Resistin increases the expression of NOD2 in mouse monocytes

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Abstract. Previous studies have indicated that resistin, a type of adipokine, contributes to the development of insulin resistance and type 2 diabetes mellitus, and mediates inflammatory reactions. However, a specific receptor for resistin has not yet been identified. In this study, the relationship between resistin and nucleotide-binding oligomerization domain-like receptors, as well as resistin signal transduction, was examined through transfection, quantitative polymerase chain reaction, western blot analysis and ELISA. The mRNA expression of nucleotide-binding oligomerization domain-containing protein 2 (NOD2), a key immune receptor related to insulin resistance, was significantly increased by resistin treatment at concentrations of 100, 150 and 200 ng/ml ($P<0.05$, $P<0.01$ and $P<0.01$, respectively). The mRNA expression of downstream signaling molecules in the NOD2 signaling pathway, receptor-interacting serine/threonine-protein kinase 2 (RIP2; $P<0.01$ at 6, 12 and 24 h) and inhibitor of NF-κB kinase subunit beta ($P<0.01$ at 3, 6, 12 and 24 h) were significantly increased by resistin treatment compared with the control. The mRNA expression of key proinflammatory cytokines, tumor necrosis factor α, IL (interleukin)-6 and IL-1β, were also significantly increased by resistin treatment compared with the control ($P<0.01$). NOD2 knockdown by small interfering RNA (siRNA) significantly decreased the expression of NOD2 and RIP2 ($P<0.01$), and there was no significant increase in the levels of cytokines, as compared with treatment with control siRNA. These findings indicate that the inflammatory reaction induced by resistin involves the NOD2-nuclear factor (NF)-κB signaling pathway. The inhibition of NF-κB significantly decreased the secretion of key inflammatory cytokines ($P<0.01$), suggesting that NF-κB signaling mechanisms are essential to the resistin-induced inflammatory response.

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Introduction

Obesity is considered an epidemic in numerous countries worldwide and is linked to insulin resistance, which constitutes a principal risk factor for type 2 diabetes (1-3). Previous studies have shown that inflammation is a key pathophysiological process linked to obesity, insulin resistance and type 2 diabetes (4,5). Insulin resistance is also associated with chronic low-grade inflammation *in vivo*, which is largely mediated by activated innate immune cells (6).

Resistin is a cysteine-rich, 12.5-kDa protein that was first identified as a mediator of insulin resistance in obese mice (7-10). Resistin promotes both inflammation and insulin resistance associated with energy homeostasis impairment (11). However, the key molecular mechanisms mediating its effects are unknown. Previous studies have investigated the function of resistin in mouse obesity and diabetes models, and have implicated resistin in the pathogenesis of obesity-mediated insulin resistance and type 2 diabetes (3,12,13). Moreover, it has been reported that resistin is closely associated with inflammation (14-16). Resistin regulates the synthesis and secretion of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin-6 (IL-6) and IL-1β in macrophages via a nuclear factor κB (NF-κB)-dependent pathway (17-20), but the specific receptor of resistin *in vivo* has not yet been identified. A previous study reported that the resistin-induced inflammatory response may involve the Toll-like receptor 4 (TLR4) signaling pathway (21). However, another study reported that the inflammatory response induced by resistin has no direct link with the TLR4 pathway (22). Other previous reports have stated that resistin is related to the insulin-like growth factor 1 receptor and receptor tyrosine kinase-like orphan receptor 1 (ROR1) (22-24). However, the specific receptor of resistin and its signaling pathway have not yet been identified *in vivo*.

NOD1 and NOD2 are cytosolic pattern recognition receptors *in vivo*. Some innate immune receptors are regulated by endogenous molecules, such as TLRs and NODs, which are capable of inducing 'aseptic inflammation' (3). NOD1 and NOD2 induce the recruitment of receptor-interacting serine/threonine-protein kinase 2 (RIP2), which promotes NF-κB-mediated proinflammatory gene expression when exposed to signal molecules (25). Previous research showed that activation of NOD1 or NOD2 contributes to insulin resistance and a proinflammatory response (26-28). On this basis, the current study explored the relationship between resistin

and NOD receptors. The results showed that resistin increased NOD2 expression in RAW 264.7 cells, but had no effect on the expression of NOD1. Resistin also promoted the secretion of inflammatory cytokines via the NF-κB signaling pathway. These findings may contribute to identifying a specific resistin receptor and understanding the underlying mechanisms of resistin in chronic inflammation and insulin resistance.

