Differential regulation of CIDEA and CIDEC expression by insulin via Akt1/2- and JNK2-dependent pathways in human adipocytes[S]

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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. Previously, we reported that CIDEA and CIDEC are differentially regulated by insulin and contribute separately to insulin-induced anti-apoptosis and lipid droplet formation in human adipocytes. However, the upstream signals of CIDE proteins remain unclear. Here, we investigated the signaling molecules involved in insulin regulation of CIDEA and CIDEC expression. The phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and PI-103 blocked both insulin-induced downregulation of CIDEA and upregulation of CIDEC. The Akt inhibitor API-2 and the c-Jun N-terminal kinase (JNK) inhibitor SP600125 selectively inhibited insulin regulation of CIDEA and CIDEC expression, respectively, whereas the MAPK/ERK kinase inhibitor U0126 and the p38 inhibitor SB203580 did not. Small interfering RNA-mediated depletion of Akt1/2 prevented insulin-induced downregulation of CIDEA and inhibition of apoptosis. Depletion of JNK2, but not JNK1, inhibited insulin-induced upregulation of CIDEC and lipid droplet enlargement. Furthermore, insulin increased both Akt and JNK phosphorylation, which was abrogated by the PI3K inhibitors.** **These results suggest that insulin regulates CIDEA and CIDEC expression via PI3K, and it regulates expression of each protein via Akt1/2- and JNK2-dependent pathways, respectively, in human adipocytes.**—Ito, M., M. Nagasawa, N. Omae, T. Ide, Y. Akasaka, and K. Murakami. **Differential regulation of CIDEA and CIDEC expression by insulin via Akt1/2- and JNK2-dependent pathways in human adipocytes.** ***J. Lipid Res.*** **2011.** 52: **1450–1460.**

Supplementary key words cell death-inducing DNA fragmentation factor-α-like effector • phosphatidylinositol 3-kinase • c-Jun N-terminal kinase • small interfering RNA • apoptosis • lipid droplet formation

White adipose tissue (WAT) is a key organ for energy homeostasis. Excessive lipid accumulation of WAT in obesity contributes to severe diseases, including type 2 diabetes, hypertension, dyslipidemia, cardiovascular disease, arthritis, and several types of cancer (1). WAT mass is determined by the number and size of adipocytes (2, 3) regulated by cell differentiation, apoptosis, and lipid droplet formation (4–6). Insulin is known to inhibit apoptosis (7, 8) and increase lipid droplet formation (9, 10) in adipocytes. Hyperinsulinemia is associated with weight gain in humans (11). Insulin signaling in adipocytes is critical for the development of obesity (12, 13). Therefore, it has been suggested that insulin is one of the determinants involved in increasing the WAT mass. However, the mechanisms underlying enlargement of insulin-induced fat mass remain unclear.

The biological actions of insulin are mediated by a tyrosine kinase receptor, i.e., the insulin receptor, and its activation results in stimulation of phosphatidylinositol 3-kinase (PI3K), Akt serine/threonine kinase, and mitogen-activated protein kinase (MAPK) pathways (14). Akt is a key downstream target of PI3K and mediates a number of the metabolic actions of insulin, such as cell survival, growth, and metabolism (15). Akt is composed of three highly homologous isoforms. Akt1 and Akt2 are expressed ubiquitously, Akt3 is expressed in the brain and testis, and many cell lines express all three isoforms (15, 16). It has been suggested that each isoform has both unique and overlapping roles. For example, Akt2, but not Akt1 or Akt3, is indispensable for glucose homeostasis (17–19), whereas all three isoforms have overlapping roles in cell survival (20, 21). The biological roles of Akt isoforms vary widely and depend on the developmental stage and cell type.

Manuscript received 26 October 2010 and in revised form 2 June 2011.

Published, JLR Papers in Press, June 2, 2011
DOI 10.1194/jlr.M012427

Abbreviations: CIDE, cell death-inducing DNA fragmentation factor-α-like effector; DAPI, 4′,6′-diamidino-2-phenylindole; Dex, dexamethasone; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; WAT, white adipose tissue.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of two figures.

On the other hand, the three major MAPKs, i.e., extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, also contribute to the actions of insulin (22) and are associated with obesity and insulin resistance (23–26). The JNK subtypes are encoded by three genes. JNK1 and JNK2 are expressed ubiquitously, and they play key roles in the development of obesity and insulin resistance (24, 25). JNK3 is expressed mainly in the brain, heart, and testis (27), and it is an important component in the pathogenesis of neurotoxicity (28). These JNK isoforms mediate cell proliferation, apoptosis in various cell types (29, 30), and lipid droplet metabolism in adipocytes (31).

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), was identified initially as a group of factors that induces apoptosis in mammalian cells (32, 33). Recent studies using mice deficient in CIDE proteins suggested that this class of protein is closely related to energy balance and obesity (34–37). In addition, lower levels of CIDEA in human WAT are observed in abdominal obesity, enlarged fat cells, and insulin resistance (38, 39). Polymorphism of CIDEA and CIDEC is associated with human obesity and partial lipodystrophy (40–42). CIDEC is localized around the lipid droplets in adipocytes and plays an important role in lipid droplet formation (43, 44). Furthermore, we recently showed that insulin decreases CIDEA and increases CIDEC expression in human adipocytes and the differential regulation of these genes is related, at least in part, to insulin-induced anti-apoptosis and lipid droplet formation (45). These results suggest that downregulation of CIDEA and upregulation of CIDEC by insulin in human adipocytes contribute to the actions of insulin and may play key roles in the development of obesity.

