OBESITY IS BECOMING A GLOBAL EPIDEMIC that affects both adults and children. Accumulating evidence indicates that obesity is associated with chronic inflammation, as characterized by macrophage infiltration into adipose tissue and elevated proinflammatory chemokine/cytokine secretion (43, 44, 47). Chronic inflammation in adipose tissue is causally linked to obesity-associated insulin resistance, type 2 diabetes, and other metabolic dysfunctions (10, 33). Innate immune receptors play critical roles in innate immune responses and inflammation. Innate immune receptors are pattern recognition receptors (PRR) that recognize the pathogen-associated molecular patterns (PAMPs) on invading pathogens and activate downstream signaling pathways leading to the upregulation of diverse arrays of proinflammatory gene expression for tailored immune responses. Two prominent families are membrane-bound Toll-like receptors (TLRs) and cytosolic nucleotide oligomerization domain-like receptors (NLRs) (40).

TLRs are transmembrane receptors composed of extracellular leucine-rich repeat (LRR) motifs and a cytoplasmic Toll/interleukin-1 receptor (TIR) homology domain. So far, 10 and 12 functional TLRs have been identified in humans and mice, respectively (20). The respective bacterial or viral PAMPs that individual TLR detects have been well characterized (40). Recent evidence has shown that TLR4 and TLR2 might be activated by nonmicrobial, endogenous molecules [e.g., saturated fatty acids (SFA)] in inducing “sterile inflammation” (27). We and others have shown that TLR4 and TLR2 (dimerized with TLR6 or TLR1) can mediate SFA-induced NF-κB activation and target gene response in murine macrophage RAW264.7 cells (26, 28) and myobute C2C12 cells (35), respectively. These studies were followed by the investigations of TLRs in 3T3-L1 adipocytes and in vivo in TLR4 or TLR2 knockout mice. SFA-induced TNF-α and IL-6 gene expression were blunted in TLR4 knockdown or TLR4 knockout (TLR4−/−) adipocytes (36). TLR4 knockout mice and C3H/HeJ mice, which carry a spontaneous mutation inactivating Tlr4, were protected against adipose inflammation and the development of insulin resistance in response to a systemic lipid infusion or a high-fat diet, respectively (30, 36, 39). Mice lacking TLR2 were protected from insulin resistance, macrophage infiltration, and adiposity induced by two dietary models that approximate contemporary diet compositions (15). Moreover, mice with TLR2 deficiency were also protected from high-fat diet-induced insulin resistance, tissue inflammation, and pancreatic β-cell dysfunction (6). Consistent with these results, a broad mRNA expression profile of TLRs and their functionalities have been demonstrated in mature 3T3-L1 adipocytes (25) and human primary adipocytes isolated from fat tissue (24).

NLRs are a family of cytosolic sensors that play important roles in innate immunity and inflammation. These NLRs display a central nucleotide-binding domain, an N-terminal protein interaction domain, and a COOH-terminal leucine-rich repeat (LRR) domain (8). Two prominent members of NLRs are nucleotide-binding oligomerization domain-containing protein-1 (NOD1) and NOD2, whose recognition motifs in bacterial peptidoglycan have been mapped. The minimal peptidoglycan structure that NOD1 recognizes is a dipeptide, L-Ala-D-Glu-meso-diaminopimelic acid (iE-DAP) (4) or a tripeptide, L-Ala-γ-D-Glu-meso-diaminopimelic acid (Tri-DAP) (12) derived mostly from gram-negative bacteria, whereas NOD2 recognizes the minimal peptidoglycan muramyl dipeptide MurNAc-t-Ala−d-isoGln (MDP), from both gram-positive and gram-negative bacteria (13, 17). Similar to TLR, NOD activa-
tion leads to the activation of signaling pathways including NF-κB and MAPK, leading to proinflammatory gene expression (9, 16).

We have studied the role of NOD1 and NOD2 in SFA-induced inflammation (49). In human intestinal epithelial HCT116 cells, which express abundant NOD1 and NOD2 but no detectable levels of TLR4 and TLR2, and in reconstituted 293T cells transfected with NOD expression plasmid, both NOD1 and NOD2 can mediate SFA-induced NF-κB activation and target gene IL-8 expression (49). However, the roles of NOD proteins on adipose inflammation and insulin resistance have not been studied.

