Overexpression of Perilipin A and B Blocks the Ability of Tumor Necrosis Factor α to Increase Lipolysis in 3T3-L1 Adipocytes*

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Perilipins, a family of phosphoproteins, are specifically located at the surface of intracellular lipid (triaclylglycerol) droplets, the site of lipolysis. Stimulation of lipolysis in 3T3-L1 adipocytes by tumor necrosis factor α (TNF-α) is associated with a decrease in total cellular expression of perilipin A and B, consistent with the hypothesis that a decrease in perilipin protein expression is required for TNF-α-induced lipolysis. Adenovirus-mediated overexpression of perilipin A or B maintains perilipin protein levels on the lipid droplet and blocks TNF-α-induced lipolysis. In contrast, overexpression of perilipin A or perilipin B does not inhibit isoproterenol-stimulated lipolysis and does not alter the isoproterenol-induced migration of perilipins from the lipid droplet. These results provide the first evidence of how perilipin functions and suggest that TNF-α regulates lipolysis, in part, by decreasing perilipin protein levels at the lipid droplet surface.

The major form of stored energy in the body is triacylglycerol contained in white adipose tissue (1). In times of energy need, such as fasting and exercise, catecholamines rapidly activate cAMP-dependent protein kinase (PKA), resulting in hydrolysis of triacylglycerol to glycerol and free fatty acids (FFA). FFA are then used as substrates for ATP generation in diverse tissues. However, in certain pathophysiologic states such as obesity and diabetes, circulating FFA are chronically elevated (1, 2). It has been suggested that chronic elevations of plasma FFA promote insulin resistance (2). The cytokine tumor necrosis factor α (TNF-α) has been associated with insulin resistance (2). TNF-α induces lipolysis only after hours (6 h) of incubation (7, 8). Infusion of TNF-α in mice results in an increase in plasma FFA and systemic insulin resistance (9). In addition, TNF-α knockout mice exhibit lower circulating FFA and are protected from the insulin resistance of obesity (10). Thus, TNF-α-induced lipolysis may be one mechanism by which this cytokine is involved in the pathogenesis of the obese/diabetic state.

Whereas hormonal regulation of lipolysis is well described, little is known about the cellular and molecular mechanisms underlying this process. Lipolysis occurs at the surface of the intracellular lipid droplet where the perilipins, a family of phosphoproteins, are specifically located (11, 12). The predominant perilipins in adipocytes, perilipin (Peri) A and B, are rise by alternative RNA splicing from a single gene, generating predicted protein products of 57 and 46 kDa, respectively (13). Peri A is the most abundant of the perilipin proteins and is also the major phosphorylation target for PKA in adipocytes (12–14). Despite their abundance and unique location at the surface of the lipid droplet, the role of the perilipins in lipolysis is unknown.

In the present study we demonstrate that TNF-α decreases the protein levels of perilipins at the surface of the lipid droplet concurrent with an increase in adipocyte lipolysis. Adenovirus-mediated overexpression of Peri A and Peri B blocks the ability of TNF-α to increase lipolysis. These results are consistent with the model in which TNF-α increases lipolysis by decreasing the expression of perilipin proteins.

EXPERIMENTAL PROCEDURES

Materials—A polyclonal antibody that recognizes both Peri A and Peri B (Ab 46) was generated as described previously (8). A specific polyclonal anti-Peri A antibody was generated using peptide: PREK-3PARRVSDFFPFRPSVC (Ab PREK). Antibodies were subsequently affinity-purified and used for Western blotting (1:1500) and for immunofluorescence (1:500). Recombinant murine TNF-α was purchased from Genzyme (Cambridge, MA), and 3T3-L1 cells were from the American Type Culture Collection (Manassas, VA). All other chemicals were purchased from Sigma.

Generation of Recombinant Adenovirus—Plasmids pJM17 (15) and pACCMVpLpa (16) have been described. Plasmids pACCMV-β-Gal, pACCMV-GFP, pACCMV-Peri A, and pACCMV-Peri B were obtained by subcloning the cDNA for β-galactosidase (β-Gal) with an SV40 nuclear localization sequence, the Aequorea victoria green fluorescent protein, mouse Peri A (gift of C. Londos, A. R. Kimmel, and J. Gruia-Gray, National Institutes of Health) and mouse Peri B (cloned by screening a murine adipocyte cDNA library) into the multiple cloning site of the vector pACCMVpLpa. Recombinant, replication-defective adenoviruses (Ad) were generated by homologous recombination as described previously (15, 17, 18). Briefly, pACCMV constructs and the pJM17 vector, which contains a modified Ad5 genome, were cotransfected into Hek 293 cells (Microbiox, Toronto, ON, Canada), which provide in trans the missing adenoviral early region 1 functions of pJM17. Clonal viral stocks were isolated by single plaque purification, and their DNA was analyzed by polymerase chain reaction, amplified in Hek 293 cells, and purified and concentrated to 10^12 plaque-forming units/ml by CsCl ultracentrifugation.

