Differential roles of CIDEA and CIDEC in insulin-induced anti-apoptosis and lipid droplet formation in human adipocytes

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Abstract Both insulin and the cell death-inducing DNA fragmentation factor-α-like effector (CIDE) family play important roles in apoptosis and lipid droplet formation. However, regulation of the CIDE family by insulin and the contribution of the CIDE family to insulin actions remain unclear. Here, we investigated whether insulin regulates expression of the CIDE family and which subtypes contribute to insulin-induced anti-apoptosis and lipid droplet formation in human adipocytes. Insulin decreased CIDEA and increased CIDEC but not CIDEB mRNA expression. Starvation-induced apoptosis in adipocytes was significantly inhibited when insulin decreased the CIDEA mRNA level. Small interfering RNA-mediated depletion of CIDEA inhibited starvation-induced apoptosis similarly to insulin and restored insulin deprivation-reduced adipocyte number, whereas CIDEC depletion did not. Lipid droplet size of adipocytes was increased when insulin increased the CIDEC mRNA level. In contrast, insulin-induced enlargement of lipid droplets was markedly abrogated by depletion of CIDEC but not CIDEA. Furthermore, depletion of CIDEC, but not CIDEA, significantly increased glycerol release from adipocytes. These results suggest that CIDEA and CIDEC are novel genes regulated by insulin in human adipocytes and may play key roles in the effects of insulin, such as anti-apoptosis and lipid droplet formation.—Minoru, I., M. Nagasawa, T. Hara, T. Ide, and K. Murakami. **Differential roles of CIDEA and CIDEC in insulin-induced anti-apoptosis and lipid droplet formation in human adipocytes.** ***J. Lipid Res.*** **2010.** 51: **1676–1684.**

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White adipose tissue (WAT) is the key organ for energy homeostasis. It also releases metabolites into the circulation and adipokines with systemic effects on insulin sensitivity and fuel partitioning in muscles and other tissues (1, 2). On the other hand, excessive accumulation of WAT in obesity contributes to severe diseases, such as type 2 diabetes, hypertension, cardiovascular disease, dyslipidemia, arthritis, and several types of cancer (3). WAT mass is determined by both number and size of adipocytes (4, 5) regulated by cell differentiation, apoptosis, and lipid formation (2, 6–10). Insulin is known to induce adipocyte differentiation (11, 12), inhibit apoptosis (13, 14), and increase lipogenesis (15, 16) in adipocytes. Hyperinsulinemia is associated with weight gain in humans (17–22). A study of adipose tissue-selective insulin receptor deficiency in mice demonstrated that insulin signaling in adipocytes is critical for the development of obesity (23). Insulin depletion leads to adipose-specific cell death in obese mice (24). Therefore, it has been suggested that insulin is one of the determinants involved in increasing the WAT mass. However, the mechanisms of insulin actions, such as anti-apoptosis and lipid accumulation in human adipocytes, remain unclear.

The cell death-inducing DNA fragmentation factor-α-like effector (CIDE) family, i.e., CIDEA, CIDEB, and CIDEC (CIDE-3 or Fat-specific protein 27), show sequence similarity with the DNA fragmentation factor DFF45 and were identified initially as factors that induce apoptosis in mammalian cells (25, 26). CIDEA is expressed at high levels in brown adipocytes in mice (27), whereas in humans, CIDEA is expressed predominantly in WAT (28). Lower levels of CIDEA in WAT were observed in abdominal obesity, enlarged fat cells, and insulin resistance (28–31). The V115F polymorphism of CIDEA is associated with human obesity (32, 33). CIDEB is strongly expressed in the liver in both mice and humans (25, 34). CIDEC is expressed at high levels in WAT and increases during adipogenesis in

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Abbreviations: CIDE, cell death-inducing DNA fragmentation factor-α-like effector; DAPI, 4',6'-diamidino-2-phenylindole; Dex, dexamethasone; PPAR, peroxisome proliferator-activated receptor; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; WAT, white adipose tissue.

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mice (35, 36). Recent studies demonstrated that CIDEC is an adipocyte lipid droplet protein that plays an important role in lipid droplet formation (35, 37). Mice deficient in each of CIDEA, CIDEB, and CIDEC show increased insulin sensitivity, decreased adipose tissue mass, and increased energy expenditure (27, 34, 38, 39). These previous studies suggested that CIDEA, CIDEB, and CIDEC are differentially expressed in tissues but are all capable of inducing apoptosis and are correlated with energy balance and obesity. However, the physiological and critical roles of the CIDE family in human adipocytes and correlations with insulin remain unclear.

To investigate whether the CIDE family contributes to the actions of insulin, we evaluated regulation of CIDE family expression by insulin and the relation of the CIDE family to apoptosis and lipid droplet formation in human adipocytes. Here, we showed that CIDEA and CIDEC gene expression are differentially regulated by insulin, and the regulation of these genes by insulin may be related, at least in part, to the actions of insulin on apoptosis and lipid droplet formation in human adipocytes.

MATERIALS AND METHODS

Materials

DMEM/F-12 (1:1, v/v) was purchased from Invitrogen (Carlsbad, CA). Human insulin was purchased from Novo Nordisk (Bagsværd, Denmark), and rosiglitazone was purchased from Alexis Biochemicals (San Diego, CA). 3-Isobutyl-1-methylxanthine, dexamethasone (Dex), pantothenate, and anti-β-actin antibody were purchased from Sigma (St. Louis, MO). Biotin was purchased from Wako Pure Chemical Industries (Osaka, Japan), and FBS was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Mouse anti-human CIDEA monoclonal antibody and mouse anti-human CIDEC polyclonal antibody were purchased from Abnova Corporation (Taipei, Taiwan).

Differentiation of human preadipocytes into adipocytes

Human preadipocytes, derived from subcutaneous adipose tissue of six male subjects, were obtained from Zen-Bio (Research Triangle Park, NC) with institutional approval of the study and informed consent from the participants. The patients were nonsmokers with a mean body mass index of 27.2 (range 26.4–28.4) and an average age of 41 years old (range 29–57). Human preadipocytes were differentiated into adipocytes according to the supplier's protocol with a few modifications. Human preadipocytes were seeded on 24-well plates and cultured in DMEM/F-12 medium with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C with 5% CO_2. Cells were grown to confluence and treated with differentiation medium composed of DMEM/F-12 medium containing 3% FBS, 500 μM 3-isobutyl-1-methylxanthine, 1 μM Rosiglitazone, 100 nM insulin, 1 μM Dex, 33 μM biotin, 17 μM pantothenate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B for 6 days. Cells were then cultured in maintenance medium composed of DMEM/F-12 medium containing 3% FBS, 100 nM insulin, 1 μM Dex, 33 μM biotin, 17 μM pantothenate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B for 5 days. Cells were treated again with differentiation medium for 6 days and then cultured in maintenance medium for 2 days. These cells were used as differentiated adipocytes in all experiments. Each medium was changed for fresh medium every 3 days.

