Bacterial endotoxin/lipopolysaccharide elicits inflammatory responses and also elevates circulating levels of free fatty acids (FFAs) and impairs insulin sensitivity. Serum FFA elevation in acute endotoxemia has long been thought to be due to endotoxin dysregulating lipid disposal and counterregulatory hormones and cytokines. Here, we investigated the direct lipolysis effect of endotoxin in rodents and in isolated primary adipocytes. Endotoxin increases lipolysis in vivo in adipose tissues, elevates circulating FFA level, induces insulin resistance in rats, and directly stimulates chronic lipolysis in vitro in adipocytes. The lipolytic action of endotoxin is mediated via its lipid A moiety and is blocked by anti-endotoxin peptides. Neither adipocytokine secretion nor nuclear factor-κB activation is involved in endotoxin-induced lipolysis. Different from catecholamine, endotoxin stimulates lipolysis without elevating cAMP production and activating protein kinase A and protein kinase C. Instead, endotoxin induces phosphorylation of Raf-1, MEK1/2, and ERK1/2. Upon inhibition of ERK1/2 but not JNK and p38 MAPK, endotoxin-stimulated lipolysis ceases. Endotoxin causes perilipin down-regulation and phosphorylation and increases the activity and protein levels of hormone-sensitive lipase and adipose triglyceride lipase but does not induce hormone-sensitive lipase translocation to intracellular lipid droplets. In TLR4 (Toll-like receptor 4)-deficient mice and adipocytes, endotoxin fails to increase in vivo and in vitro lipolysis. These findings suggest that endotoxin stimulates lipolysis via TLR4 and ERK1/2 signaling in adipocytes. The lipolytic action of endotoxin liberates FFA efflux from adipocytes to the bloodstream, which is a possible basis for systemic FFA elevation and insulin resistance in endotoxemia or Gram-negative bacterial infection.

Endotoxin/lipopolysaccharide (LPS) is a membrane component of Gram-negative bacteria that consists of three parts: a core polysaccharide, the repeating O-antigen structures, and lipid A. LPS binds and activates TLR4 (Toll-like receptor 4) on mammalian cells, which ensures transmembrane signal transduction (1, 2). During bacterial infection, endotoxin elicits immune and inflammatory responses that can result in a fatal shock syndrome (1) and also causes a series of metabolic alterations (3), such as accelerated energy expenditure, elevated serum levels of free fatty acids (FFAs), hypertriglyceridemia, and impaired insulin action in humans (4, 5) and rodents (6, 7). The dysregulated disposal of metabolic substances and level of counterregulatory hormones and cytokines, such as catecholamines, glucocorticoids, interleukins, and tumor necrosis factor-α (TNF-α) has long been thought to be responsible for these metabolic alterations in acute endotoxemia (3–6). However, the cellular basis for endotoxin-mediated metabolic changes, particularly for the elevated level of circulating FFA, is incompletely understood.

Triglyceride hydrolysis (lipolysis) in adipocytes produces glycerol and FFA. Due to the lack of glycerol kinase in normal adipose cells (8, 9), glycerol cannot be reutilized for triglyceride resynthesis, but in company with FFA, it is released to the plasma. Regulation of circulating FFA concentrations depends mainly on the lipolysis of adipocytes in response to various hormones. Catecholamines stimulate lipolysis via elevating cellular cAMP level and activating cAMP-dependent protein kinase A (PKA) (10). Cytokines such as TNF-α and interleukins induce chronic lipolysis (11–13). Although catecholamines and cytokines are increased in the circulation in endotoxemia (3–6), they seem not to be the only lipolytic stimulators that account for elevated level of serum FFA. For example, adrenergic or interleukin-1 receptor antagonists and TNF-α-neutralizing antibodies cannot sufficiently prevent increases in serum levels of FFA and triglycerides in endotoxemic rats (6, 7), which suggests that other factors or perhaps LPS itself could stimulate adipose lipolysis to liberate FFA efflux to the bloodstream. Early studies showed that hormone-stimulated lipolysis may be