Therefore, we investigated the potential roles of NOD proteins in inducing inflammation in adipocytes, leading to obesity-associated insulin resistance. Here, we report that both NOD1 and NOD2 mRNA are markedly upregulated during adipocyte conversion of murine 3T3-L1 cells and human primary adipocyte culture. NOD1 mRNA is markedly increased only in the fat tissues in diet-induced obese mice and not in genetically obese ob/ob mice. Moreover, stimulation of NOD1 with the synthetic ligand induces proinflammatory gene expression and suppresses insulin signaling and subsequent glucose uptake in 3T3-L1 adipocytes.

**MATERIALS AND METHODS**

**Reagents.** NOD1 synthetic ligands Tri-DAP and iE-DAP were purchased from invivoGen (San Diego, CA). Lipopolysaccharide (LPS), SFAs, methylisobutylxanthine, dexamethasone, and insulin were purchased from Sigma (St. Louis, MO). Pharmacological inhibitors caffeic acid phenethyl ester, Bay 117821, SB-203580, SP-600125, and PD-98054 were from Tocris Bioscience (Ellisville, MI). Antibodies anti-phospho-Akt(Ser473), anti-phospho-Akt(Thr308), anti-Akt, anti-phospho-GSK3α/β, anti-phospho-ERK1/2(Thr202/Tyr204), anti-ERK1/2, anti-phospho-p38(Thr180/Tyr182), anti-p38, anti-phospho-JNK(Thr183/Tyr185), anti-JNK, anti-phospho-p65(Ser536), anti-p65 and anti-phospho-IκBα(Ser32), anti-insulin receptor substrate 1 (IRS-1), anti-phospho-IRS-1(Ser612), and anti-phospho-IRS-1(Ser307) were purchased from Cell signaling Technology (Danvers, MA). Antibodies anti-phospho-IRS-1(Tyr632) and anti-phosphotyrosine (PY99) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin and anti-tubulin antibodies were purchased from Sigma.

**Animals.** Diet-induced obese (DIO) mice as well as ob/ob and their control mice were purchased from the Jackson Laboratory. For DIO mice, male C57BL/6J mice were fed a high-fat diet (60% kcal% from fat, Research Diets) or regular chow diet for 20 wk before being euthanized at 26 wk of age. Male ob/ob and their control mice were purchased from the Jackson Laboratory. For DIO mice, male C57BL/6J mice were fed a high-fat diet (60% kcal% from fat, Research Diets) or regular chow diet for 20 wk before being euthanized at 26 wk of age.
feder a chow diet for 8 wk before being euthanized at 14 wk of age. The
mouse studies were approved by the Institutional Animal Care and
Use Committee at the University of Tennessee.

Adipocyte differentiation. Murine 3T3-L1 fibroblasts (ATCC, Mal-
nassas, VA) were grown in Dulbecco’s modified Eagle’s medium
(DMEM) containing 10% calf serum (Hyclone) in 5% CO2, 37°C
environment until they reach confluence. Differentiation was initiated
as described (29). Briefly, on the day the cells reached confluence
(designated as day 0, D0), cells were treated with differentiation
DMEM containing 10% fetal bovine serum (FBS, Atlas Biologicals),
10 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-
methylxanthine for 3 days. The cells were then grown in maintenance
DMEM containing 10% FBS and 10 μg/ml insulin for an additional
2 days and then in DMEM containing 10% FBS until the cells were
used for NOD1 stimulation experiments. Typically, adipocyte conversion
occurred in 99% of the cells 8 days following the initiation of
differentiation (D8). Human subcutaneous fat-derived primary prea-
dipocyte culture was purchased from Zen-Bio (Research Triangle
Park, NC) and were grown and differentiated according to the man-
ufacturer’s instructions. Briefly, human primary preadipocytes were
seeded and grown in 60-mm tissue culture dishes in preadipocyte
medium until confluence. The differentiation was initiated with adi-
pocyte differentiation medium for 7 days and maintained in adipocyte
maintenance medium for an additional 7 days. All media used for
human primary adipocyte culture were purchased from Zen-Bio. The
cells were used 14 days postinitiation of differentiation.

