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Long-term leptin treatment exerts a pro-apoptotic effect on renal tubular cells via prostaglandin E_2 augmentation

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ABSTRACT

Adipokine leptin reportedly acts on the kidney in pathophysiological states. However, the influence of leptin on renal tubular epithelial cells is still unclear. Gentamicin, a widely used antibiotic for the treatment of bacterial infection, can cause nephrotoxicity. This study aims to investigate the influence of long-term leptin treatment on gentamicin-induced apoptosis in rat renal tubular cells (NRK-52E) and mice. We monitored apoptosis and molecular mechanisms using annexin V/ propidium iodide staining and small interfering RNA transfection. In NRK-52E cells, leptin reduced gentamicin-induced apoptosis at 24 h, but significantly increased apoptosis at 48 h. Long-term treatment of leptin decreased Bcl-x_L expression and increased caspase activity in gentamicin-treated NRK-52E cells. Leptin also increased the expression of cyclooxygenase-2 (COX-2) and its product, prostaglandin E_2 (PGE_2), in a dose-dependent manner. The COX-2 inhibitor, NS398 (*N*-[2-(Cyclohexyloxy)-4- nitrophenyl]methanesulfonamide), blocked PGE_2 augmentation and the pro-apoptotic effects of leptin. The addition of PGE_2 recovered the pro-apoptotic effect of leptin in NS398-treated NRK-52E cells. In a mouse animal model, a 10 day leptin treatment significantly increased gentamicin-induced apoptotic cells in proximal tubules. NS398 treatment inhibited this in vivo pro-apoptotic effect of leptin. Results reveal that long-term elevation of leptin induces COX-2-mediated PGE_2 augmentation in renal tubular cells, and then increases these cells' susceptibility to gentamicin-induced apoptosis.

1. Introduction

Leptin, a 16 kDa hormone secreted from adipocyte, has a pivotal role in regulating food intake and energy consumption. Leptin acts on hypothalamus to induces a complex response, which putatively contributes to the regulation of bodyweight and energy outlay (Ahima et al., 1996; Friedman and Halaas, 1998). In humans, blood leptin levels correlate closely with body mass and may increase from 1–3 ng/ml in non-obese subjects to as high as 100 ng/ml in obese individuals (Blum et al., 1998). One disease state in which leptin metabolism is altered in relation to adiposity is renal disease (Sharma et al., 1997; Wiesholzer et al., 1998). Patients on hemodialysis who have little residual renal function have on average higher leptin concentrations in relation to body mass than normal, with some patients possessing manifold elevations. The investigation of the influence of leptin elevation on the progression of renal failure is contributive to therapy of kidney injury.

The influence of leptin on cell physiology is varied. Leptin has inhibitory effects on cell proliferation and promotes apoptosis in breast cancer cells (Naviglio et al., 2009). In pituitary gland cells, leptin may alter DNA synthesis and stimulate apoptosis (Liu et al., 2009). Apoptosis of renal tubular epithelial cells is a component of kidney diseases which can contribute to renal failure (Havasi and Borkan, 2011). The influence of leptin on renal tubular cell apoptosis may influence the progression of renal failure. On the other hand, some studies show that leptin is functional in the activation of mitogenic and anti-apoptotic signaling pathways in many cell types. In human esophageal adenocarcinoma cells, for example, leptin stimulates proliferation and inhibits apoptosis via cyclooxygenase-2 (COX-2)-dependent, prostaglandin-E2 (PGE_2)-mediated, transforming growth factor alpha-mediated transactivation of the EGF receptor, and c-Jun NH2-terminal kinase activation (Gao et al., 2009; Ogunwobi and Beales, 2008). However, COX-2 expression and COX-2-mediated PGE_2 generation obviously increase during kidney failure (Rios et al., 2011). Studies in the 5/6 nephrectomy rat renal failure model have shown that chronic COX-2 inhibition may protect against the hyperfiltration and

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reduce proteinuria (Wang et al., 2000). Increased podocyte COX-2 expression predisposes further podocyte injury in response to antibiotics probably through PGE_2 receptor activation (Cheng et al., 2009; Martineau et al., 2004). Similarly, increased COX-2 expression has been reported in obese Zucker rats, which exhibit increased urinary prostaglandin excretion in parallel with the development of metabolic abnormalities (Komers et al., 2005). Based on these studies, leptin-induced COX-2 and PGE_2 generation are supposed to be harmful to renal injury although our recent study showed that leptin reduces gentamicin-induced apoptosis at 24 h in rat renal tubular cells in vitro (Chen et al., 2011). For obese individuals and hemodialytic patients, the long-term effect of high leptin concentration on renal tubular cells is pathologically significant. However, the influence of long-term leptin treatment on renal injury is still unclear.

