Activation of pattern recognition receptors in brown adipocytes induces inflammation and suppresses uncoupling protein 1 expression and mitochondrial respiration

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Bae J, Ricciardi CJ, Esposito D, Komarnytsky S, Hu P, Curry BJ, Brown PL, Gao Z, Biggerstaff JP, Chen J, Zhao L. Activation of pattern recognition receptors in brown adipocytes induces inflammation and suppresses uncoupling protein 1 expression and mitochondrial respiration. Am J Physiol Cell Physiol 306: C918–C930, 2014. First published March 13, 2014; doi:10.1152/ajpcell.00249.2013.—Pattern recognition receptors (PRR), Toll-like receptors (TLR), and nucleotide-oligomerization domain-containing proteins (NOD) play critical roles in mediating inflammation and modulating functions in white adipocytes in obesity. However, the role of PRR activation in brown adipocytes, which are recently found to be present in adult humans, has not been studied. Here we report that mRNA of TLR4, TLR2, NOD1, and NOD2 is upregulated, paralleled with upregulated mRNA of inflammatory cytokines and chemokines in the brown adipose tissue (BAT) of the obese mice. During brown adipocyte differentiation, mRNA and protein expression of NOD1 and TLR4, but not TLR2 and NOD2, is also increased. Activation of TLR4, TLR2, or NOD1 in brown adipocytes induces activation of NF-κB and MAPK signaling pathways, leading to inflammatory cytokine/chemokine mRNA expression and/or protein secretion. Moreover, activation of TLR4, TLR2, or NOD1 attenuates both basal and isoproterenol-induced uncoupling protein 1 (UCP-1) expression without affecting mitochondrial biogenesis and lipid accumulation in brown adipocytes. Cellular bioenergetics measurements confirm that attenuation of UCP-1 expression by PRR activation is accompanied by suppression of both basal and isoproterenol-stimulated oxygen consumption rates and isoproterenol-induced uncoupled respiration from proton leak; however, maximal respiration and ATP-coupled respiration are not changed. Further, the attenuation of UCP-1 by PRR activation appears to be mediated through downregulation of the UCP-1 promoter activities. Taken together, our results demonstrate the role of selected PRR activation in inducing inflammation and downregulation of UCP-1 expression and mitochondrial respiration in brown adipocytes. Our results uncover novel targets in BAT for obesity treatment and prevention.

Brown adipose tissue; brown adipocytes; UCP-1; pattern recognition receptor; inflammation

BROWN ADIPOSE TISSUE (BAT) is a specialized tissue whose function is to produce heat through nonshivering thermogene-

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mammals, such as rat or mice, to allow the animals to live in a cold environment without utilizing shivering mechanism to produce heat (3). The thermogenic property of BAT is conferred to mainly by the functions of uncoupling protein 1 (UCP-1), which is uniquely expressed and localized in the inner membrane of brown adipocyte mitochondria to uncouple ATP synthesis from respiration (31, 38). BAT is heavily innervated by sympathetic effenter fibers, and the release of norepinephrine by these fibers not only increases BAT thermogenic activity (i.e., heat production) but also thermogenic capacity (i.e., brown adipogenesis, mitochondrial biogenesis, and synthesis of UCP-1 and other BAT thermogenic proteins) (37).

It was thought until recently that humans only possess BAT when they were newborn infants and that BAT quickly regresses following birth. However, new evidence from positron emission tomography/computed tomography scanning has revealed symmetrical fat depots in the cervical, supracleavicular, and paraspinal regions in adult humans that have all the characteristics of BAT (5, 40, 49, 51, 55). Recent gene expression profilings have confirmed many classical BAT features in the supraclavicular regions of adult humans (6, 18). Due to its energy-dissipating activity, BAT has become a novel target for obesity treatment and prevention (50, 52).

In contrast to white adipose tissue (WAT), whose function is to store energy, brown adipocytes are multicellular with many small lipid droplets and contain a large number of mitochondria, compared with a single large lipid droplet and few mitochondria in white adipocytes. It is now recognized that obesity is associated with chronic inflammation in WAT at both local and systemic levels. Adipose inflammation, characterized by elevated proinflammatory cytokine/chemokine expression and secretions and infiltration of immune cells, including macrophages, is thought to contribute to obesity-associated comorbidities, such as insulin resistance and diabetes (13, 27, 33, 36).