Materials and methods

Cell culture and stimulation. RAW 264.7 mouse monocyte cells (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal calf serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin, 100 mg/ml streptomycin (both Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and 2 mmol/l L-glutamine at 37°C under 5% CO_2. Cells were incubated with resistin (PeproTech EC Ltd., London, UK) at concentrations of 50, 100, 150 and 200 ng/ml for 3, 6, 12 and 24 h to evaluate the impact of resistin treatment on NOD pathways in RAW 264.7 cells. Cells were stimulated by muramyl dipeptide (20 μg/ml; Sigma-Aldrich; Merck KGaA) or diaminopimelic acid (20 μg/ml; Sigma-Aldrich; Merck KGaA) as a positive control group, and a control group without any treatment was also included. For inhibitor treatment, the specific inhibitor of NF-κB, parthenolide (Sigma-Aldrich; Merck KGaA), was used. The cell culture medium was replaced with fresh medium containing inhibitor (25 μM) and incubated for 1 h prior to resistin (200 ng/ml) treatment.

Small interfering RNA (siRNA) silencing. siRNA duplexes targeting the mouse NOD2 gene (NOD2 siRNA) and non-targeting siRNA (control siRNA) were purchased from Guangzhou Ribobio Co., Ltd. (Guangzhou, China). Transfection of NOD2-siRNA and control siRNA into RAW 264.7 cells was performed using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's guidelines. Transient transfections in RAW 264.7 cells were carried out in 6-well plates, and cells were seeded overnight at $4x10^5$ cells per well for 6 h. All transfections were carried out in Opti-MEM (Invitrogen, Thermo Fisher Scientific, Inc.) medium. Cells were incubated at 37°C for 24 h after transfection prior to analysis. Protein and mRNA expression levels of NOD2 were quantified in order to detect the effect of the siRNA.

Determination of cytokine levels. The protein levels of IL-6, TNF-α, IL-1β and resistin were measured by sandwich ELISA (R&D Systems, Inc., Minneapolis, MN, USA) using a pair of mouse antibodies, and expressed in pg/ml.

RNA isolation and quantitative polymerase chain reaction (qPCR). Messenger RNA (mRNA) expression levels were determined by qPCR. Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into cDNA using standard reagents (Takara

Biotechnology Co., Ltd., Dalian, China). The cDNA was then submitted to qPCR analysis using specific primer pairs and an SYBR Premix Ex Taq™ II kit (Takara Biotechnology Co., Ltd.). Sequences of promoter-specific primers (Table I) were designed by Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by BGI Tech Solutions Co., Ltd. (Copenhagen, Denmark). The reaction conditions were set to 1 min at 95°C (first segment, one cycle), 5 sec at 95°C and 30 sec at 62°C (second segment, 39 cycles). Specific transcripts were confirmed by melting-curve profiles (cooling the sample to 65°C and heating slowly to 95°C with measurement of fluorescence) at the end of each PCR cycle using a C1000 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Relative gene expression was defined as a ratio of target gene expression vs. β-actin gene expression. The results were analyzed using a $2^{-\Delta\Delta Cq}$ assay (29).

Western blot analysis. Monoclonal antibodies against NOD1 (3545; 1:1,000), NOD2 (sc-30199; 1:800), RIP2 (4982; 1:1,000), NF-κB p65 (8242; 1:1,000) and β-actin (BA2305; 1:800) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and horseradish peroxidase (HRP)-conjugated secondary antibody (BA2305; 1:3,000) was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Total cytoplasmic and nuclear protein was sequentially extracted using a Cytoplasmic and Nuclear Protein Extraction kit (BestBio, Inc., Shanghai, China), and protein concentrations were calculated using bicinchoninic acid assay kits (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Protein lysates were kept at -20°C until used for western blot analysis. Protein lysates were fractionated through 7.5-12% SDS-PAGE and transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat-free milk powder at room temperature for 1 h and immunoblotted overnight at 4°C with primary antibodies. Next they there incubated with HRP-conjugated secondary antibody for 1 h at room temperature. After each step, the membranes were washed 5 times with PBS with Tween for 5 min. Finally, the blots were developed using the enhanced chemiluminescence (ECL) system (GE Healthcare Life Sciences).

