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ORIGINAL ARTICLE

Nucleotide-binding Oligomerization Domain-1 Ligand Induces Inflammation and Attenuates Glucose Uptake in Human Adipocytes[△]

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Key word: nucleotide-binding oligomerization domain-1; human adipocyte; inflammation;
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Objective To investigate the effects of stimulant for nucleotide-binding oligomerization domain 1 (NOD1) on secretion of proinflammatory chemokine/cytokines and insulin-dependent glucose uptake in human differentiated adipocytes.

Methods Adipose tissues were obtained from patients undergoing liposuction. Stromal vascular cells were extracted and differentiated into adipocytes. A specific ligand for NOD1, was administered to human adipocytes in culture. Nuclear factor- κ B transcriptional activity and proinflammatory chemokine/cytokines production were determined by reporter plasmid assay and enzyme-linked immunosorbent assay, respectively. Insulin-stimulated glucose uptake was measured by 2-deoxy-D-[³H] glucose uptake assay. Furthermore, chemokine/cytokine secretion and glucose uptake in adipocytes transfected with small interfering RNA (siRNA) targeting NOD1 upon stimulation of NOD1 ligand were analyzed.

Results Nuclear factor- κ B transcriptional activity and monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6, and IL-8 secretion in human adipocytes were markedly increased stimulated with NOD1 ligand (all $P < 0.01$). Insulin-induced glucose uptake was decreased upon the activation of NOD1 ($P < 0.05$). NOD1 gene silencing by siRNA reduced NOD1 ligand-induced MCP-1, IL-6, and IL-8 release and increased insulin-induced glucose uptake (all $P < 0.05$).

Conclusion NOD1 activation in adipocytes might be implicated in the onset of insulin resistance.

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INSULIN resistance, a hallmark of type 2 diabetes mellitus (T2DM), is associated with low-grade systemic inflammation characterized by up-regulated cytokine production and activated inflammatory signaling pathways.^{1, 2} Innate and adaptive immune systems have been implicated in the proinflammatory responses and have emerged as critical factors in the manifestation of insulin resistance.^{3, 4} Innate immune receptors are pattern recognition receptors (PRRs) that recognize the pathogen-associated molecular patterns (PAMPs) on invading pathogens and activate downstream signaling pathways leading to the up-regulation of diverse arrays of proinflammatory gene expression. Membrane-bound pattern recognition receptors, Toll-like receptor (TLR) 4 and TLR2 in particular, have been suggested to be involved in developing adipose inflammation and insulin resistance.⁵⁻⁸ The recently identified nucleotide-binding oligomerization domain (NOD) proteins, as cytoplasmic PRRs, recognize conserved moieties of bacterial peptidoglycan (PGN) and activate proinflammatory signaling pathways. NOD1 recognizes PGN-related molecules containing the amino acid meso-diaminopimelic acid (meso-DAP) that are produced by most Gram-negative and certain Gram-positive bacteria. Similar to TLRs, NOD1 and NOD2 activate gene transcription through the nuclear factor- κ B (NF- κ B) transcription factor and the mitogen-activated protein kinase (MAPK) signaling pathways via the adaptor molecules receptor-interacting protein (RIP)-like interacting caspase-like apoptosis regulatory protein kinase (RICK, also known as RIP2) and caspase-recruitment domains 9 (CARD9).⁹

Adipose tissue is a source of mediators of inflammation and insulin resistance. Recent researches have demonstrated that NOD1 and NOD2 mRNAs were markedly increased in differentiated murine 3T3-L1 adipocytes and human primary adipocyte culture upon adipocyte conversion.^{10, 11} However, the function of NOD1 in adipose tissue remains poorly understood. In the present study, we investigated the effects of NOD1 ligand on secretion of proinflammatory chemokine/cytokines and insulin-dependent glucose uptake in human mature adipocytes.