The mechanisms underlying adipose inflammation in obe-

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pathways leading to distinct inflammatory and immune responses in the host cells (1, 22). Toll-like receptors (TLR) and Nod-like receptors (NLR) are two prominent families of PRRs. 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. The respective bacterial or viral pathogen-associated molecular patterns (PAMP) that individual TLR detects have been characterized (1). In contrast, NLRs are a family of cytosolic sensors that play important roles in innate immunity and inflammation (16). These NLRs display a central nucleotide-binding domain, an NH2-terminal protein interaction domain, and a COOH-terminal LRR domain. Two prominent members of NLRs are NOD1 and NOD2, and each recognizes a distinct minimal peptidoglycan structure: NOD1 recognizes a dipeptide, γ-d-Glu-meso-diaminopimelic acid (iE-DAP) (4), or a tripeptide, l-Ala-γ-d-Glu-meso-diaminopimelic acid (Tri-DAP) (10) derived mostly from Gram-negative bacteria; NOD2 recognizes the peptidoglycan muramyl dipeptide MurNAc-l-Ala-isoGln (MDP) from both Gram-positive and Gram-negative bacteria (11, 17). Various PRRs, including TLR4, TLR2, NOD1, and NOD2, have been detected in WAT and/or white adipocytes, and activation of PRRs induces a proinflammatory response (7, 44, 54), resulting in adipocyte dysfunction and disturbed homeostasis of whole body metabolism (8, 14, 41, 43).

Here, we report the expression of NOD1, NOD2, TLR4, and TLR2 in classical BAT of mouse models of obesity and during brown adipocyte differentiation. Activation of NOD1, TLR4, and TLR2, but not NOD2, induces a proinflammatory response in brown adipocytes through NF-κB and MAPK signaling pathways. Moreover, the activation of these selected PRRs leads to downregulation of basal and isoproterenol-induced UCP-1 mRNA and protein expression and suppresses mito-

## MATERIALS AND METHODS

### Reagents

NOD1 synthetic ligand lauryl-γ-d-Glu-meso-diaminopimelic acid (C12-iE-DAP) and TLR2 ligand a synthetic triacylated lipoprotein Pam3CSK4 were from Invivogen (San Diego, CA). Ultra pure TLR4 ligand lipopolysaccharide (LPS) was from List Biological Laboratories (Campbell, CA). 3-Isobutyl-L-methylxanthine, T3, dexamethasone, insulin, indometacin, and isoproterenol were from Sigma-Aldrich (St. Louis, MO). The pharmacological MAPK inhibitors PD98054, SP600125, and SB203580 were from Tocris Bioscience (Ellisville, MO), and the NF-κB inhibitors QNZ and caffeic acid phenethyl ester were purchased from the Jackson Laboratory (Bar Harbor, ME). For RNA preparation and quantitative real-time PCR analysis. Total RNA was prepared from BAT of mouse models of obesity and during brown adipocyte differentiation. Activation of NOD1, TLR4, and TLR2, but not NOD2, induces a proinflammatory response in brown adipocytes through NF-κB and MAPK signaling pathways. Moreover, the activation of these selected PRRs leads to downregulation of basal and isoproterenol-induced UCP-1 mRNA and protein expression and suppresses mitochondrial respiration in brown adipocytes.

### Western blot analysis.

Total cell lysates were prepared and protein concentrations were determined by the BCA assay kit (Thermo Scientific, Waltham, MA). Fifty micrograms of total cell lysates were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20 (pH 7.4) containing 5% nonfat milk. The membrane was immunoblotted with primary antibodies at 4°C overnight followed by secondary antibody conjugated with horseradish peroxidase (GE Healthcare). The signal was quantified by densitometry using a ChemiDocXRS+ imaging system with ImageLab software (Bio-Rad).

### RNA preparation and quantitative real-time PCR analysis.

Total RNA was prepared from BAT from DIO or ob/ob mice or from brown adipocytes using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. Total RNA abundance was quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was carried out using Fermentas first strand synthesis kit (Thermo Scientific, Pittsburgh, PA) according to the manufacturer’s instructions. mRNA expression of PRRs, brown adipocyte marker genes, inflammatory cytokine/chemokine genes, and the housekeeping gene 36B4 was measured quantitatively using gene-specific TaqMan gene expression assays (Applied Biosystems) (for samples of BAT) or Absolute Blue QPCR SYBR Green ROX mix (Thermo Fisher Scientific) and gene-specific primers (for samples of brown adipocytes). Specific primer sequences can be provided upon request. PCR reactions were run in a 96-well format using an ABI 7300HT instrument. Cycle conditions were 50°C 2 min, 95°C 15 min and then 40 cycles of 95°C for 15 s/60°C for 1 min. Relative gene expression was calculated using the 2−ΔΔCT method, which normalizes against 36B4.

### Reporter gene assays. 

For NF-κB-Luc experiments, brown adipocytes in 24-well plates were infected with adenovirus encoding NF-κB-Luc reporter gene for 24 h. The cells were stimulated with C12-iE-DAP, LPS, Pam3CSK4, or the vehicle control for 15 h. For UCP-1-Luc experiments, brown adipocytes were subcultured and
transfected with a mouse 3.1 kb UCP-1 promoter luciferase construct (UCP-1-Luc) (39) and subsequently pretreated with the ligand for each PRR or the vehicle control for 1 h and then cotreated with isoproterenol for 15 h. The cell lysate was prepared and reporter luciferase activities were measured with GloMax Luminometer (Promega, Madison, WI). Relative luciferase activities were normalized by protein concentrations and expressed as fold of vehicle control.