Data analysis and statistics. Statistical analysis was performed using SPSS 5 software (SPSS, Inc., Chicago, IL USA). Analysis of variance was used to analyze differences among the groups. Data are expressed as the mean ± standard error of the mean of three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Resistin treatment increases NOD2 expression in RAW 264.7 cells. To explore the relationship between resistin and NOD, the mRNA and protein expression levels of NOD1 and NOD2 were analyzed in RAW 264.7 cells by qPCR and western blot analysis. Cells were incubated with different concentrations of resistin (50, 100, 150 or 200 ng/ml). As shown in Fig. 1A, NOD2 protein expression increased following treatment with resistin, as compared with the control. Furthermore, NOD2 mRNA expression significantly increased following treatment with resistin at concentrations of 100, 150 and 200 ng/ml (P<0.05,

Table I. Primers used for quantitative polymerase chain reaction.

Items	Sense (5' to 3')	Antisense (5' to 3')
NOD1	AGGCATTGAAGGACCACC	AGCATCTCAGCGAAGCAC
NOD2	GCTGTCTTGGGATGTGCT	GGATGAAGGGAGTGAGTGTC
RIP2	CCTCCTCGTGTTCCTTGGC	GGTCCTTGTAGGTTTGGTGCT
IKKβ	GTACACCGTGACCGTTGACT	TCCACTTCGCTCTTCTGCCG
Resistin	CTTCCTTGTCCCTGAACTGC	ACGAATGTCCCACGAGCC
β-actin	CTGTCCCTGTATGCCTCTG	ATGTCACGCACGATTTCC

NOD, nucleotide-binding oligomerization domain-containing protein; RIP2, receptor-interacting serine/threonine-protein kinase 2; IKKβ, inhibitor of nuclear factor-κB kinase subunit β.
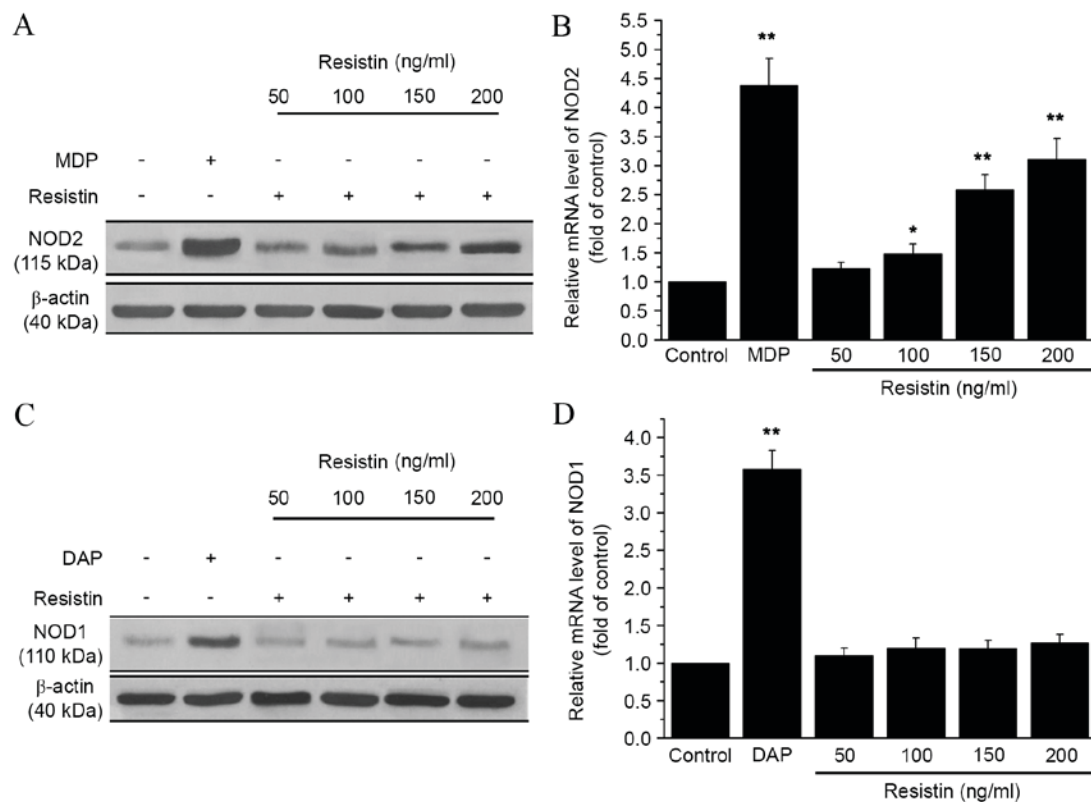