Cell Culture and Adenovirus Treatment—3T3-L1 fibroblasts placed...
Role of Perilipins on Lipolytic Actions of TNF-α

in 12-well plates were cultured in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum and differentiated using standard protocols (19). Adipocytes were infected on day 5 of differentiation, when small lipid droplets were visible, with a multiplicity of infection of ~100 plaque-forming units/cell for 18 h at 37 °C. Four days later, infected adipocytes were serum-deprived overnight in Dulbecco’s modified Eagle’s medium with 2% bovine serum albumin. For most of the assays, recombinant adenosivirus expressing β-galactosidase was used as a control (Ad β-Gal). Adenoviruses expressing either green fluorescent protein or no exogenous proteins were also used as controls, and the results were identical to those observed with Ad β-Gal. The following morning, cells were treated as described under “Results.”

Lipolysis—Glycerol content of the incubation medium was determined using a colorimetric assay (GPO-Trinder, Sigma). Protein content was determined using the BCA protein assay (Pierce).

Western Analysis—Adipocytes were rinsed briefly with 1 ml of phosphate-buffered saline. Proteins were extracted as described previously (8), separated in 10% SDS-polyacrylamide gel electrophoresis, and electrothermally transferred to nitrocellulose membranes. Equivalent amounts of protein were loaded onto the gel for each treatment as described in figure legends. Proteins were detected with the ECL system (Amersham Pharmacia Biotech).

Immunofluorescence—For determination of perilipin fluorescence, cells were cultured in 35-mm coverslip bottomed dishes (MatTek Corp., Ashland, MA) and infected as described above. After treatment, adipocytes were fixed in 2% paraformaldehyde for 10 min at 25 °C, washed, and treated with either polyclonal antiserum that recognizes both Peri A and Peri B or a specific anti-Peri A polyclonal antibody (Ab. PREK, 1:500 dilution) and a donkey-anti-rabbit Cy-5 labeled antibody (Jackson Immunoresearch). Cy-5 fluorescence imaging was assessed by confocal microscopy as described (20).

Statistical Analysis—Results are expressed as the means ± S.E. Effects were assessed by using single-factor analysis of variance. Tukey’s honestly significant differences were used to make pairwise comparisons. All calculations were performed using SYSTAT version 7 for Windows (SPSS, Inc., Chicago, IL).

RESULTS

**TNF-α Decreases the Expression of Perilipins**—TNF-α begins to increase glycerol release by 6 h of treatment, and the rate of lipolysis is maximal (~6.5-fold increase) by 24 h (Fig. 1A) (7, 8). Therefore, we investigated the effects of TNF-α (10 ng/ml) on perilipin expression by incubating 3T3-L1 adipocytes with TNF-α for 24 h. Western blotting analyses of adipocyte lysates demonstrated ~70% reduction (densitometry analyses) in both Peri A and Peri B levels in response to TNF-α (Fig. 1B).

**Overexpression of Peri A and Peri B Blocks TNF-α-induced Lipolysis**—To ascertain the role of perilipin in TNF-α-induced lipolysis, we generated two recombinant type 5 adenoviruses expressing either Peri A or Peri B (Ad Peri A or Ad Peri B). 3T3-L1 adipocytes were infected with adenovirus control (Ad β-Gal) and Ad Peri A and Ad Peri B (see “Experimental Procedures”) and incubated in the presence or absence of 10 ng/ml TNF-α for 24 h. Western blotting of protein lysates revealed

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**Fig. 1.** TNF-α increases lipolysis and decreases perilipins expression. 3T3-L1 adipocytes were serum depleted and incubated in the absence (C, control) or presence of TNF-α (10 ng/ml) for 24 h. A, to measure lipolysis, media were collected and assayed for glycerol. Data are expressed as the means ± S.E. (n = 8 experiments performed in triplicate). *, p < 0.001. White bar, control; black bar, TNF-α (10 ng/ml). B, Western blotting analyses of the cell lysates using Ab 46, which recognizes Peri A and Peri B. Data shown are representative of four experiments.

