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Resistin aggravates the expression of proinflammatory cytokines in cerulein-stimulated AR42J pancreatic acinar cells

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Abstract. Resistin, an adipocytokine secreted by fat tissues, has been shown to be associated with increased local and systemic complications in acute pancreatitis (AP). However, the mechanism underlying the effect of resistin in the aggravation of AP remains to be elucidated. The aim of the present study was to investigate the functional consequences of exposing rat pancreatic acinar cells to resistin and to determine whether it amplifies proinflammatory signaling in an *in vitro* AP model. AR42J cells pretreated with recombinant resistin were activated by cerulein as an *in vitro* model of AP. The secretion of amylase was measured to evaluate the cytotoxic effect. The mRNA expression levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 were determined using reverse transcription-quantitative polymerase chain reaction analysis. The nuclear protein expression levels of the nuclear factor (NF)-κB p65 subunit were determined using western blot analysis. Resistin treatment significantly increased the secretion of amylase, and the mRNA expression levels of TNF-α and IL-6 in the cerulein-induced *in vitro* AP model. High protein levels of the NF-κB p65 subunit were observed in the nuclei of cells in the resistin-treated AP model, compared with the untreated AP model. Pretreatment of the *in vitro* resistin-treated AP model with the NF-κB inhibitor, pyrrolidine dithiocarbamate decreased the protein expression of the NF-κB p65 subunit in nuclei, and significantly attenuated the increased mRNA expression levels of TNF-α and IL-6 induced by resistin. The results of the present study showed that resistin increased the production of the TNF-α and IL-6 proinflammatory cytokines via the NF-κB-dependent pathway during AP. Thus, the overproduction of obesity-associated resistin and the associated amplification of the inflammatory response may result in the aggravation of AP severity.

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Introduction

The increasing incidence of obesity, leading to metabolic complications or metabolic syndrome, is now recognized as a major public health problem (1). Investigations have focused on the association between obesity and various chronic diseases; however, few studies have specifically defined its effect in acute inflammatory diseases (2). Previous studies have confirmed obesity as a negative prognostic factor in acute pancreatitis (AP) (3-5). Patients with AP who are obese have a higher rate systemic inflammatory response and poorer outcomes (4,6). Meta-analysis has suggested that obesity is a risk factor for the development of local and systemic complications, and mortality rates in AP (7,8). Novel scoring systems for the severity of AP have also been suggested, including obesity as an independent essential predictive factor (9).

Over the past decade, fat tissue has been viewed not only as a site of energy storage, but also as an active endocrine organ, which secretes a variety of bioactive substances known as adipocytokines (10). Adipocytokines have a wide variety of endocrine, paracrine and autocrine effects, including the regulation of energy metabolism, immunity and inflammation (11,12). Resistin, a 108-amino-acid peptide hormone secreted by adipocytes and macrophages, is a member of the resistin-like molecule family of cysteine-rich proteins (13-15). Originally, resistin was implicated as a factor linking obesity and diabetes by impairing insulin sensitivity and glucose tolerance in mice (16). Elevated levels of resistin have been used as an early marker of inflammation in patients with AP due to its association with major local and systemic components of the inflammatory response (17). Resistin has also been shown to affect inflammatory cell infiltration of the pancreas and peripancreatic visceral fat tissues, thus affecting the severity of the clinical symptoms of AP (18,19). Several previous studies have examined and characterized the association between resistin and inflammatory factors (16,20-22) and have suggested that resistin may have an effect on the severity of AP.

It is generally considered that inflammation in AP is crucial in the pathogenesis of local and systemic damage (5). The activation of nuclear factor kappa B (NF-κB), a transcription factor associated with the activation of inflammatory genes, results in the overexpression of inflammatory genes in pancreatic acinar cells (23). The ultimate severity of the resulting pancreatitis may be determined by the inflammatory responses, which occur subsequent to acinar cell injury (24).

As resistin has potent immunomodulatory and metabolic activities (15), the present study hypothesized that the proinflammatory alterations observed in AP may be, in part, associated with the adipocytokine resistin.

In the present study, the effects of resistin were investigated on a cerulein-induced *in vitro* model of AP using the AR42J cell line. In addition, the mechanism underlying resistin-induced AP aggravation associated with NF-κB activation was determined using this model.