Figure 1. Effect of resistin treatment on the mRNA and protein expression levels of NOD1 and NOD2. RAW 264.7 cells were incubated with MDP (20 µg/ml), DAP (20 µg/ml) or resistin (50, 100, 150 and 200 ng/ml) for 24 h. (A and C) The protein levels of NOD1 and NOD2 were detected by western blot analysis. (B and D) The relative mRNA levels of NOD1 and NOD2. At 24 h of treatment, qPCR was conducted to measure NOD1 and NOD2 mRNA levels. *P<0.05 and **P<0.01 vs. control. NOD, nucleotide-binding oligomerization domain-containing protein; MDP, muramyl dipeptide; DAP, diaminopimelic acid; qPCR, quantitative polymerase chain reaction.

P<0.01 and P<0.01, respectively; Fig. 1B), as compared with the control. However, the expression of NOD1 did not change significantly as compared with the control. The expression level of NOD2 reached its peak at 12 h after adding resistin compared with the control group (P<0.01; Fig. 2). These data indicated that resistin treatment increased the expression of NOD2, but not NOD1.

Resistin treatment activates NOD2-NF-κB pathways. To investigate the potential impact of resistin on NOD2-NF-κB pathways, the expression levels of downstream signaling molecules, RIP2 and inhibitor of NF-κB kinase subunit β

(IKKβ), were determined by qPCR and western blot analysis. The western blot data showed an increase in RIP2 protein expression, and an increase of NF-κB in the nucleus was also observed (Fig. 2A). At the mRNA level, there was a significant increase in both RIP2 and IKKβ expression at 6, 12 and 24 h, as compared with the control (P<0.01; Fig. 2B). Furthermore, we evaluated the effect of resistin treatment on the expression of three key proinflammatory cytokines: TNF-α, IL-6 and IL-1β. There was a significant increase in the protein levels of TNF-α, IL-6 and IL-1β as compared with the control (P<0.01, Fig. 2C). These data indicate that the stimulation of resistin activates NOD2-NF-κB pathways.
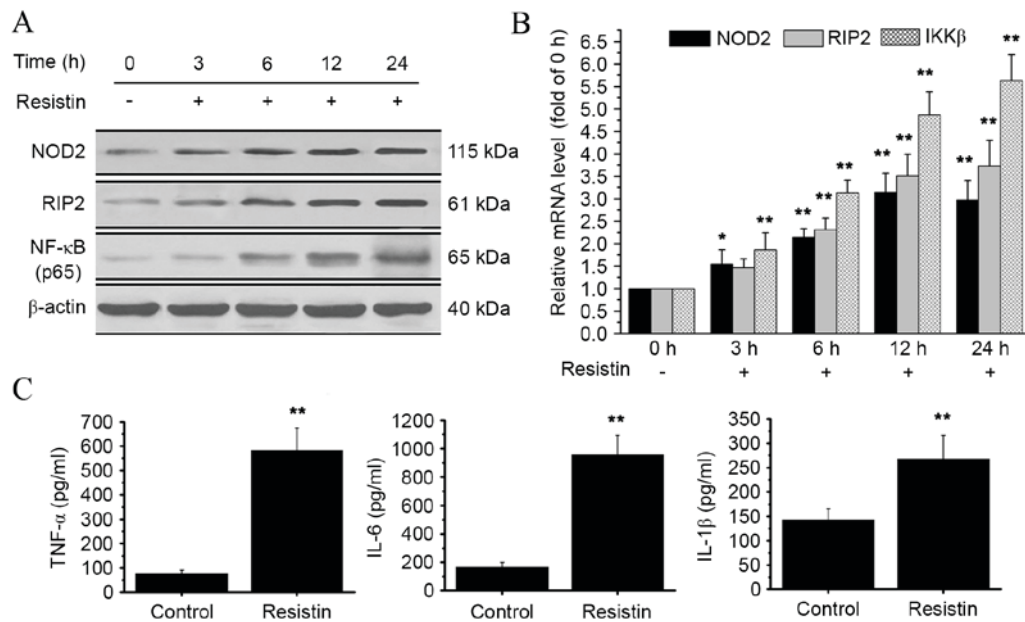
Figure 2. Effect of resistin treatment on NOD2-NF-κB pathways. RAW 264.7 cells were treated with resistin (200 ng/ml) for 0, 3, 6, 12 and 24 h. (A) Protein levels of NOD2, RIP2 and NF-κB p65 were detected by western blot analysis. The NF-κB p65 protein was obtained from the nucleus. (B) Relative mRNA levels of NOD2, RIP2 and IKKβ were analyzed using qPCR at the end of each experimental period of resistin treatment. mRNA levels at 3, 6, 12 and 24 h were calculated as a fold change of the mRNA level at 0 h. (C) At the end of the experimental period of resistin treatment, the cell supernatant was collected and the levels of key proinflammatory cytokines (TNF-α, IL-6 and IL-1β) were detected by ELISA. $^*P<0.05$ and $^{**}P<0.01$ vs. control. NOD2, nucleotide-binding oligomerization domain-containing protein 2; NF-κB, nuclear factor-κB; RIP2, receptor-interacting serine/threonine-protein kinase 2; IKKβ, inhibitor of NF-κB kinase subunit β; TNF-α, tumor necrosis factor α; IL, interleukin; qPCR, quantitative polymerase chain reaction.