However, the upstream mechanisms by which insulin differentially regulates CIDEA and CIDEC expression remain unclear. In this study, we investigated signaling molecules involved in insulin regulation of CIDEA and CIDEC in human adipocytes. Here, we show that PI3K mediates both CIDEA and CIDEC regulation by insulin and that Akt1/2 and JNK2 mediate the insulin regulation of CIDEA and CIDEC, which are related to insulin-induced anti-apoptosis and lipid droplet formation, respectively, 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), and pantothenate 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). Wortmannin, PI-103, API-2, U0126, SP600125, and SB203580 were purchased from Calbiochem (San Diego, CA). Mouse anti-human CIDEA monoclonal antibody (H00001149-M01) and mouse anti-human CIDEC polyclonal antibody (H00063924-B01P) were purchased from Abnova Corporation (Taipei, Taiwan). Anti-Akt, anti-phospho-Akt (Ser473), anti-phospho-Akt (Thr308), anti-JNK, anti-phospho-JNK, and anti-phospho-c-Jun antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-c-Jun antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Differentiation of human preadipocytes into adipocytes

Human preadipocytes, derived from subcutaneous adipose tissue of six female subjects, were obtained from Zen-Bio (Research Triangle Park, NC). Institutional approval was obtained for the study and all participants gave their informed consent. The patients were nonsmokers with a mean body mass index of 27.9 (range 25.7-29.9) and an average age of 40 years (range, 29-52 years). Human preadipocytes were differentiated into adipocytes as described previously (45). 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 under an atmosphere of 5% CO_2. Cells were grown to confluence and treated with differentiation medium consisting 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 consisting 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. The medium was changed for fresh medium every 3 days in all cases.

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 are shown as the mRNA levels relative to control. PCR was performed using Hs00154455_m1 for CIDEA, Hs00535723_m1 for CIDEC, Hs00178289_m1 for Akt1, Hs00609846_m1for Akt2, Hs00178533_m1 for Akt3, Hs00177083_m1 for JNK1, Hs00177102_m1 for JNK2, and Hs99999901_s1 for 18S rRNA (Applied Biosystems).

siRNA study

Differentiated adipocytes were transfected with 10 nM control siRNA (12935-110; Invitrogen), Akt1 siRNA (12935-001 Duplex1; Invitrogen), Akt2 siRNA (12937-40 Duplex2; Invitrogen), Akt3 siRNA (HSS115177; Invitrogen), JNK1 siRNA (12936-42 Duplex1; Invitrogen), or JNK2 siRNA (12936-44 Duplex1; Invitrogen) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Transfection was performed once 5 days prior to the assays.

Western blot analysis

Western blot analysis was performed as described previously (45). 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 17800 *g* for 10 min at 4°C. The supernatants were separated on 10-15% SDS-polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore Corporation, Bedford, MA). Membranes were blocked for 1 h with 5% BSA in TBS with 0.05% Tween-20 and incubated overnight at 4°C with antibodies specific to CIDEA, CIDEC, Akt, phospho-Akt (Ser473), phospho-Akt (Thr308), JNK, phospho-JNK, c-Jun, and phospho-c-Jun. The blots were then treated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 1 h. Proteins were visualized using ECL detection reagents (GE Healthcare).

Analysis of apoptosis

Analysis of apoptosis was performed as described previously (45). 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 48 h. 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 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 ProLong Gold Antifade Reagent (Invitrogen) and visualized by confocal laser microscopy (LSM 700; Carl Zeiss, Jena, Germany). Photomicrographs were captured under green (TUNEL), blue (DAPI), and red (Nile

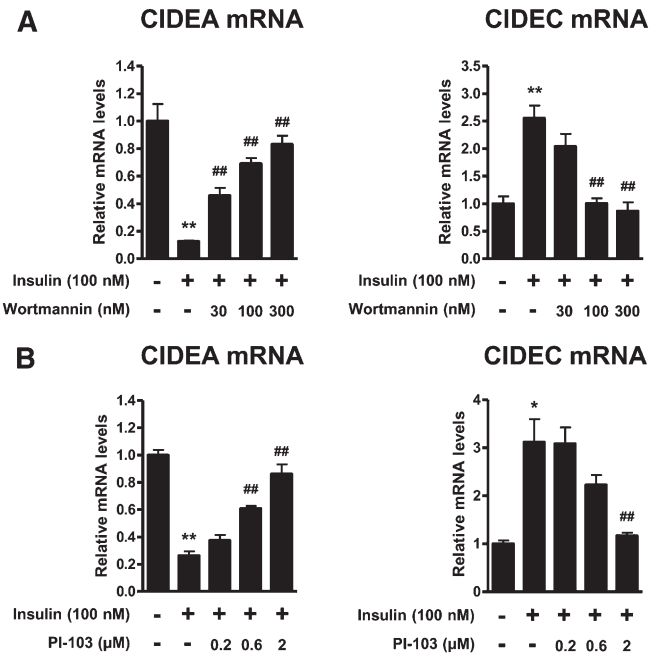

Fig. 1. PI3K inhibitors block the regulation of both CIDEA and CIDEC expression by insulin. A and B: Concentration-response effect of PI3K inhibitors on CIDEA and CIDEC mRNA expression. Differentiated adipocytes were starved in serum/Dex/insulin-free maintenance medium for 16 h. Cells were then treated with wortmannin or PI-103 at the indicated concentrations for 30 min followed by stimulation with or without 100 nM insulin for 24 h. The mRNA expression levels of CIDEA and CIDEC 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. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control without insulin; $^{\#\#}P < 0.01$ versus control with insulin.