Quantitative real-time PCR

Total RNA was isolated and treated with DNase using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (100–200 ng) was reverse transcribed to cDNA in 20 μl reactions using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Quantitative gene expression analysis was performed on an ABI 7500 Fast instrument (Applied Biosystems) by TaqMan gene expression assay. Gene expression levels were normalized relative to 18S rRNA and shown as the mRNA levels relative to control. PCR was performed using Hs00154455_m1 for CIDEA, Hs00205339_m1 for CIDEB, Hs00535723_m1 for CIDEC, and Hs99999901_s1 for 18S rRNA (Applied Biosystems).

Western blot analysis

Total cell lysates were prepared with a lysis buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 50 mM sodium fluoride, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The cell lysates were centrifuged at 17,800 *g* for 10 min at 4°C. The supernatants were separated on 12.5% SDS-polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore Corporation, Bedford, MA). Membranes were blocked for 1 h with 5% BSA and 5% skim milk in TBS with 0.05% Tween-20 and incubated overnight at 4°C with antibodies specific to CIDEA and CIDEC. The blots were then treated with horseradish peroxidase anti-mouse secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 1 h. Proteins were visualized using ECL detection reagents (GE Healthcare).

Small interfering RNA study

Differentiated adipocytes were transfected with 10 nM each of control small interfering RNA (siRNA) (12935-200; Invitrogen), CIDEA siRNA (HSS141577; Invitrogen), or CIDEC siRNA (HSS127223; Invitrogen) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Transfection was performed once 5 days prior to assays.

Analysis of apoptosis

Preadipocytes were grown and differentiated into adipocytes on glass coverslips as described above. Differentiated adipocytes were incubated in serum/Dex-free maintenance medium in the presence or absence of 100 nM insulin for the indicated times. After treatment, the cells were fixed with 2% paraformaldehyde in PBS for 20 min at room temperature and washed once with PBS, followed by permeabilization with 0.2% Triton X-100 in 0.1% sodium citrate for 10 min on ice. After fixation, cells were incubated in terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) reaction mixture for 60 min at 37°C. Cells were then washed, incubated with 0.2 μg/ml of 4',6'-diamidino-2-phenylindole (DAPI; Sigma) and 0.1 μg/ml Nile Red (Sigma) in PBS for 5 min at room temperature, and washed three times. After the final washes, cells were mounted on slides with Fluoromount-G (Southern Biotech, Birmingham, AL) and visualized with a confocal laser microscope (FV500-D; Olympus, Tokyo, Japan). Photomicrographs were captured under green (TUNEL), blue (DAPI), and red (Nile Red) channels at ×20 magnification and merged using ImageJ software (http://rsb.info.nih.gov/ij/). Apoptotic adipocytes were triple-stained with TUNEL, DAPI, and Nile Red, and apoptosis was quantified by counting the number of triple-stained cells. The total adipocyte number was quantified as described below. The results are expressed as the number of TUNEL-positive adipocytes per 1,000 adipocytes (minimum 1,000 cells counted) in 12 random fields. The TUNEL assay was performed using an in situ cell death detection kit (Roche) according to the manufacturer's instructions.

Analysis of adipocyte number

Cells were fixed and stained with DAPI and Nile Red as described above. Cells were photographed at ×20 magnification. The adipocyte number was quantified by counting the number of DAPI/Nile Red-stained cells. The results are expressed as the number of adipocytes per square millimeter in 12 random fields.

Analysis of lipid droplet size

Cells were fixed and stained with Nile Red as described above. Cells were photographed at ×100 magnification. The size of Nile Red-stained lipid droplets was calculated using ImageJ software (100 lipid droplets in 10 random fields examined).

Measurement of glycerol release

Differentiated adipocytes were incubated in serum/Dex-free maintenance medium in the presence or absence of insulin for 24 h. After incubation, the medium was collected and glycerol contents were measured using free glycerol reagent (Sigma) according to the manufacturer's instructions. The results were corrected for cellular proteins, which were quantified using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) and are expressed as micrograms of glycerol per milligram of protein.

Statistical analyses

The significance of differences was assessed by Student's *t*-test. $P < 0.05$ was considered statistically significant. Data are expressed as the means ± SEM of three independent experiments.

RESULTS

Insulin downregulates CIDEA and upregulates CIDEC but not CIDEB expression in human adipocytes

To investigate whether insulin regulates expression of the CIDE family, we measured the mRNA expression levels of CIDEA, CIDEB, and CIDEC in human-differentiated adipocytes treated with insulin. Insulin decreased the levels of CIDEA mRNA in a time-dependent manner with the maximal effect observed at 24 h (**Fig. 1A**, left panel) and in a concentration-dependent manner with its maximal effect observed over 100 nM insulin (Fig. 1B, left panel). Treatment with 100 nM insulin for 24 h decreased CIDEA mRNA levels by 90%. In contrast, insulin increased CIDEC mRNA levels in a time-dependent manner with its maximal effect observed at 24 h (Fig. 1A, right panel) and in a concentration-dependent manner with its maximal effect observed over 100 nM insulin (Fig. 1B, right panel). Treatment with 100 nM insulin for 24 h led to an increase of approximately 2-fold in CIDEC mRNA level. Insulin did not affect the levels of CIDEB mRNA (Fig. 1A, B, middle panel).

Next, we examined the effects of insulin on CIDEA and CIDEC protein expression. Insulin decreased the levels of CIDEA protein (Fig. 1C, left panel) but increased those of CIDEC (Fig. 1C, right panel). Together, these results suggest that insulin downregulates CIDEA and upregulates CIDEC but not CIDEB expression in human adipocytes.

Insulin inhibits starvation-induced apoptosis in adipocytes

We next examined whether insulin inhibits starvation-induced apoptosis in human adipocytes. Differentiated adipocytes were starved by incubation in serum/Dex-free maintenance medium in the presence or absence of insulin and analyzed for apoptosis by TUNEL/DAPI/Nile Red-staining. DAPI and TUNEL staining revealed nuclear chromatin condensation and fragmentation in Nile Red-stained adipocytes after starvation for 48 h (**Fig. 2A**). After starvation for 72 h, numbers of adipocytes were decreased by 18.0 ± 7.7% and 6.6 ± 0.6% of adipocytes were TUNEL-positive (Fig. 2B). Insulin significantly decreased the number of TUNEL-positive adipocytes at the indicated times (Fig. 2B). After incubation in normal culture medium, staining with DAPI revealed normal nuclear morphology, and the number of TUNEL-positive adipocytes was very low (data not shown). These results indicate that starvation-induced apoptosis is suppressed by insulin in human adipocytes.