Materials and methods

Cell cultures. Rat AR42J pancreatic acinar cells were purchased from the China Center for Type Culture Collection (Wuhan, China). The AR42J cells were maintained in Ham's F-12 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were routinely plated at a density of $1x10^5$ cells/ml in 6-well cluster dishes, and incubated in a humidified incubator at 37˚C with 95% air and 5% CO_2. The cells were divided into a control group, model group (treated with cerulein) and resistin treatment group (treated with cerulein and resistin). The drugs were dissolved in PBS and added at the following concentrations: 100 nM cerulein (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and 8 nM resistin (ProSpecTany TechnoGene, Ltd., Rehovot, Israel). In a previous study, it was found that resistin exhibits maximal effects on pancreatic acinar cells at a concentration of 8 nM (21) and this concentration is also close to the levels of resistin in patients with AP on admission (19). For the treatment groups, the cells ($1x10^5$ cells/ml) were pre-treated with resistin at 37˚C for 30 min prior to the addition of cerulein. AR42J cells treated with PBS alone were used as negative controls. After 24 h, the cells and culture media were harvested for further analysis. To determine the effect of NF-κB, AR42J cells were incubated for 2 h at 37˚C with 60 μM pyrrolidine dithiocarbamate (PDTC) (Sigma-Aldrich; Merck Millipore), an antioxidant, which acts as a specific inhibitor of NF-κB activation (25), prior to stimulation with cerulein or resistin.

Estimation of amylase secretion. The culture media was collected and amylase assays were performed. The secretion of amylase was measured using the 2-chloro-4-nitrophenyl-α-m altotrioside method according to the manufacturer's protocols (Kehua Bio, Shanghai, China). Absorbance data were measured at 405 nm, and amylase secretion was expressed in units per liter using the standard curve provided by the manufacturer.

Total RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from the AR42J cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA synthesis was performed using a One Step PrimeScript miRNA cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China). Specific mRNA quantification was performed using qPCR analysis using SYBR Premix Ex TaqTM II (Takara Biotechnology Co., Ltd.) in a Lightcycler 480 Real-Time PCR system (Roche Diagnostics, Meylan, France) as previously described (26). The tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were designed using Primer Express 2.0 computer software (Applied Biosystems; Thermo Fisher Scientific, Inc.). The gene-specific primers used were as follows: TNF-α, sense 5'-TGAACTTCGGGGTGATCG-3' and antisense 5'-GGGCTTGTCACTCGAGTTTT-3'; IL-6, sense 5'-TCGAGCCCACCAGGAACGAAAGT-3' and antisense 5'-AGTAGGGAAGGCAGTGGCTGTCA-3'; GAPDH, sense 5'-CTCAACTACATGGTCTACATGTTCCA-3' and antisense: 5'-CTTCCCATTCTCAGCCTTGACT-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 Cq value was defined as the number of PCR cycles in which the fluorescence signal exceeded the detection threshold value. First, ΔCq=Cq Gene-Cq GAPDH. Then, ΔΔCq=ΔCq treated-ΔCq control. Lastly, $2^{\Delta\Delta Cq}$ was calculated to represent the relative mRNA expression of target genes (27). The relative quantity of mRNA for each gene was normalized based on that of the housekeeping gene, GAPDH.

Western blot analysis. Variations in the protein expression levels of the NF-κB p65 subunit in the nuclei of AR42J cells were detected using western blot analysis. Cell lysates and nuclear extracts were prepared using a Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Intitute of Biotecnology, Nantong, China) according to the manufacturer's protocols. The proteins were quantified using a BCA Protein Assay kit (Beyotime Intitute of Biotecnology). For the western blot assays, equal quantities of the protein samples (18 μl) were separated by 10% SDS-PAGE, following which they were electrophoretically transferred onto PVDF membranes (Invitrogen; Thermo Fisher Scientific, Inc.). The non-specific sites on each blot were blocked for 1 h at room temperature with 5% milk powder diluted in TBS with 0.05% Tween-20 (TBST). The membranes were then incubated with the following antibodies: Rabbit polyclonal anti-NF-κB p65 (diluted 1:1,000; Abcam, Cambridge, MA, USA) and mouse monoclonal anti-lamin-B (diluted 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Following incubation at 4˚C overnight with primary antibodies, the blots were washed four times with TBST buffer. The blots were then finally incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (1:10,000; Santa Cruz Biotechnology, Inc.) and HRP-conjugated goat anti-mouse secondary antibodies (1:1,000; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. The proteins were detected using an enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.). Band intensity was quantified using Bandscan 5.0 software (Glyko, Novato, CA, USA). Protein expression was normalized to lamin B.

Statistical analysis. All statistical analyses were performed using SPSS version 12.0 (SPSS, Inc., Chicago, CA, USA). Data are expressed as the mean ± standard deviation of three independent experiments performed in duplicate. Statistical significance was evaluated using one-way analysis of variance with Student-Newman-Keuls test for post hoc analysis. $P<0.05$ was considered to indicate a statistically significant difference.

