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# Long-Term Treatment with Insulin Induces Apoptosis in Brown Adipocytes: Role of Oxidative Stress

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**Trying to define the precise role played by insulin regulating the survival of brown adipocytes, we have used rat fetal brown adipocytes maintained in primary culture. The effect of insulin on apoptosis and the mechanisms involved were assessed. Different from the known effects of insulin as a survival factor, we have found that long-term treatment (72 h) with insulin induces apoptosis in rat fetal brown adipocytes. This process is dependent on the phosphatidylinositol 3-kinase/mammalian target of rapamycin/p70 S6 kinase pathway. Short-term treatment with the conditioned medium from brown adipocytes treated with insulin for 72 h mimicked the apoptotic effect of insulin. During the process, caspase 8 activation, Bid cleavage, cytochrome *c* release, and activation of caspases 9 and 3 are sequentially produced. Treatment with the caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp (Z-VAD), prevents activation of this apoptotic cascade. The antioxidants, ascorbic acid and superoxide dismutase, also impair this process of apoptosis. Moreover, generation of reactive oxygen species (ROS), probably through reduced nicotinamide adenine dinucleotide phosphate oxidases, and a late decrease in reduced glutathione content are produced. According to this, antioxidants prevent caspase 8 activation and Bid cleavage, suggesting that ROS production is an important event mediating this process of apoptosis. However, the participation of uncoupling protein-1, -2, and -3 regulating ROS is unclear because their levels remain unchanged upon insulin treatment for 72 h. Our data suggest that the prolonged hyperinsulinemia might cause insulin resistance through the loss of brown adipose tissue. (*Endocrinology* 144: 5390–5401, 2003)**

THE FUNCTION OF brown adipose tissue (BAT) is the production of heat by the mechanism called nonshivering thermogenesis. Thus, lipids accumulated in BAT are used as a source of fatty acids that are oxidized in the mitochondria, generating a proton electrochemical gradient, which is dissipated as heat by the mitochondrial uncoupling protein-1 (UCP-1). This thermogenic mechanism is only activated under particular circumstances such as cold exposure, high-fat diet feeding, and during the perinatal period (for review, see Ref. 1). Hence, under these circumstances, a development and recruitment of BAT is induced. This involves processes of proliferation and differentiation to increase the number of highly differentiated brown adipocytes. An increase in the number of cells is produced that is accompanied by the expression of UCP-1 and adipogenic-related genes (1).

Among the positive signals regulating BAT, noradrenaline, IGF-I, and insulin are probably the most relevant. Noradrenaline activates proliferation (2–3), thermogenic differentiation (3–5), and survival (6–7). IGF-I is also a mitogen with a positive effect on thermogenic and adipogenic differentiation of BAT (8–9), as well as on survival (10). On the other hand, insulin is also an important mitogen with a potent adipogenic effect (9), which is able to protect cells from apoptosis induced by the expression of the oncogene H-ras (H-ras$^{Lys12}$) in brown adipocyte-derived cell lines upon serum deprivation (11). Similarly, insulin protects from apoptosis induced by TNF-$\alpha$ in white adipocytes (12). In addition, insulin is also a survival factor in other cell types such as neurons (13–15). However, recent data indicate that insulin is unable to counteract the TNF-$\alpha$-induced apoptosis of cultured brown adipocytes from mice (16). Trying to define the precise role played by insulin regulating the survival of brown adipocytes, we have performed experiments using rat fetal brown adipocytes maintained in primary culture. We have found that insulin is not only unable to protect from TNF-$\alpha$-induced apoptosis in rat fetal brown adipocytes maintained in culture but also that insulin alone is able to induce apoptosis after long-term treatment. We have characterized the role played by insulin in the regulation of apoptosis of brown adipocytes and the mechanisms involved.

Abbreviations: BAT, Brown adipose tissue; DPI, diphenyleneiodonium; GSH, reduced glutathione; JNK, Jun-kinase; MEK, MAPK kinase; mTOR, mammalian target of rapamycin; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NF-$\kappa$B, nuclear factor $\kappa$B; PI3K, phosphatidylinositol 3-kinase; p70S6K, p70 S6 kinase; ROS, reactive oxygen species; UCP, uncoupling protein; Z-VAD, benzyloxycarbonyl-Val-Ala-Asp.