CIDEA depletion inhibits starvation-induced apoptosis in adipocytes

To evaluate the roles of CIDEA and CIDEC in apoptosis in human adipocytes, experiments using siRNA-mediated gene suppression of CIDEA and CIDEC were performed. Differentiated adipocytes were transfected with siRNA, then starved by incubation in serum/Dex-free maintenance medium in the presence or absence of insulin for 48 h, and analyzed for apoptosis. CIDEA siRNA significantly inhibited starvation-induced apoptosis (**Fig. 3A**). The levels of inhibition of apoptosis by CIDEA siRNA were similar to those by insulin. There was no additive effect of CIDEA siRNA on insulin-reduced apoptosis. In contrast, CIDEC siRNA showed no effect on adipocyte apoptosis. Each siRNA-mediated knockdown resulted in specific reductions in the levels of CIDEA and CIDEC mRNAs, respectively (Fig. 3B). These results indicate that CIDEA, but not CIDEC, is a key factor in starvation-induced apoptosis and suggest that the antiapoptotic effects of insulin and CIDEA depletion may act, at least in part, through the same pathway.

CIDEA depletion restores insulin deprivation-reduced adipocyte number

To examine the effects of CIDEA and CIDEC on adipocyte number, differentiated adipocytes were treated with siRNA in the presence or absence of insulin, and adipocyte number was determined. The presence of insulin significantly increased adipocyte number compared with the absence of insulin (**Fig. 4A**, B). There was no change in the number of preadipocytes in the cultures under these conditions (data not shown), suggesting that insulin deprivation had no effect on preadipocyte death. CIDEA siRNA markedly restored adipocyte number reduced by insulin deprivation, whereas CIDEC siRNA had no such effect (Fig. 4A, B). Insulin, alone or in combination with

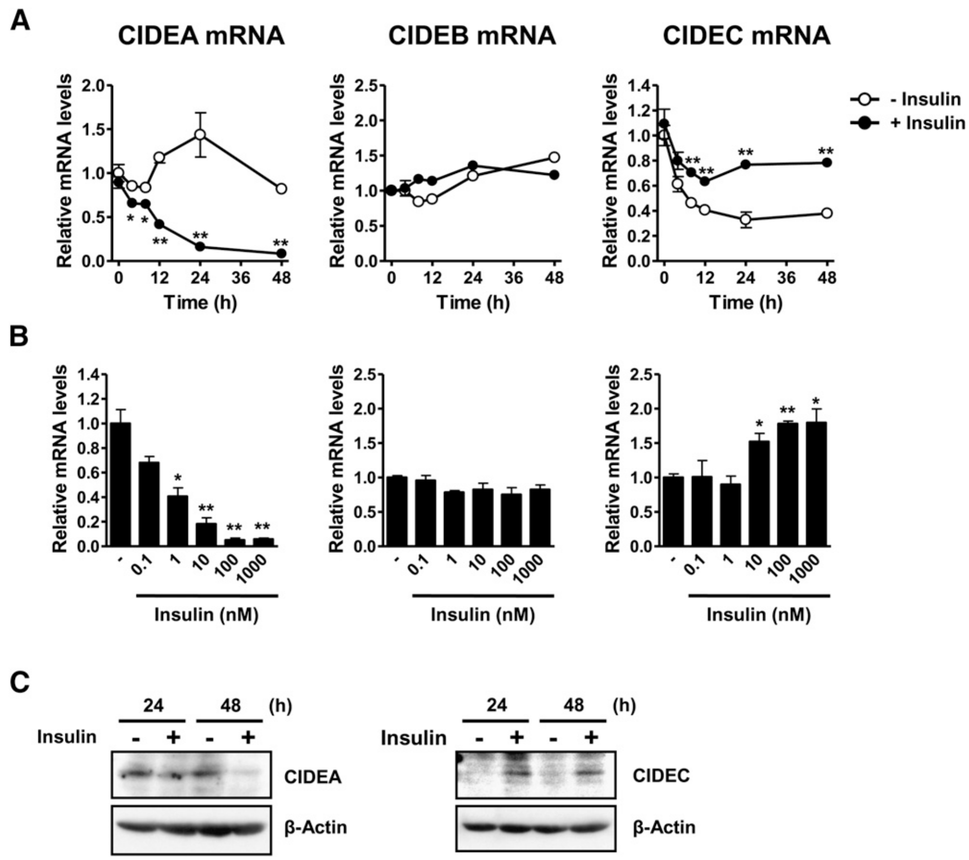

Fig. 1. Differential expression of CIDE family genes regulated by insulin in human adipocytes. A: Time course of insulin-regulated CIDE family gene expression. Differentiated adipocytes were starved in serum/Dex/insulin-free maintenance medium for 16 h. Cells were then incubated in serum/Dex-free maintenance medium in the presence or absence of 100 nM insulin for the indicated times. The mRNA expression levels of each gene were measured by quantitative real-time PCR, normalized relative to 18S rRNA expression, and shown as mRNA levels relative to zero-time control without insulin. Data are presented as means ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$. B: Concentration response effect of insulin on CIDE family gene expression. Differentiated adipocytes were starved in serum/Dex/insulin-free maintenance medium for 16 h and then incubated in serum/Dex-free maintenance medium in the presence or absence of insulin at the indicated concentrations for 24 h. The mRNA expression levels of each gene were measured by quantitative real-time PCR, normalized relative to 18S rRNA expression, and shown as relative mRNA levels. Data are presented as means ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$. C: Western blot analysis of CIDEA and CIDEC expression. Differentiated adipocytes were starved in serum/Dex/insulin-free maintenance medium for 16 h. Cells were then incubated in serum/Dex-free maintenance medium in the presence or absence of 100 nM insulin for the indicated times. β-Actin served as a loading control. These experiments were performed three times and the results of one representative experiment are shown.

CIDEA siRNA, similarly increased adipocyte number. Each siRNA-mediated knockdown resulted in specific reductions in the levels of CIDEA and CIDEC mRNAs, respectively (Fig. 4C). These data indicate that both insulin and CIDEA depletion maintain the number of adipocytes and suggest that these effects may act, at least in part, through the same pathway.

