Preadipocytes Mediate Lipopolysaccharide-Induced Inflammation and Insulin Resistance in Primary Cultures of Newly Differentiated Human Adipocytes

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Recent data suggest that proinflammatory cytokines secreted from adipose tissue contribute to the morbidity associated with obesity. However, characterization of the cell types involved in inflammation and how these cells promote insulin resistance in human adipocytes are unclear. We simulated acute inflammation using the endotoxin lipopolysaccharide (LPS) to define the roles of nonadipocytes in primary cultures of human adipocytes. LPS induction of the mRNA levels of proinflammatory cytokines (e.g. IL-6, TNF-α, and IL-1β) and chemokines (e.g. IL-8, monocyte chemoattractant protein-1) occurred primarily in the nonadipocyte fraction of newly differentiated human adipocytes. Nonadipocytes were characterized as preadipocytes based on their abundant mRNA levels of preadipocyte markers preadipocyte factor-1 and adipocyte enhancer protein-1 and only trace levels of markers for macrophages and myocytes. The essential role of preadipocytes in inflammation was confirmed by modulating the degree of differentiation in the cultures from approximately 0–90%. LPS-induced proinflammatory cytokine/chemokine expression and nuclear factor-κB and MAPK signaling decreased as differentiation increased. LPS-induced cytokine/chemokine expression in preadipocytes was associated with: 1) decreased adipogenic gene expression, 2) decreased ligand-induced activation of a peroxisome proliferator activator receptor (PPARγ)-γ reporter construct and increased phosphor-ylation of PPARγ, and 3) decreased insulin-stimulated glucose uptake. Collectively, these data demonstrate that LPS induces nuclear factor-κB- and MAPK-dependent proinflammatory cytokine/chemokine expression primarily in preadipocytes, which triggers the suppression of PPARγ activity and insulin responsiveness in human adipocytes. (Endocrinology 147: 5340–5351, 2006)

Obesity and its associated metabolic pathologies are the most common metabolic diseases in the United States, affecting over 50% of the adult population. One emerging feature of obesity is the linkage between obesity and chronic inflammation characterized by increased cytokine and chemokine production and acute-phase inflammatory signaling in adipose tissue (1, 2). Thus, white adipose tissue (WAT) is no longer considered an inert depot of stored energy but also an active endocrine organ secreting a diverse array of proinflammatory adipokines such as leptin, IL-1β, IL-6, IL-8, TNF-α, monocyte chemoattractant protein (MCP)-1, and macrophage migration inhibitory factor, all of which have been linked to insulin resistance. However, the exact role of cells comprising adipose tissue in mediating inflammation and causing insulin resistance is still unclear.

Adult human WAT has been reported to be composed of approximately 50–70% adipocytes, approximately 20–40% stromal vascular (SV) cells (i.e. preadipocytes, fibroblasts, nondifferentiated mesenchymal cells), and approximately 1–30% infiltrated macrophages (3). However, less is known about the localization and secretory pattern of cytokines in WAT. It has been suggested that nonadipocytes (e.g. SV cells and/or cells from the supporting matrix) in human WAT are the major producers of IL-6 and TNF-α rather than adipocytes (4, 5). Similarly, preadipocytes have been reported to act as macrophage-like cells and secrete an array of cytokines (6). Conversely, it has been proposed that macrophages residing in adipose tissue are responsible for most of the secreted cytokines (7). Weisberg et al. (8) reported that adipose tissue recruits circulating monocytes/macrophages from bone. Intriguingly, Charrière et al. (9) reported plasticity of preadipocytes showing evidence that 3T3-L1 cells have the ability to acquire phagocytic phenotypes and properties in the presence of macrophages.

Given these emerging data linking cross talk between nonadipocytes and adipocytes with the development of obesity and insulin resistance, the use of primary cultures of...
newly differentiated human adipocytes as a cell model to investigate this linkage is timely. These heterogeneous cultures contain various percentages of nonadipocytes and adipocytes, depending on the isolation, growth, and differentiation protocols used. However, data on the types of cells in these cultures, and their role in triggering inflammation and insulin resistance are lacking.

Based on our previous findings demonstrating that cultures of newly differentiated human adipocytes robustly secrete cytokines/chemokines that impair insulin sensitivity (10, 11) and reports showing that preadipocytes are targets of inflammatory stimuli (6, 12), we focused on delineating the role of nonadipocytes from WAT in inflammation and insulin resistance. To simulate acute inflammation, we treated the cultures with the bacterial endotoxin lipopolysaccharide (LPS). LPS has been reported to induce nuclear factor-κB (NFκB) signaling through Toll-like-receptors (TLRs) in macrophages (13) and (pre)adipocytes (14, 15) and has been linked to insulin resistance. However, the mechanism by which LPS induces inflammation and insulin resistance in human WAT is less clear.