## Materials and Methods

### *Isolation and culture of fetal brown adipocytes*

Fetal brown adipocytes from 20-d-old rat fetuses were isolated as described (10, 17) and maintained in primary culture. Cells were grown in MEM supplemented with 10% fetal bovine serum for 24 h. Then cells were serum starved overnight and maintained in the absence or in the presence of insulin (20 nM) and or TNF-$\alpha$ (10 ng/ml) for different time periods as indicated. To inhibit ERKs, cells were pretreated for 1 h before the addition of the signals with the MAPK kinase (MEK)-1 inhibitor PD98059 (Calbiochem, San Diego, CA) at a concentration of 20 $\mu$M. Similarly, to inhibit phosphatidylinositol 3-kinase (PI3K), cells were

pretreated with the specific inhibitor LY 294002 (Calbiochem) at 5 μM, and to inhibit mammalian target of rapamycin (mTOR)/p70 S6 kinase (p70S6K) pathway, cells were pretreated with rapamycin (Calbiochem) at 10 μM.

Caspases were inhibited by the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (Z-VAD)-fmk at doses of 10–40 μM. As scavengers of reactive oxygen species (ROS), the antioxidants, ascorbic acid (Sigma A-4034; Sigma, St. Louis, MO) at 10 mM and superoxide dismutase (Roche no. 567680, Roche, Nutley, NJ) at 60 μg/ml, were used.

To inhibit cellular reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, brown adipocytes maintained with either control-conditioned medium or conditioned medium from insulin-treated cells were treated or not with diphenyleneiodonium chloride (DPI) at a dose of 10 μM for the last 2 h before ROS quantification.

### *Flow cytometric analysis*

Analysis of DNA content and cell cycle was performed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cells were fixed with ethanol at −20 C (2 min), washed with PBS, and treated with ribonuclease A for 30 min at 37 C. Then, DNA was stained with propidium iodide. Measurements were carried out using a double discriminator module to discriminate doublets. Ten thousand cells were acquired per sample. The percentage of cells with DNA content lower than 2 C was calculated using Multicycle software (Phoenix software, Los Angeles, CA).

### *Analysis of DNA fragmentation*

DNA from the extranuclear fraction was isolated as previously described (10). Then, DNA was electrophoresed in a 1.5% agarose gel.

### *Nuclear factor κB (NF-κB) gel mobility shift assay*

Nuclear extracts, obtained essentially as described previously (17), were used for the NF-κB mobility shift assay, which was performed as described (17), using the double-stranded oligonucleotide corresponding to the κB distal motif present in the murine inducible nitric oxide synthase promoter: TGCTAGGGGGATTTTCCCTC TCT CTGT.

### *Jun-kinase (JNK) assay*

JNK activity was determined in total cell lysates as previously described (17). A pull down of JNKs was performed with glutathione *S*-transferase-c-jun 79 protein (containing the first 79 amino acids fused to glutathione *S*-transferase) provided by Dr. Silvio Gutkind (NIH, Bethesda, MD). Then, kinase assay was carried out in a kinase buffer containing 0.3 μCi [$\gamma^{32}$P]ATP and 20 μM of cold ATP. Reaction was stopped by adding Laemmli sample buffer. Phosphorylated c-Jun was visualized by autoradiography after electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels.

### *Purification of mitochondrial and cytosolic extracts*

Mitochondrial and cytosolic extracts were obtained by a modified version of the method described by Porras *et al.* (4). First, cells were incubated for 5 min in a hypotonic buffer [1 mM EDTA, 10 mM HEPES, 50 mM sucrose (pH 7.6)] at 37 C and homogenized. Then, after the addition of a hypertonic solution [1 mM EDTA, 10 mM HEPES, 1 M sucrose (pH 7.6)], cells were centrifuged at 2000 rpm at 4 C. The supernatant was recovered and subsequently centrifuged at 10,000 rpm at 4 C, obtaining a supernatant corresponding to the cytosolic fraction and a pellet with mitochondria. Mitochondria were then resuspended in an isotonic buffer [1 mM EDTA, 10 mM HEPES, 250 mM sucrose (pH 7.6)].