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4 The abbreviations used are: LPS, lipopolysaccharide; ATGL, adipose triglyceride lipase; BNEP, synthetic bacterial endotoxin-neutralizing peptide; ERK, extracellular signal-regulated kinase; MEK, extracellular signal-regulated kinase; HSL, hormone-sensitive lipase; iKB, inhibitory iKB; IkB, iKB kinase; NFκB, nuclear factor-κB; PDTC, pyrrolidine dithiocarbamate; FFA, free fatty acid; HSL, hormone-sensitive lipase; iKBa, inhibitory iKB; IkBa, iKB kinase; NFκBa, nuclear factor-κBa; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; TNF-α, tumor necrosis factor-α; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase.
enhanced in adipocytes isolated from LPS-injected rats (14, 15) or in adipocytes briefly exposed to endotoxin (16); in addition, LPS seemed to elevate lipolysis in the fragments of adipose tissues around lymph nodes of guinea pigs (17). These observations imply that endotoxin could directly act on adipose tissues to modulate lipolysis. Nevertheless, other studies showed that short term (2-h) stimulation with LPS could not induce lipolysis in pig adipocytes (18), and incubation with a conditioned medium of LPS-stimulated macrophages increased lipolysis, but the effect was thought to result from LPS-elevated TNF-α secretion in the medium (19). Therefore, the direct effect of LPS in adipose lipolysis and its association with an elevated level of serum FFAs in endotoxemia remain unclear.

In an effort to understand the underlying mechanism of LPS-induced FFA elevation, this study was designed to investigate the in vivo and in vitro lipolytic actions of LPS in primary adipocytes and adipose tissues in rodents. LPS directly stimulated chronic lipolysis, which did not rely on elevation of cellular cAMP level and activation of PKA, PKC, or nuclear factor-κB (NFκB). Instead, LPS-stimulated lipolysis was mediated by TLR4 and extracellular signal-regulated kinase-1/2 (ERK1/2) signaling and was involved in down-regulation and phosphorylation of perilipins and increased activity and protein levels of HSL and adipose triglyceride lipase (ATGL). In TLR4-deficient adipocytes and mice, LPS failed to activate lipolysis and was unable to elevate serum FFA level. These findings reveal a novel pathway of LPS stimulating lipolysis via TLR4 and ERK1/2 signaling and increasing FFA efflux from adipocytes to the bloodstream, thus elevating systemic FFA levels and thereby impairing insulin sensitivity in acute endotoxemia or Gram-negative bacterial infection.

**EXPERIMENTAL PROCEDURES**

**Materials**—LPS from *Escherichia coli* serotype O55:B5 and O127:B8, detoxified LPS from *E. coli* O127:B8, diphosphoryl lipid A derived from *E. coli* F583 (Rd mutant), and phenol red-free Dulbecco’s modified Eagle’s medium were from Sigma. LPS from *Salmonella* Minnesota rough mutant strain 595 was from List Biological Laboratories (Campbell, CA). Antibodies against rat perilipin and HSL were generous gifts from Dr. Londo at the United States National Institutes of Health (Bethesda, MD).

**Animals**—The animal studies were approved by the Animal Care and Use Committee of Peking University Health Science Center. C3H/HeJ mouse is a TLR4 mutant because of a point mutation within the coding region of the *Tlr4* gene, whereas the C3H/HeN substrain mouse is TLR4 normal (20) and served as the control. The C3H/HeJ and C3H/HeN mice were from the Jackson Laboratory (Bar Harbor, ME). Male mice and male

**FIGURE 1. In vivo and in vitro lipolytic actions of LPS.** A and B, the fed (*n* = 5) and fasted (*n* = 5) rats were sacrificed 12 h after the intravenous injection with saline or LPS (*E. coli* O127:B8; 100 μg/kg, body weight). Serum concentrations of FFA, glycerol, triglycerides (TAG), and total cholesterol (TC) were measured (A). The epididymal adipose tissue fragments were isolated from the rats injected with saline or LPS and incubated for 60 min, and then the ex vivo release of glycerol in the medium was determined and served as an index of lipolysis (B). The data are mean ± S.E. of five animals. *, *p* < 0.05; **, *p* < 0.01 versus saline. C, the epididymal adipose tissues isolated from normal rats were incubated for 6 or 24 h in the presence or absence of 1 μg/ml LPS. The glycerol release in the medium was assayed. D, primary adipocytes isolated from normal rats were incubated for 24 h with LPS at 0, 0.01, 0.1, 1, and 10 μg/ml. The glycerol released in the medium was assayed in triplicate. LPS stimulated lipolysis in a dose-dependent manner. E and F, LPS at 1 μg/ml increased FFA (E) and glycerol (F) release from primary adipocytes in a time-dependent manner. The results are mean ± S.E. and represent at least four separate experiments. **, *p* < 0.01 versus control. PCV, packed cell volume.
Sprague-Dawley rats (180–220 g) were injected intravenously with LPS or saline and sacrificed at 12 h after administration. The blood samples were collected for bioassays with the use of commercial kits. Subsequently, epididymal fat tissues were isolated and used for lipolysis assay.