CIDEC depletion prevents insulin-induced enlargement of lipid droplets in adipocytes

To investigate the roles of CIDEA and CIDEC in lipid droplet formation in human adipocytes, differentiated adipocytes were treated with siRNA in the presence or absence of insulin. Insulin markedly increased the size of lipid droplets (**Fig. 5A**, B). Insulin-induced enlargement of lipid droplets was dramatically abrogated by CIDEC siRNA but not CIDEA siRNA. Insulin had no effect on glycerol release (Fig. 5C). CIDEA siRNA also had no effect on glycerol release, whereas CIDEC siRNA significantly increased glycerol release in the presence of insulin (Fig. 5C). Each siRNA-mediated knockdown resulted in specific reductions in the mRNA levels of CIDEA and CIDEC, respectively (Fig. 5D). These data suggest that CIDEC, but not CIDEA, is a key factor in lipid droplet formation, and insulin-promoted formation of lipid droplets is mediated by CIDEC upregulation in human adipocytes.

DISCUSSION

In this study, we evaluated the regulation of CIDE family expression by insulin and found that insulin downregu-

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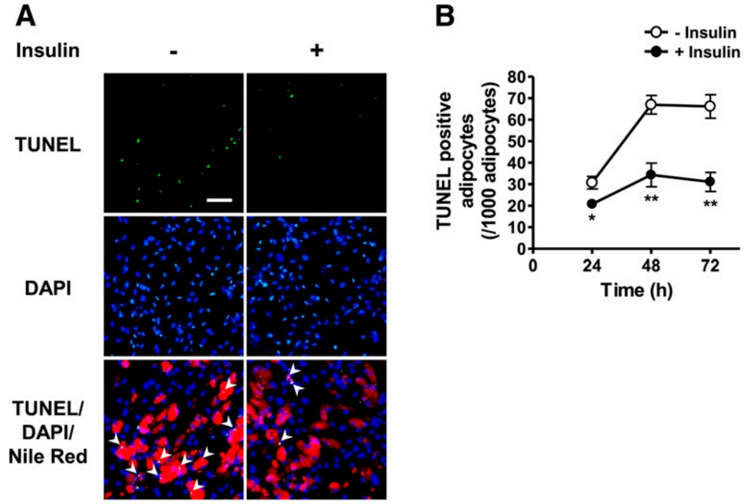

Fig. 2. Insulin inhibits starvation-induced apoptosis in human adipocytes. A: Fluorescence microscopy of adipocytes stained with TUNEL, DAPI, and Nile Red. Differentiated adipocytes were incubated in serum/Dex-free maintenance medium in the presence or absence of 100 nM insulin for 48 h. Cells were triple-stained with TUNEL (green), DAPI (blue), and Nile Red (red). TUNEL-positive adipocytes are indicated by the arrowheads. Scale bar, 100 μm. B: Quantification of TUNEL-positive adipocytes. Differentiated adipocytes were starved in serum/Dex-free maintenance medium in the presence or absence of 100 nM insulin for the indicated times. Data are presented as means ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

lates CIDEA and upregulates CIDEC mRNA expression, but not that of CIDEB, in human adipocytes (Fig. 1). This is the first report to observe that each CIDE family protein is differentially regulated by insulin in human adipocytes. Insulin did not affect the levels of adipocyte fatty acid binding protein (aP2) and peroxisome proliferator-activated receptor (PPAR)γ mRNAs, which are representative marker genes of adipocyte differentiation (data not shown). The aP2 is also a PPARγ-regulated gene. These results suggest that the observed effects of insulin on CIDE mRNA expression may not result from differentiation or PPARγ-directed transcription. It has recently been reported that insulin suppresses CIDEA expression in bovine mammary epithelial cells (40) and increases CIDEC expression in mouse 3T3-L1 adipocytes (36). These reports support our findings in human adipocytes. CIDEA and CIDEC are predominantly expressed in human WAT (32, 35, 36), whereas CIDEB is strongly expressed in the liver (25, 34). Accordingly, CIDEA and CIDEC, but not CIDEB, would be important factors associated with the actions of insulin in human adipocytes. CIDEA and CIDEC are proapoptotic factors (25, 26) and are correlated with lipid droplet formation (30, 35, 37). Previous studies suggested that CIDEA is localized in endoplasmic reticulum (41), nucleus (42), mitochondria (27), or around the lipid droplet (43) in various situations, whereas CIDEC is localized around the lipid droplet (35, 37). The study on the subcellular localizations of CIDEA and CIDEC is very important to predict the functions of these genes. However, it is not clearly understood, and further study will be needed to elucidate it.

We next examined the contributions of the CIDE family to the anti-apoptosis and lipid droplet formation actions of insulin. Mimicking CIDEA depletion by siRNA instead of by insulin significantly inhibited apoptosis (Fig. 3) and restored insulin deprivation-reduced cell number (Fig. 4). Insulin, alone or combined with CIDEA siRNA, similarly inhibited apoptosis (Fig. 3) and restored insulin deprivation-reduced cell number (Fig. 4), suggesting that insulin and CIDEA may act through the same pathway. Taken together, these results suggest that the antiapoptotic effects of insulin are, at least in part, mediated by downregulation of CIDEA. In contrast, CIDEC depletion by siRNA did not affect apoptosis in either the presence or absence of insulin (Fig. 3). Although it has been reported that overex-

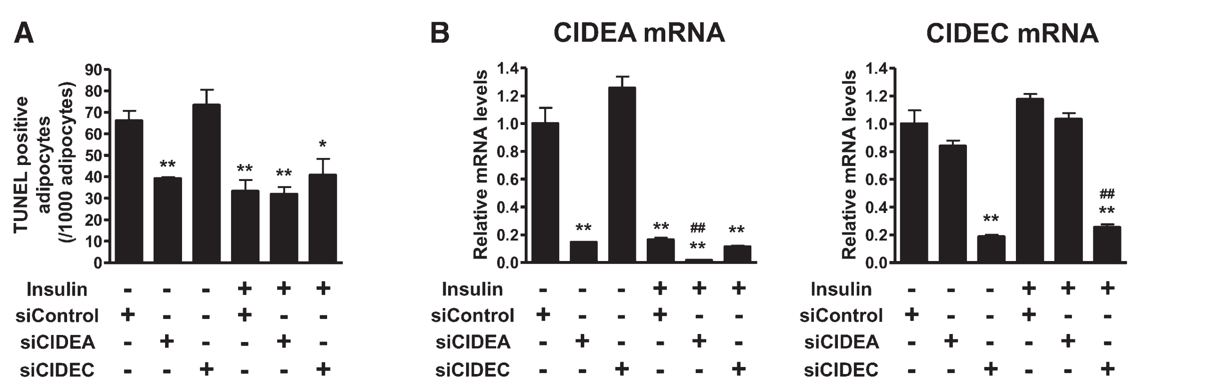

Fig. 3. Suppression of CIDEA expression inhibits starvation-induced apoptosis in human adipocytes. A: Quantification of TUNEL-positive adipocytes. Differentiated adipocytes were treated with control siRNA (siControl), CIDEA siRNA (siCIDEA), or CIDEC siRNA (siCIDEC) in maintenance medium for 7 days. Cells were then incubated in serum/Dex-free maintenance medium in the presence or absence of 100 nM insulin for 48 h and analyzed for apoptosis. Data are presented as means ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$. B: Expression analysis of CIDEA and CIDEC mRNA by real-time PCR. The mRNA expression levels of each gene were normalized relative to 18S rRNA expression and shown relative to control siRNA (siControl) without insulin. Data are presented as means ± SEM of three independent experiments. **, $P < 0.01$ versus siControl without insulin; ##, $P < 0.01$ versus siControl with insulin.