Measurement of cytokine/chemokine. The supernatant from brown adipocytes that were stimulated with PRRs was collected and the levels of monocyte chemotactic protein 1 (MCP-1), interleukin 6 (IL-6), and regulated upon activation, normal T-cell expressed, and secreted (RANTES) were determined by ELISA using Quantikine kits (R&D Systems, Minneapolis, MN).

Oil red O staining and quantification. Differentiated brown adipocytes were serum-starved overnight and were treated with C12-iEDAP, LPS, Pam3CSK4, or the vehicle control for 12 h, followed by costimulated with isoproterenol (1 μM) in the presence of the ligand or another for 6 h. The cells were fixed, and lipid accumulation in brown adipocytes was stained with oil red O (ORO) and quantified by ORO absorbance at 500 nm.

Cellular bioenergetics measurements. D6 2.5 × 10^4 brown adipocytes were subcultured and seeded in 24-well XF assay plates overnight in the differentiation medium and subjected to real-time measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) using XF24 Extracellular Flux Analyzer ( Seahorse Biosciences, North Billerica, MA). To study the effects of PRR ligands on bioenergetics of brown adipocytes, cells were pretreated with C12-iEDAP (10 μg/ml), LPS (0.1 μg/ml), Pam3CSK4 (1 μg/ml), or the vehicle control for 12 h, rinsed once, changed to 500 μl of XF assay buffer (DMEM without NaHCO₃, 10 mM glucose, 2 mM pyruvate, and 2 mM GlutaMAX, pH 7.4), and equilibrated at non-CO₂ incubator and 37°C for 1 h. Following basal measurements of OCR and ECAR, all cells were injected either with the vehicle control or isoproterenol (1 μM) and the subsequent readings were taken over the 6-h period. At the end of the experiment, mitochondrial complex inhibitors were injected to all treatments sequentially in the following order: oligomycin (1 μM), carbonyl cyanide-4-trifluoromethoxyphenylnhydrazone (FCCP; 0.75 μM), and antimycin A/rotenone (1 μM each), and three readings were taken after each inhibitor. OCR and ECAR were automatically recorded by XF24 software v1.8 provided by the manufacturer.

Basal and isoproterenol-stimulated OCR and ECAR rates were determined by averaging the measurements. Calculations of maximal OCR, ATP-coupled OCR, OCR from proton leak, spare respiration capacity, and coupling efficiency were performed according to the manufacturer’s instructions.

Analysis of mitochondrial content by MitoTracker green staining. Mitochondria were labeled using the mitochondria-specific dye MitoTracker Green (Life Technologies, Carlsbad, CA) according to manufacturer’s protocol. Briefly, the differentiated brown adipocytes were washed and trypsinized from the cultured plate and incubated with MitoTracker green at 100 nM for 30 min at 37°C. The fluorescence intensity was measured with Accuri C6 flow cytometry (BD, Franklin Lakes, NJ). Background autofluorescence from nonstained cells was averaged and subtracted from the mean fluorescence intensity values.

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

RESULTS

mRNA expression of selected PRRs is upregulated in brown fat tissue of DIO and ob/ob mice. We first examined selected PRRs NOD1, NOD2, TLR4, and TLR2 mRNA expression in the classical interscapular BAT from DIO and ob/ob mice, compared with their respective controls (Fig. 1, A and B). In DIO mice, NOD1 and TLR4 were two highly expressed PRRs, followed by NOD2 and TLR2, similar to those of their chow-
fed controls. Moreover, NOD1, TLR4, and TLR2 mRNA was significantly upregulated \((P < 0.05)\) in DIO mice compared with their controls (Fig. 1A). Similar relative expression patterns were observed in BAT of ob/ob mice compared with their wild-type (WT) controls (Fig. 1B). Further, we examined inflammatory gene expression in BAT from DIO and ob/ob mice. mRNA of MCP-1, IL-6, and TNF-\(\alpha\), but not RANTES, was significantly upregulated in BAT of DIO mice compared with the chow-fed controls \((P < 0.01\) for MCP-1 and TNF-\(\alpha\) and \(P < 0.05\) for IL-6; Fig. 1C). mRNA of all four genes were significantly upregulated in BAT of ob/ob mice compared with the WT controls \((P < 0.01\) for MCP-1 and \(P < 0.05\) for IL-6, TNF-\(\alpha\), and RANTES; Fig. 1D). These results demonstrate that upregulation of selected PRRs is correlated with the upregulation of mRNA of proinflammatory genes in BAT in mice models of obesity.