Isolation and Culture of Primary Adipocytes—Adipocytes were isolated from epididymal fat pads of normal or injected Sprague-Dawley rats (150–180 g) and C3H/HeJ and C3H/HeN mice according to our laboratory method (21, 22). The minced fat pads were digested in Krebs-Ringer solution containing 0.75 mg/ml type I collagenase, 200 nM adenosine, 25 mM Hepes, pH 7.4, and 1% defatted bovine serum albumin. After incubation for 40 min at 37 °C in a water bath with shaking at 100 cycles/min, adipocytes were filtered through a nylon mesh and washed three times with warmed phenol red-free Dulbecco’s modified Eagle’s medium containing 200 nM adenosine. Adipocytes floating on the top of the tube were packed by centrifuging at 200 × g for 3 min. The packed cell volume of adipocytes was determined (21, 22). Adipocytes were preincubated in an atmosphere of 5% CO₂ at 37 °C for 1 h prior to treatments.

Fatty Acid Assay—The FFA content in the culture medium was determined by colorimetric assay as described (13). Briefly, 50 μl of culture medium was mixed with 120 μl of isooctane and 80 μl of cupric acetate-pyridine and centrifuged for 10 min at 12,000 × g at room temperature. The upper organic phase (80 μl) was mixed with 180 μl of the color development reagent, and the absorbance at 540 nm was measured.

Glycerol Assay—Glycerol content in culture medium served as an index of lipolysis and was determined at the absorption at 490 nm (13, 21) with the use of a colorimetric assay kit from Applygen Technologies (Beijing, China). Lipolysis data were expressed as μmol of glycerol or FFA/ml of packed cell volume of adipocytes.

Immunoblotting—Adipocytes were packed and lysed in sample buffer containing 62 mM Tris-HCl, pH 6.8, 4% SDS, 0.1 mM sodium orthovanadate, and 50 mM sodium fluoride (23, 24). After centrifugation at 12,000 × g for 10 min at 4 °C, the lysate was collected, and the protein content was determined with use of a bicinchoninic acid protein assay kit (Applygen Technologies, Beijing, China). For immunoblot detection of phosphorylated perilipin, a low bis concentration polyacrylamide gel (10% acrylamide and 0.07% N,N-methylene-bisacrylamide) was used, which provides better resolution of proteins in the 60–70-kDa range (21). After electrophoresis and transferring, nitrocellulose membranes were blocked with nonfat milk and immunoblotted with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The blots were developed with the use of enhanced chemiluminescence detection reagents (Applygen Technologies, Beijing, China). If required, the blots were stripped and reprobed. Densitometric analysis of protein bands involved the use of NIHImage software.

cAMP Radioimmunoaasay—According to our previous method (13), adipocytes (20 μl) were lysed in 150 μl of ice-cold
buffer containing 50 mM Tris-HCl, pH 7.4, and 1 mM EDTA. After vortexing and centrifugation at 12,000 × g for 15 min at 4 °C, 90 μl of cytosol fraction was mixed with 30 μl of 40% trichloroacetic acid. The tubes were incubated on ice for 5 min, vortexed, and centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was collected and used for cAMP assay according to the protocol of the commercial 125I-radioimmunoassay kit (Isotope Laboratory of Shanghai University of Chinese Medicine, Shanghai, China). Cellular cAMP concentration was expressed as pmol of cAMP/mg of proteins.

