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Effect of ghrelin on chronic liver injury and fibrogenesis in male rats: Possible role of nitric oxide

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ABSTRACT

Recent studies have revealed that ghrelin may be an antioxidant and anti-inflammatory agent in many organs, however its role in chronic liver injury (CLI) remains unclear. The role of nitric oxide (NO) in CLI is controversial as evidence suggests that NO is either a primary mediator of liver cell injury or exhibits a protective effect against injurious stimuli. Recent evidence demonstrated that the therapeutic potential for ghrelin was through eNOS activation and increase in NO production. However, its role on NO production in the liver has not been previously investigated. The aim of this study was to investigate the role of ghrelin in treatment of CLI, and whether this action is mediated through NO. Forty male rats were divided into four groups: Group I: Control; Group II: chronic liver injury (CLI); Group III: CLI + Ghrelin; and Group IV: CLI + Ghrelin + L-NAME. Liver enzymes and tumor necrosis factor alpha (TNF-α), were measured to assess hepatocellular injury. Liver tissue collagen content, malondialdehyde (MDA), gene expression of Bax, Bcl-2, and eNOS were assessed to determine the mechanism of ghrelin action. Results showed that ghrelin decreased serum liver enzymes and TNF-α levels. Ghrelin also reduced liver tissue collagen, MDA, and Bax gene expression, and increased Bcl-2 and eNOS gene expression. The effects on TNF-α, collagen, MDA, Bax, and eNOS were partially reversed in Group IV, suggesting that ghrelin's action could be through modulation of NO levels. Therefore, ghrelin's hepatoprotective effect is partially mediated by NO release.

1. Introduction

The liver plays a crucial role in the metabolic elimination of most drugs and other foreign compounds, thus making it an important target for toxicity. Rodent models of toxin-induced hepatotoxicity are used to elucidate the biochemical processes involved in many forms of liver disease and to evaluate the potential candidates for hepatoprotectants [51]. Thioacetamide (TAA) is a thiono-sulphur-containing compound, which has been frequently used in industry in the past. Administration of one dose of TAA leads to acute hepatic toxicity [7,28], while chronic exposure causes hepatic cirrhosis and possible development of liver tumors [36]. After metabolic activation of TAA by cytochrome P450, TAA-intermediates and reactive oxygen species (ROS) can covalently bind to biologically important molecules and increase cellular oxidative stress [8,40], lipid peroxidation, and deplete glutathione [45]. Both necrosis and apoptosis appear in the process of cell death after TAA application [1].

The influences of nitric oxide (NO) on hepatic injury are controversial, mainly derived from its diverse functions. NO may be protective or toxic at various concentrations [17]. Intrinsic hepatic NO generation attenuates sinusoidal perfusion failure, improves liver tissue oxygenation [41], and ameliorates apoptotic liver damage [29]. In contrast, excessive NO production can be detrimental since it may downregulate cytochrome P450, suppress liver protein and DNA synthesis, thus induce apoptosis and necrosis [32]. NO also reacts with superoxide radical to form peroxynitrite, a potent oxidant that inhibits cellular respiration and triggers apoptosis [52]. NO is generated from L-arginine by a reaction which is catalyzed by three different nitric oxide synthases (NOSs). The constitutive NO synthases are the endothelial NO synthase (eNOS) and the neuronal NO synthase. Both synthesize low amounts of NO and regulate physiological NO homeostasis and cellular signaling. In contrast, the inducible form of NO synthase (iNOS) produces high amounts of

Abbreviations: ALP, alkaline phosphatase; ALT, alanine transferase; AST, aspartate transferase; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; CCl4, carbon tetrachloride; CLI, chronic liver injury; eNOS, endothelial nitric oxide synthase; GHS, growth hormone secretagogue receptor; L-NAME, nitro-L-arginine methylester; MDA, malondialdehyde; NO, nitric oxide; NOS, nitric oxide synthase; PI3-kinase/Akt, phosphatidylinositol 3-kinase/protein kinase B; TAA, thioacetamide; TNF-α, tumor necrosis factor alpha.

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NO induced by cytokines, such as interferon-gamma (INF-γ), tumor necrosis factor-alpha (TNF-α) or interleukin 1-alpha (IL-1α) [24].

Ghrelin is a recently discovered hormone that has been shown to be a natural ligand of the orphan growth hormone secretagogue (GHS) receptor type 1a (GHS-R1a) [23]. Two thirds of circulating ghrelin is produced by X/A-like cells of the oxyntic mucosa of the stomach, while the remainder originates in X/A-like cells of the small intestine [10]. In addition to its GH releasing effect, ghrelin has also been described to have a potent orexigenic effect [25], as well as a beneficial effect on gastrointestinal, cardiovascular, reproductive, immune, and coagulation systems [2,13,19,21]. Recent studies have revealed that ghrelin may be an antioxidant and anti-inflammatory agent in many organs such as the rat ovary [22], erythrocytes, and brain of rats [37]. However, its role as a possible hepatoprotectant still remains unclear. Ghrelin has been reported to have several effects through NO modulation in other organs, such as ghrelin induced feeding by increasing NOS in the hypothalamus [14], and regulation of gastric acid secretion [4]. However, the link between ghrelin and NO modulation in liver injury has not been previously investigated.