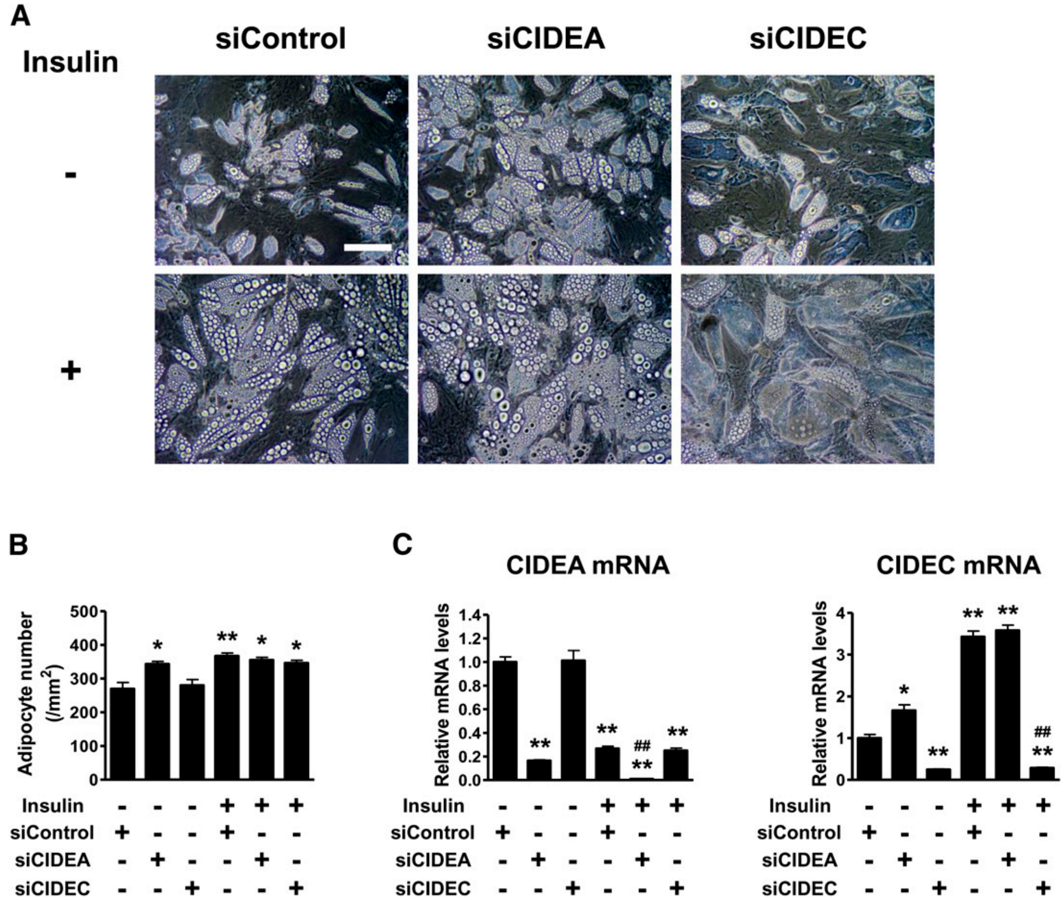

Fig. 4. Suppression of CIDEA expression restores insulin deprivation-reduced adipocyte number. A: Phase contrast microscopy of adipocytes. Differentiated adipocytes were treated with control siRNA (siControl), CIDEA siRNA (siCIDEA), or CIDEC siRNA (siCIDEC) in the maintenance medium in the presence or absence of 100 nM insulin for 15 days. Scale bar, 100 μm. B: Quantification of adipocyte number. Data are presented as means ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$. C: Expression analysis of CIDEA and CIDEC mRNA by real-time PCR. The mRNA expression levels of each gene were normalized relative to 18S rRNA expression and shown relative to siControl without insulin. Data are presented as means ± SEM of three independent experiments. *, $P < 0.05$, **, $P < 0.01$ versus siControl without insulin; ##, $P < 0.01$ versus siControl with insulin.

pression of CIDEC increases apoptosis of 3T3-L1 adipocytes (26), the moderate upregulation of CIDEC expression by insulin may not be associated with apoptosis in human adipocytes.

On the other hand, CIDEC depletion by siRNA abrogated insulin-induced lipid droplet formation and stimulated lipolysis (Fig. 5). These results suggest that insulin induces lipid droplet formation by upregulation of CIDEC in human adipocytes. Our results were consistent with previous reports indicating that the suppression of CIDEC in 3T3-L1-differentiated adipocytes stimulates lipolysis and reduces the size of lipid droplets (35, 37). CIDEA depletion by siRNA did not affect lipolysis or the size of lipid droplets in differentiated human adipocytes (Fig. 5). It has been reported that overexpression of CIDEA increases formation of lipid droplets in 3T3-L1 preadipocytes (30, 35) and depletion of CIDEA stimulates lipolysis in human preadipocytes (31). One of the reasons might be differences between preadipocytes and differentiated adipocytes, though it is not clearly understood. In differentiated adipocytes containing large lipid droplets, the basal levels of lipolysis would be much greater than those of preadipocytes. Therefore, minor changes in lipolysis may not have been observed because of the high background in differentiated adipocytes. Consequently, it is suggested that insulin-induced downregulation of CIDEA expression may not be highly associated with lipid droplet formation and lipolysis in human adipocytes. The differentiated human adipocytes we used in this study were very useful for long time culture and siRNA study. However, these cell cultures were mixtures of adipocytes and preadipocytes. Therefore, further study will be needed to verify the key points of these data in primary human adipocytes if the cells can endure the experiments.