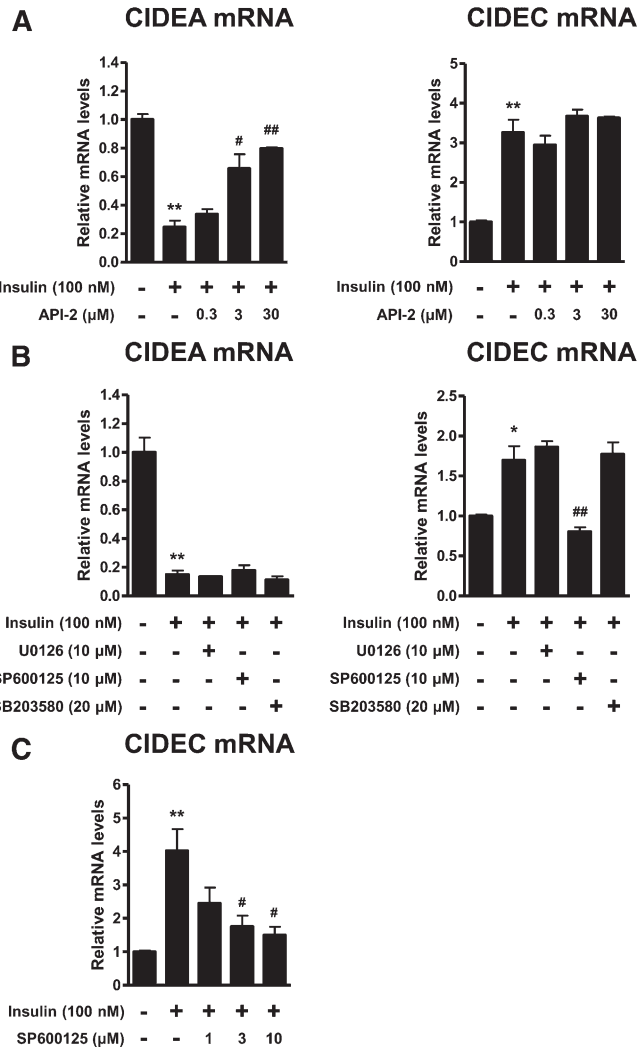

Fig. 2. The Akt inhibitor API-2 and the JNK inhibitor SP600125 selectively block regulation of CIDEA and CIDEC expression by insulin, respectively. A: Concentration-response effect of the Akt inhibitor on CIDEA and CIDEC mRNA expression. Differentiated adipocytes were starved in serum/Dex/insulin-free maintenance medium for 16 h. Cells were then treated with API-2 at the indicated concentrations for 30 min followed by stimulation with or without 100 nM insulin for 24 h. B: Effects of the MAPK inhibitors on CIDEA and CIDEC mRNA expression. Differentiated adipocytes were starved in serum/Dex/insulin-free maintenance medium for 16 h. Cells were then treated with 10 μ U0126, 10 μ SP600125, or 20 μ SB203580 for 30 min followed by stimulation with or without 100 nM insulin for 24 h. C: Concentration-response effect of the JNK inhibitor on CIDEC mRNA expression. Differentiated adipocytes were starved in serum/Dex/insulin-free maintenance medium for 16 h. Cells were then treated with SP600125 at the indicated concentrations for 30 min followed by stimulation with or without 100 nM insulin for 24 h. The mRNA expression levels of CIDEA and CIDEC 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. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control without insulin; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control with insulin.