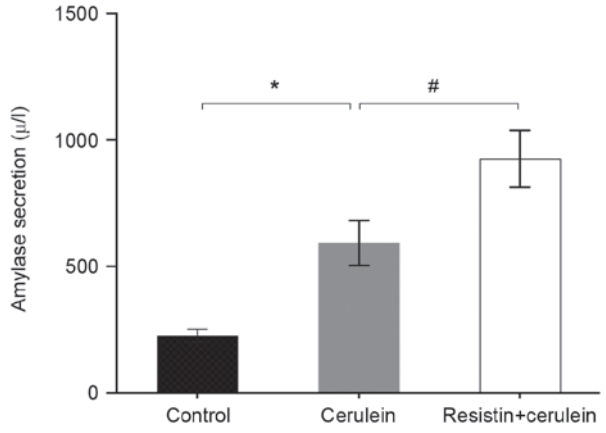

Figure 1. Amylase secretion in AR42J cells. AR42J cells were incubated with cerulein alone or with resistin, and amylase secretion was examined. The data are presented as the mean ± standard deviation of three independent experiments, each performed in duplicate. *P<0.01; #P<0.05.

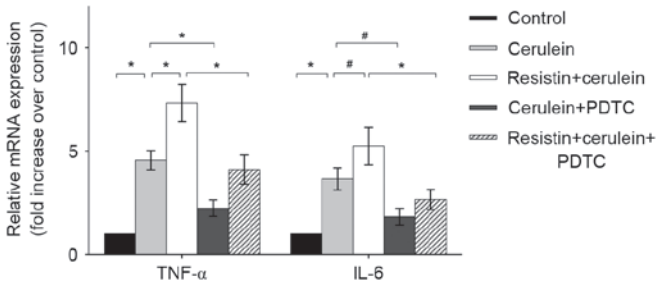

Figure 2. mRNA expression of TNF-α and IL-6 in AR42J cells. AR42J cells were incubated with or without the NF-κB inhibitor, PDTC, for 2 h, followed by stimulation with cerulein or cerulein+resistin. Total RNA was extracted from the AR42J cells and assayed for the mRNA expression of TNF-α and IL-6 using reverse transcription-quantitative polymerase chain reaction analysis. GAPDH was used as an internal control. The data are presented as the mean ± standard deviation of three independent experiments, each performed in duplicate. *P<0.01; #P<0.05. TNF-α, tumor necrosis factor α; IL-6, interleukin-6; NF-κB, nuclear factor kappa B; PDTC, pyrrolidine dithiocarbamate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

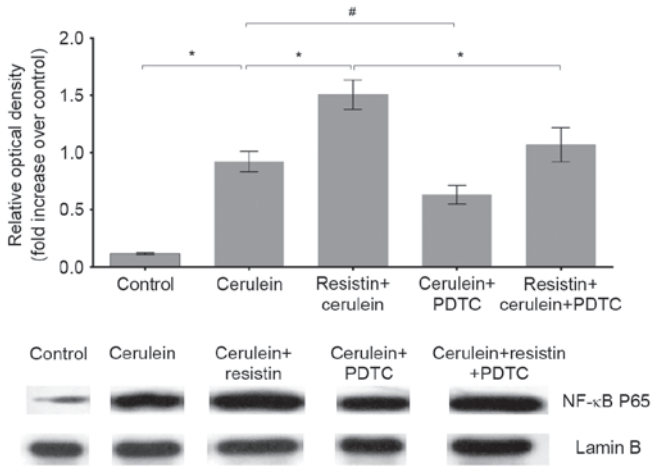

Figure 3. Western blot analysis of protein levels of the NF-κB p65 subunit in the nuclear fraction of AR42J cells. AR42J cells were incubated with or without NF-κB inhibitor PDTC for 2 h, followed by stimulation with cerulein or cerulein+resistin. Subsequently, cells were subjected to nuclear extraction to detect protein levels of the NF-κB p65 subunit. Protein expression was normalized to lamin B. The data are presented as the mean ± standard deviation of three independent experiments, each performed in duplicate. *P<0.01 and #P<0.05. NF-κB, nuclear factor-κB; PDTC, pyrrolidine dithiocarbamate.

Results

Effects of resistin on amylase secretion in cerulein-treated AR42J cells. Treatment of the AR42J cells with cerulein increased the secretion of amylase into the culture media, compared with the untreated control cells (P<0.01; Fig. 1). In the resistin-pretreated *in vitro* AP model, a significantly higher concentration of amylase secretion was produced, compared with that in the cerulein-treated group (P<0.05; Fig. 1).