To this end, we tested the hypothesis that LPS induces proinflammatory cytokine/chemokine expression predominantly in nonadipocytes in our cultures, which subsequently triggers insulin resistance in adipocytes. We found that cytokine/chemokine expression was predominantly in the nonadipocyte fraction, which were primarily preadipocytes based on marker analyses. We also demonstrated that LPS-stimulated endotoxemia activated proinflammatory cytokine/chemokine production via NFκB and MAPK signaling, predominantly in preadipocytes, and decreased peroxisome proliferator activated receptor (PPAR)-γ activity and insulin responsiveness in adipocytes. These data demonstrate that human preadipocytes play a pivotal role in the development of insulin resistance in human adipocytes via increasing proinflammatory cytokine/chemokine expression involving NFκB and MAPK signaling.

Materials and Methods

Materials

All cell cultureware and scintillation cocktail (ScintiSafe) were purchased from Fisher Scientific (Norcross, GA). Fetal bovine serum (FBS) was purchased from Cambrex/BioWhittaker (Walkersville, MD). Monoclonal antibody for CD68 was purchased from Research Diagnostics Inc. (Cincinnati, OH) was added to each fraction for RNA extraction. Gene-specific primers for real-time PCR were purchased from IDT (Coralville, IA). 6-Dimethylaminopurine (DMAP) and MCI12 were purchased from Calbiochem (San Diego, CA). SYBR400, U0126, and primary antibodies for p-inhibitory-κB (IkB) kinase (IKK)-α/β (Ser 180/181, rabbit), p-stress-activated protein kinase/Jun-N-terminal kinase (JNK) (Thr183/Tyr185, mouse), p-AKT (protein kinase B) (Ser473, rabbit), p-Erk-1/2 (Thr202/Tyr204 rabbit) were purchased from Cell Signaling Technology (Beverly, MA). Preadipocyte factor-1 (Pef-1/Dlk1) monoclonal antibody was obtained from R&D Systems (Minneapolis, MN). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Cell cultures

Abdominal adipose tissue was obtained from females with a body mass index (BMI) of 30 or less during elective surgery with approval from the Institutional Review Board at University of North Carolina-Greensboro. SV cells were isolated and cultured as previously described with minor modification (10). To induce approximately 50% differentiation (e.g. 50% of cells containing visible lipid droplets), confluent cultures of SV cells were supplemented with differentiation media-1 [DM1; 97% DMEM/Ham F-12, 3% FBS, 1 μM rosiglitazone, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 33 μM biotin, 17 μM panthothenate, 100 μM insulin] for the first 3 d (referred to as DM1 in Fig. 1 or AD50 in the other figures). To obtain maximum differentiation, cultures of SV cells were exposed to DM1 for 6 d, which resulted in approximately 90% differentiation (AD90). To generate cultures that did not differentiate into adipocytes (referred to as –DM1 in Fig. 1 or AD0 in other figures), cultures of SV cells were supplemented with adipocyte media (AM1; 97% DMEM/Ham F-12, 3% FBS, 1 μM dexamethasone, 33 μM biotin, 17 μM panthothenate, 100 μM insulin) beginning on d 1 of differentiation until the assays were performed.

For the coculture experiment (see Fig. 4C), SV cells were initially seeded as a monoculture in individual cell culture inserts (Falcon 0.4 μm pores catalog no. 3090; Fisher Scientific) and suspended in six-well Multiwell plates containing AM1 for 12 d (AD0). On d 14, inserts containing the AD0 cultures were transferred to six-well Multiwell plates and cocultured above AD50 cultures for 8 h, thereby allowing the AD0 and AD50 cultures to communicate with one another by sharing the same media during treatment with LPS. For the positive controls of macrophages, the U937 human monocyte line (CRL1593; American Type Culture Collection, Manassas, VA) was used. U937 cells were supplemented with RPMI 1640 media containing 10% FBS and induced to differentiation by adding 10 ng phorbol 12-myristate 13-acetate for 72 h.

Fractionation of SV cells and adipocytes using density gradient

For fractionation of lipid-laden adipocytes from the nondifferentiated SV cells, cultures grown in 100-mm plates (~3 million cells) were washed with Hanks’ balanced salt solution (HBSS)/0.5 mM EDTA and trypsinized with trypsin-like enzyme at 37°C. Cells were layered onto the 6% iodixanol (Optiprep; Axis-Shield, Oslo, Norway; –1.03 g/ml) in 0.5% BSA/HBSS in a 15-ml centrifuge tube and centrifuged at 650 g for 20 min at 4°C. SV cells were collected from the pellet. The floating adipocytes were harvested from the top and delivered to microfuge tubes. To remove SV cell contamination and dead cell debris, adipocytes were resuspended with ice-cold HBBS and centrifuged at 5000 × g for 5 min. Adipocytes were collected from the top of the microfuge tube in which fat cells formed a fat film. TriReagent (Molecular Research Center Inc., Cincinnati, OH) was added to each fraction for RNA extraction.