**Fig. 2.** Maintenance of perilipin levels by overexpression blocks TNF-α-induced lipolysis. 3T3-L1 cells were transduced with Ad β-Gal or Ad Peri A or Ad Peri B (see “Experimental Procedures”). After serum depletion, 3T3-L1 adipocytes were treated with TNF-α (10 ng/ml) for 24 h. A, cell lysates were collected and analyzed by Western blotting using Ab 46 that recognizes Peri A and Peri B. Data shown are representative of four experiments. B, glycerol accumulated during 24 h. White bars, control; black bars, TNF-α (10 ng/ml). C, glycerol accumulated during the last 3 h of a 24-h treatment. Data are expressed as the means ± S.E. (n = 8 experiments performed in triplicate). *, p < 0.001, significantly different from adipocytes transduced with Ad β-Gal. White bars, control; black bars, TNF-α (10 ng/ml).
that Peri A and Peri B expression were increased by 5- and 49-fold, respectively, as compared with Ad β-Gal-transduced cells (Fig. 2A, lane 1 versus lanes 3 and 5). Importantly, in adipocytes transduced with Ad Peri A or Ad Peri B and subsequently incubated with TNF-α, the expression of Peri A or B was not decreased as compared with untreated cells (Fig. 2A, lane 3 versus lane 4 and lane 5 versus lane 6). In fact, perilipin levels were higher with TNF-α-treated cells than in controls. This is presumably because of up-regulation of the CMV promoter by TNF-α-induced activation of stress-activated mitogen-activated protein kinases (21–23).

To determine whether maintenance of elevated perilipin protein levels blocked TNF-α-stimulated adipocyte lipolysis, media from the treated adipocytes described in Fig. 2A were assayed for glycerol release, an index of lipolysis. In adipocytes overexpressing Peri A or Peri B, TNF-α-induced lipolysis was reduced by 80 and 72%, respectively (Fig. 2B). This decrease was observed even when the rate of lipolysis was maximal, i.e. during the last 3 h of a 24-h incubation (Fig. 2C).

Overexpression of Perilipins Does Not Alter Isoproterenol-induced Lipolysis—To determine whether maintenance of elevated perilipin protein levels blocked TNF-α-stimulated adipocyte lipolysis, media from the treated adipocytes described in Fig. 2A were assayed for glycerol release, an index of lipolysis. In adipocytes overexpressing Peri A or Peri B, TNF-α-induced lipolysis was reduced by 80 and 72%, respectively (Fig. 2B). This decrease was observed even when the rate of lipolysis was maximal, i.e. during the last 3 h of a 24-h incubation (Fig. 2C).

**Fig. 3.** Overexpression of perilipins does not block Iso-stimulated lipolysis. A, 3T3-L1 cells were transduced with Ad β-Gal or Ad Peri A or Ad Peri B (see “Experimental Procedures”). After serum depletion, 3T3-L1 adipocytes were treated with Iso (10 μM) for 3 h, and media were collected and assayed for glycerol. Data are expressed as the means ± S.E. (n = 8). White bars, control; black bars, Iso (10 μM). B, the electrophoretic pattern of Iso-induced hyperphosphorylation of Peri A (65 kDa) is maintained in cells overexpressing perilipins. Cell lysates of 3T3-L1 adipocytes transduced with Ad β-Gal, Ad Peri A, or Ad Peri B and treated as described above and analyzed by Western blotting using antibody against perilipins. Data shown are representative of four experiments.

**Fig. 4.** Differential localization of perilipins in nontransduced adipocytes following TNF-α and Iso treatment. 3T3-L1 adipocytes were incubated in the absence (panels A and D) or presence (panels B and E) of TNF-α (10 ng/ml) for 24 h or with Iso (10 μM) for 3 h (panels C and F) and either analyzed for perilipin immunofluorescence (IF) by confocal microscopy using Ab 46 (A, B, and C) or for DIC (D, E, and F). Data shown are representative of four experiments. LD, lipid droplets; n, nucleus. Magnification, 1000×.