**Lipase Activity of [3H]Triolein Hydrolysis**—150 μl of packed adipocytes or 150 mg of minced adipose tissues was homogenized in 150 μl of cold buffer containing 20 mM Tris-HCl, pH 7.4, and 1 mM EDTA. The homogenate was centrifuged at 15,000 × g for 15 min at 4 °C. The cytosol fraction (150 μl) was added to the reaction consisting of triolein, [3H]triolein, and arabic gum. The reaction was incubated at 30 °C for 60 min, when the adipose lipases hydrolyze emulsified [3H]triolein to produce [3H]oleic acid. The released [3H]oleic acid was partitioned in methanol/chloroform/heptane solvent and separated to a potassium carbonate-boric acid buffer (25) then quantified by a liquid scintillation counter. The radioactive quantification represented lipase activity and was normalized to mg of tissue protein or ml of PVC of adipocytes.

**Differentiation of Rat Preadipocytes and Immunostaining of HSL**—Preadipocytes were isolated from rat epididymal fat pads and differentiated into adipocytes for 3 days in serum-free Dulbecco’s modified Eagle’s medium/F-12 (1:1) supplemented with 5 μg/ml insulin, 33 μM biotin, and 200 pM triiodothyronine, as we previously described (21, 22). The differentiated adipocytes (day 5) were stimulated with LPS. Immunostaining of HSL was performed according to our prior method. The cells were fixed and immunostained with rabbit antisera against HSL and then with fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (22, 24). Immunofluorescent signaling was observed with use of a Nikon Eclipse TE2000-U microscope.

**Statistical Analysis**—Data are expressed as mean ± S.E. One-way analysis of variance Tukey’s test or Student’s t test was used for statistical analysis. p < 0.05 was considered statistically significant.

**RESULTS**

**In Vivo and in Vitro Lipolytic Effects of LPS**—We first investigated the effect of LPS on adipose lipolysis and its relation with circulating lipids alteration in rats. The rats were *ad libitum* fed (*n* = 5) or fasted (*n* = 5) for 8 h and then injected intravenously with saline or LPS (*E. coli* O127:B8, 100 μg/kg body weight) and were sacrificed 12 h after injection. The serum concentrations of triglycerides and total cholesterol were increased in fasted rats but were decreased in *ad libitum* fed rats treated with LPS. LPS treatment elevated the serum concentrations of FFA and glycerol slightly in fasted rats but
markedly in fed rats (Fig. 1A). After a 60-min incubation, the rate of glycerol release was increased in epididymal adipose tissues isolated only from LPS-treated rats but not from saline-injected rats (Fig. 1B), indicating that LPS increased lipolysis in vivo in adipose tissues. Next, we determined whether LPS directly stimulates lipolysis ex vivo in adipose tissues and in primary adipocytes isolated from normal rats. Epididymal adipose tissue pieces stimulated by 1/20 g/ml LPS showed a slight increase of glycerol release at 6 h but a significant 2-fold increase at 24 h (Fig. 1C). Primary rat adipocytes were preincubated for 24 h with 1/20 µg/ml LPS or lipid A or with 1.5 µM PMA. Adipocytes were then washed and incubated for 60 min in fresh medium. Glycerol release in the media was assayed and expressed as mean ± S.E. of three separate experiments. **, p < 0.01 versus control; ††, p < 0.01 versus isoproterenol or phorbol myristate acetate. PCV, packed cell volume.