Gentamicin, an antibiotic of aminoglycoside, is widely used to treat Gram-negative bacterial infection because of its low cost. But gentamicin can cause acute renal failure with acute tubular necrosis in about 20% of patients (Leehey et al., 1993). Treatment of experimental animals with gentamicin produces apoptosis (Li et al., 2009) of tubular epithelial cells in vivo and also in cultured cells (Pessoa et al., 2009). Previous studies show that gentamicin enhances the generation of reactive oxygen species (ROS) through the mitochondria-mediated signaling pathway (Denamur et al., 2011; Juan et al., 2007; Servais et al., 2005). ROS-mediated apoptosis signaling results in the permanent formation of toxic lipid peroxidation and the activation of caspases (Denamur et al., 2011; Servais et al., 2005). In mammalian cells, a major caspase activation pathway is the cytochrome *c*-initiated pathway, which is regulated by Bcl-2 family proteins, including Bcl-2, BAD and Bcl-x_L (Martinou and Youle, 2011). In this pathway, various apoptotic stimuli cause cytochrome *c* release from mitochondria, resulting in activating caspases to cause subsequent cell death (Jiang and Wang, 2004; Martinou and Youle, 2011).

The present study investigated the influence of leptin on gentamicin-induced apoptosis in rat renal tubular cells, and found that long-term leptin treatment exerts a pro-apoptotic effect. Leptin-induced PGE_2 augmentation played a critical role in the pro-apoptotic effect of leptin in rat renal tubular cells. Similar trend was also demonstrated in mice received 10 day leptin pretreatment before gentamicin.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and tissue culture reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD, USA). Leptin, *N*-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide (NS398), PGE_2 and other chemical reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture

Rat renal proximal tubular cells (NRK-52E) were purchased from Bioresource Collection and Research Center (Taiwan), and cultured in DMEM culture medium supplemented with antibiotic/antifungal solution and 10% fetal bovine serum. Cell growth was allowed to continue until the monolayer became confluent. The medium for the cultured cells was then changed to the serum-free medium, and the cells were incubated overnight before experimental assays.

2.3. Lactate dehydrogenase assay

For lactate dehydrogenase (LDH) assays, NRK-52E cells were plated, at a cell density of 10,000 cells per well, in 96-well plates and grown overnight. After treatment with gentamicin, the culture medium was collected and assayed using the LDH cytotoxicology detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Each data point was determined in triplicate.

2.4. Apoptosis detection

FITC-annexin V/propidium iodide (PI) double staining was used to detect apoptosis induced by gentamicin treatment. Treated NRK-52E cells were harvested and washed twice with ice-cold PBS. Specific binding of FITC-annexin V and staining with PI were performed using an apoptosis detection kit (BD Pharmingen Franklin Lakes, NJ, USA) according to the manufacturer's instructions. The cells were then analyzed using flow cytometry.

2.5. Western blot analysis

Twenty micrograms of NRK-52E lysate proteins were applied to each lane and analyzed using western blotting. The antibody of Ob-R (M-18) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and diluted to 1:200 for assay. The antibodies of Akt, phospho-Akt, Bcl-x_L, caspase-3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Danvers, MA, USA), and diluted to 1:1000 for assay. GAPDH was detected as a loading control.

2.6. Measurement of prostaglandin by enzyme immunoassay

The cultured medium was collected for PGE_2 detection, and the cells were sonicated in ice-cold buffer (0.05 M Tris at pH 7.0, 0.1 M NaCl, and 0.02 M EDTA) for PGI_2 detection. Production of PGI_2 was typically monitored using measurement of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), because 6-keto-$PGF_{1\alpha}$ is a stable product of the non-enzymatic hydration of PGI_2. Samples were analyzed using PGE_2 EIA kits and 6-keto-$PGF1_{1\alpha}$ EIA kits from Cayman Chemical Company (Ann Arbor, MI, USA).

2.7. Animals and treatments

Male BALB/c mice weighing 20–25 g and aged eight weeks were obtained from BioLasco Taiwan Co., Ltd., Taipei, Taiwan. Animals were housed in a central facility, subjected to a 12 h light-dark cycle, and provided with regular mouse chow and tap water. The mice ($n=6$) for gentamicin treatment received intraperitoneal (i.p.) gentamicin injection (20 mg/kg/day) for 10 day. The mice ($n=6$) for gentamicin and leptin treatment received intravenous (i.v.) injection of recombinant mouse leptin (Sigma), dissolved in PBS, at a dose of 5 mg/kg/day 30 min before each gentamicin injection. The mice ($n=6$) for gentamicin, leptin and NS398 treatment received i.p. injection of NS398, dissolved in PBS, at a dose of 15 mg/kg/day 30 min before each leptin injection. Control mice received i.p. and i.v. injection of PBS. Treated and control mice were killed 24 h after the last treatment. Kidneys were harvested by laparotomy and the renal cortex tissue was snap-frozen in dry ice and stored at $-80\,^{\circ}C$ until commencement of in situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.