**Expression of selected PRRs is upregulated during brown adipocyte differentiation.** To study the relationships between the upregulation of PRRs and inflammatory genes in BAT, we employed an immortalized brown fat cell line generated from the classical interscapular brown fat from C57BL6 mice (23). In vitro brown adipocyte differentiation was validated by the increasing mRNA expression of known brown adipocyte markers UCP-1, PGC-1\(\alpha\), and PPAR\(\gamma\) (Fig. 2A, bottom). mRNA expression of NOD1 and TLR4, but not NOD2 and TLR2, was upregulated during differentiation (Fig. 2A, top).

Fig. 2. Expression of selected PRRs are upregulated during brown adipocyte differentiation. Brown fat cells were differentiated as described in MATERIALS AND METHODS. Total RNA was isolated at confluency (D0), 2 (D2), 4 (D4), and 6 day (D6) postinitiation of differentiation. A: mRNA expression of NOD1, NOD2, TLR4, and TLR2 and brown adipocyte markers peroxisome proliferator-activated receptor-\(\gamma\) coactivator 1a (PGC-1a), proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)), and uncoupling protein 1 (UCP-1) was evaluated by semiquantitative RT-PCR. The relative mRNA expression was normalized to 36B4 and expressed as fold of NOD2 value (set as 1). B: relative mRNA expression of NOD1, NOD2, TLR4, and TLR2 before differentiation (D0) and after differentiation (D6) was compared. The relative mRNA expression was normalized to 36B4 and expressed as fold of NOD2 value (set as 1). C: protein expression of NOD1, TLR4, and TLR2 was analyzed at D0, 2, 4, and 6 by Western analysis with primary antibodies against NOD1, TLR4, and TLR2. ERK1/2 protein was used as a loading control. This experiment has been performed independently three times, and the representative results are shown (left). Relative quantification of Western analysis was done by densitometry. The data are expressed as fold to the D0 level (set as 1) \((\bar{x} \pm SE\) \(n = 3\)). Different letters indicate significant differences between bars. * \(P < 0.05\), ** \(P < 0.01\), significantly different from the controls.
the relative PRR expression pattern, NOD1 was the highest expressed PRR examined in brown fat cells before differentiation, followed by TLR4 and TLR2, and NOD2 had the lowest expression. In contrast, both NOD1 and TLR4 were among the highest expressed PRRs, followed by TLR2 and NOD2 after differentiation (Fig. 2B). Western analysis and relative quantification confirmed that NOD1 and TLR4 were increased, whereas TLR2 was decreased upon differentiation (Fig. 2C). NOD2 protein expression was undetectable by Western analysis (data not shown). These results suggest NOD1, TLR4, and TLR2 may be involved in mediating inflammation and modulating functions in brown adipocytes.

PRR activation in brown adipocytes induces phosphorylation and activation of NF-κB and MAPK signaling. Activation of PRR leads to NF-κB and MAPK signaling pathways in various PRR expressing cells, including white adipocytes. Because of the relative higher expression levels of NOD1, TLR4, and TLR2 than NOD2 in brown adipocytes, we focused on the signaling pathways downstream of NOD1, TLR4, and TLR2 activation in brown adipocytes by stimulating the cells with their respective synthetic ligands. The NF-κB pathway was activated as shown by the phosphorylation of p65 and IκBα when stimulated with the NOD1 ligand C12-iEDAP, TLR4 ligand LPS, or TLR2 ligand Pam3CSK4 in a time-dependent manner (Fig. 3A). Moreover, MAPK pathways were also activated within 60 min after the stimulation, as shown by the phosphorylation of p38, JNK, and ERK when stimulated with C12-iEDAP, LPS or Pam3CSK4 (Fig. 3A).

We further confirmed that activation of NOD1, TLR4, and TLR2 induced NF-κB activation with NF-κB-responsive luc.
ciferase reporter assays in brown adipocytes by transiently infecting the brown adipocytes with the adenovirus encoding NF-κB-Luc receptor gene. Activation of NOD1, TLR4, and TLR2 dose dependently induced NF-κB reporter activation (Fig. 3B). In contrast, the NOD2 ligand MDP induced minimal NF-κB activation even when used up to 100 μg/ml in brown adipocytes (data not shown).

PRR activation in brown adipocytes upregulates the mRNA expression and protein secretion of proinflammatory cytokines/chemokines. Consequently, we found that activation of NOD1, TLR4, and TLR2 significantly increased mRNA expression of the chemokine MCP-1, cytokine IL-6, RANTES, and tumor necrosis factor-α (TNF-α; $P < 0.01$ or $P < 0.05$; Fig. 4A). Other than TNF-α, which was below detection limit, protein secretion in the supernatant of the brown adipocytes was confirmed for MCP-1, IL-6, and RANTES by ELISA (Fig. 4B).