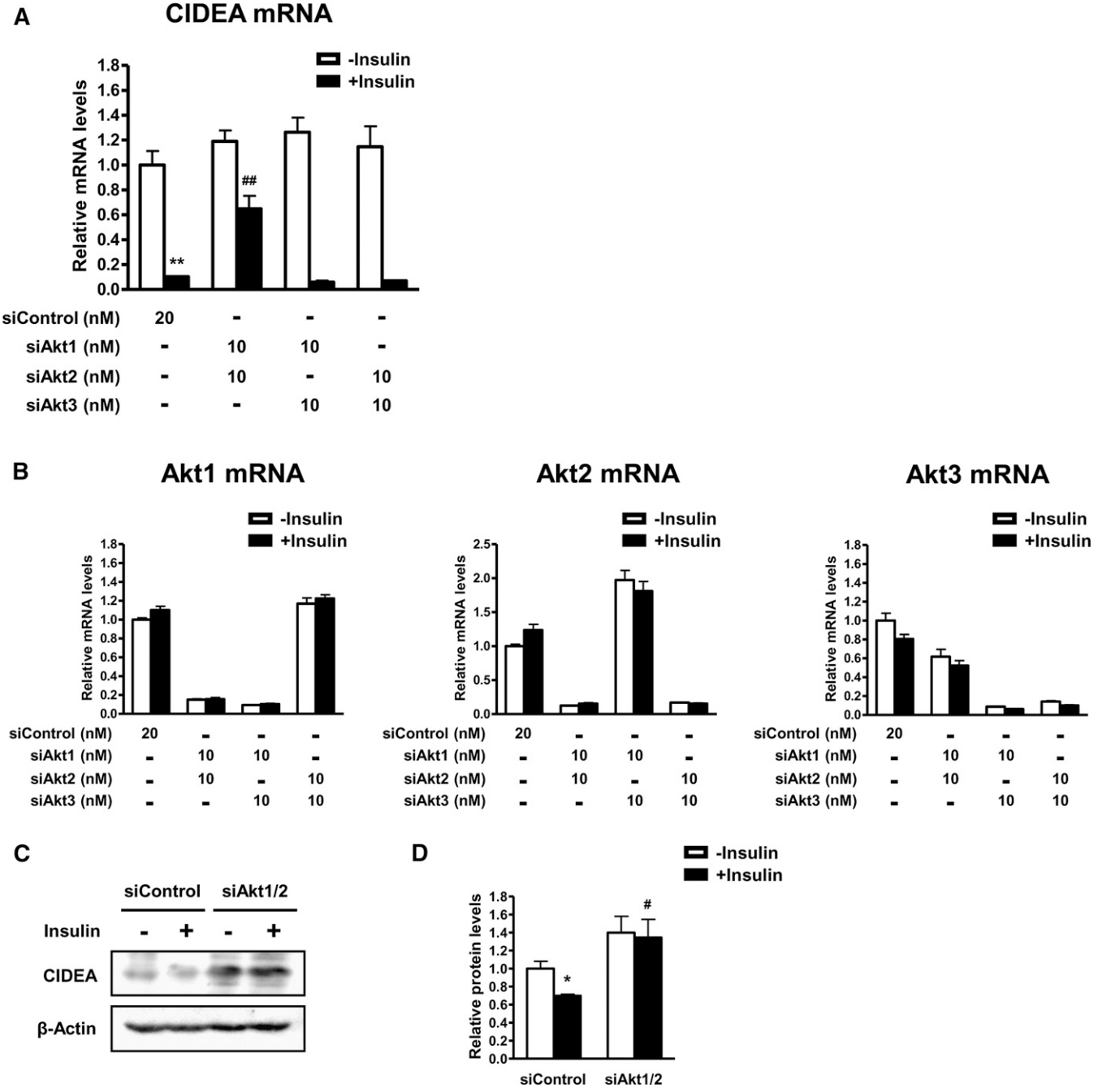

Fig. 3. Depletion of both Akt1 and Akt2 enhances insulin-reduced CIDEA expression. A and B: Differentiated adipocytes were treated with control siRNA (siControl), Akt1 siRNA (siAkt1), Akt2 siRNA (siAkt2), and/or Akt3 siRNA (siAkt3) in maintenance medium for 7 days. Cells were then starved in serum/Dex/insulin-free maintenance medium for 16 h followed by stimulation with or without 100 nM insulin for 24 h. The mRNA expression levels of CIDEA (A), Akt1, Akt2, and Akt3 (B) 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. $^{**}P < 0.01$ versus siControl without insulin; $^{\#\#}P < 0.01$ versus siControl with insulin. C: Western blot analysis of CIDEA protein expression. Differentiated adipocytes were treated with control siRNA or Akt1/2 siRNA in maintenance medium for 7 days. Cells were then starved in serum/Dex/insulin-free maintenance medium for 16 h followed by stimulation with or without 100 nM insulin for 48 h. β-Actin served as a loading control. These experiments were performed three times, and the results of one representative experiment are shown. D: Quantification of protein expression levels of CIDEA. The protein expression levels of CIDEA were normalized relative to β-actin protein expression and shown as relative protein levels. Data are presented as means ± SEM of three independent experiments. $^{*}P < 0.01$ versus siControl without insulin; $^{\#}P < 0.01$ versus 10 nM siControl with insulin.

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. Total adipocyte number was quantified by counting the number of DAPI/Nile Red-stained cells. The results are expressed as percentages of TUNEL-positive adipocytes compared with total adipocytes (minimum 500 cells counted) in 12 random fields. The

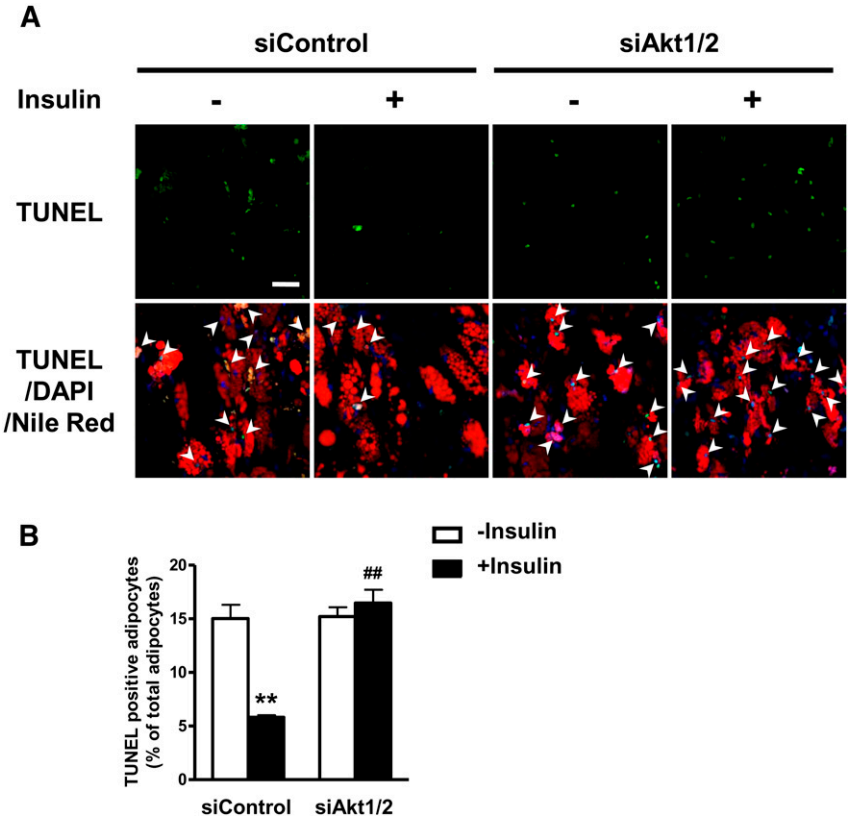

Fig. 4. Depletion of both Akt1 and Akt2 restores insulin-reduced apoptosis. A: Fluorescence microscopy of adipocytes stained with TUNEL, DAPI, and Nile Red. Differentiated adipocytes were treated with control siRNA (siControl) or Akt1/2 siRNA (siAkt1/2) in maintenance medium for 7 days. Cells were then incubated in serum/Dex-free maintenance medium in the presence or absence of 100 nM insulin for 48 h. Cells were triple-stained with TUNEL (green), DAPI (blue), and Nile Red (red). TUNEL-positive adipocytes are indicated by the arrowheads. Scale bar, 50 μm. B: Quantification of TUNEL-positive adipocytes. Data are presented as means ± SEM of three independent experiments. $^{**}P < 0.01$ versus siControl without insulin; $^{\#\#}P < 0.01$ versus siControl with insulin.

TUNEL assay was performed using an in situ cell death detection kit (Roche) according to the manufacturer's instructions.