MATERIALS AND METHODS

Subjects

Abdominal subcutaneous white adipose tissue samples were obtained from overweight or obese patients who underwent liposuction under general anaesthesia for cosmetic purposes. The patients were randomly selected from Fourth Affiliated Hospital of China Medical University

during the period of July 2010 and June 2011. A total of 14 samples were obtained from 14 patients. All patients were female (aged between 25 and 48 years, mean 37 years). Their mean body mass index was 27.1 ± 2.7 kg/m². The study was performed with the approval of the Ethics Committee of China Medical University, and patients provided informed consents.

Primary human adipocytes culture

Adipose tissue was digested with collagenase (2 mg/mL; Worthington Biochemical, NJ, USA) to isolate the preadipocytes. Human preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Zen-Bio Inc., Research Triangle Park, NC, USA), 100 U/mL penicillin, and 50 μ g/mL streptomycin at 37°C in 5% CO₂. In order to induce differentiation, confluent human preadipocytes were subsequently cultured in serum-free DMEM containing 50 nmol/L insulin, 100 nmol/L dexamethasone (Sigma-Aldrich, St Louis, MO, USA), 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma-Aldrich), and 100 μ mol/L rosiglitazone (Sigma-Aldrich). The medium was changed every 2 days for the first 4 days. Thereafter, the medium was replaced by serum-free DMEM containing 50 nmol/L insulin and 100 nmol/L dexamethasone, which was changed every 2 days until lipid droplets had accumulated (days 14-17).

NF- κ B reporter assay

NF- κ B is an inducible transcription factor that plays a central role in regulation of inflammatory. To evaluate whether NF- κ B is activated by NOD1 ligand in human adipocytes, we determined the NF- κ B transcriptional activity by NF- κ B reporter plasmid assay. Four μ g of a NF- κ B-luciferase reporter plasmid (BD Pharmingen, CA, USA) were transfected into primary human adipocytes. After 24 hours of transfection, the cells were incubated with or without 10 μ g/mL NOD1 synthetic ligand (D-lactyl-L-Ala- γ -D-Glu-meso-DAP, FK156; InvivoGen, San Diego, CA; $n=10$) or with 10 μ g/mL NOD2 ligand (L18-muramyl dipeptide, L18-MDP; InvivoGen; $n=10$). After incubated with NOD1 ligand or NOD2 ligand for 24 hours, adipocytes were washed twice with PBS, and lysed with 80 μ L of reporter lysis buffer (Promega, WI, USA). After quantification using Bradford reagent (Bio-Rad, CA, USA), the lysates (20 μ L) were transferred into a 96-well plate for luciferase activity analysis using the dual luciferase assay kit (Promega). Luciferase activity was measured using a Mithras LB 940 luminescence reader (Berthold Technologies, Germany). NF- κ B activity was estimated as relative luminescence unit (RLU) corresponding to equal protein

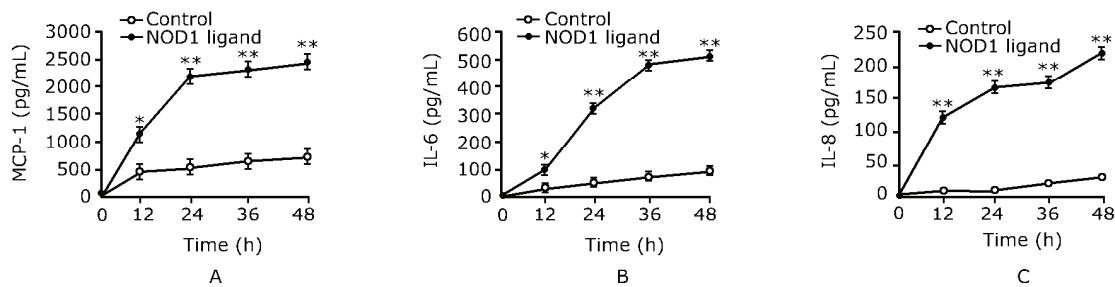


Figure 1. Secretion of MCP-1 (A), IL-6 (B), and IL-8 (C) by human mature adipocytes induced by NOD1 ligand. Differentiated adipocytes were treated with 10 $\mu\text{g/mL}$ NOD1 ligand for 0, 12, 24, 36, and 48 hours, respectively. MCP-1, IL-6, and IL-8 secretions in the medium of adipocytes treated with NOD1 ligand (NOD1 ligand group, $n=10$) or not with NOD1 ligand (control group, $n=10$) were determined by ELISA.

