hepatic tissue of Control group (A), I/R group (B) and Obestatin group (C). H&E stain, original magnification ×200. For more details see Material and Methods.
tion of inflammatory cytokines (TNF-α and IL-6). These findings are in line with a recent study of Sözen et al. (2011). During I/R, the antioxidant enzyme levels rapidly decrease and ROS is produced and begins to damage various cellular molecules, contributing to further pathological complications (Czubkowski et al. 2010; Klune and Tsang 2010). Lee and Lee (2006) and Gedik et al. (2008) suggested that oxidant stress is the major cause of hepatic I/R injury and that excessive ROS cause tissue damage and cell death by binding and altering cellular macromolecules, including DNA, proteins and lipids, and affect their function. Hepatic I/R results in an enhanced spontaneous release of inflammatory cytokines such as TNF-α and interleukins by Kupffer cells early after reperfusion (Wanner et al. 1996; Mehany et al. 2013). These mediators activate the chemotaxis of neutrophils, which in-turn produce more ROS and increase the production of TNF-α from Kupffer cells and consequently cause more hepatocyte damage (Mehany et al. 2013; Perry et al. 2013).

Obestatin treatment significantly ameliorated the I/R injury of the liver, as shown by decreased oxidative parameters (TOS and OSI), increased anti-inflammatory parameter (TAS) and markedly reduced pro-inflammatory cytokines (TNF-α and IL-6). This conforms to the recent data published by Koç and colleagues (2014) regarding the anti-inflammatory and anti-inflammary effect of obestatin.

Furthermore, I/R group in the present model showed a significant increase in iNOS expression and a significant decrease in eNOS expression. These results match those reported by Mizar et al. (2015). Emerging evidence suggests that NO has an important role in ischemia injury; however there are conflicting reports regarding the action of NO in reperfusion damage. Several reports suggested that moderate levels of NO, generated by eNOS, may be beneficial for its vasodilator action, whereas high levels of NO, produced by iNOS, interacting with superoxide anion to produce peroxynitrite, a potent oxidant associated to pathological liver conditions (Beckman et al. 1990; Koken and Inal 1999). Moreover, NO generated by iNOS, may induce inflammatory cell infiltration and parenchyma cell dysfunction (Lanteri et al. 2003). It is considered that endogenous (basal) NO, produced by eNOS, protects both hepatocytes and endothelial cells against reperfusion injury in the liver (Cottart et al. 1999). NO counteracts the vasoconstriction caused by endothelin-1, which is involved in microvascular dysfunction, particularly during the early stages of liver I/R (Datta et al. 2013). Therefore, eNOS expression seems to have a cytoprotective effect through maintaining basal levels of NO production and acts protectively against the early phase of I/R injury by preservation of the sinusoidal structure and maintenance of blood flow through the hepatic microcirculation, thus limiting the extent of I/R injury (Serracino-Inglott et al. 2003; Kukreja et al. 2005).

Obestatin treatment resulted in amelioration of NO expression changes reported in I/R group, an effect that is in accordance with the work of Koç et al. (2014). Obestatin has been shown to upregulate eNOS expression, thus directly enhancing NO bioavailability. Obestatin ameliorates renal I/R injury through modulation of NO metabolism. Vascular relaxation caused by obestatin appears to be mediated by endothelium-dependent NO release via a signaling cascade involving an adenylyl cyclase-linked GPCR, PI3K/PKB and Ca⁺²-dependent eNOS activation. Obestatin binds to an adenylyl cyclase (AC)-linked GPCR, thereby promoting PI3K/PKB-, Ca⁺²-dependent eNOS activation (Agnew et al. 2012).

Conclusion

Obestatin, a newly discovered peptide provides a hepatoprotective effect against I/R-induced liver damage via reducing oxidative stress and inflammatory process and improving antioxidant activity thus restoring normal live architecture. The significant protective role provided by obestatin against I/R injury appears to be closely related to NO production.

Declaration of interest statement. The author declares that there is no conflict of interest.

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