Insulin is generally a prosurvival factor (44–50) and has been shown to inhibit apoptosis in mouse and rat adipocytes (13, 14). The present study also provided the first evidence that insulin inhibits starvation-induced apoptosis in human adipocytes (Fig. 2). Insulin is a lipogenic factor that increases adipocyte differentiation (11, 12), lipid stor-

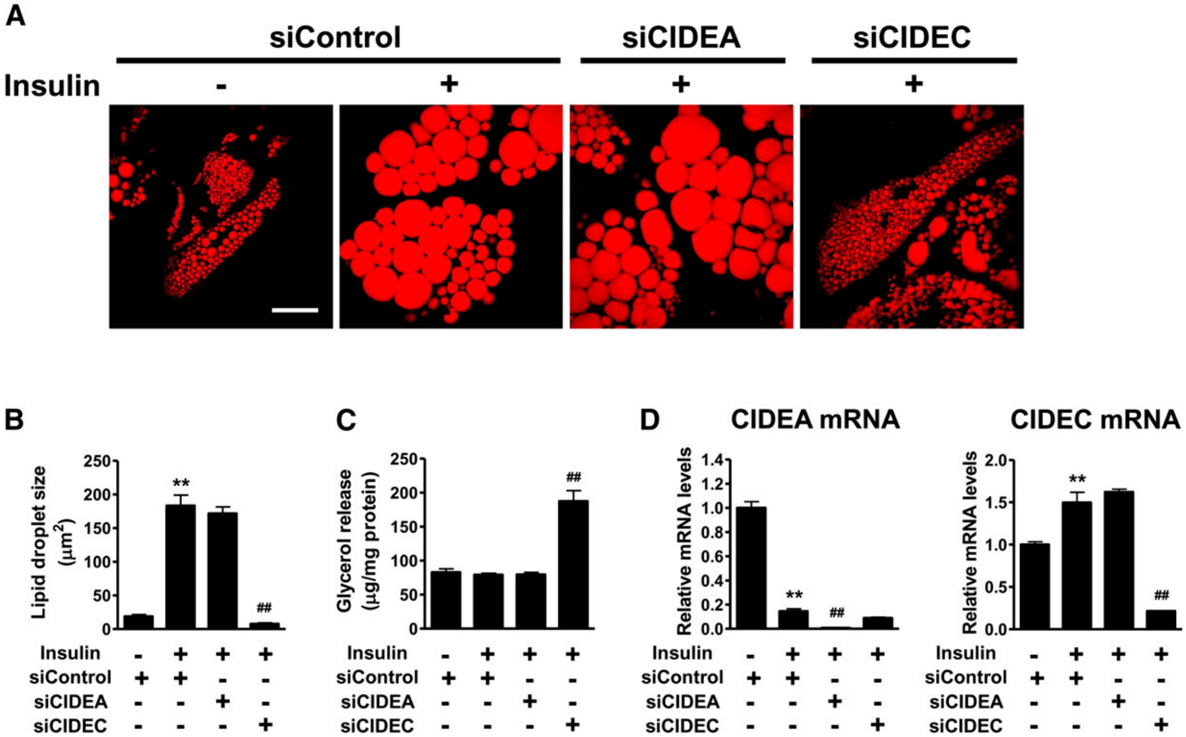

Fig. 5. Suppression of CIDEC expression inhibits insulin-induced enlargement of lipid droplets and increases glycerol release in human adipocytes. A: Fluorescence microscopy of adipocytes stained with Nile Red. Differentiated adipocytes were treated with control siRNA (siControl), CIDEA siRNA (siCIDEA), or CIDEC siRNA (siCIDEC) in maintenance medium in the presence or absence of 100 nM insulin for 10 days and then stained with Nile Red (red). Scale bar, 30 µm. B: Quantification of lipid droplet size. Data are presented as means ± SEM of three independent experiments. **, $P < 0.01$ versus siControl without insulin; ##, $P < 0.01$ versus siControl with insulin. C: Lipolysis assay. Glycerol released into the medium over 24 h was measured after siRNA-mediated depletion of CIDEA and CIDEC. Data are presented as means ± SEM of three independent experiments. ##, $P < 0.01$ versus siControl with insulin. D: Expression analysis of CIDEA and CIDEC mRNA by real-time PCR. The mRNA expression levels of each gene were normalized relative to 18S rRNA expression and shown relative to siControl without insulin. Data are presented as means ± SEM of three independent experiments. **, $P < 0.01$ versus siControl without insulin; ##, $P < 0.01$ versus siControl with insulin.

age, and lipid droplet formation (15, 16). A study of adipose tissue-selective insulin receptor deficiency in mice indicated that insulin signaling in adipocytes is critical for the development of obesity (23). Therefore, the actions of insulin in anti-apoptosis and enlargement of lipid droplets in adipocytes may lead to expansion of WAT mass. Accordingly, we speculate that insulin decreases CIDEA and increases CIDEC expression, which cause inhibition of apoptosis and enlargement of lipid droplets in adipocytes, respectively, and thus may lead to an increase of WAT mass (**Fig. 6**).

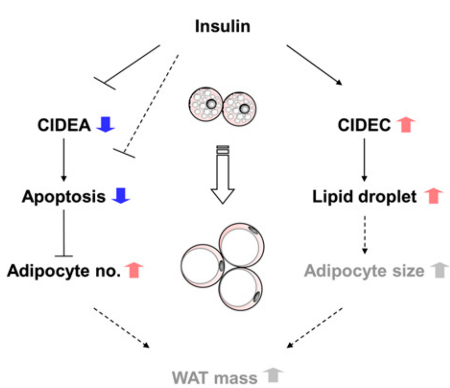

Fig. 6. Schematic diagram of insulin action in human adipocytes. Insulin suppresses apoptosis, at least in part, through downregulation of CIDEA mRNA expression but increases lipid droplet formation through upregulation of CIDEC mRNA expression. Chronic insulin action would increase adipocyte number and size through gene regulation of CIDEA and CIDEC and increase the mass of WAT in humans.

In conclusion, we identified the regulation of CIDEA and CIDEC by insulin and found that these genes make different contributions to insulin-induced anti-apoptosis and lipid droplet formation in human adipocytes. These findings provide a greater understanding of the roles of insulin in regulating the physiological functions of human adipocytes, most notably anti-apoptosis and lipid accumulation. Furthermore, the identification of novel genes that respond to insulin, such as CIDEA and CIDEC, will provide additional targets for the development of effective therapeutics to combat obesity and its associated disorders.

REFERENCES

1. Gesta, S., Y. H. Tseng, and C. R. Kahn. 2007. Developmental origin of fat: tracking obesity to its source. *Cell.* **131:** 242–256.
2. Rosen, E. D., and B. M. Spiegelman. 2006. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature.* **444:** 847–853.
3. Haslam, D. W., and W. P. James. 2005. Obesity. *Lancet.* **366:** 1197–1209.