NOD1: nucleotide-binding oligomerization domain-1; MCP-1: monocyte chemoattractant protein-1; IL: interleukin.

* $P<0.01$, ** $P<0.001$ compared with the control group.

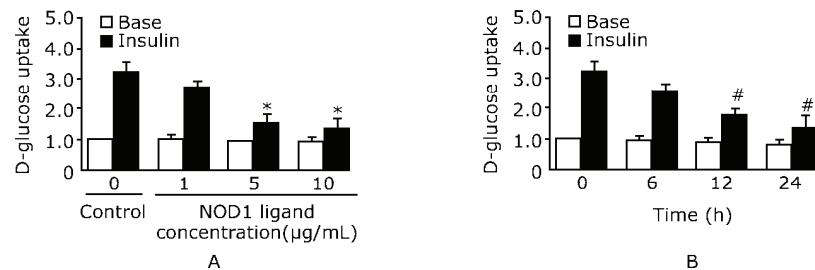


Figure 2. NOD1 ligand reduces insulin-stimulated glucose uptake in human adipocytes.

Adipocytes were incubated with NOD1 ligand (1, 5, 10 $\mu\text{g/mL}$) for 24 hours (A) or with 10 $\mu\text{g/mL}$ NOD1 ligand for indicated times (B). After incubation, adipocytes were left untreated or stimulated with 100 nmol/L insulin for 30 minutes, followed by determination of 2-D-glucose uptake. Results are expressed as fold stimulation above control or base. $n=10$

* $P<0.05$ compared with the control group; # $P<0.05$ compared with the base group.

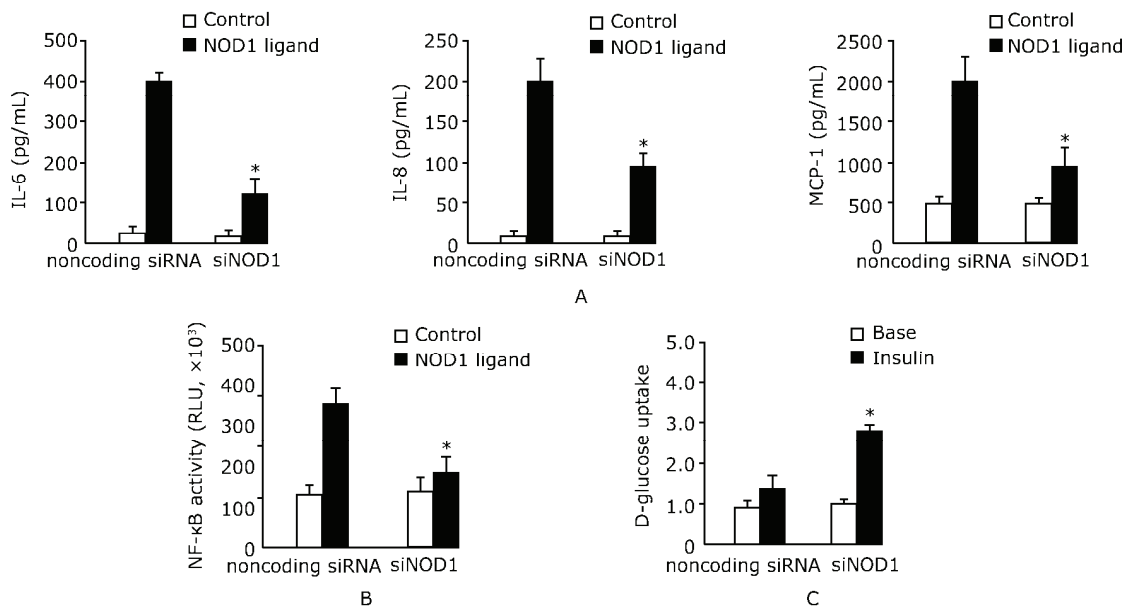


Figure 3. NOD1 ligand-induced proinflammatory response and decrease in insulin-induced glucose uptake in adipocytes are via NOD1.