Immunostaining

Cells were cultured on coverslips for immunofluorescence microscopy and stained as described previously (10). For double staining of Pref-1 and adipose tissue fatty acid binding protein (aP2), coverslips were first incubated with mouse-anti Pref-1 (1:10) overnight and stained with fluorescein isothiocyanate-conjugated secondary antibody (1:500). Then coverslips were blocked again and incubated with rabbit-anti aP2 for 2 h and stained with Rodamine red-conjugated secondary antibodies (1:500). For MAC-1 and CD68 immunostaining, 1:10 diluted antibodies were incubated overnight at 4°C. Fluorescent images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI) mounted on a BX60 fluorescence microscope (Olympus, Tokyo, Japan).

Immunoblotting and 4 μm urea-SDS-PAGE

Immunoblotting was conducted as we previously described (10) using NuPage precasted gels (Invitrogen, Carlsbad, CA). To resolve PPARγ phosphoproteins, total cell extracts (75 μg protein) were subjected to 10% SDS-PAGE [acylamide to bisacrylamide 100:1 (wt/wt)] containing 4 μm urea and electrophoresis at 80 V for 20 h. Separated proteins were subsequently transferred to polyvinylidene difluoride membranes and immunoblotted with a monoclonal PPARγ antibody (Santa Cruz Inc., Santa Cruz, CA). The abundance of PPARγ was quantified from exposed x-ray film using the Kodak image station 440 (Eastman Kodak Co., Rochester, NY).
Fig. 1. Primary cultures of newly differentiated human adipocytes are composed of adipocytes and preadipocytes. SV cells were isolated from human sc adipose tissue. Confluent SV cells were either induced to differentiation (+ DM1) or kept in adipocyte media (- DM1) for 12 d. A, Morphological changes obtained using phase contrast microscopy (×10). B, Gene expression of Pref-1 and aP2 using RT-PCR. C, Immunolocalization of Pref-1 (green) and aP2 (red). D, Protein expression of PPARγ, aP2, Pref-1, and actin using Western blotting. E, Differentiated cultures of human adipocytes (+ DM1) were immunostained with the macrophage-specific markers CD68 and MAC-1. Differentiated U937 cells (human macrophage cell line) were used as positive controls. F, Gene expression profiles of CD68 from RNA differentiated cultures (+ DM1), compared with RNA from freshly isolated, floating human adipocytes (Floater AD), differentiated human U937 cells (U937), human muscle, and human primary hepatocytes by real-time qPCR, G, Gene expression profiles of the muscle marker MyoD in differentiated cultures (+ DM1) were compared with RNA from the cultures or tissues described in F. Data are representative of at least two independent experiments in all panels. Data in F and G were obtained using RNA from a single subject.

[2-3H deoxyglucose] uptake

Basal and insulin-stimulated glucose uptakes were measured as we described previously (11).

RNA isolation and PCR

Total RNA was isolated from the cultures using TriReagent according to manufacturer’s protocol for RT-PCR. Total RNA (0.5 μg) from each RNA sample was used with the One-Step RT-PCR kit (QIAGEN, Valencia, CA). Primer sets for aP2 were previously described (42). Primer sequences for Pref-1 (accession no. NM_003836) were forward (5'-TAC-GAGTGTCTGTGCAAGC), reverse (5'-TACAGTGTCGTGCAAGC), reverse (5'-ACACAAAGATAACCGAA-CAC) and running conditions were 37 cycles of 95°C for 30 sec, 56°C for 18 sec, and 72°C for 18 sec. For real time quantitative PCR (qPCR), 1 μg total RNA was converted into first-strand cDNA using Omniscript RT kit (QIAGEN). qPCR was performed in a Smartcycler (Cepheid, Sunnyvale, CA) using the QuantiTect SYBR Green PCR kit (QIAGEN) for 40 cycles. To account for possible variation related to cDNA input amounts or the presence of PCR inhibitors, the endogenous reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was simultaneously quantified in a separate tube for each sample. Initial real-time amplifications were examined by agarose gel electrophoresis to confirm the sizes of the products. After PCR amplification, a melting curve was generated for each PCR product to check the specificity of the PCR. Primer sequences and running conditions are summarized in Table 1.

Transient transfection and PPAR activity

For measuring PPAR activity, primary human adipocytes were transiently transfected with the PPAR-responsive luciferase reporter construct pTK-PPRE3x-luc (16) using the Amaxa Nucleofactor (Amaya, Cologne, Germany) according to the manufacturer’s protocol. On d 6 of differentiation, 1 × 10^6 cells from a 60-mm plate were trypsinized and resuspended in 100 μl of nucleofector solution (Amaya) and mixed with 2 μg of pTK-PPRE3x-luc and 25 ng pRL-CMV for each sample. Electroporation was performed using the V-33 nucleofector program (Amaya). Cells were replated in 96-well plates after 10 min recovery in calcium-free RPMI 1640 media. Two hours later, cultures were supplemented with charcoal-stripped AM1. LPS stimulation was performed 20 h after transfection for 3 h. Firefly luciferase activity was measured using the Dual-Glo luciferase kit (Promega, Madison, WI) and normalized to Renilla luciferase activity from the cotransfected control pRL-CMV vector. All luciferase data are presented as a ratio of firefly luciferase to Renilla luciferase activity.