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2 Y. Kang and A. S. Greenberg, unpublished data.
cells showing a punctate pattern and others a diffuse cytoplasmic immunostaining (Fig. 4, C and F). Although reducing perilipin levels at the surface of intracellular lipid droplets appears to be required for both TNF-α and Iso to stimulate lipolysis, this reduction is achieved by different mechanisms. TNF-α decreases the total amount of perilipin protein, whereas Iso does not.

To determine the subcellular localization of the perilipin proteins in adipocytes overexpressing Peri A, IF analyses were performed using antiserum specific for Peri A. Adipocytes overexpressing Peri A generated stronger fluorescence (Fig. 5, B, C, and D), relative to Ad β-Gal-transduced adipocytes (Fig. 5A). Overexpressed perilipins localized to the lipid droplet similar to the endogenous perilipin (compare Figs. 4A with 5B). In addition, perilipin immunostaining at the surface of the lipid droplet did not decrease in cells transduced with Ad Peri A that were treated with 10 ng/ml of TNF-α for 24 h (Fig. 5, B versus C). In contrast, perilipin immunostaining in Iso-treated cells was similar to that observed with endogenous perilipins (compare Figs. 5D with 4C). The pattern of immunofluorescence in adipocytes overexpressing Peri B was similar to that observed with overexpression of Peri A (data not shown). Thus, maintaining high levels of perilipin at the surface of the lipid droplet impairs the ability of TNF-α to increase lipolysis, whereas Iso-stimulated lipolysis and its apparent redistribution of perilipins to the cytoplasm are not altered.

**DISCUSSION**

The perilipin phosphoproteins are highly expressed in adipocytes and are located at the surface of the lipid droplet, the site of lipolysis in adipocytes. Despite the location of the perilipins, their role in lipid hydrolysis is unknown. Using TNF-α-induced lipolysis as a model of reduced perilipin expression in adipocytes, the experiments described in this paper demonstrate for the first time that Peri A and B can regulate the hydrolysis of triacylglycerol. We observed that a decrease in perilipin protein levels at the lipid droplet surface is associated with TNF-α-induced lipolysis. Adenovirus-mediated overexpression of Peri A and B blocked TNF-α-induced lipolysis in 3T3-L1 adipocytes, thus delineating a mechanism whereby this cytokine could increase the breakdown of triacylglycerol. Moreover, this effect is specific for TNF-α, because Iso-stimulated lipolysis was not altered when Peri A or B was overexpressed.

Our IF studies confirm that Peri A and B are located at the surface of lipid droplets in untreated cells and indicate that TNF-α and Iso-stimulated lipolysis are associated with reduced perilipin protein levels at the surface of the lipid droplet. TNF-α reduces total cellular perilipins and thus lipid droplet-associated perilipin protein levels while increasing adipocyte lipolysis. Overexpression of the perilipins prevents both the TNF-α-mediated reduction in perilipin protein accumulation at the surface of the lipid droplet and the ability of the cytokine to increase lipolysis. However, in Iso-treated adipocytes, the pattern of perilipin immunostaining is consistent with a reduction of perilipins at the lipid droplet surface and redistribution in the cell. Consistent with the confocal experiments, in cell fractionation studies, Iso induces a redistribution of the perilipins from the fat (fat cake) to the infranatant. PKA-mediated phosphorylation, secondary to Iso stimulation, is presumably required for the redistribution of the perilipin proteins. Based on these data we propose a model of lipolysis in which perilipin accumulation at the lipid droplet surface may limit lipid hydrolysis. TNF-α reduces perilipin protein levels at the lipid droplet surface, resulting in an increase in lipolysis. In contrast, Iso-induced phosphorylation of perilipins would cause relocation of the perilipins, potentially increasing the accessibility of stored triacylglycerol to lipases. Further studies are necessary to extend our understanding of the role of perilipins in catecholamine-stimulated lipolysis and to delineate their mechanisms in lipid hydrolysis.

The thiazolidinediones (TZDs) are a class of antidiabetic agents that, by unknown mechanisms, ameliorate insulin resistance and type II diabetes mellitus. One suggested mechanism is that the TZDs antagonize the actions of TNF-α, resulting in decreased plasma FFA levels (9, 25). Increased plasma FFA have been suggested to induce systemic insulin resistance (2). We have previously demonstrated that the TZDs partially blocked both the TNF-α-mediated reduction of Peri A and TNF-α-induced lipolysis (8). The results of this prior study are consistent with the data presented in this paper and suggest a possible role for the perilipins and TNF-α-induced lipolysis in the pathogenesis of the obese-diabetic state.

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