SiRNA-mediated NOD2 knockdown impairs resistin-induced activation of NOD2 pathways, but has no effect on the nuclear translocation of NF-κB. To investigate the potential link between resistin and NOD2-NF-κB proinflammatory pathways and assess the effect of NOD2 on resistin signaling, NOD2-siRNA was used to generate NOD2-deleted RAW 264.7 cells (Fig. 3). Treatment with NOD2-siRNA resulted in a significant decrease in both NOD2 protein level (P<0.01; Fig. 3A) and NOD2 mRNA level (P<0.01; Fig. 3C), as compared with treatment with control siRNA.

As shown in Fig. 2, treatment of RAW 264.7 cells with resistin increased the expression of RIP2, NF-κB and IKKβ as well as the release of TNF-α, IL-6 and IL-1β. However, as shown in Fig. 3B, NOD2 knockdown reversed the resistin-induced increase in RIP2 protein expression. NOD2 knockdown also significantly decreased the mRNA expression level of RIP2 after resistin treatment compared with the control (P<0.01, Fig. 3C). The expression of IKKβ and the nuclear translocation of NF-κB were not affected (Fig. 3B and C). No significant changes in cytokine secretion levels were detected by ELISA assay (Fig. 3D).

Resistin induces a proinflammatory effect through the NF-κB pathway. The aforementioned data suggested that resistin induced activation of the NF-κB pathway, and that this was necessary for a resistin-induced inflammatory response. To further evaluate the role of the NF-κB pathway in resistin-mediated effects, parthenolide, an inhibitor of NF-κB, was introduced to the cell cultures 1 h prior to stimulation with resistin. Inhibiting NF-κB resulted in significantly decreased resistin-induced protein expression of TNF-α, IL-6 and IL-1β compared with the control group (P<0.01, Fig. 4).

This suggested that the NF-κB pathway is involved in resistin signaling.