Inhibition of the NF-κB or MAPK pathways differentially attenuates proinflammatory mRNA expression in brown adipocytes. To determine if NF-κB or MAPK signaling pathway underlies proinflammatory cytokine or chemokine mRNA expression, we examined the effects of the pharmacological inhibitors of NF-κB or MAPK.

Fig. 4. PRR activation in brown adipocytes upregulates the expression and secretion of proinflammatory cytokines/chemokines. Brown adipocytes were stimulated with C12-iEDAP (10 μg/ml), LPS (0.1 μg/ml), Pam3CSK4 (1 μg/ml), or the vehicle control for 12 h. Total RNA was isolated for mRNA expression of MCP-1, IL-6, RANTES, and TNF-α by semiquantitative RT-PCR (A), and the supernatant was collected for protein measurements of MCP-1, IL-6, and RANTES by ELISA (B). Data are means ± SE ($n = 3$). *$P < 0.05$, **$P < 0.01$, significantly different from the controls.

Fig. 5. Inhibition of NF-κB attenuates mRNA expression of proinflammatory cytokines/chemokines induced by PRR activation. Brown adipocytes were pretreated with NF-κB inhibitor QNZ (20 nM) and caffeic acid phenethyl ester (CAPE; 10 μM) for 1 h and costimulated with C12-iEDAP (10 μg/ml), LPS (0.1 μg/ml), Pam3CSK4 (1 μg/ml), or the vehicle control. mRNA expression of MCP-1, IL-6, RANTES, and TNF-α was evaluated by semiquantitative RT-PCR. The relative mRNA expression was normalized to 36B4 and expressed as fold of the vehicle value (set as 1). Data are means ± SE ($n = 3$). *$P < 0.05$, **$P < 0.01$, significantly different from the controls.
inhibitors of these pathways on mRNA expression on the cytokines/chemokines. The NF-κB inhibitor QNZ (47) and CAPE (29) significantly attenuated C12-iEDAP, LPS, or Pam3CSK4-induced MCP-1, IL-6, RANTES, and TNF-α in brown adipocytes (Fig. 5), suggesting that NF-κB pathway is involved in NOD1-, TLR4-, or TLR2-mediated inflammatory cytokine/chemokine expression.

Among the inhibitors of MAPKs, only the p38 inhibitor SB203580 significantly attenuated C12-iEDAP-induced MCP-1, IL-6, TNFα, and RANTES mRNA expression (Fig. 6). In contrast, the JNK inhibitor SP600125 significantly attenuated LPS-induced IL-6, TNFα, and RANTES mRNA but not MCP-1 mRNA, whereas p38 inhibitor SB203580 did not affect any of the four mRNA induced by LPS. The p38 inhibitor SB203580 significantly attenuated Pam3CSK4-induced IL-6 and RANTES mRNA, and the ERK inhibitor PD98054 only attenuated Pam3CSK4-induced MCP-1 and IL-6 mRNA, whereas the JNK inhibitor SP600125 attenuated Pam3CSK4-induced IL-6, TNFα, RANTES, but not IL-6 (Fig. 6).

**PRR activation suppresses both basal and isoproterenol-induced UCP-1 expression, but not mitochondrial biogenesis, in brown adipocytes.** The main function of brown adipocytes is mediated by UCP-1 to uncouple oxidative phosphorylation from ATP synthesis to produce heat, and UCP-1 is known to be predominantly regulated at transcriptional level (37). We next examined whether PRR activation in brown adipocytes affects UCP-1 mRNA expression. Activation of NOD1, TLR4, and TLR2 suppressed both basal and isoproterenol, a nonspecific β-adrenergic agonist, -induced UCP-1 mRNA expression (Fig. 7A). We also examined other brown adipocyte markers. Similar suppression was observed for cell-death inducing DFFA-like effector a (Cidea), and distinct but different changes were observed for PGC-1α and PPARγ. PPARγ mRNA was suppressed by activation of NOD1, TLR4, or TLR2 under basal and isoproterenol -induced conditions. In contrast, PGC-1α mRNA was only suppressed by the activa-
tion of the PRR under the basal condition. Moreover, no changes were observed for nuclear respiratory factor 1 (Nrf-1) and transcription factor A (Tfam), and two nuclei encoded mitochondrial genes, cytochrome c oxidase subunit IV a (Cox4a) and cytochrome b-1 complex subunit 6 (Uqcrh) (Fig. 7A). Western analysis and relative quantification confirmed that UCP-1 expression was suppressed by the activation of NOD1, TLR4, and TLR2 in brown adipocytes under both conditions but more pronounced under isoproterenol-stimulated condition (Fig. 7B). PPARγ protein expression was markedly suppressed by the PRR activation under both conditions, similar to that of PPARγ mRNA. In contrast, the PGC-1α protein was not affected by PRR activation under both conditions in brown adipocytes. We further examined the effects of PRR activation on brown adipocyte cell morphology and lipid accumulation. Under our experimental conditions, there were no significant changes in cell morphology (data not shown) and lipid accumulation, revealed by ORO staining, by the activation of NOD1, TLR4, or TLR2 under both conditions (Fig. 7C).