Analysis of lipid droplet size

Cells were fixed and stained with Nile Red as described above. Cells were photographed at 63× magnification, and 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

Measurement of glycerol release was performed as described previously (45). 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 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 unpaired *t*-test. In all analyses, $P < 0.05$ was taken to indicate statistical significance. Data are expressed as the means ± SEM of three independent experiments.

RESULTS

Insulin regulates CIDEA and CIDEC expression via a PI3K-dependent pathway

To investigate whether regulation of CIDEA and CIDEC by insulin in human adipocytes is mediated by PI3K, differentiated adipocytes were treated with the PI3K inhibitors wortmannin or PI-103 followed by stimulation with or without insulin. Wortmannin and PI-103 blocked both insulin-induced downregulation of CIDEA and upregulation of CIDEC expression in a concentration-dependent manner (**Fig. 1A**, B). These results suggest that the regulation of CIDEA and CIDEC expression by insulin in human adipocytes is mediated via a PI3K-dependent signaling pathway.

Akt and JNK selectively mediate insulin regulation of CIDEA and CIDEC, respectively

We next examined the contributions of Akt and MAPK pathways to the regulation of CIDEA and CIDEC expression by insulin. Differentiated adipocytes were treated with the Akt inhibitor API-2, which suppresses growth factor-induced phosphorylation of Akt1, Akt2, and Akt3 (46), the MAPK/ERK kinase inhibitor U0126, the JNK inhibitor SP600125, or the p38 inhibitor SB203580 followed by

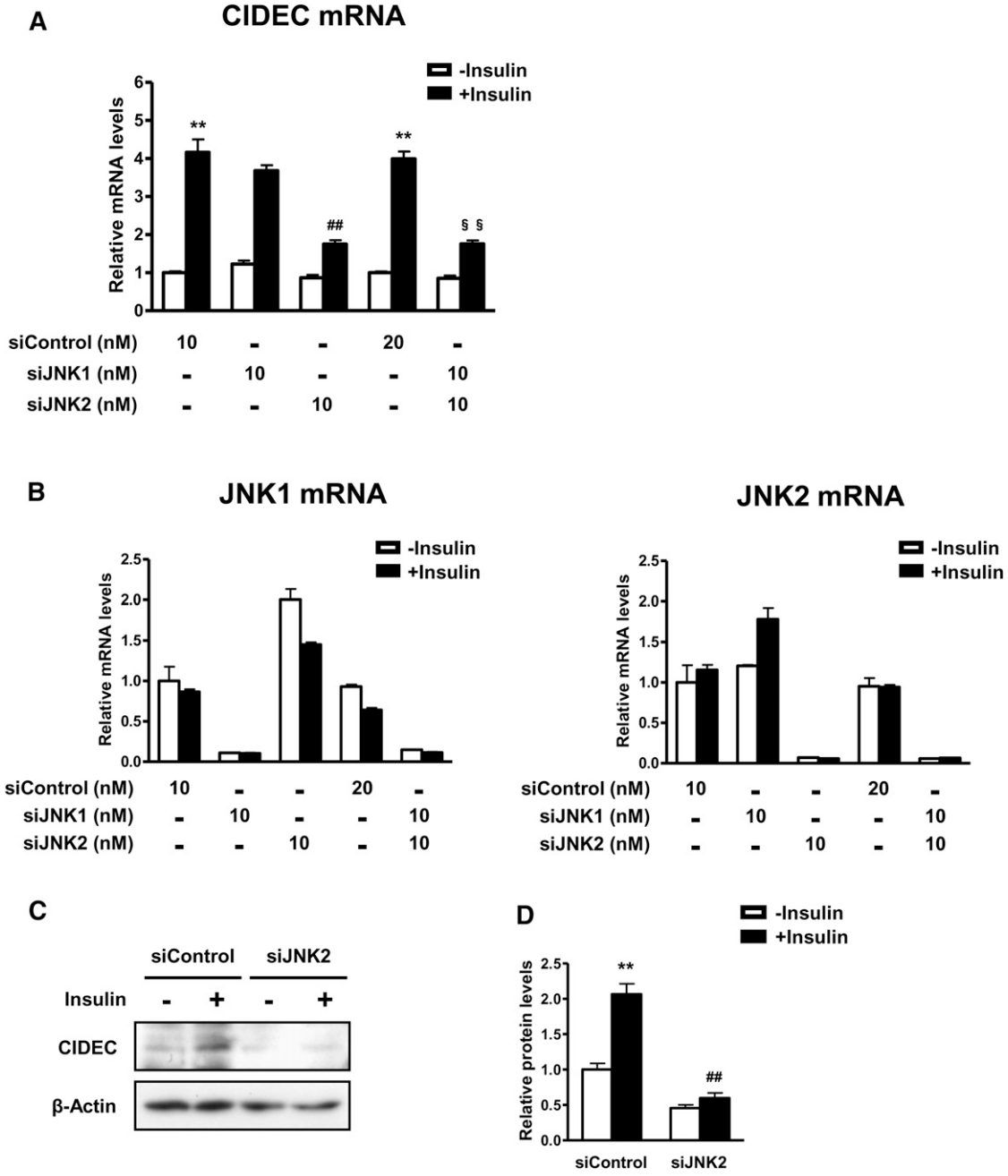

Fig. 5. Depletion of JNK2, but not JNK1, attenuates insulin-induced CIDEC expression. A and B: Differentiated adipocytes were treated with control siRNA (siControl), JNK1 siRNA (siJNK1), and/or JNK2 siRNA (siJNK2) in maintenance medium for 7 days. Cells were then starved in serum/Dex/insulin-free maintenance medium for 16 h followed by stimulation with or without 100 nM insulin for 24 h. The mRNA expression levels of CIDEC (A), JNK1, and JNK2 (B) 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. $^{**}P < 0.01$ versus siControl without insulin; $^{\#\#}P < 0.01$ versus 10 nM siControl with insulin; $^{\S\S}P < 0.01$ versus 20 nM siControl with insulin. C: Western blot analysis of CIDEC protein expression. Differentiated adipocytes were treated with control siRNA or JNK2 siRNA in maintenance medium for 7 days. Cells were then starved in serum/Dex/insulin-free maintenance medium for 16 h followed by stimulation with or without 100 nM insulin for 48 h. β-Actin served as a loading control. These experiments were performed three times, and the results of one representative experiment are shown. D: Quantification of protein expression levels of CIDEC. The protein expression levels of CIDEC were normalized relative to β-actin protein expression and shown as relative protein levels. Data are presented as means ± SEM of three independent experiments. $^{**}P < 0.01$ versus siControl without insulin; $^{\#\#}P < 0.01$ versus 10 nM siControl with insulin.