Discussion

A previous study has shown that resistin promotes both inflammation and insulin resistance (11). However, its specific receptor has not yet been identified, and little is known about the molecular mechanisms mediating resistin effects. In the current study, resistin treatment increased the expression of NOD2, RIP2 and IKKβ and promoted the nuclear translocation of NF-κB. This indicated that the resistin-induced inflammatory reaction is induced through the NOD2-NF-κB signaling pathway. It was noted that resistin treatment had no effect on NOD1 expression. SiRNA-mediated NOD2 knockdown attenuated the expression of NOD2 and RIP2 at both mRNA and protein level. Furthermore, the inflammatory reaction induced by resistin was slightly inhibited, which indicated that NOD2-NF-κB pathway is involved in resistin signaling. These findings suggested that there may be a synergistic effect between NOD2 and another receptor for resistin.

Resistin is a member of the resistin-like molecule (RELM) hormone family, which includes RELMα and RELMβ. Resistin and RELMβ contain an additional cysteine near the amino terminus, and crystal structures of both proteins reveal an unusual multimeric structure. RELMβ is known to contribute to local immune responses (30). Furthermore, resistin and RELMβ specifically inhibit insulin action in the liver, resulting in insulin resistance (13).

Until now, four receptors for resistin have been reported: TLR4, an isoform of decorin, ROR1 and adenylyl cyclase-associated protein 1 (CAP1). However, none of these are considered
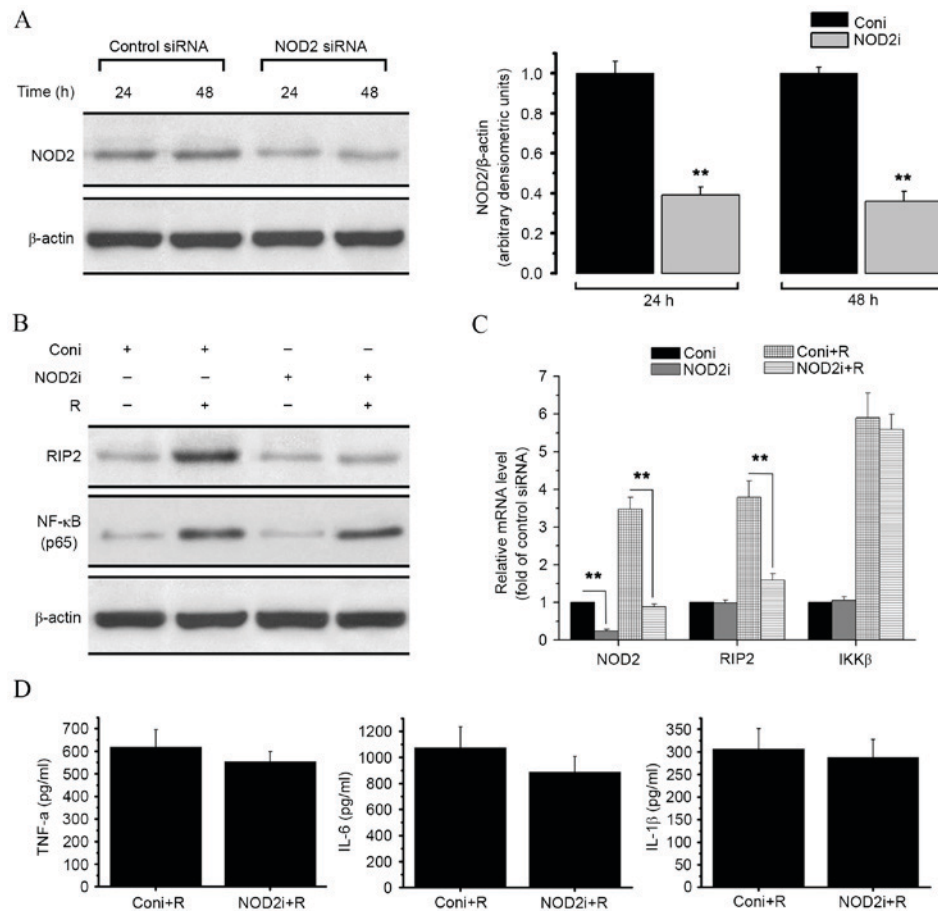
Figure 3. Effect of siRNA-mediated NOD2 knockdown on resistin signaling in RAW 264.7 cells. RAW 264.7 cells were treated with control siRNA or specific siRNA duplexes targeting NOD2. Proteins and RNA were then extracted from the cells for western blot analysis and qPCR. (A) Western blot analysis of NOD2 content in control and NOD2 siRNA-treated cells. The results are expressed as the ratio of NOD2/β-actin. (B) Western blot analysis of the expression level of RIP2, and the NF-κB p65 protein level within the nucleus, in the presence or absence of resistin (200 ng/ml for 24 h) in RAW 264.7 cells titrated with control or NOD2 siRNA. (C) Relative mRNA levels of NOD2, RIP2 and IKKβ in the presence or absence or resistin (200 ng/ml for 24 h) in RAW 264.7 cells titrated with control or NOD2 siRNA were measured by qPCR. (D) Protein level of TNF-α, IL-6 and IL-1β in each group were detected by ELISA. **P<0.01 vs. control. NOD2, nucleotide-binding oligomerization domain-containing protein 2; NF-κB, nuclear factor-κB; RIP2, receptor-interacting serine/threonine-protein kinase 2; IKKβ, inhibitor of NF-κB kinase subunit β; TNF-α, tumor necrosis factor α; IL, interleukin; qPCR, quantitative polymerase chain reaction; siRNA, small interfering RNA; coni, control siRNA; NOD2i, NOD2-siRNA; R, resistin.
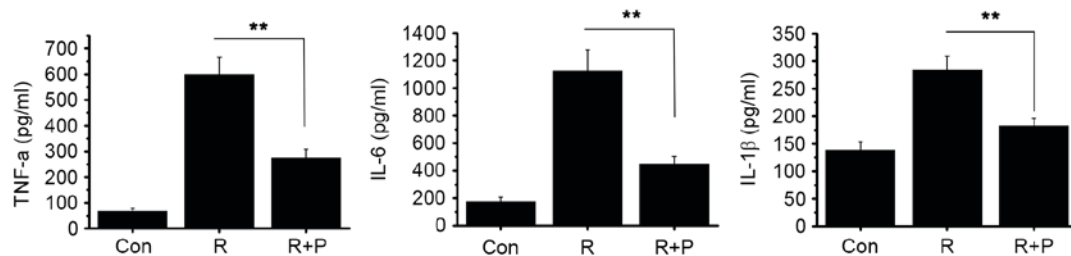