NOD1 ligand stimulation. Differentiated 3T3-L1 adipocytes (at
D9) were serum starved in DMEM containing 0.25% FBS for 12–15
h and stimulated with vehicle control, Tri-DAP, or iE-DAP as indicated.
Total RNA and media were collected for chemokine/cytokine
mRNA expression and protein secretion analysis. For insulin-stimu-
lated Akt experiments, cells were pretreated with Tri-DAP as indi-
cated and then stimulated with insulin (20 nM) for 20 min. For IRS-1
experiments, the cells were then stimulated with insulin for 5 min.
The cells were lysed with 1× RIPA buffer (Cell signaling) for 15 min on
ice followed by vortex and sonication for 5 s. Total cell lysates were
prepared after centrifugation at 4°C for 15 min to remove the insol-
uble materials and fat layer and were subjected to Western blot
analysis.

RNA preparation and quantitative real-time PCR analysis. At
indicated times, total RNA was prepared from adipocytes using
TRizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s
instruction. Total RNA abundance was quantified using a NanoDrop
ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington,
DE). Reverse transcription was carried out using a high-capacity
reverse transcription kit (Applied Biosystems, Foster City, CA) ac-
cording to the manufacturer’s instruction. mRNA expression of NOD1,
NOD2, TLR4, TLR2, various chemokines/cytokines, and loading
genes were measured and then in DMEM containing 10% FBS until the cells were
used for NOD1 stimulation experiments. Typically, adipocyte conversion
occurred in 99% of the cells 8 days following the initiation of
differentiation (D8). Human subcutaneous fat-derived primary prea-
dipocyte culture was purchased from Zen-Bio (Research Triangle
Park, NC) and were grown and differentiated according to the man-
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ice followed by vortex and sonication for 5 s. Total cell lysates were
prepared after centrifugation at 4°C for 15 min to remove the insol-
uble materials and fat layer and were subjected to Western blot
analysis.

Measurement of chemokines/cytokines. The supernatant from
3T3-L1 and human primary adipocyte cultures that were stimulated
with different stimuli were collected, and the levels of monocyte
chemoattractant protein-1 (MCP-1), regulated upon activation, normal
T cell expressed and secreted (RANTES), IL-6, and IL-8 were
determined by enzyme-linked immunosorbent assay (ELISA) using
Quantikine kits (R&D Systems, Minneapolis, MN).

Small RNA interference. Small RNA interference against NOD1,
NOD2, TLR4, and TLR2 were performed in differentiated 3T3-L1
adipocytes. siRNA oligo targeting against murine NOD1, NOD2,
TLR4, and TLR2 were from Silencer Select from Ambion and were transfected into 3T3-L1 adipocytes with DeliverX plus system (Parnomics) according to the manufacturer’s protocol.

Glucose uptake assay. Insulin-stimulated glucose uptake in 3T3-L1 adipocytes was determined by measuring deoxy-[3H]glucose uptake as described (14). Briefly, differentiated 3T3-L1 adipocytes (D9) were washed twice with PBS and incubated in serum free, low-glucose DMEM for 2–3 h at 37°C. The cells were washed once and incubated with Krebs-Ringer phosphate buffer containing 0.25% BSA for 3 or 24 h in the presence or absence of NOD1 ligand Tri-DAP (10 μg/ml). The cells were pretreated with cytochalasin B (a cell-permeable mycotoxin that blocks the translocation of glucose transporters) or vehicle control, followed by stimulation with or without insulin (20 nM) for 15 min. Glucose uptake was initiated by the addition of deoxy-[3H]glucose (PerkinElmer, Waltham, MA) into the adipocyte culture for 5 min. The reaction was terminated by immersing the dish in ice-cold PBS and washing with ice-cold PBS four times. The cells were extracted by 0.05% SDS in 1× PBS and subjected to scintillation counting for 3H radioactivity. The specific insulin-induced glucose uptakes were calculated by subtracting the radioactivities of cytochalasin B treated samples from those of the vehicle treated samples. The radioactivity of each sample was normalized by the protein concentration. The data were expressed as counts per minute per microgram of protein. For siRNA experiments, differentiated 3T3-L1 adipocytes were transfected with siRNA oligo targeting against NOD1 or nontargeting controls on days 1 and 3, and the glucose uptake assays were performed after 72 h of siRNA transfection.