stimulation with or without insulin. API-2 blocked insulin-induced downregulation of CIDEA in a concentration-dependent manner, but it did not affect insulin-induced upregulation of CIDEC (**Fig. 2A**). The MAPK inhibitors did not alter the effects of insulin on CIDEA expression (Fig. 2B). In contrast, SP600125 blocked the effects of insulin on CIDEC expression, whereas U0126 and SB203580 did not (Fig. 2B). SP600125 also inhibited insulin-induced upregulation of CIDEC in a concentration-dependent manner (Fig. 2C). These results suggest that the regulatory

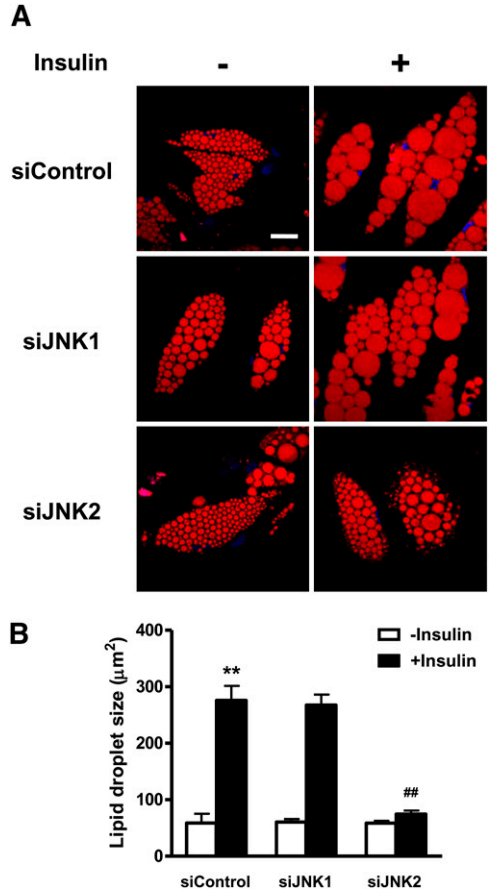

Fig. 6. Depletion of JNK2, but not JNK1, inhibits insulin-induced enlargement of lipid droplets. A: Fluorescence microscopy of adipocytes stained with Nile Red and DAPI. Differentiated adipocytes were treated with control siRNA (siControl), JNK1 siRNA (siJNK1), or JNK2 siRNA (siJNK2) in maintenance medium in the presence or absence of 100 nM insulin for 15 days and then stained with Nile Red (red) and DAPI (blue). 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.

effects of insulin on CIDEA and CIDEC expression are selectively mediated by Akt and JNK, respectively, in human adipocytes.

Both Akt1 and Akt2 mediate insulin-induced downregulation of CIDEA and inhibition of apoptosis

To further verify the specific involvement of Akt in insulin regulation of CIDEA, experiments using siRNA-mediated gene suppression of Akt1, Akt2, and/or Akt3 were performed. Differentiated adipocytes were treated with siRNA and then stimulated with or without insulin. Treatment with Akt1, Akt2, or Akt3 siRNA alone did not affect insulin-induced downregulation of CIDEA (data not shown). In contrast, combined treatment with Akt1/2 siRNA recovered CIDEA mRNA levels reduced by insulin by 61%, whereas Akt1/3 or Akt2/3 did not affect the CIDEA mRNA levels (**Fig. 3A**). Each siRNA-mediated knockdown resulted in specific reductions in the levels of Akt1, Akt2, and Akt3 mRNAs, respectively (Fig. 3B). Furthermore, Akt1/2 siRNA enhanced CIDEA protein levels reduced by insulin (Fig. 3C, D). These results suggest that insulin-induced downregulation of CIDEA is mediated by both Akt1 and Akt2 in human adipocytes.

We next investigated the contributions of Akt to the anti-apoptotic actions of insulin in human adipocytes. Combined treatment with Akt1/2 siRNA restored apoptosis reduced by insulin (**Fig. 4A**, B). These results indicate that insulin-induced inhibition of apoptosis is mediated by both Akt1 and Akt2 in human adipocytes.

JNK2, but not JNK1, is required for insulin-induced upregulation of CIDEC and enlargement of lipid droplets

To further verify the specific involvement of JNK in insulin regulation of CIDEC, differentiated adipocytes were treated with JNK1 and/or JNK2 siRNA, and then stimulated with or without insulin. JNK1 siRNA alone had no effect on CIDEC expression, whereas either treatment with JNK2 siRNA alone or combined treatment with JNK1/2 siRNA similarly attenuated CIDEC expression induced by insulin (**Fig. 5A**). Each siRNA-mediated knockdown resulted in specific reductions in the levels of JNK1 and JNK2 mRNAs, respectively (Fig. 5B). Furthermore, JNK2 siRNA attenuated CIDEC protein levels induced by insulin (Fig. 5C, D).

To investigate the roles of JNK in insulin-induced lipid droplet formation in human adipocytes, differentiated adipocytes were treated with siRNA in the presence or absence of insulin. Insulin-induced enlargement of lipid droplets was markedly inhibited by JNK2 but not JNK1 siRNA (**Fig. 6A**, B). These results indicate that insulin-induced upregulation of CIDEC and enlargement of lipid droplets require JNK2 but not JNK1 in human adipocytes.