Statistical analysis

Unless otherwise indicated, data are expressed as the mean ± SEM (n = 3–8) using a pool of cells obtained from three to five different human subjects. Data were analyzed using one-way ANOVA, followed by Student’s t tests for each pair for multiple comparisons. Differences were considered significant if P < 0.05. All analyses were performed using JMP IN 4.04 software (SAS Institute, Cary, NC).

Results

Primary cultures of newly differentiated adipocytes contain preadipocytes and adipocytes

Our normal differentiation protocol using DM1 for the first 3 d of differentiation resulted in a cell population on d 12 containing approximately 50% adipocytes and approximately 50% nonadipocytes (cells without visible lipid droplets). Based on our findings that the nonadipocyte fraction of our cultures robustly express and/or secrete cytokines (e.g., IL-6, TNF-α) and chemokines (e.g., IL-8) in response to trans-10, cis-12 CLA treatment (10, 11), we wanted to know whether preadipocytes were present in this nonadipocyte or SV fraction. To answer this question, we first cultured the
cells in the absence or presence of DM1 for the first 3 d of differentiation, followed by 9 d of exposure to AM1 used to maintain the adipocyte phenotype. As shown in Fig. 1A, approximately 50% of the cells were differentiated into lipid-containing adipocytes by d 12 when exposed to DM1. In contrast, cells not supplemented with DM1 had few lipid-containing adipocytes by d 12 when exposed to AM1. In approximately 50% of the cells were differentiated into lipid-containing adipocytes by d 12 when exposed to DM1. In contrast, cultures receiving DM1 for 3 d and then AM1 for 9 more days contain both adipocytes (generously provided by Zen Bio Inc.) and preadipocytes (generously provided by Zen Bio Inc., Research Triangle Park, NC) as negative controls for macrophages and myocytes, respectively. We measured CD68 and MyoD in RNA from muscle and hepatocytes (generously provided by Zen Bio Inc., Research Triangle Park, NC) as negative controls for macrophages and myocytes, respectively. Very little mRNA or protein for CD68 (Fig. 1, E and F) or MAC-1 (Fig. 1E) were detected in our newly differentiated cultures of human adipocytes. mRNA levels of the myocyte marker MyoD were not detectable in our differentiated cultures (Fig. 1G). Interestingly, RNA obtained from freshly isolated floating adipocytes (generously provided by Zen Bio Inc.) expressed significant amounts of mRNA for CD68, suggesting the presence of monocytes or lipid-laden macrophages in this fraction (Fig. 1F). Collectively, these data suggest that our cultures of newly differentiated adipocytes contain negligible amounts of macrophages or myocytes.

**Primary cultures of newly differentiated adipocytes do not express markers of macrophages or myocytes**

Next, we wanted to determine which cell types (other than preadipocytes) with the potential to produce cytokines/chemokines were present in our cultures. To answer this question, we measured the expression and/or localization of markers of human macrophages (e.g. CD68, MAC-1) and myocytes (e.g. MyoD), cells known to secrete cytokines/chemokines, in our differentiated cultures (+DM1). We measured CD68/MAC-1 and MyoD in differentiated human macrophages (U937 cells) and RNA obtained from muscle as positive controls for macrophages and myocytes, respectively. We measured CD68 and MyoD in RNA from muscle and hepatocytes (generously provided by Zen Bio Inc., Research Triangle Park, NC) as negative controls for macrophages and myocytes, respectively. Very little mRNA or protein for CD68 (Fig. 1, E and F) or MAC-1 (Fig. 1E) were detected in our newly differentiated cultures of human adipocytes. mRNA levels of the myocyte marker MyoD were not detectable in our differentiated cultures (Fig. 1G). Interestingly, RNA obtained from freshly isolated floating adipocytes (generously provided by Zen Bio Inc.) expressed significant amounts of mRNA for CD68, suggesting the presence of monocytes or lipid-laden macrophages in this fraction (Fig. 1F). Collectively, these data suggest that our cultures of newly differentiated adipocytes contain negligible amounts of macrophages or myocytes.

