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Protective effects of ghrelin on cisplatin-induced nephrotoxicity in mice

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ABSTRACT

Cisplatin is a potent chemotherapeutic agent that has activity against malignant tumors. However, cisplatin causes various adverse effects, such as nephrotoxicity, which are associated with high morbidity and mortality. Recent studies have revealed that the mechanism of cisplatin nephrotoxicity includes a robust inflammatory response. Since ghrelin has been shown to have anti-inflammatory properties, we hypothesized that ghrelin might have protective effects against cisplatin nephrotoxicity. Mice were randomly divided into three groups: control, cisplatin with vehicle, and cisplatin with ghrelin. Ghrelin (0.8 μg/kg/min via osmotic-pump, subcutaneously) or vehicle administration was started one day before cisplatin injection. At 72 h after cisplatin administration (20 mg/kg, intraperitoneally), we measured serum blood urea nitrogen and creatinine, urine albumin/creatinine, renal mRNA levels of monocyte chemoattractant protein-1, interleukin-6, tumor necrosis factor-α, interleukin-1β, kidney injury molecule-1, and neutrophil gelatinase-associated lipocalin by real-time polymerase chain reaction, and histological changes. Ghrelin significantly attenuated the increase in serum blood urea nitrogen and creatinine induced by cisplatin. Ghrelin tended to attenuate the increase in urine albumin/creatinine, although not significantly. Cisplatin-induced renal tubular injury and apoptosis were significantly attenuated by ghrelin pretreatment. Consequently, ghrelin significantly attenuated renal mRNA levels of monocyte chemoattractant protein-1, interleukin-6, kidney injury molecule-1, and neutrophil gelatinase-associated lipocalin. In conclusion, ghrelin produces protective effects in cisplatin-induced nephrotoxicity through inhibition of inflammatory reactions. Pretreatment with ghrelin may become a new prophylactic candidate for cisplatin-induced nephrotoxicity.

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1. Introduction

Cisplatin is an effective chemotherapeutic agent used in the treatment of a wide variety of malignancies. Acute kidney injury (AKI) is the major toxicity associated with this compound, sometimes requiring dose reduction or discontinuation of the treatment [1,2]. In addition, AKI induced by cisplatin is associated with high morbidity and mortality [1]. The mechanisms involved in cisplatin-induced AKI appear to be multifactorial, involving inflammation, oxidative stress and apoptosis [1–3]. Previous studies have suggested that inflammatory mechanisms may play an important role in the pathogenesis of cisplatin nephrotoxicity [2,4]. Therefore, modulation of the renal inflammatory reaction after cisplatin treatment may help prevent cisplatin-induced AKI.

Ghrelin is a growth hormone-releasing peptide originally isolated from the rat stomach in 1999 [5]. Ghrelin acts at the growth hormone secretagogue receptor (GHS-R), which is expressed in the hypothalamus, pituitary, heart, and blood vessels [5,6]. The actions of ghrelin include appetite stimulation, carbohydrate utilization, and growth hormone release. Recent studies have shown that ghrelin has various anti-inflammatory effects in various experimental models [7,8]. We have previously reported that ghrelin exhibited protective effects on the cardiovascular system through the cholinergic anti-inflammatory pathway [8]. On the basis of

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these previous studies, we hypothesized that ghrelin may protect the kidney against cisplatin-induced AKI by reducing renal inflammation in mice. The objective of the present study was to investigate the protective effects of ghrelin on cisplatin-induced AKI.

2. Materials and methods

2.1. Animals and experimental design

Seven-week-old C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Ghrelin was obtained from the Peptide Institute Inc. (Osaka, Japan). Cisplatin was purchased from Yakult Co., Ltd. (Tokyo, Japan). The mice were randomly divided into four groups: vehicle (n = 6), ghrelin (n = 6), vehicle with cisplatin (n = 8), and ghrelin with cisplatin (n = 6) groups. One day before cisplatin administration, ghrelin administration was initiated using an osmotic mini-pump, as previously reported [9–11]. The osmotic mini-pump (Alzet Model 1003D; Duret Corporation, Cupertino, CA) containing either saline (vehicle) or ghrelin in saline (0.8 μg/kg/minute) was implanted subcutaneously under anesthesia in the upper back of each mouse. Cisplatin was administered as a single intraperitoneal dose of 20 mg/kg, and an identical volume of sterile saline was administered to control mice. The mice were allowed free access to water and food. Daily food intake was measured by weighing the pellets between 9:00 and 10:00 a.m. Mice were sacrificed three days after cisplatin injection under general anesthesia, and the kidneys were rapidly removed. The right kidney was immediately frozen in liquid nitrogen and stored at −80 °C until analysis, and the left kidney was fixed in 4% paraformaldehyde. The experimental protocol was approved by the Animal Care Ethics Committee of the National Cerebral and Cardiovascular Center Research Institute.

2.2. Renal function analysis

On the last day of the experiment, urine samples were collected after spontaneous urination, and mice were anesthetized with inhaled isoflurane (Escain; Mylan Seiyaku Co., Tokyo, Japan). Blood samples were collected via the femoral vein and centrifuged (2000*g* for 5 min), and the sera were collected and stored at −80 °C until analysis. Serum blood urea nitrogen (BUN) and creatinine levels and urine protein and creatinine levels were measured as previously reported [10].