FIGURE 4. cAMP/PKA and PKC signaling are not involved in lipolytic action of LPS or lipid A. A, cAMP 125I-radioimmunoassay. Rat adipocytes were stimulated with 1 µg/ml LPS for 24 h or with 1 µM isoproterenol (ISO) for 30 min. The cytosolic fractions of lysed adipocytes underwent cAMP measurement with 125I-radioimmunoassay. The pmol/mg cAMP concentrations were normalized against cytosolic protein content and expressed as mean ± S.E. of three experiments in sextuplicate. B and C, PKA activity. Adipocytes were stimulated with 1 µM isoproterenol for 30 min (B) or with 1 µg/ml LPS for 0.5, 2, 6, and 24 h (C). Adipocyte lysates underwent immunoblotting analysis (IB) with a primary antibody against PKA phosphosubstrate motif (RRX(S/T)). The blot was stripped and reprobed with an anti-actin antibody. D, PKA inhibitor did not inhibit the lipolysis response to LPS. Adipocytes were preincubated for 1 h with 20 µM H89 and stimulated with 1/20 g/ml LPS for 24 h or with 1/20 µM isoproterenol for 30 min. Glycerol release in the medium was assayed, and the data are expressed as percentage of the control value. E–G, PKC inhibition did not attenuate the lipolysis induced by LPS (E) or lipid A (F) but suppressed the lipolysis stimulated by phorbol myristate acetate (PMA), a PKC activator (G). Adipocytes were preincubated for 1 h with 50 nM chelerythrine (CHE) or 5 µM Ro-31-8220 and then treated for 24 h with 1 µg/ml LPS or lipid A or with 1.5 µM PMA. Adipocytes were then washed and incubated for 60 min in fresh medium. Glycerol release in the media was assayed and expressed as mean ± S.E. of three separate experiments. **, p < 0.01 versus control; ††, p < 0.01 versus isoproterenol or phorbol myristate acetate. PCV, packed cell volume.
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with three different species of LPS: from the Salmonella minnesota rough mutant strain 595 and from E. coli serotype O55:B5 and O127:B8. These LPS species stimulated glycerol release to a similar magnitude (Fig. 2A). In contrast, detoxified LPS (E. coli O127:B8) devoid of the active lipid A part failed to increase glycerol and FFA release, whereas diphosphoryl lipid A (E. coli F583) alone sufficiently stimulated robust lipolysis similar to that with LPS-induced lipolysis (Fig. 2, B and C). Polymyxin B (Sigma) is a peptide antibiotic that can bind and neutralize LPS (26). BNEP (Hybio Engineering Co., Shenzhen, China) is a synthetic endotoxin-neutralizing peptide consisting of the 148–161 amino acids of bactericidal permeability-increasing protein (27). To investigate the specificity of the lipolytic action of LPS, adipocytes were preincubated for 1 h with polymyxin B or BNEP and then stimulated for 24 h with LPS or TNF-α. Polymyxin B at 500 units/ml attenuated LPS-induced glycerol release by 71%, and at 1,000 units/ml, it completely blocked the lipolysis (Fig. 2D). Similarly, BNEP efficiently abrogated the lipolysis response to LPS but did not affect TNF-α-induced lipolysis (Fig. 2E).

Adipokine or NFκB Activation Is Not Involved in LPS-stimulated Lipolysis—An early study showed that the conditioned medium of LPS-stimulated macrophages increased lipolysis, but this effect may have resulted from increased TNF-α secretion from LPS-stimulated microphages (19). We therefore investigated whether the cytokine secretion from adipocytes accounts for LPS-stimulated lipolysis. The conditioned medium and parallel control medium were collected after primary adipocytes were incubated for 24 h with or without 100 ng/ml LPS, and glycerol accumulation was monitored (Fig. 3A). Fresh primary adipocytes were then incubated for 24 h in the conditioned and control media in the presence or absence of BNEP to neutralize the original LPS residues. Then the media were removed, and the adipocytes were incubated for another 60 min in the fresh medium. In the conditioned medium with LPS, glycerol release was elevated by 44%, but this effect was completely abolished when the original LPS in the conditioned medium was neutralized by BNEP (Fig. 3B), which suggests that adipocytokine production of LPS-stimulated adipocytes was not sufficient to induce detectable lipolysis. The parallel control medium without LPS did not affect lipolysis (Fig. 3B). Since NFκB activation modulates TNF-α-induced lipolysis (28), we examined whether NFκB signaling participates in LPS-stimulated lipolysis. Immunoblot analysis showed that the addition of 1 μg/ml LPS slightly promoted phosphorylation of 1κB kinase α/β (Ikkα/β) and inhibitory 1κB α (IkBα), two upstream kinases responsible for NFκB activation (Fig. 3C). Adipocytes were pretreated for 1 h with the NFκB inhibitors, pyrrolidine dithiocarbamate (PDTC), or SN50, and incubated for 24 h with 1 μg/ml LPS. PDTC at 1, 10, or 100 μM did not inhibit glycerol release (Fig. 3D) and FFA (Fig. 3E) release from LPS-stimulated adipocytes.
PKC activator, 1.5 μM phorbol myristate acetate (Fig. 4G), as a positive control.