**Preadipocytes play an essential role in LPS-induced cytokine gene expression and insulin resistance**

To determine the capacity of preadipocytes and adipocytes in our differentiated cultures to express cytokine/chemokine genes (and secrete cytokines/chemokines) reported to cause insulin resistance, we first developed a procedure to separate SV cells (preadipocytes) from adipocytes obtained from our differentiated cultures (Fig. 2A). Next, we treated the cultures with LPS; separated the SV cells from the adipocytes; and measured the mRNA levels for several cyto-
Fig. 2. LPS stimulates inflammatory cytokine gene expression predominantly in the SVF obtained from primary cultures of newly differentiated human adipocytes. Differentiated cultures of human adipocytes were fractionated using 6% iodixanol (1.03 g/ml). The lipid-laden ADF (■) was floated, leaving the SVF (□) as pellets. A, Fractionations were verified by measuring the gene expression of aP2 and AEBP-1 using real-time qPCR and Pref-1 using RT-PCR. B, Cultures of differentiated human adipocytes (d 14) were incubated in the presence or absence of LPS (10 ng/ml) for 3 h before fractionation. Relative mRNA expression of IL-6, IL-8, TNF-α, IL-1β, COX-2, APM-1, and PPARγ were investigated using qPCR. Means (±SEM, n = 4) not sharing a common superscript differ significantly (P < 0.05).

kines, preadipocyte markers, and adipogenic genes in these two fractions (Fig. 2B). As shown in Fig. 2A, our fractionation procedure using 6% iodixanol yielded an SV fraction (SVF) in the pellet containing cells with little mRNA for the adipocyte marker aP2 and significantly more mRNA for the preadipocyte markers adipocyte-enhancer binding protein (AEBP-1) and Pref-1, compared with the buoyant adipocyte fraction (ADF), which had more aP2 and less AEBP-1 and Pref-1. LPS robustly induced the expression of IL-6, IL-8, TNF-α, IL-1β, and cyclooxygenase (COX)-2, genes positively associated with inflammation and NFκB activation, in the SVF, compared with the ADF (Fig. 2B). Conversely, the expression levels of adiponectin (APM-1) and PPARγ, almost exclusively expressed in the ADF, were attenuated by LPS treatment. These data demonstrate the capacity of preadipocytes to generate inflammatory signals and their associa-
tion with the suppression of markers of insulin sensitivity in human adipocytes.

To further investigate the role of preadipocytes in inflammation, we established three human (pre)adipocyte models by manipulating the duration and exposure to DM1. Using this protocol, cultures on d 14 had 0, 50, or 90% adipocytes (Fig. 3A). LPS-stimulated TNF-α, IL-6, and IL-8 expression decreased as the degree of differentiation increased to 90% (Fig. 3B). A similar trend was observed for TNF-α and IL-6 mRNA levels under basal conditions.

The capacity of preadipocytes to recruit monocytes was examined in the same cultures by measuring the mRNA levels of MCP-1, a chemokine associated with monocyte recruitment and inflammation. LPS robustly induced MCP-1 gene expression in cultures containing exclusively preadipocytes (AD0) but decreased as the degree of differentiation increased (Fig. 3C). These data provide further support for the hypothesis that preadipocytes are important inducers of inflammation in primary cultures of newly differentiated human adipocytes. In addition, these data also suggest that preadipocytes have the capacity to initiate macrophage infiltration into adipose tissue given their ability to express MCP-1.

Given the role of TLRs in mediating inflammation induced by LPS, the relative mRNA levels of TLR4 and TLR2 were determined in our cultures (Fig. 3D). In the absence of LPS, AD0 (preadipocyte) cultures expressed approximately 3.0 and approximately 1.9 times more TLR4 and TLR2 mRNA, respectively, compared with the AD90 (adipocyte) cultures. Whereas LPS stimulation had only a marginal impact on TLR4 expression, TLR2 mRNA levels were robustly increased by LPS. The expression of both TLR4 and TLR2 decreased as the degree of differentiation increased, consistent with the proinflammatory capacity of preadipocytes demonstrated in Fig. 3, B and C.

To determine the impact of LPS-induced cytokine production in preadipocytes on insulin responsiveness in adipocytes, we measured [2-3H] deoxyglucose uptake in our three models (Fig. 4). As expected, AD0 cultures, which are primarily preadipocytes, showed a blunted response to insulin-stimulated glucose uptake (Fig. 4, A and B). However, even this small increase in insulin-stimulated glucose uptake

**Fig. 3.** LPS induction of cytokine gene expression decreases as the degree of adipocyte differentiation increases. Three human (pre)adipocyte cell models containing 0, 50, or 90% adipocytes were established by modulating exposure to DM1 (see Materials and Methods for details). A, On d 14, each culture was stained with oil-red-O to show neutral lipid accumulation and are representative of two independent experiments. B–D, On d 14, cultures of human (pre)adipocytes were incubated in the absence (□) or presence (■) with LPS for 3 h and total RNA was harvested for the mRNA analyses of TNF-α, IL-6, and IL-8 (B), MCP-1 (C), or TLR4 and TLR2 (D) using qPCR in each cell model. Data in B–D are normalized to the basal (−LPS) gene expression level of AD0. Means (±SEM, n = 4) not sharing a common superscript differ significantly (P < 0.05).
pressed LPS-mediated glucose uptake by another 30%, compared with LPS-treated cultures without inserts (Fig. 4C). Collectively, these data demonstrate that preadipocytes are required for LPS suppression of insulin-stimulated glucose uptake and suggest that proinflammatory cytokines originating in preadipocytes mediate insulin resistance in adipocytes.