Statistical analysis. All data are presented as means ± SE. Each experiment was repeated at least three times. Within an experiment, measurements were performed in triplicates. Data were log transformed when appropriate. Statistical analysis was performed using SigmaPlot 11.0 (Systat Software). One-way ANOVA with repeated measures followed by a multiple comparisons test (Student-Newman-Keuls method) were performed to determine the differences between the treatment groups and/or time points. Student’s t-test was performed when appropriate. The level of significance was set at P < 0.05.

RESULTS

NOD1 and NOD2 mRNA are markedly increased during differentiation of 3T3-L1 and human primary culture derived from subcutaneous fat. TLR4 (32, 37) and TLR2 (32) mRNA level in pre- and adipocyte states have been studied in 3T3-L1 cells and their functionalities demonstrated in 3T3-L1 (25, 31, 32).

Fig. 3. NOD1 ligand Tri-DAP (L-Ala-γ-D-Glu-meso-diaminopimelic acid)-induced proinflammatory chemokine and cytokine expression in 3T3-L1 adipocytes in a dose-dependent manner. 3T3-L1 adipocytes were stimulated with increasing doses of Tri-DAP (0, 1, 10, 50 μg/ml) for 15 h. A: relative mRNA expression of (MCP-1), RANTES (regulated upon activation, normal T cell expressed and secreted), TNF-α, MIP-2 (human IL-8 homolog), and IL-6 were evaluated by quantitative RT-PCR. Relative gene expression was normalized to 18S. Data are expressed as fold of vehicle value (set as 1). B: MCP-1 and RANTES protein secretion in supernatant of adipocytes stimulated with Tri-DAP were evaluated by ELISA. Data are means ± SE; n = 3. NS, not significant.
Although NOD1 and NOD2 mRNA expression in 3T3-L1 preadipocytes and human primary adipocytes have been reported (38), their expression in adipocyte state have not been studied. Therefore, we examined the changes of mRNA expression of NOD1 and NOD2 upon differentiation in 3T3-L1 cells and compared their mRNA expression to that of TLR4 and TLR2 in preadipocyte and adipocyte state. The levels of gene expression are relative to 36B4, a gene that was reported not changed upon 3T3-L1 differentiation (41). Upon differentiation, NOD1 mRNA level was markedly increased and reached the highest peak at about sixfold at D5, compared with D0. It reached about fourfold that of RAW264.7 cells (a murine monocytic/macrophage cell line) at D8 (Fig. 1A). NOD2 mRNA level was also remarkably increased upon differentiation. It reached the highest peak of ~40-fold at D5 compared with D0 (Fig. 1A). The relative mRNA expression profiles of NOD1, NOD2, TLR4, and TLR2 changed with differentiation state. The relative NOD1 mRNA expression was ~68- and ~10-fold that of NOD2 in pre- and adipocyte state, respectively (Fig. 1B). TLR4 and TLR2 mRNA were expressed in both pre- and adipocytes, consistent with other reports (31, 32, 37). TLR4 and TLR2 mRNA levels were two- and sixfold that of NOD1, respectively, in preadipocytes, and was about two- and less than onefold that of NOD1 in adipocytes (Fig. 1B). Moreover, we examined NOD1 and NOD2 expression in pre-, adipocyte state and during adipocyte conversion of human primary preadipocyte culture derived from the subcutaneous fat. NOD1 mRNA was increased by 85% at D14; NOD2 mRNA was barely detectable in preadipocytes and was increased by 338% at D14 compared with D0 (P < 0.05; Fig. 1C). Similar upregulation of TLR4 and TLR2 mRNA level was observed during human adipocyte conversion (P < 0.01; Fig. 1C).