**ERK1/2 Activation Mediates Lipolysis Stimulation by LPS and Lipid A**—ERK1/2 activation is another important event during lipolysis. Immunoblot results showed that the addition of 1 μg/ml LPS in adipocytes caused a rapid phosphorylation of Raf-1, an upstream serine/threonine kinase in the mitogen-activated protein kinase (MAPK) cascade (Fig. 5A). Subsequently, LPS increased the phosphorylation of MEK1/2 (ERK1/2 MAPK kinase) and ERK1/2 (Fig. 5, A and B). By contrast, LPS did not alter c-Jun-NH$_2$-terminal kinase (JNK) phosphorylation but slightly decreased the level of native JNK and phosphorylated stress/cytokine-activated p38 MAPK (Fig. 5C). MEK-ERK1/2 inhibition with PD98059 suppressed basal and LPS-induced ERK1/2 phosphorylation (Fig. 5B). Adipocytes were preincubated for 1 h with different MAPK inhibitors and then stimulated for 24 h with 1 μg/ml LPS or lipid A. Two MEK-ERK1/2 inhibitors, PD98059 and U0126, slightly decreased basal lipolysis in unstimulated adipocytes but greatly inhibited 24-h glycerol accumulation (Fig. 5D) and 1-h glycerol release (Fig. 5E) in adipocyte culture with LPS or lipid A. In contrast, SP600125 and SB203580, the MAPK inhibitors specific for JNK or p38, did not suppress lipolysis stimulation by LPS (Fig. 5D).

**Regulation of LPS on Lipid Droplet-associating Protein Perilipins**—Perilipins coat the lipid droplets in adipocytes (31). Down-regulation (13, 32) and/or phosphorylation (21, 33) of perilipins facilitate lipolysis. After a 24-h incubation with LPS or lipid A, perilipin protein level was significantly down-regulated in isolated adipose tissues (Fig. 6A). When primary adipocytes were incubated for 16 h with LPS at 0.01, 0.1, or 1 μg/ml, the level of perilipins was gradually decreased (Fig. 6B). MEK1/2-ERK1/2 inhibition with 25 μM PD98059 or 10 μM U0126 but not PKA inhibitor H89 or proteasome inhibitor MG132 attenuated LPS-mediated perilipin down-regulation. D, immunoblot analysis of perilipin phosphorylation by SDS-PAGE on low bis concentration polyacrylamide gels. Primary (upper image) and differentiated (lower image) adipocytes were incubated for 1 μg/ml LPS. Phosphorylated perilipin (67 kDa) migrated more slowly than its native species (65 kDa) on low bis concentration polyacrylamide gels. Note that for better showing of the shifted bands of the phosphorylated perilipins, the film was overexposed, and the down trend of native perilipin bands could be artificially masked. In primary adipocytes, long-term stimulation with LPS at 24–32 h slightly promoted perilipin phosphorylation, as indicated by a weak band shift from 65 kDa (native) to 67 kDa (phosphorylated) (Fig. 6C). Next, we examined whether LPS treatment induces the phosphorylation of perilipins. In primary adipocytes, long-term stimulation with LPS at 24–32 h slightly promoted perilipin phosphorylation, as indicated by a weak band shift from 65 (native) to 67 kDa (phosphorylated) on specifically prepared low bis concentration polyacrylamide gels (21) (Fig. 6D, upper image). However, in differentiated rat adipocytes, LPS caused an obvious switch of native perilipins (65 kDa) to the phosphorylated species (67 kDa) (Fig. 6D, lower image); this effect was...
only slightly inhibited by PKA inhibitor H89 (data not shown). Consistent with increased perilipin phosphorylation, LPS-stimulated lipolysis was more robust in differentiated adipocytes than in primary adipocytes (Fig. 6E).

**LPS Promotes Activity and Protein Level of Adipose Lipases but Does Not Induce HSL Translocation**—HSL and ATGL are two major lipases in adipocytes (34, 35). We measured lipase activity by determining the hydrolysis rate of [3H]triolein in the extracts of adipose tissues (A) or primary adipocytes (C). The data are mean ± S.E. of three or four separate experiments. **, p < 0.01 versus control; †, p < 0.05 versus LPS. Protein levels of HSL and ATGL in adipose tissues (B) and in adipocytes (D and E) were detected by immunoblotting analysis. Rat differentiated adipocytes were treated for 24 h with 1 μg/ml LPS or stimulated for 30 min with 1 μM isoproterenol as a positive control. The adipocytes were fixed and immunostained with anti-HSL antibodies and fluorescein isothiocyanate-conjugated second antibodies (F). The dark circles inside the cells represent intracellular lipid droplets. HSL fluorescence appeared throughout the cytosol but did not surround the lipid droplets in LPS-treated adipocytes; HSL was translocated from the cytosol to the lipid droplets surface on isoproterenol stimulation (F).