Owing to its anti-inflammatory and anti-oxidant properties mentioned above, we investigated the possible protective effect of ghrelin against chronic TAA-induced hepatic injury and fibrosis and whether ghrelin's effect is mediated through NO release.

2. Materials and methods

2.1. Materials

1. Thioacetamide was purchased from (Epico Co., Cairo, Egypt) in the form of light yellow powder supplied in a dark brown bottle. The calculated TAA dose was dissolved in hot water and an injected volume of 1 ml per rat was given i.p. Control rats were injected the same amount of saline only.
2. Ghrelin was purchased from (Sigma–Aldrich, Germany) in the form of a white powder. The calculated dose was dissolved in distilled water; given subcutaneously (S.C.) with an injected volume of 1 ml.
3. L-Name was obtained from Sigma (St. Louis, MO, USA) in the form of powder. The calculated dose was dissolved in saline; given orally with an injected volume of 1 ml.

2.2. Animals

Forty adult male albino rats weighing between 150 g and 200 g were used in this study. All experimental procedures were carried out in accordance with the guide for the care and use of laboratory animals published by the U.S. National Institute of Health (NIH 1985). All animals were housed in plastic cages (German University in Cairo), kept in a conditioned atmosphere at 25 °C, humidity 50–55%, with a 12 h light/dark cycles for one week for stabilization. Animals were fed with a standard diet of water and rat chow.

2.3. Experimental design

Rats were randomly divided into 4 groups; each consisted of 10 rats. In the Control Group (I), rats were injected with saline (1 ml i.p.) twice a week for 6 weeks. In the CLI Group (II); rats received TAA (200 mg/kg/i.p.) twice a week for 6 weeks [38]. In the CLI + Ghrelin Group (III); CLI was induced by the same dose of TAA as Group II, plus rats received treatment of S.C. Ghrelin (10 ng/kg/day) for 6 weeks [18]. Rats in the CLI + Ghrelin + L-NAME Group (IV) received the same doses of TAA and ghrelin as Group III, plus concomitant treatment with L-NAME (20 mg/kg/day) orally for 6 weeks [27].

2.4. Experimental procedure

Blood samples were collected from the retro-orbital plexus for biochemical analysis. Animals were sacrificed; livers were excised, cut into small pieces, and stored at −80 °C for analysis. Serum samples were used for the measurement of aspartate transferase (AST), alanine transferase (ALT), and alkaline phosphatase (ALP), as the markers of hepatic injury; as well as measuring the pro-inflammatory marker tumor necrosis factor-alpha (TNF-α). Liver samples were used for the measurement of malondialdehyde (MDA), collagen content, the pro-apoptotic marker Bax, the anti-apoptotic marker Bcl-2, and eNOS gene expression. Additional liver samples were fixed with 10% formaldehyde and stained with Hematoxylin & Eosin (H&E) and Masson's Trichome for histological analysis.

2.5. Determination of liver enzymes

Serum ALT, AST, and ALP were determined according to manufacturer's instructions using test reagent kits (Quimica Clinica Aplicada S.A., Spain). The analysis was performed on a Shimadzu UVPC 2401v 3.9 spectrophotometer (Shimadzu, Kyoto, Japan).

2.6. Determination of tumor necrosis factor-α by solid phase ELISA

TNF-α was determined by the Quantikine Rat/Mouse Immunoassay ELISA commercial kit according to the manufacturer's instructions. Zero point five gram liver tissue was added to 2 ml saline, and the tissues were scissor minced and homogenized on ice using the Teflon Potter homogenizer. Liver homogenate was centrifuged at $1000 \times g$ for 15 min, and the supernatant was used for the assay. TNF-α concentration was determined as optical density using the Vector multiple ELISA plate reader (Perkin-Elmer, Covina, CA, USA) set to 450 nm. Values were calculated as pg/ml from the constructed standard curve, and then expressed as pg/mg tissue protein. Total protein analysis was performed using the total protein diagnostic kit.

2.7. Determination of lipid peroxides

Lipid peroxidation was measured in 10% liver homogenate by the thiobarbituric acid (TBA) assay according to the method of Mihara and Uchiyama [31]. Thiobarbituric acid reactive substances (TBARS) content was calculated according to the standard curve using 1,1,3,3-tetraethoxypropane as a standard and expressed in nmol/gm wet tissue. The absorbance was measured at 535 nm using a Shimadzu UVPC 2401v 3.9 spectrophotometer.

2.8. Determination of liver collagen content

Hydroxyproline in tissue hydrolysates is a direct measure of the amount of collagen or gelatin present. Liver tissue samples (50 gm) were homogenized in dH_2O, using 100 μl H_2O for every 10 mg of tissue. To a 100 μl sample of homogenate, 100 μl concentrated HCL were added in a capped vial and hydrolyzed at 120 °C for 3 h. 10 μl of each hydrolyzed sample were transferred to a 96 well plate and evaporated to dryness under vacuum. 100 μl of the Chloramine-T reagent were added to each sample and standard and incubated at room temperature for 5 min. 100 μl of the DMAB reagent were added to each well and incubated for 90 min at 60 °C. Measurement of the absorbance at 560 nm in a microplate reader was preformed.