Effects of resistin on the mRNA expression of proinflammatory cytokines in cerulein-treated AR42J cells. The results of the RT-qPCR analysis demonstrated that the mRNA expression levels of TNF-α and IL-6 in the cerulein-treated group were increased significantly, compared with the levels observed in the untreated controls (P<0.01; Fig. 2). Resistin treatment augmented the increased mRNA levels of TNF-α and IL-6 mRNA elicited by cerulein (TNF-α, P<0.01; IL-6, P<0.05; Fig. 2). Notably, pretreatment of cells with PDTC significantly attenuated the mRNA expression levels of TNF-α and IL-6 in the resistin+cerulein-treated group (P<0.01; Fig. 2) and the cerulein-treated group (TNF-α, P<0.01; IL-6, P<0.05; Fig. 2).

Effects of resistin on the protein levels of NF-κB p65 in the nuclei of cerulein-treated AR42J cells. Cerulein treatment significantly increased the protein levels of the NF-κB p65 subunit in the nuclei of the AR42J cells, compared with the untreated controls (P<0.01; Fig. 3). Resistin treatment augmented the increase in the protein level of NF-κB p65 elicited by cerulein (P<0.01; Fig. 3). PDTC pretreatment significantly recovered the protein levels of NF-κB p65 in the cerulein+resistin-treated group (P<0.01; Fig. 3) and cerulein-treated group (P<0.05; Fig. 3).

Discussion

The present study aimed to investigate the functional consequences of exposing rat pancreatic acinar cells to resistin and to determine whether it amplifies proinflammatory signaling in an *in vitro* AP model. Treatment of the AR42J cells with cerulein, which induced the secretion of amylase and mRNA expression levels of TNF-α and IL-6, led to the successful development of the *in vitro* model of AP. Pancreatic AR42J cells are derived from acinar cells and are the only currently available cell line that maintains normal pancreatic acinar cell characteristics (28). The expression of AR42J cell receptor and signal transduction mechanisms have been demonstrated to parallel the mechanisms found in human pancreatic acinar cells (29). Therefore, these cells are now widely applied in investigations of cellular secretion, growth, proliferation and apoptosis of the exocrine pancreas (30).

In the present study, pretreatment with resistin augmented the secretion of amylase and the expression levels of proinflammatory molecules in the *in vitro* model of AP. These findings correlate well with clinical observations suggesting the validity of reports linking the clinical severity and

outcomes of AP with circulating levels of resistin (17,19). Daniel *et al* (18) also demonstrated that the levels of resistin may provide a useful tool for the prognosis and monitoring of AP. Resistin is widely considered to be an obesity-associated adipocytokine, based on numerous studies, which have demonstrated high circulating levels of resistin in obese rodents and are associated with several pathological conditions (16,31,32). The novel feature of resistin as a proinflammatory molecule has been acknowledged. Several studies have reported that resistin can trigger a proinflammatory state (16,22,33,34). Silswal *et al* (35) incubated macrophages with recombinant resistin and found that the production of TNF-α and IL-12 increased. The exposure of hepatic stellate cells to resistin has been also found to result in increased expression levels of monocyte chemoattractant protein-1 and IL-8 (36). In our previous study, it was demonstrated that resistin treatment was capable of inducing the expression of TNF-α and IL-6 in pancreatic acinar cells (21).

Inflammation is the hallmark of human and experimental pancreatitis (37). It is well known that proinflammatory responses occurring subsequent to acinar cell injury can determine the ultimate severity of the resulting pancreatitis. Elevated TNF-α and IL-6 have been reported to be correlated with disease severity in patients with AP (38). Preventing the expression of TNF-α attenuates the stress response and leads to decreases in the mortality rates of patients with AP (39). The overexpression of IL-6 has been shown to increase susceptibility to AP (40). In the present study, the augmented expression of inflammatory cytokines induced by resistin exposure in the AP model led to an increase in the severity of injury to pancreatic acinar cells, which contributed evidence to the previously reported association between obesity and increased severity in patients with AP (39).

NF-κB has the ability to upregulate the expression of inflammatory molecules induced in experimental pancreatitis models (41). The present study further investigated the role of NF-κB inflammatory response augmentation induced by resistin in an *in vitro* model of AP. It was shown that the increasing production of proinflammatory cytokines was accompanied by NF-κB activation. Pretreatment with the NF-κB inhibitor, PDTC, completely attenuated the production of proinflammatory cytokines induced by resistin in the *in vitro* AP model. This suggested that the effect of resistin on augmenting the inflammatory response in the AP model was specifically dependent on NF-κB activation. This finding is consistent with previous studies, demonstrating that NF-κB activation is a key mediator of the inflammatory response in patients with AP (24,41,42).

In conclusion, the results of the present study showed that resistin amplified the expression of proinflammatory cytokines via the NF-κB pathway in the cerulein-induced *in vitro* AP model. These preliminary indications suggested that the overproduction of obesity-associated resistin and associated inflammatory response may result in aggravation of the severity of AP. However, further investigations are required for future application of these findings in humans.

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