**LPS decreases the activity and increases the phosphorylation of PPARγ**

LPS suppression of adipogenic gene expression (Fig. 2B) and insulin-stimulated glucose uptake (Fig. 4) suggested that LPS may decrease the activity of PPARγ, which is essential for insulin-stimulated glucose uptake and triglyceride (TG) synthesis in adipocytes. To answer this question, basal and ligand-induced activation of PPARγ activity were measured in AD50 cultures transfected transiently with a luciferase reporter construct containing a multimerized peroxisome proliferator-responsive element (PPRE). We consistently obtained approximately 65% transfection efficiency revealed by parallel transfections with a green fluorescent protein reporter construct (data not shown). Both adipocytes and preadipocytes were equally transfectable using this protocol, based on aP2 immunostaining and 4′,6′-diamino-2-phenylindole nuclear staining. Although basal levels of PPARγ activity were not affected by LPS, it decreased rosiglitazone (BRL49653)-stimulated PPARγ activity in a dose-dependent manner (Fig. 5A).

To determine the extent to which LPS decreased PPARγ activity by increasing PPARγ phosphorylation, AD50 cultures were treated with and without LPS for 3 h, and the isolated cell proteins were separated by SDS-PAGE-urea gel electrophoresis to detect band shifts in PPARγ. As seen in Fig. 5B, LPS caused a band shift of PPARγ 1 and 2, which was attenuated by treatment with alkaline phosphatase. These data indicate that LPS may decrease the activity of PPARγ by increasing its degree of phosphorylation, and suggest a mechanism by which LPS impairs insulin responsiveness. However, the upstream signaling mechanism linking LPS-induced cytokine production in preadipocytes to decreased PPARγ activity and insulin sensitivity in adipocytes remains unknown.

**LPS-induced cytokine/chemokine gene expression depends on NFκB signaling in preadipocytes**

Although well characterized in macrophages, the regulation of NFκB activation and signaling in adipose tissue is less clear. Berg et al. (15) reported altered NFκB sensitivity to LPS-induced signaling in adipocytes during differentiation using the murine 3T3-L1 cell line. However, NFκB sensitivity to LPS in primary cultures of human adipocytes has not yet been established. To determine the extent to which preadipocytes and adipocytes contribute to NFκB and MAPK activation and subsequent cytokine/chemokine expression in the mixed cultures (AD50), we measured protein phosphorylation kinetics associated with NFκB and MAPK (e.g., JNK, ERK1/2) signaling in AD0, AD50, and AD90 cultures treated with LPS. Consistent with the inflammatory cytokine expression profile in Fig. 3B, the degree to which LPS induced...
the phosphorylation of IKK, JNK, and ERK, and the degradation of IκB decreased as the degree of differentiation increased from approximately 0 to 90% (Fig. 6). NFκB and MAPK activation reached its maximum after 1 h of LPS treatment in all three models, albeit at much lower levels in the AD50 and AD90 cultures, compared with the AD0 cultures. These data provide additional evidence that the presence of preadipocytes in cultures of human adipocytes modulates the susceptibility of LPS-induced NFκB and MAPK activation that trigger cytokine/chemokine production.

Next, we determined the extent to which NFκB and MAPK activation contributed to the LPS-induced cytokine/chemokine expression using selective chemical inhibitors of NFκB and MAPK. LPS is known to act as an agonist for TLR4/2 in (pre)adipocytes (14), which triggers NFκB activation through MAPK and phosphatidylinositol 3-kinase (PI3K)/AKT pathway (17, 18). As seen in Fig. 7A, the proteasome inhibitor MG132 abolished LPS-induced TNF-α gene expression, suggesting that proteasomal degradation of IκB is crucial for NFκB activation and subsequent induction of TNF-α gene expression in human adipocytes. MG132 treatment also decreased the expression of IL-6 and IL-8, genes also regulated by NFκB, but not to the extent of TNF-α (Fig. 7, B and C). The MAPK kinase (MEK)/ERK inhibitor U0126, and the JNK inhibitor DMAP also attenuated LPS-induced cytokine production. The PI3K inhibitor LY-294002 blocked LPS-induced TNF-α gene expression by approximately 50% but had only minimal effects on IL-6 or IL-8. Collectively, these results suggest that NFκB and MAPK activation and signaling play an essential role in LPS-mediated proinflammatory cytokine/chemokine expression in preadipocytes and insulin resistance in human adipocytes.