**NOD1 mRNA is markedly increased in fat tissues in DIO mice but not in genetically obese ob/ob mice.** To probe the role of NOD1 and NOD2 in vivo, we studied their mRNA expression in DIO and genetically obese mice models. NOD1 mRNA was increased to 2.5-fold (P < 0.01) and 2.2-fold (P < 0.05) of the chow-fed controls in the epididymal and subcutaneous fat tissues, respectively, of the DIO mice (Fig. 2A). NOD2 mRNA level was slightly increased in the epididymal fat of DIO mice (Fig. 2A) but was undetectable in the subcutaneous fat of the chow-fed controls and barely detectable in the DIO mice (data not shown). Both TLR4 and TLR2 mRNA were significantly increased in the fat tissues of the DIO mice (P < 0.05). In contrast, NOD1 mRNA was not increased in leptin-deficient ob/ob mice in both fat tissues compared with the controls (Fig. 2B). On the other hand, NOD2, TLR4, and TLR2 mRNA were significantly increased in both fat tissues of the ob/ob mice (P < 0.05 and P < 0.01; Fig. 2B). In 3T3-L1 preadipocytes, NOD1, but not NOD2 activation, by its synthetic ligand was shown to induce proinflammatory cytokine expression (38). Because of the higher mRNA level of NOD1 in adipocytes and in fat tissues of the DIO mice and that the role of NOD1 in adipocytes has not been studied, we chose to focus on NOD1 in adipocytes in this study.

**NOD1 activation induces proinflammatory chemokine and cytokine expression in adipocytes.** We examined whether activation of NOD1 by the synthetic ligands can induce a proinflammatory chemokine/cytokine response in 3T3-L1 adipocytes. To confirm the stimulating specificity of Tri-DAP and iE-DAP preparation we used, we tested them in 293T reconstituted systems as described previously (49). Consistent with being reported (4, 12), the preparation of Tri-DAP and iE-DAP stimulated NF-κB activation in 293T transfected with NOD1 expression plasmid but not with TLR4 or TLR2 expression.
plasmid (data not shown), demonstrating the specificity of NOD1 activation by Tri-DAP or iE-DAP in our experiments. We focused on proinflammatory MCP-1, TNF-α, and IL-6, which are implicated in insulin resistance (10, 33), as well as RANTES and MIP-2 (human IL-8 homolog), known target genes of NOD1 activation (2). Tri-DAP dose-dependently induced MCP-1, RANTES, TNF-α, and MIP-2 mRNA expression \( (P < 0.05; \text{Fig. 3A}) \). The dose-dependent changes in MCP-1 and RANTES protein secretion were confirmed by ELISA \( (P < 0.05; \text{Fig. 3B}) \). The protein secretions of TNF-α, MIP-2, and IL-6, however, were under detection limits (data not shown). Moreover, Tri-DAP also dose-dependently induced proinflammatory MCP-1, IL-6, and IL-8 protein secretion in human primary adipocytes differentiated from preadipocyte culture derived from subcutaneous fat \( (P < 0.05) \) (Fig. 4A). Similar protein secretion patterns were observed when another NOD1 synthetic ligand, iE-DAP, was used \( (P < 0.05; \text{Fig. 4B}) \). We chose the dose of 10 μg/ml, the lower dose with significant effects, in further studies. Tri-DAP (10 μg/ml), not vehicle control, induced MCP-1, RANTES, TNF-α, and MIP2 in a time-dependent manner in 3T3-L1 adipocytes \( (P < 0.001; \text{Fig. 5A}) \). The time-dependent changes of MCP-1 and RANTES protein secretion by Tri-DAP were also confirmed by ELISA \( (P < 0.001; \text{Fig. 5B}) \). Furthermore, Tri-DAP (10 μg/ml) time-dependently induced MCP-1, RANTES, IL-6, IL-8, and TNF-α mRNA expression \( (P < 0.001; \text{Fig. 6A}) \) and MCP-1, IL-6, and IL-8 protein secretion \( (P < 0.001; \text{Fig. 6B}) \) in human primary adipocytes. RANTES and TNF-α protein secretion in human primary adipocytes were under detection limits (data not shown).

