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 Ito, Michiaki Nagasawa, Tomoko Hara, Tomohiro Ide, and Koji Murakami

Supplementary key words  cell death-inducing DNA fragmentation factor-α-like effector • small interfering RNA • lipolysis

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...
mice (35, 36). Recent studies demonstrated that CIDE 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 regulation 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 (Bagvaerd, Denmark), and rosiglitazone was purchased from Alexius 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 Centers of Taiwan). The patients were non-smokers 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 seeded on 24-well plates and cultured in DMEM/F-12 medium with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium orthovanadate, and 0.25 μg/ml amphotericin B at 37°C with 5% CO₂. These 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 pantotenate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C with 5% CO₂. Cells were grown for 5 days prior to analyses. Each medium was changed for fresh medium every 3 days.

Quantitative real-time PCR

Total RNA was isolated and treated with DNase using an RNAeasy 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, and Hs00535723_m1 for CIDEC, and Hs99999901_s1 for 18S rRNA (Applied Biosystems).

Western blot analysis

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.

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 adipocytes in all experiments. Each medium was changed for fresh medium every 3 days.

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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.

**CIDEC 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
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). CIDEC siRNA also had no effect on glycerol release, whereas CIDEA 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-
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 forma-

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 then 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 incubated 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 forma-

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 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.
Differential roles of CIDEA and CIDEC in insulin action

In 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. 4.](image)

**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.
Insulin 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).

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.

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