Discussion

Characterization of nonadipocytes in the cultures

Adult WAT is composed of several cell types. Of the cells residing in WAT, mature adipocytes are by far the largest in size, but their abundance depends on the specific adipose
FIG. 7. Inhibitors of NFκB attenuate LPS induction of cytokine gene expression. Cultures of newly differentiated human adipocytes on d 14 were preincubated for 1 h in the absence or presence of the Akt inhibitor of LY-294002 (LY, 10 µM), the MEK-ERK inhibitor U0126 (10 µM), the JNK inhibitor DMAP (250 µM), or the proteasome inhibitor MG132 (10 µM) before LPS treatment (10 ng/ml, 3 h). Total RNA was harvested, and mRNA levels of TNF-α (A), IL-6 (B), and IL-8 (C) were analyzed using qPCR. Means (± SEM, n = 4) not sharing a common superscript differ significantly (P < 0.05).

depot and species. For example, Hauner (3) reported that human adipocytes represent approximately 50–70% of the cells in human WAT. In contrast, Fain et al. (4) reported that approximately 70% of the protein from human WAT digestes is associated with tissue matrix, and the remaining 30% was equally divided between 3V cells and floating adipocytes. Interestingly, nonadipocytes from human WAT, especially from the tissue matrix, accounted for approximately 90% of the cytokine secretion (4). Furthermore, the more robust cytokine secretion from WAT obtained from morbidly obese subjects (e.g. BMI = 45), compared with obese subjects (BMI = 32) was from the nonadipocytes. In contrast, Gran-
adipocytes in our cultures and are associated with attenuated expression of adiponectin and PPARγ, two markers of insulin sensitivity. Using a second approach, we manipulated the degree of differentiation of the cultures (e.g. AD0, AD50, or AD90) before LPS treatment and then measured cytokine/chemokine expression (Fig. 3), glucose uptake (Fig. 4), PPARγ activity and phosphorylation (Fig. 5), and NFκB and MAPK activation (Fig. 6) and signaling (Fig. 7). Both of these approaches gave consistent results, demonstrating that the presence of preadipocytes in the cultures was positively associated with the degree of inflammatory gene expression, implicating preadipocytes as important sources of cytokines/chemokines that adversely affect PPARγ activity and insulin responsiveness involving NFκB and MAPK activation and signaling. Studies are underway to compare the relative abundance of cytokine/chemokine mRNAs from human macrophages, compared with human preadipocytes.

Role of PPARγ in inflammation and insulin resistance

Despite increasing evidence of the casual link between inflammation and insulin resistance, elucidating the precise mechanism by which cytokines/chemokines impair glucose uptake has proved difficult (reviewed in Refs. 21, 22). Our data highlight the importance of preadipocytes in mediating insulin resistance. One possible explanation for this observation is the suppression of adiponectin gene expression by LPS, which is exclusively secreted from adipocytes and positively associated with insulin sensitivity (23). In addition to its role in the modulation of glucose and lipid metabolism, adiponectin has been reported to have potent antiinflammatory properties due to its ability to induce the production of anti-inflammatory cytokines (i.e. IL-10), and inhibit proinflammatory cytokine production (reviewed in Ref. 24). Thus, it seems reasonable to presume that LPS attenuation of insulin responsiveness in AD50 model is due, at least in part, to the suppression of adiponectin expression. Consistent with this notion, LPS administration to cultures containing almost exclusively adipocytes (AD90) did not adversely affect insulin-stimulated glucose uptake (Fig. 4) or adiponectin gene expression (data not shown). Ajuwon and Spurlock (23) reported direct induction of PPARγ by adiponectin, coupled with suppression of NFκB activation, suggesting mutual transcriptional activation of PPARγ and adiponectin may determine adipocyte susceptibility to inflammatory stimuli.

The PPAR subfamily of nuclear receptors controls many different target genes involved in both lipid metabolism and glucose homeostasis. Loss-of-function PPARγ mutations in humans cause insulin resistance (25–27), and activation of PPARγ by thiazolidinediones act as insulin sensitizers (reviewed in Ref. 28). However, detailed mechanisms describing how inflammation suppresses PPARγ activity in human WAT are unclear. In our study, LPS suppressed ligand-induced, but not basal, PPARγ activity. Similarly, the PPARγ antagonist GW9662 decreased ligand-dependent, but not basal, PPARγ activity (our unpublished data), implicating that ligand-inducible PPARγ activity is critical in regulating insulin sensitivity.

One of the putative mechanisms modulating PPARγ activity is phosphorylation. It has been suggested that phosphorylation of PPARγ does one of the following: 1) impairs PPARγ affinity for its ligand (28, 29), 2) controls interactions between PPARs and corepressors and/or coactivators of transcription (30), or 3) alters PPARγ binding to the PPRE (reviewed in 31). ERK1/2 and JNK are two candidate transcription factors reported to phosphorylate PPARγ (32, 33). Thus, LPS-mediated impairment of insulin responsiveness (Fig. 4) and PPARγ activity (Fig. 5A) may be due to changes in PPARγ affinity for its ligand via phosphorylation, as suggested by data in Fig. 5B. We propose that posttranscriptional modification of PPARγ activity through phosphorylation may be one of the mechanisms by which cytokines affect the transcription of genes involved in glucose and lipid metabolism. However, other potential mechanisms (i.e. recruitment/dismissal of corepressors/activators or binding affinity to the PPRE) remain to be examined.