NOD1 activation induces activation of NF-κB and MAPK signaling pathways, leading to proinflammatory gene expression in adipocytes. It has been shown that NOD1 activation induces activation of NF-κB and MAPK signaling pathways, leading to proinflammatory responses in many cell types (8, 16). Therefore, we determined whether NOD1 activation by Tri-DAP in 3T3-L1 adipocytes induced activation of these signaling events. NOD1 activation by Tri-DAP induced NF-κB activation, as revealed by phosphorylation of NF-κB p65 and IκBα (Fig. 7A) and activation of NF-κB reporter gene (Fig. 7B). NOD1 activation also induced phosphorylation of p38, JNK, and ERK1/2 in adipocytes (Fig. 7D). To determine whether activation of these signaling pathways is required for the proinflammatory gene expression induced by Tri-DAP, we employed the pharmacological inhibitors. NF-κB inhibitor caffeic acid phenethyl ester (CAPE) and Bay 117821 (Bay) significantly inhibited Tri-DAP-induced MCP-1 and RANTES mRNA \( (P < 0.01; \text{Fig. 7C}) \). Similarly, the pharmacological inhibitor of p38 (SB-203580), JNK (SP-600125), or ERK (PD-98054) also significantly inhibited Tri-DAP-induced MCP-1 \( (P < 0.01) \) and RANTES mRNA \( (P < 0.01; \text{Fig. 7E}) \). In contrast, the pharmacological inhibitor of p38 and JNK, but not the inhibitor of ERK, significantly inhibited Tri-DAP-induced RANTES mRNA \( (P < 0.01; \text{Fig. 7E}) \). Together, these results demonstrate that Tri-DAP induces proinflammatory gene expression via activation of NF-κB and MAPK signaling pathways in adipocytes.
and that proinflammatory MCP-1 and RANTES gene expression may be controlled by distinct intracellular signaling pathways downstream of NOD1 activation.

**NOD1 activation suppresses insulin signaling and glucose uptake in 3T3-L1 adipocytes.** To further explore the role of NOD1 in adipose inflammation and insulin resistance, we sought to examine the effects of NOD1 activation on insulin signaling. Insulin-stimulated phosphorylation of Akt on Ser473 and Thr308 residues, the commonly used markers of insulin signaling (21, 42, 45), were evaluated. Tri-DAP suppressed insulin-induced phosphorylation of Akt on Ser473 and Thr308 at 24 h, with more suppression observed on Ser473 than on Thr308. Consistently, the phosphorylation of Akt downstream target GSK3β was also suppressed at 24 h (Fig. 8A). No apparent suppression of insulin-induced phosphorylation of Akt and GSK3β were observed by NOD1 stimulation at 3 h (Fig. 8A). We also examined upstream insulin signaling events. Tri-DAP decreased pan-tyrosine phosphorylation of IRS-1 at both 3 and 24 h; decreased specific tyrosine phosphorylation of IRS-1 on Tyr632 only at 24 h; and increased inhibitory serine phosphorylation of IRS-1 on Ser612 at 3 h (Fig. 8B). No change on IRS-1 Ser637 phosphorylation was observed (data not shown). Together, these results demonstrate that NOD1 activation impairs IRS-1 tyrosine phosphorylation, leading to impaired downstream phosphorylation of Akt and GSK.

To explore whether attenuated insulin signaling by NOD1 activation leads to insulin resistance, we evaluated insulin-induced glucose uptake by measuring insulin-stimulated deoxy-[3H]glucose uptake into the 3T3-L1 adipocytes. Insulin treatment (20 nM) induced around twofold glucose uptake in the control cells, but was suppressed by ~40% (P < 0.05) after NOD1 stimulation for 24 h (Fig. 9B). Three-hour treatment of Tri-DAP did not significantly suppress insulin-induced glucose uptake (Fig. 9A). These data are consistent with the effects of Tri-DAP on insulin signaling shown in Fig. 8.