To test whether suppression of UCP-1 mRNA by PRR activation is a direct effect on the UCP-1 promoter or due to the effects on mitochondrial biogenesis, we performed UCP-1 promoter-driven reporter assays and confirmed that the effects of PRR activation was via suppressing UCP-1 promoter activities (Fig. 7D). Consistent with the above mRNA expression results, PRR activation did not affect mitochondrial biogenesis as revealed by the staining of mitochondrial specific fluorescence mitoTracker Green (Fig. 7E). Taken together, these results suggest that PRR activation in brown adipocytes suppresses both basal and isoproterenol-induced UCP-1 expression at least in part through downregulation of the UCP-1 promoter but not through modulation of mitochondrial biogenesis.

**PRR activation suppresses mitochondrial respiration in brown adipocytes.** To further determine whether the suppression of UCP-1 by PRR activation leads to changes in mitochondrial respiration, we performed cellular bioenergetics measurements in brown adipocytes using XF24 Extracellular Flux Analyzers (Fig. 8). It has been determined that the activation of NOD1, TLR4, or TLR2 did not change cell viability as measured by the MTT assays (data not shown). The basal oxygen consumption rate (OCR) of brown adipocytes was 109 ± 10.02 pmol/min, which was suppressed to 93 ± 8.4, 79.9 ± 5.5, and 82.5 ± 9.79 pmol/min by C12-iEDAP (P < 0.05), LPS (P < 0.01), and Pam3CSK4 (P < 0.01), respectively (Fig. 8C). Injection of isoproterenol (1 μM) resulted in a gradual increase in OCR that peaked at 142.2 ± 21.1 pmol/min (P < 0.05), which was significantly reduced to 99.3 ± 10, 92 ± 8.7, and 76.8 ± 3.4 pmol/min by C12-iEDAP (P < 0.05), LPS (P < 0.01), and Pam3CSK4 (P < 0.01), respectively (Fig. 8C). The ECAR, an indirect measure of glycolysis and lactate production, was measured simultaneously with OCR. Brown adipocytes treated with C12-iEDAP (P < 0.05), LPS (P < 0.01), and Pam3CSK4 (P < 0.05) all showed a significant increase in basal ECAR, with LPS inducing the highest level. Injection of isoproterenol induced a modest but significant increase in ECAR over the baseline (P < 0.05); however, both C12-iEDAP and Pam3CSK4 significantly suppressed isoproterenol-induced ECARs (P < 0.05). LPS induced a suppressive, but not significant, trend on isoproterenol-induced ECAR (Fig. 8D).

Further calculations revealed that PRR activation did not affect isoproterenol-induced maximal respiration (Fig. 8E) and ATP-generating OCR (Fig. 8F) but significantly suppressed isoproterenol-induced uncoupled OCR from proton leak (Fig. 8G) and increased coupling efficiency (Fig. 8H). Taken together, these results show that PRR activation significantly attenuates basal and isoproterenol-induced OCR and isoproterenol-induced uncoupled respiration from proton leak but does not affect maximal respiration and ATP-coupled respiration, consistent with the suppressive effects of PRR activation on UCP-1 expression in brown adipocytes.

**DISCUSSION**

It has been well recognized that obesity is associated with chronic inflammation, both systemically and locally in WAT. Here we report, for the first time, that mRNA of major PRR NOD1, TLR4, and TLR2, but not NOD2, was upregulated in BAT, which was paralleled by the mRNA expression of the inflammatory chemokine MCP-1 and cytokines IL-6 and TNF-α in both DIO and ob/ob mice compared with their respective controls (Fig. 1). Using an immortalized brown cell line derived from the classical interscapular BAT, we show that both mRNA expression and protein expression of NOD1 and TLR4, but not TLR2 or NOD2, were increased upon brown adipocyte differentiation (Fig. 2); the activation of NOD1, TLR4, and TLR2 induced activation of the NF-κB and MAPK signaling pathways (Fig. 3), leading to upregulation of mRNA and/or protein expression of the proinflammatory gene MCP-1, IL-6, RANTES, and TNF-α in the cultured brown adipocytes (Figs. 4–6). Moreover, activation of these PRRs in the brown adipocytes suppressed basal and isoproterenol-induced UCP-1 mRNA and protein expression (Fig. 7, A and B), at least in part through suppressing UCP-1 promoter activities (Fig. 7D). Further, PRR activation suppressed both basal and isoproterenol-induced oxygen consumption rates (Fig. 8C) and isoproterenol-induced uncoupled respiration from proton leak (Fig. 8G) and increased coupling efficiency (Fig. 8H) but did not affect the maximal respiration (Fig. 8E) and ATP-generating respiration (Fig. 8F).