4. Hirsch, J., and B. Batchelor. 1976. Adipose tissue cellularity in human obesity. *Clin. Endocrinol. Metab.* **5:** 299–311.
5. Sakai, T., H. Sakaue, T. Nakamura, M. Okada, Y. Matsuki, E. Watanabe, R. Hiramatsu, K. Nakayama, K. I. Nakayama, and M. Kasuga. 2007. Skp2 controls adipocyte proliferation during the development of obesity. *J. Biol. Chem.* **282:** 2038–2046.
6. Fischer-Posovszky, P., H. Tornqvist, K. M. Debatin, and M. Wabitsch. 2004. Inhibition of death-receptor mediated apoptosis in human adipocytes by the insulin-like growth factor I (IGF-I)/IGF-I receptor autocrine circuit. *Endocrinology.* **145:** 1849–1859.
7. Despres, J. P., and I. Lemieux. 2006. Abdominal obesity and metabolic syndrome. *Nature.* **444:** 881–887.
8. Prins, J. B., C. U. Niesler, C. M. Winterford, N. A. Bright, K. Siddle, S. O'Rahilly, N. I. Walker, and D. P. Cameron. 1997. Tumor necrosis factor-alpha induces apoptosis of human adipose cells. *Diabetes.* **46:** 1939–1944.
9. Prins, J. B., and S. O'Rahilly. 1997. Regulation of adipose cell number in man. *Clin. Sci. (Lond.).* **92:** 3–11.
10. Prins, J. B., N. I. Walker, C. M. Winterford, and D. P. Cameron. 1994. Apoptosis of human adipocytes in vitro. *Biochem. Biophys. Res. Commun.* **201:** 500–507.
11. Farmer, S. R. 2006. Transcriptional control of adipocyte formation. *Cell Metab.* **4:** 263–273.
12. Rosen, E. D., and O. A. MacDougald. 2006. Adipocyte differentiation from the inside out. *Nat. Rev. Mol. Cell Biol.* **7:** 885–896.
13. Urso, B., C. U. Niesler, S. O'Rahilly, and K. Siddle. 2001. Comparison of anti-apoptotic signalling by the insulin receptor and IGF-I receptor in preadipocytes and adipocytes. *Cell. Signal.* **13:** 279–285.
14. Qian, H., D. B. Hausman, M. M. Compton, R. J. Martin, M. A. Della-Fera, D. L. Hartzell, and C. A. Baile. 2001. TNFalpha induces and insulin inhibits caspase 3-dependent adipocyte apoptosis. *Biochem. Biophys. Res. Commun.* **284:** 1176–1183.
15. Kahn, B. B., and J. S. Flier. 2000. Obesity and insulin resistance. *J. Clin. Invest.* **106:** 473–481.
16. Kersten, S. 2001. Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Rep.* **2:** 282–286.
17. Cancello, R., J. Tordjman, C. Poitou, G. Guilhem, J. L. Bouillot, D. Hugol, C. Coussieu, A. Basdevant, A. Bar Hen, P. Bedossa, et al. 2006. Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. *Diabetes.* **55:** 1554–1561.
18. Cancello, R., C. Henegar, N. Viguerie, S. Taleb, C. Poitou, C. Rouault, M. Coupaye, V. Pelloux, D. Hugol, J. L. Bouillot, et al. 2005. Reduction of macrophage infiltration and chemo attractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes.* **54:** 2277–2286.
19. Arvidsson, E., N. Viguerie, I. Andersson, C. Verdich, D. Langin, and P. Arner. 2004. Effects of different hypocaloric diets on protein secretion from adipose tissue of obese women. *Diabetes.* **53:** 1966–1971.
20. Engeli, S., M. Feldpausch, K. Gorzelniak, F. Hartwig, U. Heintze, J. Janke, M. Mohlig, A. F. Pfeiffer, F. C. Luft, and A. M. Sharma. 2003. Association between adiponectin and mediators of inflammation in obese women. *Diabetes.* **52:** 942–947.
21. Ziccardi, P., F. Nappo, G. Giugliano, K. Esposito, R. Marfella, M. Cioffi, F. D'Andrea, A. M. Molinari, and D. Giugliano. 2002. Reduction of inflammatory cytokine concentrations and improvement of endothelial functions in obese women after weight loss over one year. *Circulation.* **105:** 804–809.
22. Wajchenberg, B. L., C. E. Leme, A. C. Lerario, I. T. Toledo e Souza, H. W. Rodbard, and D. Rodbard. 1984. Insulin resistance in Cushing's disease. Evaluation by studies of insulin binding to erythrocytes. *Diabetes.* **33:** 455–459.
23. Bluher, M., M. D. Michael, O. D. Peroni, K. Ueki, N. Carter, B. B. Kahn, and C. R. Kahn. 2002. Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. *Dev. Cell.* **3:** 25–38.
24. Loftus, T. M., F. P. Kuhajda, and M. D. Lane. 1998. Insulin depletion leads to adipose-specific cell death in obese but not lean mice. *Proc. Natl. Acad. Sci. USA.* **95:** 14168–14172.
25. Inohara, N., T. Koseki, S. Chen, X. Wu, and G. Nunez. 1998. CIDE, a novel family of cell death activators with homology to the 45 kDa subunit of the DNA fragmentation factor. *EMBO J.* **17:** 2526–2533.
26. Liang, L., M. Zhao, Z. Xu, K. K. Yokoyama, and T. Li. 2003. Molecular cloning and characterization of CIDE-3, a novel member of the cell-death-inducing DNA-fragmentation-factor (DFF45)-like effector family. *Biochem. J.* **370:** 195–203.
27. Zhou, Z., S. Yon Toh, Z. Chen, K. Guo, C. P. Ng, S. Ponniah, S. C. Lin, W. Hong, and P. Li. 2003. Cidea-deficient mice have lean phenotype and are resistant to obesity. *Nat. Genet.* **35:** 49–56.
28. Gummesson, A., M. Jernas, P. A. Svensson, I. Larsson, C. A. Glad, E. Schele, L. Gripeteg, K. Sjoholm, T. C. Lystig, L. Sjostrom, et al. 2007. Relations of adipose tissue CIDEA gene expression to basal metabolic rate, energy restriction, and obesity: population-based and dietary intervention studies. *J. Clin. Endocrinol. Metab.* **92:** 4759–4765.
29. Dahlman, I., K. Linder, E. Arvidsson Nordstrom, I. Andersson, J. Liden, C. Verdich, T. I. Sorensen, and P. Arner. 2005. Changes in adipose tissue gene expression with energy-restricted diets in obese women. *Am. J. Clin. Nutr.* **81:** 1275–1285.
30. Puri, V., S. Ranjit, S. Konda, S. M. Nicoloro, J. Straubhaar, A. Chawla, M. Chouinard, C. Lin, A. Burkart, S. Corvera, et al. 2008. Cidea is associated with lipid droplets and insulin sensitivity in humans. *Proc. Natl. Acad. Sci. USA.* **105:** 7833–7838.
31. Nordstrom, E. A., M. Ryden, E. C. Backlund, I. Dahlman, M. Kaaman, L. Blomqvist, B. Cannon, J. Nedergaard, and P. Arner. 2005. A human-specific role of cell death-inducing DFFA (DNA fragmentation factor-alpha)-like effector A (CIDEA) in adipocyte lipolysis and obesity. *Diabetes.* **54:** 1726–1734.
32. Dahlman, I., M. Kaaman, H. Jiao, J. Kere, M. Laakso, and P. Arner. 2005. The CIDEA gene V115F polymorphism is associated with obesity in Swedish subjects. *Diabetes.* **54:** 3032–3034.
33. Zhang, L., K. Miyaki, T. Nakayama, and M. Muramatsu. 2008. Cell death-inducing DNA fragmentation factor alpha-like effector A (CIDEA) gene V115F (G→T) polymorphism is associated with phenotypes of metabolic syndrome in Japanese men. *Metabolism.* **57:** 502–505.
34. Li, J. Z., J. Ye, B. Xue, J. Qi, J. Zhang, Z. Zhou, Q. Li, Z. Wen, and P. Li. 2007. Cideb regulates diet-induced obesity, liver steatosis, and insulin sensitivity by controlling lipogenesis and fatty acid oxidation. *Diabetes.* **56:** 2523–2532.
35. Keller, P., J. T. Petrie, P. De Rose, I. Gerin, W. S. Wright, S. H. Chiang, A. R. Nielsen, C. P. Fischer, B. K. Pedersen, and O. A. Macdougald. 2008. Fat-specific Protein 27 Regulates Storage of Triacylglycerol. *J. Biol. Chem.* **283:** 14355–14365.
36. Kim, J. Y., K. Liu, S. Zhou, K. Tillison, Y. Wu, and C. M. Smas. 2008. Assessment of fat-specific protein 27 in the adipocyte lineage suggests a dual role for FSP27 in adipocyte metabolism and cell death. *Am. J. Physiol. Endocrinol. Metab.* **294:** E654–E667.
37. Puri, V., S. Konda, S. Ranjit, M. Aouadi, A. Chawla, M. Chouinard, A. Chakladar, and M. P. Czech. 2007. Fat-specific protein 27, a novel lipid droplet protein that enhances triglyceride storage. *J. Biol. Chem.* **282:** 34213–34218.
38. Nishino, N., Y. Tamori, S. Tateya, T. Kawaguchi, T. Shibakusa, W. Mizunoya, K. Inoue, R. Kitazawa, S. Kitazawa, Y. Matsuki, et al. 2008. FSP27 contributes to efficient energy storage in murine white adipocytes by promoting the formation of unilocular lipid droplets. *J. Clin. Invest.* **118:** 2808–2821.
39. Toh, S. Y., J. Gong, G. Du, J. Z. Li, S. Yang, J. Ye, H. Yao, Y. Zhang, B. Xue, Q. Li, et al. 2008. Up-regulation of mitochondrial activity and acquirement of brown adipose tissue-like property in the white adipose tissue of fsp27 deficient mice. *PLoS ONE.* **3:** e2890.
40. Yonezawa, T., S. Haga, Y. Kobayashi, K. Katoh, and Y. Obara. 2009. Saturated fatty acids stimulate and insulin suppresses CIDE-A expression in bovine mammary epithelial cells. *Biochem. Biophys. Res. Commun.* **384:** 535–539.
41. Qi, J., J. Gong, T. Zhao, J. Zhao, P. Lam, J. Ye, J. Z. Li, J. Wu, H. M. Zhou, and P. Li. 2008. Downregulation of AMP-activated protein kinase by Cidea-mediated ubiquitination and degradation in brown adipose tissue. *EMBO J.* **27:** 1537–1548.
42. Valouskova, E., K. Smolkova, J. Santorova, P. Jezek, and M. Modriansky. 2008. Redistribution of cell death-inducing DNA fragmentation factor-like effector-a (CIDEa) from mitochondria to nucleus is associated with apoptosis in HeLa cells. *Gen. Physiol. Biophys.* **27:** 92–100.
43. Hallberg, M., D. L. Morganstein, E. Kiskinis, K. Shah, A. Kralli, S. M. Dilworth, R. White, M. G. Parker, and M. Christian. 2008. A functional interaction between RIP140 and PGC-1alpha regulates the expression of the lipid droplet protein CIDEA. *Mol. Cell. Biol.* **28:** 6785–6795.
44. Tseng, Y. H., K. Ueki, K. M. Kriauciunas, and C. R. Kahn. 2002. Differential roles of insulin receptor substrates in the anti-apoptotic function of insulin-like growth factor-1 and insulin. *J. Biol. Chem.* **277:** 31601–31611.