**Tri-DAP-induced proinflammatory response and decrease in insulin-induced glucose uptake in adipocytes were via NOD1.** To further confirm whether the observed effects of Tri-DAP in inducing proinflammatory gene expression and reducing insulin-induced glucose uptake in adipocytes is mediated through NOD1, we employed siRNA against NOD1, NOD2, TLR4, and TLR2 in adipocytes. As shown in Fig. 10A, all three siRNA targeting against NOD1, but not the other siRNA oligos, reduced NOD1 mRNA by 60–80% in 3T3-L1 adipocytes. All three siRNA targeting NOD1, but not the siRNA targeting TLR4, TLR2, and NOD2, significantly reduced Tri-DAP-induced MCP-1 by 50–65% (P < 0.01) and RANTES mRNA by 62–78% (P < 0.01) (Fig. 10B). MCP-1 and RANTES protein levels were consistent with the mRNA expression (Fig. 10C). Tri-DAP-induced TNF-α and MIP-2 mRNA were also reduced by siRNA targeting NOD1 (P < 0.01) but not by siRNA targeting TLR4, TLR2, and NOD2 (data not shown). Moreover, the siRNA targeting NOD1 decreased Tri-DAP-induced NF-κB activation by ~50% (P < 0.05), as revealed by NF-κB reporter gene (Fig. 10D). Furthermore, the siRNA targeting NOD1 reversed Tri-DAP-induced suppression of insulin-induced glucose uptake (P < 0.05; Fig. 10E). Together, these results demonstrate the specific role of
NOD1 in observed proinflammatory response and insulin resistance induced by Tri-DAP in adipocytes.

DISCUSSION

Chronic inflammation, as characterized by macrophage infiltration and proinflammatory cytokine/chemokine secretion, is associated with obesity and insulin resistance (43, 44, 47); however, the underlying mechanisms are not fully understood. Membrane-bound pattern recognition receptors, TLR4 and TLR2 in particular, have been suggested to be involved in developing adipose inflammation and insulin resistance (15, 30, 36, 39). Here, we show, for the first time, the role of NOD1, a cytosolic PRR, in proinflammatory response and insulin resistance in adipocytes. NOD1 and NOD2 mRNA are markedly increased upon differentiation of murine 3T3-L1 adipocytes and human primary adipocytes; NOD1 mRNA is increased in the fat tissues of the DIO mice, but not in the ob/ob mice, compared with controls; activation of...
NOD1 by the synthetic ligand Tri-DAP induces MAPK- and NF-κB-mediated proinflammatory gene expression and suppression of insulin signaling and insulin-induced glucose uptake in adipocytes. To confirm the specificity of the role of NOD1, we show that Tri-DAP-induced MCP-1 and RANTES mRNA and protein expression are blocked by the siRNA targeting NOD1 but not by the siRNA targeting TLR4, TLR2, or NOD2. Moreover, Tri-DAP-induced NF-κB activation and suppression of insulin-induced glucose uptake are attenuated by the siRNA targeting NOD1. Together, our results suggest that NOD1 may play an important role in adipose inflammation and insulin resistance in diet-induced obesity.

NOD1 activation by Tri-DAP induces proinflammatory MCP-1, RANTES, TNF-α, IL-6, and IL-8 mRNA expression both in 3T3-L1 adipocytes and in human primary adipocyte culture (Figs. 3–6). MCP-1, a chemoattractant factor for monocyte/macrophage, is critical for macrophage infiltration into adipose tissue, which is thought to be the initial step leading to adipose inflammation (18, 19, 47). TNF-α and IL-6 are known cytokines that can induce insulin resistance. In addition, RANTES has been reported to be upregulated and associated with T cell accumulation in murine and human obesity (46), which contributes to adipose inflammation and metabolic syndrome. Recently, it has been reported that neutrophils transiently infiltrate intra-abdominal fat early under high-fat feeding in mice (7). CXC chemokines, including IL-8, and MIP-2, are among the most critical inflammatory mediators for recruitment of neutrophils (23). Our results suggest that NOD1 activation may facilitate the infiltration of macrophages as well as T cells and neutrophils into adipose tissue, leading to adipose inflammation. Moreover, NOD1-mediated secretions of proinflammatory cytokines from adipocytes may directly contribute to insulin resistance. Consistently, we show that NOD1 activation induces attenuation of insulin-signaling, which leads to suppression of insulin-induced glucose uptakes.

