The adipocytokine resistin stimulates the production of proinflammatory cytokines TNF-α and IL-6 in pancreatic acinar cells via NF-κB activation
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INTRODUCTION
The adipokine resistin, found in inflammatory zone 3 (Fizz3) and also known as adipocyte-specific secretory factor, is a member of a class of cytokines primarily produced by visceral fat that are important in mediating the pathologic effects of obesity (1). Resistin, a 108-amino-acid peptide hormone produced by adipocytes as well as peripheral blood mononuclear cells (PBMC), bone marrow cells, and macrophages, is an adipocyte-derived polypeptide member of the resistin-like molecule (RELM) family of cysteine-rich proteins (2-4). The compound was originally identified in diabetic mice, where it was found to be involved in insulin sensitivity impairment (5). Recently, elevated resistin level has been used as an early marker of inflammation in patients with acute pancreatitis (AP) due to its association with both major local and systemic aspects of the inflammatory response (1, 6). Resistin has also been shown to effect inflammatory cell infiltration of the pancreas and peripancreatic visceral fat tissues, thus affecting the severity of clinical AP symptoms (7, 8).

AP is a common, non-infectious, inflammatory disorder of the pancreas with characteristically high mortality and morbidity, a wide range of clinical presentations, and an etiology that is not fully elucidated (6). Pancreatologists have long recognized obesity as a risk factor associated with poor outcomes in AP patients, though few studies have specifically defined its effect in acute inflammatory diseases (9). In fact, obesity has been proposed as an independent and essential predictive factor for AP in modern severity scoring methods (10) and negative prognostic factor in AP (11). Several recent studies have further explored and characterized the association between resistin and inflammatory factors (12-14), suggesting that resistin may play a role in the severity of AP.

Pancreatic acinar cells are secretory cells with altered behavioral patterns in the presence of noxious stimuli, exhibiting the inflammatory cell characteristic ability to activate signal transduction pathways involved in inflammatory mediator expression (15). Throughout the course of AP, reactive oxygen species (ROS) overproduction in acinar cells plays a role in activating the mitogen-activated protein kinase (MAPK) cascade and thus elevating the expression of inflammatory genes (16-18). The kinase cascade mediates the activation of nuclear factor-κB (NF-κB), a transcription factor commonly associated with the activation of inflammatory genes (19, 20), and other signal transducers and activators of transcription that have been demonstrated to result in overexpression of inflammatory genes in pancreatic acinar cells (21).

During early AP, intra-acinar cell activation of genes associated with digestive zymogen may cause acinar cell injury (22), further impacting AP severity by altering the inflammatory response in these damaged cells (23).
mor necrosis factor α (TNF-α) was the first cytokine revealed to be overexpressed in a non-inflammatory cell type (24). Since then, multiple studies have shown that TNF-α, interleukin 6 (IL-6), and interleukin 1β (IL-1β) are all overexpressed in pancreatic acinar cells (16, 25). Because resistin has potent immunomodulatory and metabolic activities (26), proinflammatory changes in pancreatic acinar cells may be, in part, due to dysregulation of the adipocytokine resistin.

The current study investigates the functional consequences of exposing rat pancreatic acinar cells to resistin in order to determine the effects of resistin exposure on mRNA and protein expressions of the proinflammatory signaling molecules TNF-α and IL-6 in rat pancreatic acinar AR42J cells. This study also provides further information pertaining to the mechanism of resistin-induced production of proinflammatory cytokines associated with NF-κB activation.

**MATERIALS AND METHODS**

**Cell cultures**

Rat pancreatic acinar AR42J cells were purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China). AR42J cells were maintained in Ham’s F-12 medium (F-12K) (Invitrogen, Gaithersburg, MA, USA) supplemented with 10% fetal bovine serum (Sijiqing, Hangzhou, China), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were routinely plated at a density of 1×10^5 cells/ml in 6-well cluster dishes and incubated in a humidified incubator at 37 °C with 95% normal air and 5% CO₂. AR42J cells were cultured to confluence and treated with variant concentrations of recombinant rat resistin (ProSpec, Rehovot, Israel). Resistin was dissolved in PBS for these experiments, and AR42J cells treated with PBS alone were used as negative controls.

**Amylase secretion**

AR42J cells were incubated with variant concentrations (1, 10, and 100 ng/ml) of recombinant rat resistin for 45 min at 37 °C, and the supernatants were used in amylase assays. Amylase secretion was measured using the 2-chloro-4-nitrophenyl-alpha-maltotrioside (CNPG3) (Kehua Bio, Shanghai, China) method, according to the instructions provided by the manufacturer. Absorbance data were measured at 405 nm, and amylase secretion was expressed in units per liter using the standard curve provided by the manufacturer.