PI3K mediates insulin-induced phosphorylation of Akt and JNK

To confirm whether Akt and JNK act downstream of PI3K in human adipocytes, we examined the effects of PI3K inhibitors on Akt and JNK phosphorylation. Time course study showed that insulin induced Akt Ser473, Akt Thr308, and JNK phosphorylation (**Fig. 7A**). Akt and JNK phosphorylation, which were apparent 30 min after initiation of insulin treatment, were prevented by treatment with either wortmannin or PI-103 (Fig. 7B). Furthermore, API-2 and SP600125 did not affect insulin-induced phosphorylation of JNK and Akt, respectively, suggesting that neither Akt nor JNK activation may be required for modulation of each other by insulin (Fig. 7C, D). These results suggest that insulin-induced phosphorylation of Akt and JNK is mediated via a PI3K-dependent pathway in human adipocytes.

DISCUSSION

We recently reported that differential gene regulation of CIDEA and CIDEC by insulin contributes to insulin-induced anti-apoptosis and lipid droplet formation in human adipocytes. The results of the present study provided the first evidence regarding the signaling pathways involved

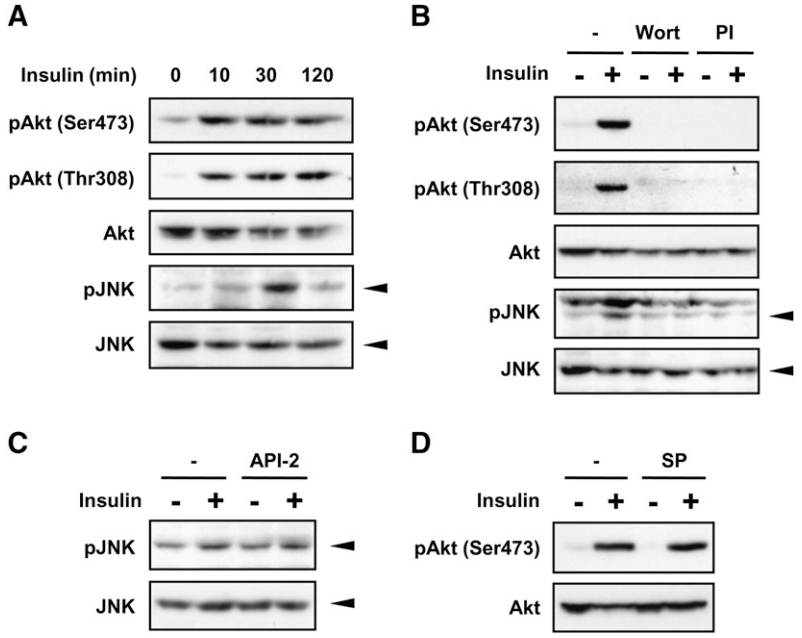

Fig. 7. PI3K inhibitors block both Akt and JNK phosphorylation induced by insulin. A: Time course of insulin-induced Akt and JNK phosphorylation. Differentiated adipocytes were starved in serum/Dex/insulin-free maintenance medium for 16 h followed by stimulation with or without 100 nM insulin for the indicated times. B: Effects of PI3K inhibitors on insulin-induced Akt and JNK phosphorylation. Differentiated adipocytes were starved in serum/Dex/insulin-free maintenance medium for 16 h. Cells were then treated with 0.2 μM wortmannin (Wort) or 2 μM PI-103 (PI) for 30 min followed by stimulation with or without 100 nM insulin for 30 min. C: Effects of the Akt inhibitor on insulin-induced JNK phosphorylation. Differentiated adipocytes were starved in serum/Dex/insulin-free maintenance medium for 16 h. Cells were then treated with 30 μM API-2 for 30 min followed by stimulation with or without 100 nM insulin for 30 min. D: Effects of the JNK inhibitor on insulin-induced Akt phosphorylation. Differentiated adipocytes were starved in serum/Dex/insulin-free maintenance medium for 16 h. Cells were then treated with 10 μM SP600125 (SP) for 30 min followed by stimulation with or without 100 nM insulin for 30 min. Akt Ser473, Akt Thr308, and JNK phosphorylation were determined by Western blot analysis. Total Akt and total JNK served as a loading control. Arrowheads indicate the 54 kDa isoform of JNK. These experiments were performed three times, and the results of one representative experiment are shown.

in insulin-mediated regulation of CIDEA and CIDEC in human adipocytes. PI3K inhibitors blocked both insulin-induced downregulation of CIDEA and upregulation of CIDEC (Fig. 1). Upregulation of CIDEC by insulin by PI3K was consistent with previous reports in mouse 3T3-L1 adipocytes (47). These results suggest that the regulatory effects of insulin on both CIDEA and CIDEC expression are mediated via a PI3K-dependent pathway.

We next examined the contributions of Akt, a key target of PI3K, and MAPK to regulation of CIDEA and CIDEC expression by insulin. Inhibition of Akt, but not ERK, JNK, or p38, by specific inhibitors selectively blocked insulin-induced downregulation of CIDEA (Fig. 2). Although siRNA-mediated depletion of Akt1/3 or Akt2/3 did not affect the regulatory effects of insulin on CIDEA expression, Akt1/2 depletion prevented insulin-induced downregulation of CIDEA expression (Fig. 3). These results suggest that insulin-induced downregulation of CIDEA is mediated by both Akt1 and Akt2, which may play overlapping roles in the insulin regulation of CIDEA. As our previous findings suggested that CIDEA contributes to insulin-induced inhibition of apoptosis (45), we investigated the role of Akt in apoptosis. Depletion of Akt1/2 by siRNA restored apoptosis reduced by insulin, suggesting that Akt1/2 mediates insulin-induced inhibition of apoptosis in human adipocytes (Fig. 4). Consistent with our results, it has been reported that combined loss of Akt1/2 causes increased apoptosis after stimulation in thymocytes (20). Inhibition of Akt signaling induces apoptosis in 3T3-L1 adipocytes (48). These results suggest that Akt1/2 contributes to the anti-apoptotic effect of insulin and the effect of insulin via Akt1/2 is mediated at least in part by downregulation of CIDEA in human adipocytes. Future studies will be required to clarify the precise contribution of CIDEA to the anti-apoptotic effect of insulin via Akt1/2.