**TLR4 Mediates the in Vivo and in Vitro Lipolysis Response to LPS**—TLR4 is the endotoxin receptor responsible for transmembrane signaling transduction (1). To investigate whether TLR4 mediates LPS-induced lipolysis, we examined the lipolysis effects of LPS in vivo in the fasted TLR4 mutant C3H/HeJ mice and TLR4 normal C3H/HeN mice (20). The mice were sacrificed at 12 h after injection with LPS (E. coli O127:B8; 100 μg/kg body weight). LPS administration increased glycerol release in adipose tissue isolated from TLR4 normal mice but not TLR4 mutant mice (Fig. 8A), which indicates that TLR4 mutation blunted the in vivo lipolysis response to LPS. LPS treatment elevated the serum concentrations of FFA, glycerol, triglyceride, and glucose in TLR4 normal mice but not in TLR4 mutant mice (Fig. 8B and C), which is consistent with lipolysis deficiency in adipose tissue. Finally, we examined the lipolytic action of LPS in isolated mouse primary adipocytes. Isoproterenol (Fig. 9A) and TNF-α (Fig. 9B) induced robust lipolysis in adipocytes isolated from the ad libitum-fed TLR4 normal and TLR4 mutant mice. In contrast, LPS or lipid A stimulated lipolysis in adipocytes isolated from TLR4 normal mice but not TLR4 mutant mice (Fig. 9C), which indicates that TLR4 deficiency muted the in vitro lipolysis response to LPS or lipid A. Immunoblot results showed that LPS treatment resulted in ERK1/2 phosphorylation, perilipin down-regulation, and HSL or ATGL up-regulation only in TLR4 normal
DISCUSSION

Although acute bacterial endotoxemia is associated with systemic FFA elevation, the effect of the LPS response in adipose lipolysis is unknown. In this study, we show that LPS directly stimulates lipolysis in adipocytes through TLR4 and MEK1/2-ERK1/2 pathways, which increases FFA efflux to the bloodstream and therefore could be a cellular basis of systemic FFA elevation in acute endotoxemia. In rats and mice, LPS administration increased FFA and glycerol release from adipose tissues, thereby elevating circulating FFA levels and causing hyperlipidemia. In isolated primary adipocytes, LPS efficiently stimulated robust lipolysis. Different from the effect of catecholamine (10) but similar to that of TNF-α (12, 13, 32), LPS stimulated chronic lipolysis that was detectable at 6–8 h. This “chronic” feature may explain why short term (2-h) stimulation with LPS could not increase lipolysis in adipocytes, as noted previously (18).

The chemical structure of the polysaccharide part of LPS varies greatly, but the lipid A fraction is highly conserved among different bacterial species. Lipid A serves as the active moiety for many biological effects of endotoxin (1, 2). In our study, three kinds of LPS species, different in categories and serotypes, stimulated lipolysis to a similar magnitude, which implicated that the polysaccharide and O-antigen chains of LPS were not the key parts responsible for lipolysis. Indeed, lipolysis was sufficiently induced by pure lipid A alone but not by the detoxified LPS devoid of the lipid A moiety. Anti-endotoxin peptides, polymyxin B and BNEP, which neutralize endotoxin by binding and sequestering lipid A (26, 27), completely blocked the lipolysis response to LPS but not to TNF-α. Thus, the lipid A is an essential moiety responsible for the specific lipolysis stimulation of LPS.