Human adipocytes were transfected with NOD1-specific or a non-coding control siRNA for 24 hours followed by incubation with (NOD1 ligand group, $n=10$) or without (control group, $n=10$) 10 $\mu\text{g/mL}$ NOD1 ligand for an additional 24 hours.

siRNA: small interfering RNA; siNOD1: siRNA targeting NOD1.

A. MCP-1, IL-6, and IL-8 levels in the supernatant of adipocytes were evaluated by ELISA.

B. NF- κ B activity of adipocytes was analyzed using luciferase reporter assays.

C. After NOD1 ligand induction, D-glucose uptake was measured in adipocytes stimulated with 100 nmol/L insulin for 30 minutes.

* $P<0.05$ compared with NOD1 ligand stimulated adipocytes transfected with non-coding control siRNA.

DISCUSSION

The chronic inflammation observed in obesity appears to be a critical factor in the development of insulin resistance. Since insulin sensitivity is decreased in patients with endotoxemia,¹³ it appears plausible that this response is mediated by PRRs in insulin target organs like muscles and fat tissue.^{14, 15} Adipose tissue is increasingly recognized as a key site of inflammation that can propagate responses leading to systemic insulin resistance. Here we investigated NOD1 proteins that recognize molecular patterns derived from pathogens other than Gram negative bacteria are also expressed in *in vitro* matured human adipocytes and may contribute to insulin resistance.

In present study, we demonstrated that pure selective NOD1 ligand FK156 (a synthetic tetrapeptide originally isolated from culture filtrates of *Streptomyces* strains, with a molecular mass of 519.5 Da) induced a strong NF- κ B activation. It is well known that NF- κ B is a key regulator of inflammatory responses.¹⁶ Following exposure to NOD1 ligand, adipocytes generated inflammatory cytokine IL-6 and chemokines such as MCP-1, IL-8. In agreement with our findings, NOD1 synthetic ligands Tri-DAP and iE-DAP have been shown to induce proinflammatory chemokine gene expression in differentiated human adipocytes through NOD1 and NF- κ B action.¹⁰ In this study, we additionally revealed that human adipocytes responded robustly to agonists stimulation such as NOD1 ligand. Recently, it has been reported that the proinflammatory chemokines are increased during insulin resistance in humans and rodents, and the cytokines have been directly linked to impaired insulin resistance.^{17, 18} We further found that NOD1 ligand suppressed the ability of insulin-stimulated glucose uptake in adipocytes, suggesting a possible role of bacterial components in the pathogenesis of insulin resistance.

To confirm the specificity of NOD1 in adipose inflammation and insulin resistance, we used siRNA targeting NOD1 to inhibit the effects of NOD1 ligand. The results revealed that NOD1 ligand-induced chemokine/cytokine protein expression are blocked by the siRNA targeting NOD1 but not by the non-coding siRNA. Moreover, NOD1 ligand-induced NF- κ B activation and suppression of insulin-induced glucose uptake are attenuated by the siRNA targeting NOD1. Together, these results suggest that NOD1 might play an important role in adipose inflammation and insulin resistance.

We found here that NOD1 activation induces NF- κ B activation and up-regulates cytokine release, which leads to suppression of insulin-induced glucose uptakes. But

these data are not able to elucidate whether NOD1 ligand interacts directly with NOD1 in adipocytes. Therefore, it is necessary to further investigate the effects of NOD1 overexpression or NOD1 knock down in adipocytes in the absence or presence of its ligands.

In conclusion, we demonstrate that NOD1 ligand can activate the innate immune pathway in isolated human adipocytes to stimulate secretion of proinflammatory chemokine/cytokines. NOD1 activation by NOD1 ligand evidently reduced insulin-dependent glucose uptake in adipocytes, which eventually leads to insulin resistance. These results further strengthen the argument that NOD1 is a new modulator in the cross-talk between innate immunity and metabolic pathways. We, therefore, suggest that a selective interference with NOD1 might provide novel strategies to prevent insulin resistance.

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