Role of NFκB and MAPK in inflammation and insulin resistance

In addition to controlling gene expression, PPARγ has been linked to NFκB regulation through physical interactions that block its transcriptional activity (34). Conversely, cytokine-induced NFκB activation suppresses PPARγ DNA binding (35). Consistent with these data, activation of NFκB (34, 36, 37) and MAPK (33, 38, 39) hinders PPARγ DNA binding affinity or transcriptional activation, providing a mechanism by which LPS-induced cytokine production suppresses PPARγ activity. The antiinflammatory role of PPARγ is also demonstrated in this work, showing that the more adipocytes in the culture, and consequently more PPARγ activity, the less robust NFκB signaling observed in Fig. 6. These data support recent findings showing that PPARγ mediates transcriptional repression of NFκB target gene expression (40). Also consistent with data from Berg et al. (15) comparing 3T3-L1 preadipocytes to mature adipocytes, LPS activation of NFκB was substantially attenuated in human adipocytes, compared with preadipocytes in our study. However, constitutive NFκB activation found in 3T3-L1 adipocytes was absent in our human adipocyte cultures (15).

The mechanism by which LPS signals to its downstream targets in adipocytes has yet to be clearly established. We showed in Fig. 3D that TLR4 mRNA appears to be constitutively expressed in both preadipocytes (AD0) and adipocytes (AD90). In contrast, TLR2 expression was robustly induced by LPS, particularly in preadipocytes, which could be partially responsible for the higher proinflammatory responsiveness of preadipocytes to LPS (Fig. 3C). In macrophages, LPS signals through TLR4 and TLR2. TLR4 and TLR2 activate at least two downstream pathways, PI3K/AKT and IL-1 receptor-associated kinase 1/ TNF receptor-associated factor 6/NFκB-inducing kinase/IKK pathway, which depend on adaptor protein MyD88 (13). Additionally, Covert et al. (41) suggested that MyD88-independent, but interferon-regulatory factor 3-dependent, pathways are involved in LPS activation of NFκB. Based on their work in macrophages, we used specific inhibitors to block potential pathways involved LPS signaling. Consistent with these data, activation of the MAPK pathway, including ERK and JNK, and NFκB were critical for LPS-induced cytokine expression in our cultures.
PI3K/AKT pathway appeared to play a minor role judged by TNF-α gene expression, implicating IL-1 receptor-associated kinase 1/TNF receptor-associated factor 6/NFκB-inducing kinase pathway may be the major signaling pathway by which LPS induces cytokine synthesis in human (pre)adipocytes. However, the role of MyD88-independent pathways in this study was not examined.

Proposed model

Adipose tissue is a source of mediators of inflammation and insulin resistance. Factors suggested to cause obesity-induced inflammation and insulin resistance include dietary fatty acids (i.e. trans fats and/or saturated fats), circulating free fatty acids, adipokines, and stress-induced hormones and/or viral/bacterial infection. Currently a chicken-or-egg debate is being waged concerning which factors associated with obesity initiate the inflammatory cascade that promote insulin resistance, i.e. do enlarged and/or inflamed adipocytes instigate inflammation that leads to insulin resistance, or do nonadipocytes (e.g. immune cells, preadipocytes) initiate the inflammatory cascade that promotes insulin resistance? In either case, we demonstrated by inducing acute inflammation with LPS that human preadipocytes, rather than adipocytes, are the primary source of LPS-induced proinflammatory cytokines/chemokines in these cultures that lack macrophages (Figs. 1–3). Clearly human preadipocytes transmit paracrine signals to neighboring adipocytes that suppress glucose uptake (Fig. 4) and PPARγ activity (Fig. 5) via NFκB and MAPK signaling (Figs. 6 and 7). Based on these data, we propose a working model in Fig. 8 in which LPS initiates proinflammatory signaling through TLRs primarily in preadipocytes, which triggers activation of NFκB, MAPK, and PI3K pathways resulting in cytokine (i.e. TNF-α, IL-6) and chemokine (e.g. IL-8, MCP-1) production in preadipocytes. These cytokines/chemokines, in turn, activate their cognate cell surface receptors on both adipocytes and preadipocytes, further augmenting cytokine production. In adipocytes, cytokine/chemokine activation of NFκB, MEK/ERK, and JNK leads to decreased PPARγ phosphorylation, thereby attenuating PPARγ target gene expression and insulin-stimulated glucose uptake. We also speculate, given the robust LPS induction of MCP-1 in preadipocytes, that human preadipocytes are involved in the recruitment of monocytes to adipocytes, thereby augmenting the inflammatory cascade.

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