The mechanisms underlying the proinflammatory gene expression downstream of NOD1 activation in adipocytes seem to involve both NF-κB and MAPK signaling pathways. The fact that the NF-κB pathway is essential for both MCP-1 and...
RANTES mRNA expression but ERK1/2 is only necessary for MCP-1, not RANTES. mRNA expression (Fig. 7, C and E) suggests that proinflammatory gene expression downstream of NOD1 activation is controlled by distinct intracellular pathways in adipocytes, consistent with NOD1 results in other systems (2).

How the signaling events downstream of NOD1 activation interact with insulin signaling, leading to insulin resistance in adipocytes, remain to be determined. NOD1 activation induces activation of p38, JNK, and ERK1/2 MAPK as well as NF-κB pathway in 3T3-L1 adipocytes (Fig. 7). It has been reported that JNK, ERK1/2, and IKKβ (a component of IKK complex that activates NF-κB) can phosphorylate IRS-1 on serine residues (1, 5, 11), which in turn inhibits tyrosine phosphorylation of IRS-1, required for the activation of insulin signaling. It remains to be determined whether activation of JNK, ERK1/2, and p38 MAPK and IKKβ upon NOD1 activation directly attenuates insulin signaling or whether NOD1-mediated production of proinflammatory cytokines/chemokines, e.g., TNF-α, IL-6, and MCP-1, interferes with insulin signaling in auto- and/or paracrine fashion, leading to insulin resistance in adipocytes. TNF-α, IL-6, and MCP-1 are known to alter insulin signaling through activation of ribosomal protein S6 kinase...
NOD1 activation leads to insulin resistance

(S6K) (48), mammalian target of rapamycin (mTOR) (22), and ERK1/2 (34), respectively. Understanding the signaling events downstream of NOD1 activation as it interfaces with insulin signaling may provide novel targets for treating obesity-associated inflammation and insulin resistance.

Previously, we (49) have shown that NOD1 can be activated by SFA, leading to NF-κB activation and proinflammatory target gene expression in human intestinal epithelial HCT116 cells and 293T reconstituted with NOD1 expression plasmid. High FFAs found in obese subjects are thought to be the metabolic signal to induce inflammation in the obese state (3). But the primary molecular mechanisms by which FFAs cause inflammation and insulin resistance are not fully understood, and the roles of TLRs, TLR4 and TLR2 in particular, in diet-induced obesity, adipose inflammation, and insulin resistance have been suggested (15, 36). In light of our early studies and the results that NOD1 is expressed and functional in adipocytes, it warrants further investigation whether NOD1 plays an important role in SFA- and/or high-fat diet-induced obesity, inflammation, and insulin resistance. Consistently, NOD1 mRNA is found to be increased in the fat tissues of DIO mice but not in genetically obese ob/ob mice, whereas TLR4 and TLR2 mRNA are induced in both obesity models (Fig. 2), suggesting a unique role for NOD1 in diet-induced obesity. Although the relative mRNA level of NOD1 is lower than or similar to that of TLR4 or TLR2 in 3T3-L1 pre- and adipocytes (Fig. 1B), further studies are warranted to dissect the relative contributions of each PRR to adipose inflammation and insulin resistance in diet-induced obesity.

In conclusion, we show that NOD1 activation induces MAPK- and NF-κB-mediated proinflammatory gene expression and suppresses insulin signaling and subsequent glucose uptake in adipocytes. Our results lay the groundwork for future studies on the role of NOD1 in diet-induced obesity, inflammation, and insulin resistance. Understanding the role of NOD1 in obesity-associated inflammation and insulin resistance may provide novel strategies to prevent and/or treat obesity and insulin resistance.

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DISCLOSURES

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