45. Diaz, B., J. Serna, F. De Pablo, and E. J. de la Rosa. 2000. In vivo regulation of cell death by embryonic (pro)insulin and the insulin receptor during early retinal neurogenesis. *Development.* **127:** 1641–1649.
46. Boehm, J. E., O. V. Chaika, and R. E. Lewis. 1999. Rac-dependent anti-apoptotic signaling by the insulin receptor cytoplasmic domain. *J. Biol. Chem.* **274:** 28632–28636.
47. Bertrand, F., C. Desbois-Mouthon, A. Cadoret, C. Prunier, H. Robin, J. Capeau, A. Atfi, and G. Cherqui. 1999. Insulin antiapoptotic signaling involves insulin activation of the nuclear factor kappaB-dependent survival genes encoding tumor necrosis factor receptor-associated factor 2 and manganese-superoxide dismutase. *J. Biol. Chem.* **274:** 30596–30602.
48. Yenush, L., C. Zanella, T. Uchida, D. Bernal, and M. F. White. 1998. The pleckstrin homology and phosphotyrosine binding domains of insulin receptor substrate 1 mediate inhibition of apoptosis by insulin. *Mol. Cell. Biol.* **18:** 6784–6794.
49. Hallmann, D., K. Trumper, H. Trusheim, K. Ueki, C. R. Kahn, L. C. Cantley, D. A. Fruman, and D. Horsch. 2003. Altered signaling and cell cycle regulation in embryonal stem cells with a disruption of the gene for phosphoinositide 3-kinase regulatory subunit p85alpha. *J. Biol. Chem.* **278:** 5099–5108.
50. Iida, K. T., H. Suzuki, H. Sone, H. Shimano, H. Toyoshima, S. Yatoh, T. Asano, Y. Okuda, and N. Yamada. 2002. Insulin inhibits apoptosis of macrophage cell line, THP-1 cells, via phosphatidylinositol-3-kinase-dependent pathway. *Arterioscler. Thromb. Vasc. Biol.* **22:** 380–386.