Figure 4. Effect of NF-κB inhibition on the resistin-induced inflammation response. RAW 264.7 cells were treated with a specific inhibitor of the NF-κB signaling pathway (parthenolide) prior to stimulation with resistin. Cell cultures were examined by ELISA for the expression of cytokines TNF-α, IL-6 and IL-1β. **P<0.01 vs. R group. NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor α; IL, interleukin; con, control; R, resistin; P, parthenolide.

to be the specific receptor of resistin *in vivo* (22). Previous research found no evidence for a direct interaction between resistin and TLR4 in a biochemical binding assay (22). Decorin and ROR1 were only expressed at low levels in human monocytes and neither expression level increased following resistin stimulation (22). Therefore, the inflammatory response induced by resistin was not directly related to TLR4, ROR1 or decorin. Furthermore, the study found that resistin could

combine with CAP1, but as CAP1 lacks a transmembrane domain, it is not clear how the signal would be transmitted to the cell interior (22).

NOD2 is a key receptor in the inflammation reaction, which has been associated with insulin resistance in previous studies (26,28). These physiological functions are similar to those of resistin, therefore the links between resistin and NODs were investigated in the current study. Resistin treatment

significantly increased the expression level of NOD2, and the resistin-induced inflammatory response was found to be induced at least partly through the NOD2-NF-κB signaling pathway.

NF-κB is a key transcription factor in the process of inflammatory reactions, and many inflammatory cytokines are regulated by it, including TNF-α, IL-6 and IL-1β (31,32). In the current study, resistin treatment promoted NF-κB translocation into the nucleus and significantly increased the levels of key inflammatory cytokines. Treatment with a specific inhibitor of NF-κB confirmed that resistin-induced signals are mediated through NF-κB signaling mechanisms. These findings are consistent with recent studies, which have reported that NF-κB signaling mechanisms are essential for the resistin-induced inflammatory response.

In summary, the current study demonstrated that resistin treatment increases NOD2 expression and that the inflammatory response induced by resistin involves the NOD2-NF-κB signaling pathway.

Acknowledgements

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