2.3. ELISA

Serum tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) levels were determined using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

2.4. Measurements of plasma ghrelin concentrations

Blood samples for ghrelin measurement were collected before and three days after cisplatin injection in chilled tubes containing EDTA-2Na (2 mg/ml) and aprotinin (500 KIU/ml; Sigma-Aldrich, St Louis, MO), and centrifuged at 4 °C. Hydrogen chloride was added to the samples at a final concentration of 0.1 N immediately after separation of plasma. Plasma was immediately frozen and stored at −80 °C until further analyses. Plasma concentrations of total ghrelin were determined using the radioimmunoassay system as previously reported [12].

2.5. Histological analysis of kidney

Kidney tissue was fixed in 4% formaldehyde for 24 h and embedded in paraffin. The samples were cut on a microtome into 4 μm sections and stained with hematoxylin-eosin (HE) and periodic acid–Schiff (PAS) reagents for histological examination. Tubular injury in PAS-stained sections was examined under a microscope and scored according to the percentage of cortical tubules having epithelial necrosis: 0%, normal; 1, <10%; 2, 10–25%; 3, 26–75%; or 4, >75% [13].

For Mac-3 staining, the tissue sections were deparaffinized, and the endogenous peroxidase was blocked with 3% H_2O_2 for 30 min. After each step, the tissue sections were rinsed twice in phosphate-buffered saline (PBS) for 5 min. The deparaffinized tissue sections were incubated with Protein Block (DakoCytomation, Glostrup, Denmark) for 15 min. The rat anti-mouse Mac-3 antibody was diluted in an antibody diluent buffer (dilution 1:500; BioLegend, San Diego, CA, USA) and applied at room temperature for 90 min. After incubation with primary antibodies, the slides were incubated with biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) for 30 min, followed by incubation with peroxidase-conjugated avidin-biotin complex (Vectastain ABC kit; Vector Laboratories) for 30 min. The sections were visualized with 0.5% diaminobenzidine (DakoCytomation) and 0.3% hydrogen peroxide, and counterstained with hematoxylin.

Apoptosis in renal tissues was assessed by terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick-end labeling (TUNEL) assay with an in situ apoptosis detection kit (Takara Bio Inc., Tokyo, Japan) according to the manufacturer's protocol. The number of apoptotic cells in each section was calculated by counting the number of TUNEL-positive apoptotic cells in 10 random, non-overlapping fields at 400× magnification. The histological examinations were performed in a blinded manner.

2.6. Quantitative real-time PCR analysis

Total RNA was isolated using an RNeasy mini kit (Qiagen, Hilden, Germany). The obtained RNA was reverse-transcribed into cDNA using a Quantitect Reverse Transcription kit (Qiagen). PCR amplification was performed using SYBR Premix Ex Taq (Takara Bio Inc.). Quantitative PCR was performed in a 96-well plate using a Light Cycler 480 System II (Roche Applied Science, Indianapolis, IN, USA). The primers used were as follows: for MCP-1, sense 5′- GCAGGTGTCCCAAAGAAGCTGTAGT-3′ and antisense 5′- CAGAAGTGCTTGAGGTGGTTGTGGA-3′; for IL-6, sense 5′- CCAGTTGCCTTCTTGGGACTGATG-3′ and antisense 5′- GTAATTAAGCCTCCGACTTGTGAAG-3′; for TNF-α, sense 5′- TGGCCCAGACCCTCACACTCAGATC-3′ and antisense 5′- GCCTTGTCCCTTGAAGAGAACCTGG-3′; for interleukin-1β (IL-1β), sense 5′- AGCACCTTCTTTCCCTTCATCTTTG-3′ and antisense 5′- GAGGTGGAGAGCTTTCAGTTCATAT-3′; for kidney injury molecule-1 (KIM-1), sense 5′- CCCTACTAAGGGCTTCTATGTTGGC-3′ and antisense 5′- AATGTAGATGTTGTCTTCAGCTCGGG-3′; for neutrophil gelatinase-associated lipocalin (NGAL), sense 5′- CGCTACTGGATCAGAACATTTGTTCC −3′ and antisense 5′- GAGGCCCAGAGACTTGGCAAAGC −3′; for GHS-R, sense 5′- GTTTGCTTTCATCCTCTGCTGGCTG −3′ and antisense 5′- AGGCCCGGGAACTCTCATCCTTCAG −3′; and for 36B4, sense 5′-TCATTGTGGGAGCAGACAATGTGGG-3′ and antisense 5′- AGGTCCTCCTTGGTGAACACAAAGC-3′. The PCR settings were as follows: initial denaturation at 95 °C, followed by 35 cycles of amplification for 15 s at 95 °C and 20 s at 58–62 °C (optimized for each primer pair), with subsequent melting curve analysis, increasing the temperature from 72 °C to 98 °C. Quantification of gene expression was calculated relative to the housekeeping gene 36B4.

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