On the other hand, inhibition of JNK, but not Akt, ERK, or p38, selectively blocked insulin-induced upregulation of CIDEC (Fig. 2). The JNK inhibitor SP600125, which inhibits both JNK1 and JNK2 with similar potency (49), blocked the effects of insulin on CIDEC expression in a concentration-dependent manner (Fig. 2C). Interestingly, depletion of JNK2 or JNK1/2 similarly attenuated insulin-induced upregulation of CIDEC, whereas JNK1 depletion did not (Fig. 5). These results indicated that insulin-induced upregulation of CIDEC requires JNK2, but not JNK1. As our previous findings suggested that CIDEC contributes to insulin-induced enlargement of lipid droplets (45), we investigated the role of JNK in lipid droplet

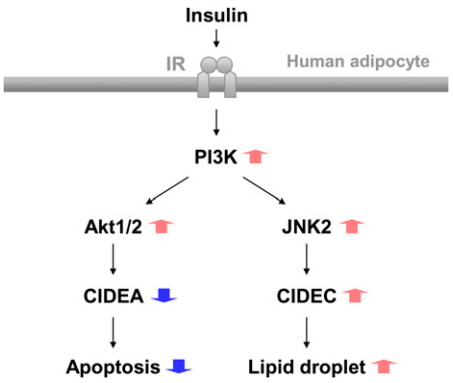

Fig. 8. Hypothesis of insulin signaling pathways associated with the regulation of CIDEA and CIDEC expression in human adipocytes. The regulation of CIDEA and CIDEC expression by insulin is mediated by PI3K signaling. However, the insulin signaling pathway involved in the regulation of CIDEA and CIDEC diverges into different pathways downstream of PI3K. Akt1/2 mediates insulin-induced downregulation of CIDEA, whereas JNK2 mediates insulin-induced upregulation of CIDEC. The insulin regulation of CIDEA and CIDEC via different signaling pathways would contribute separately to insulin-induced anti-apoptosis and lipid droplet formation in human adipocytes.

formation. Depletion of JNK2, but not JNK1, inhibited insulin-induced enlargement of lipid droplets (Fig. 6). This is the first report indicating that JNK2, but not JNK1, is required for insulin-induced lipid droplet formation in human adipocytes. Taken together, these results suggest that JNK2 is involved in insulin-mediated regulation of CIDEC, which plays a critical role in lipid droplet formation. siRNA screening revealed that JNK2 is a regulator of lipid droplet homeostasis in HeLa cells (50). JNK1 but not JNK2 deficiency resulted in reduced adiposity and increased insulin sensitivity in mouse models of obesity (24). However, mice with the phenotype $JNK1^{+/-}$ $JNK2^{-/-}$, but not $JNK1^{+/-}$ $JNK2^{+/+}$, were resistant to diet-induced obesity and insulin resistance, suggesting that JNK2 is involved in metabolic regulation (25). These observations implied a role of JNK2 in lipid-related pathologies, such as obesity and insulin resistance.

A recent study showed that insulin regulates adipocyte lipolysis via an Akt-independent but PI3K-dependent signaling pathway in rodents (51). Therefore, we examined lipolysis in human adipocytes treated with JNK1 or JNK2 siRNA. Consistent with previous studies (45, 52), insulin did not affect the basal levels of glycerol release (supplementary Fig. I). Depletion of neither JNK1 nor JNK2 affected glycerol release in either the presence or absence of insulin, suggesting that the observed effects of JNK2 depletion on insulin-induced lipid droplet formation may not be related to lipolysis. It has been shown that CIDEC stabilization is associated with triacylglycerol synthesis and lipid droplet formation (53). Thus, insulin-induced lipid droplet formation via a JNK2-dependent pathway may be related not to lipolysis but to triacylglycerol synthesis and accompanying regulation of CIDEC expression. However, the mechanism is unclear, and further studies are required to explain these observations.

We also showed that insulin induces JNK phosphorylation via the PI3K pathway, which is the first such evidence reported to date in human adipocytes. Insulin has been shown to stimulate Akt and JNK activation via PI3K in other cells (14, 54, 55). Consistent with these results, insulin stimulated both Akt and JNK phosphorylation in human adipocytes (Fig. 7A). The PI3K inhibitors abrogated both Akt and JNK phosphorylation induced by insulin (Fig. 7B). These results suggest that PI3K mediates insulin-induced activation of Akt and JNK in human adipocytes. Insulin also induced c-Jun phosphorylation in human adipocytes (supplementary Fig. II). It has been demonstrated that c-Jun is a major substrate for JNK and regulates transcription of multiple genes responsive to the hormone (56). It will be very important to verify signals, such as c-Jun, between JNK and CIDEC in future studies.

In conclusion, the present study provides the first evidence that Akt1/2 and JNK2, which act downstream of PI3K, selectively mediate regulation of CIDEA and CIDEC expression by insulin, respectively, in human adipocytes. We speculate that CIDEA and CIDEC are differentially regulated by insulin via different signaling pathways and, thus, contribute separately to insulin-induced anti-apoptosis and lipid droplet formation (**Fig. 8**). These findings reinforce our observations regarding the roles of insulin in regulating the physiological functions of human adipocytes. Furthermore, the identification of novel signaling pathways mediating insulin regulation of CIDEA and CIDEC will provide additional targets for the development of effective therapeutic strategies to combat obesity and its associated disorders.

The authors thank Prof. Kiyoto Motojima of Meiji Pharmaceutical University for helpful advice and comments on the manuscript.

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