An early study showed that the conditioned medium of LPS-stimulated macrophages increased lipolysis in adipocytes, an effect thought to result from elevated TNF-α secretion by LPS, not from LPS itself, although this conditioned medium contained at least 0.05 μg/ml LPS (19). We observed that when original LPS residues in the conditioned medium of LPS-stimulated adipocytes were neutralized by the anti-endotoxin BNEP, the conditioned medium could not elevate lipolysis. A previous study showed that after a 24-h stimulation with 1 μg/ml LPS, interleukin-6 and TNF-α secretion...
was only 2 and 4.5 ng/ml, respectively, from J774 macrophages but was much lower (0.1 and 0.02 ng/ml, respectively) from differentiated 3T3-L1 adipocytes (36). These low levels of cytokines could not elicit any measurable lipolysis. In our system, each 0.5-ml reaction vial contained 25,000 adipocytes, which are too few to produce sufficient cytokines to stimulate lipolysis. NFκB activation could modulate TNF-α-induced lipolysis (28). However, to date, very little is known about the transcriptional machinery of NFκB in adipocytes. In differentiated 3T3-L1 adipocytes (compared with undifferentiated preadipocytes), the responsiveness of NFκB transcriptional activation is sensitive to TNF-α but surprisingly is insensitive to LPS (36), implying that NFκB signaling may not be critical for the LPS responses in mature adipocytes, especially in the primary adipocytes used in the present study. We showed that LPS-induced FFA and glycerol release could not be suppressed by the NFκB inhibitors, PDTC and SN50, although LPS slightly promoted Ikκα/β and IκBα phosphorylation. Therefore, neither adipocytokine nor NFκB signaling is involved in LPS-stimulated lipolysis in primary adipocytes.

Elevated level of cellular cAMP and activation of PKA and ERK1/2 are major events during lipolytic stimulation of catecholamine or TNF-α (10, 12, 13); PKC modulates lipolysis in a separate pathway (29, 30). Different from the effect with catecholamine and TNF-α, LPS stimulated lipolysis without increasing cAMP production and activating PKA. Further, Inhibition of PKA or PKC did not attenuate glycerol release induced by LPS or lipid A, so LPS-stimulated lipolysis is not regulated by PKA and PKC. In macrophages, ERK1/2 is the major signaling kinase phosphorylated and activated upon LPS stimulation (37, 38). In adipocytes, we observed that LPS promoted phosphorylation of Raf-1, MEK1/2, and ERK1/2 but not JNK and p38 MAPK. When LPS-induced ERK1/2 phosphorylation was inhibited by PD98059, LPS-stimulated lipolysis ceased. In contrast, inhibition of JNK or p38 MAPK failed to suppress the lipolysis response to LPS or lipid A. Thus, ERK1/2 is the major signaling pathway that mediates LPS-stimulated lipolysis.

Perilipins coat the intracellular lipid droplet surface as a barrier to restrict lipase hydrolysis of triglycerides (31, 33). Perilipin down-regulation (13, 32, 39) and/or phosphorylation (21, 33) could impair the barrier function, thus facilitating lipolysis (31). LPS or lipid A down-regulated perilipins in adipose tissues and adipocytes in our study. Although perilipins are degraded by ubiquitin/proteasome (23), LPS-mediated perilipin down-regulation was not restored by inhibiting proteasome and not affected upon PKA inhibition but, rather, prevented upon MEK1/2-ERK1/2 inhibition. Further, LPS stimulated phosphorylation of perilipin, as evidenced by a migration shift of the proteins from 65 to 67 kDa on low bis concentration polyacrylamide gels (21). This effect, parallel to increased lipolysis, occurred slightly in primary adipocytes but more obviously in differentiated adipocytes and seemed not to be blunted upon PKA inhibition (data not shown). These discrepancies are unclear but might be attributed to differences in cellular contexts between primary and differentiated adipocytes.
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In conclusion, this study reveals a novel pathway whereby bacterial endotoxin directly stimulates lipolysis to liberate FFA efflux from adipocytes, thus elevating FFA level in the bloodstream. This intrinsic strategy of endotoxin manipulating adipose FFA efflux to various tissues ensures accelerated energy expenditure in endotoxemia. However, an elevated level of circulating FFA induces dyslipidemia and insulin resistance (44); moreover, not only LPS (3, 4) but also fatty acids themselves (43) induce TLR4-mediated inflammation and impair insulin signaling. Therefore, endotoxin-elevated lipolysis and FFA efflux may also produce a feed-forward machinery to further enhance the inflammatory response, dyslipidemia, and insulin resistance in obesity and diabetes with Gram-negative bacterial infection. Further research is required to elaborate knowledge of this process.

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