**Lactate dehydrogenase release**

A Cyto Tox-96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) was used to determine percent release of lactate dehydrogenase (LDH), a stable cytosolic enzyme released upon cell lysis. Briefly, AR42J cells were treated with 100 ng/ml resistin for 24 h. Then, 50 μl of cell culture medium was collected from each well, diluted to 1:1 ratio with fresh medium, and plated on a sterile microtiter plate. Next, 50 μl of substrate solution was added to each well, and the plates were incubated at room temperature for 30 min in the dark. Absorbance data were measured using an automated 96-well plate reader with an absorbance of 490 nm. The LDH activity obtained in the supernatant of cells without any treatment served as a blank. The blank value was subtracted in each case, and the percentage LDH release was calculated taking 0.2% Triton X-100-lysed cells as 100% (27).

**Enzyme-linked immunosorbent assay**

To investigate the effect of resistin on proinflammatory cytokines in rat pancreatic acinar AR42J cells, cells were treated with resistin (1, 10, 100 ng/ml) for 24 h. Commercially available enzyme-linked immunosorbent assay kits were used to measure protein levels of proinflammatory cytokines TNF-α (RayBio, Norcross, GA, USA) and IL-6 (Bender, Vienna, Austria) in the supernatant of the culture medium, according to the manufacturers’ directions.

**Total RNA isolation and real-time RT-PCR**

AR42J cells were incubated with 100 ng/ml resistin for 24 h. Total RNA was isolated from AR42J cells using TRizol (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using the One Step PrimeScript mRNA cDNA Synthesis Kit (Takara Bio, Dalian, China), according to the manufacturer’s directions. Specific mRNA quantification was performed by real-time PCR (RT-PCR) using SYBR Premix Ex Taq™ II (Takara Bio, Dalian, China) in a Lightcycler 480 RT-PCR System (Roche Diagnostics, Meylan, France), according to the manufacturer’s guidelines. TNF-α, IL-6, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were designed using the computer software Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA). The gene-specific primers used were as follows: TNF-α sense: 5'-TGAACCTTCGGGT-GATCG-3', antisense: 5'-GGGCTTGTACACTGAGTTTT-3'; IL-6 sense: 5'-TGCAGCACCAGGAACGAAATG-3', antisense: 5'-AGTAGGGAGGCAG TGGCGTGTA-3'; GAPDH sense: 5'-CTCAACTACAGGGTCTACATGG-3', antisense: 5'-CCTTCGATTTCTGACT-3'. All reactions involved initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 10 sec, 60 °C for 20 sec, and 79 °C for 20 sec. The C<sub>T</sub> value was defined as the number of PCR cycles in which the fluorescence signal exceeded the detection threshold value. First, ΔC<sub>T</sub> = Ct Gene – Ct GAPDH. Then, ΔΔC<sub>T</sub> = ΔCt treated – ΔCt control. Lastly, 2<sup>-ΔΔCt</sup> was calculated to represent the relative mRNA expression of target genes (28). GAPDH was used as an internal control.

**Western blot analysis**

Variations in the NF-κB p65 subunit in nuclei of AR42J cells were detected by western blot analysis. AR42J cells were stimulated with 100 ng/ml resistin for 24 h. Cell lysates and nuclear extracts were prepared using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Bio, Nantong, China), according to the manufacturer’s guidelines. Proteins were quantified using a BCA Protein Assay Kit (Beyotime Bio, Nantong, China). For western blot assay, equal amounts of protein (18 μg) were separated by 10% SDS-PAGE and electrophotorectomically transferred to polyvinyliden fluoride (PVDF) membranes (Invitrogen, Grand Island, NY, USA). The non-specific sites on each blot were blocked with 5% milk powder diluted in TBS with 0.05% Tween 20 (TBST). Proteins were detected by western blot using the following antibodies: rabbit polyclonal antibody for NF-κB p65 (diluted 1:1000; Abcam, Cambridge, MA, USA) and mouse monoclonal antibody for lamin-B (diluted 1:1000; Santa Cruz Bio, CA, USA). Following overnight incubation with the primary antibody, each blot was washed four times with TBST buffer. Blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000, Santa Cruz Bio, Santa Cruz, USA) and HRP-conjugated goat anti-mouse secondary antibodies (1:1000, Santa Cruz Bio, Santa Cruz, USA). Proteins were detected using an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA). Band intensity was quantified using Bandscan 5.0 software (Glyko, Novato, CA, USA). NF-κB p65 nuclear expression was normalized to lamin-B.