BAT has emerged as a novel target for obesity treatment and prevention, due to its unique role in energy expenditure. Two...
PRR-MEDIATED INFLAMMATION IN BROWN ADIPOCYTES

A

B

C

D

E

F

G

H

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sources of brown adipocytes have been identified. One is from classical BAT depots, such as interscapular region, the other one is from UCP-1 expressing, brown-like adipocytes from WAT depots, such as subcutaneous depot. While it is still unclear about the formation of the brown-like adipocytes in typical WAT, it is less debated that classical brown adipocytes share a common progenitor with skeletal muscle, different from that of brown-like adipocytes from WAT. The findings that adult humans do have classical BAT argue for research on classical BAT depots in rodents as they also have relevance to human physiology (30). To our knowledge, this is the first report demonstrating the upregulation of mRNA expression of the selected PRRs in the classical BAT in obesity and the regulation of PRR expression at both mRNA and protein levels in differentiating brown adipocytes in culture. Our report further shows that activation of selected PRRs induces the proinflammatory response and suppresses UCP-1 expression, which are accompanied by attenuated mitochondrial respiration in cultured brown adipocytes. Our results suggest that PRR-mediated inflammation in BAT may be targets for modulation of brown adipose function to combat against obesity.

It is well recognized that PRRs are capable of sensing the disturbance from not only the immune system but also metabolism, and the homeostasis of both processes is important for the survival of the organisms (15, 19). It is believed that the disturbances in obesity, i.e., the ligands for the PRRs, are excess of nutrients [e.g., saturated fatty acids (24)], production of endogenous molecules from metabolic stress and tissue injuries [e.g., ceramides (9), and high mobility group box 1 (28)], or modulation of intestinal microbiota (2, 32). Whether these ligands activate PRR in BAT in obesity remains to be determined in the future.

Mouse models of global deficiency of the PRRs have been instrumental in determining the role of PRRs in linking inflammation and metabolic stress. The effects of global deficiency of TLR4 and TLR2 on DIO, tissue inflammation, and insulin resistance have been reported (7, 8, 14, 43, 48). Shi et al. (43) reported that only female mice lacking TLR4 were partially protected from high-fat DIO, and insulin resistance, accompanied by less inflammation in the liver and fat (with no specification of fat type) compared with the WT control mice; however, energy expenditure was not reported. Male C3H/HeJ mice, which have a loss-of-function mutation in TLR4, were reported to be protected against the development of DIO with increased oxygen consumption (indicating the increased metabolic rate) and decreased respiratory exchange ratio (indicating the preferential use of fat as an energy source), improved insulin sensitivity, and enhanced insulin-signaling in white adipose tissue, muscle, and liver, compared with the controls. Inflammation of brown adipose was not reported in this study (48). Moreover, global TLR2 deficiency protected the mice from DIO (8, 14, 26), insulin resistance, and inflammation of WAT (8, 14, 26), liver, muscle (8, 26), and pancreatic β-cells (8). TLR2-deficient mice showed an increase in energy expenditure as measured by oxygen consumption ($V_o_2$) in the dark cycle (8, 26). In these studies, inflammation in BAT was not reported. Furthermore, mice with double knockout of NOD1 and NOD2 were shown to be protected from high-fat DIO, insulin resistance, inflammation in the white adipose and the liver (41). Energy expenditure and inflammation in brown adipose were not reported in this study (41). Therefore, the role of PRR-mediated inflammation in BAT in diet-induced systemic inflammation, insulin resistance, and altered energy expenditure in obesity remains to be determined.

Our results show that mRNA of selected PRR (NOD1, TLR4, TLR2, and NOD2) was upregulated in BAT in obesity, which correlated with increased mRNA expression of proinflammatory cytokines/chemokines, i.e., MCP-1, IL-6, TNF-α, and RANTES (Fig. 1). MCP-1 has been reported to be a critical chemotactic factor for macrophage infiltration into the adipose tissue, leading to adipose inflammation (20, 21). TNF-α and IL-6 are known cytokines that induce insulin resistance in skeletal muscle and liver (36). RANTES has also been reported to be upregulated and is associated with T-cell accumulation in murine and human obesity (53). Activation of NOD1, TLR4, and TLR2 is capable of inducing MCP-1, IL-6, RANTES, and TNF-α mRNA as well as protein secretion (except for TNF-α, which is below detection limit) in brown adipocytes (Fig. 4). The differences in patterns between protein secretion and mRNA expression downstream of PRRs may reflect the differences in posttranscriptional modulation of those cytokines/chemokines downstream of the PRR activation in brown adipocytes. Using pharmacological inhibitors of NF-κB and MAPK, we show these pathways were differentially involved in mRNA expression of MCP-1, IL-6, TNF-α, and RANTES downstream of NOD1, TLR4, and TLR2 (Figs. 5–6). While the NF-κB pathway appeared to be involved in all four examined cytokines/chemokines induced by C12-iEDAP, LPS, or Pam3CSK4, the involvement of MAPK seemed to differ among the activation of NOD1, TLR4, and TLR2. NOD1 activation primarily induced p38 MAPK as inhibition of p38 MAPK, but not other MAPKs, attenuated mRNA of all four cytokines/chemokines. In contrast, differential yet distinct involvements of MAPKs downstream of activation of TLR4 and TLR2 were noted in brown adipocytes. The JNK inhibitor SP600125 significantly attenuated, whereas the p38 inhibitor SB203580 did not affect the mRNA of the four proinflammatory genes induced by LPS. The ERK inhibitor PD98054 significantly attenuated LPS-induced IL-6, TNF-α, and RANTES mRNA but not MCP-1 mRNA. On the other hand, the JNK inhibitor SP600125 attenuated Pam3CSK4-induced mRNA of IL-6, TNF-α, RANTES, but not IL-6, whereas the p38 inhibitor SB203580 only significantly attenuated Pam3CSK4-induced IL-6 and RANTES mRNA and the ERK inhibitor PD98054 only attenuated Pam3CSK4-induced MCP-1 and IL-6 mRNA. That distinct intracellular signaling pathways downstream of various PRRs control cytokines/chemokine expression, which has been reported in other cell types (25, 29), make it possible to design specific strategies to target individual PRR or cytokine/chemokine for the benefits of enhancing the functions of brown adipocytes.

Moreover, our results demonstrate that PRR activation led to downregulation of both basal and isoproterenol-stimulated UCP-1 mRNA as well as protein expression with more pronounced suppression under isoproterenol-stimulated condition (Fig. 7, A and B). These were accompanied by attenuated basal and isoproterenol-stimulated oxygen consumption and uncoupled respiration from proton leak but not maximal respiration and ATP-coupled respiration (Fig. 8). Initial mechanistic studies show that PRR-mediated downregulation of UCP-1 mRNA was not due to suppression of mitochondrial biogenesis as there were no changes in mRNA of two other mitochondrial...
genes (Cox4a and Uqcrh) (Fig. 7A) and no changes of mitochondrial biogenesis by mitoTracker staining (Fig. 7E). In contrast, similar degrees of suppression of UCP-1 promoter activities were observed (Fig. 8D). Taken together, these results demonstrate that PRR activation suppresses UCP-1 expression, leading to attenuated mitochondrial respiration, via at least in part downregulation of UCP-1 promoter activities. One of the possible mechanisms for PRR-mediated suppression of UCP-1 promoter is through NF-κB pathway, since we have shown that PRR activation leads to NF-κB activation in brown adipocytes, and a few putative NF-κB transcriptional factor binding sites have been identified upstream of the transcription start site using bioinformatics tools. The molecular mechanisms underlying the cross talk between the NF-κB pathway downstream of PRR activation and UCP-1 transcription under the basal or β-adrenergic stimulation need to be explored further.

Compared with UCP-1 expression, PRR activation at the same time also differentially affected PPARγ and PGC-1α expression, both of which are important for the expression of brown adipocyte markers, such as UCP-1. Evidence has emerged from studies that PPARγ not only controls white adipocyte differentiation but also controls brown adipocyte differentiation (12, 34, 42). PGC-1α coactivates transcription factor PPARγ bound to the promoters of target genes, such as UCP-1, to control gene transcription (35, 46). The results show PPAR activation suppressed both PPARγ mRNA and protein expression under the basal and isoproterenol-stimulated conditions whereas PRR activation only suppressed PGC-1α mRNA under the basal condition but did not suppress PGC-1α protein under both conditions (Fig. 7, A and B). The differences between mRNA and protein expression of PGC-1α may be due to the protein stability. Moreover, we also examined the brown adipocyte cell morphology and lipid accumulation under the same conditions. Both the cell morphology (data not shown) and lipid accumulation by ORO staining and quantification (Fig. 7C) did not seem to be affected by PRR activation. Taken together, our results demonstrate that PRR activation in differentiated brown adipocytes selectively suppressed UCP-1 and some other brown adipocyte markers expression (e.g., PPARγ and Cidea) without affecting mitochondrial biogenesis and lipid accumulation, which is sufficient to lead to suppressed mitochondrial oxygen consumption and uncoupled respiration.

In summary, we have demonstrated the role of selected PRRs in mediating inflammation and downregulation of UCP-1 expression without affecting mitochondrial biogenesis and lipid accumulation, leading to suppressed mitochondrial respiration in the classical brown adipocytes. Our results suggest that PRR-mediated inflammation in brown adipocytes may be potential targets to modulate BAT function for obesity treatment and prevention.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