### *Western blot analysis*

Active ERK (ERK1/2) levels were quantified in total cell extracts by Western blot analysis, using an antiphospho-ERK antibody (9101S; Cell Signaling Technology, Beverly, MA). Total ERK levels with the anti-ERK1/2 antibody (06–182; Upstate Biotechnology, Waltham, MA) as described (9). Similarly, the levels of active Akt, p70S6K, and mTOR were determined by Western blot analysis using antiphospho-antibodies from Cell Signaling Technology (9271, 9204, and 2971, respectively) and total Akt and p70S6K (9272 and 9202, respectively) to normalize.

Antibody against UCP-1 was generously given by Dr. Rial (CIB, Madrid, Spain), and the antibodies for UCP-2 and -3 were from Santa Cruz Biotechnology (sc-6525 and sc-7756, respectively; Santa Cruz, CA).

The decrease in the level of Bid proform and the level of the active form of caspases 3 and 9 were quantified in total cell extracts by Western blot analysis using an anti-Bid antibody from R&D Systems (RD-AF860; Minneapolis, MN) and antiactive caspase 3 and 9 antibodies from Cell Signaling Technology (9661 and 9507, respectively).

Cytochrome *c* levels in mitochondria and cytosol were measured by Western blot analysis using an antibody from Pharmingen (P-65981A; San Diego, CA) after purification of these cellular fractions.

### *Fluorometric analysis of caspase 8 and 3 activities*

Caspase 8 and 3 activities were measured as previously described (18). Cells were lysed at 4 C in a buffer containing 5 mM Tris/HCl (pH 8.0), 20 mM EDTA, and 0.5% Triton X-100. Lysates were clarified by centrifugation at 13,000 × *g* for 10 min. For caspase 3 activity, reaction mixture contained 20 mM HEPES (pH 7.0), 10% glycerol, 2 mM dithiothreitol, 30 μg protein/condition, and 20 μM Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin) as substrate.

Reaction mixture for caspase 8 activity contained 20 mM piperazine-*N*,*N*′-bis(2-ethane sulfonic acid) (pH 7.2), 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% (wt/vol) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 10% sucrose, 70 μg protein/condition, and 22.6 μM Ac-IETD-AFC (N-acetyl-Ile-Glu-Thr-Asp-7-amino-trifluoromethyl-coumarin) as substrate. After 1.5 h of incubation at 37 C in the dark, enzymatic activity was measured in a Microplate Fluorescence Reader FL600 (Bio-Tek, Winooski, VT).

### *Measurement of intracellular ROS*

Cells were washed twice with PBS and then incubated for 30 min at 37 C in the dark with the oxidation-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR) at 2.5 μM (19). After washing twice with PBS, PBS was added and fluorescence from the plates were read in a Microplate Fluorescence Reader FL600 (Bio-Tek) using a wavelength of 486 nm for excitation and 530 nm for emission.

### *Determination of the level of glutathione*

Cells were lysed in a buffer containing 0.2% Triton X-100 and 2.5% sulfosalicylic acid and then centrifuged at 10,000 × *g* for 10 min at 4 C. The supernatant was used for the determination of total [reduced glutathione (GSH) + oxidized glutathione] glutathione by a modified version of the Griffith's method (19). Using glutathione as standard, glutathione is expressed as pmol/μg protein and represented in figures as fold increase of control level.

## Results

### *Insulin induces apoptosis in brown adipocytes after long-term treatment*

There are many published data about the role of insulin as a survival signal in different cell types (13–15). However, insulin did not protect from apoptosis induced by TNF-α plus cycloheximide in brown adipocytes from mice (16). Based on these data, we wanted to check and deeply analyze whether insulin could prevent the process of apoptosis induced by TNF-α in rat fetal brown adipocytes, which we have previously described and characterized (10, 17). Upon quantification of apoptotic cells (cells with DNA content lower than 2C), we found that insulin partially protects from TNF-α-induced apoptosis at 24 h but not at 48 h (Fig. 1A). Moreover, treatment with insulin for 48 h induced apoptosis,

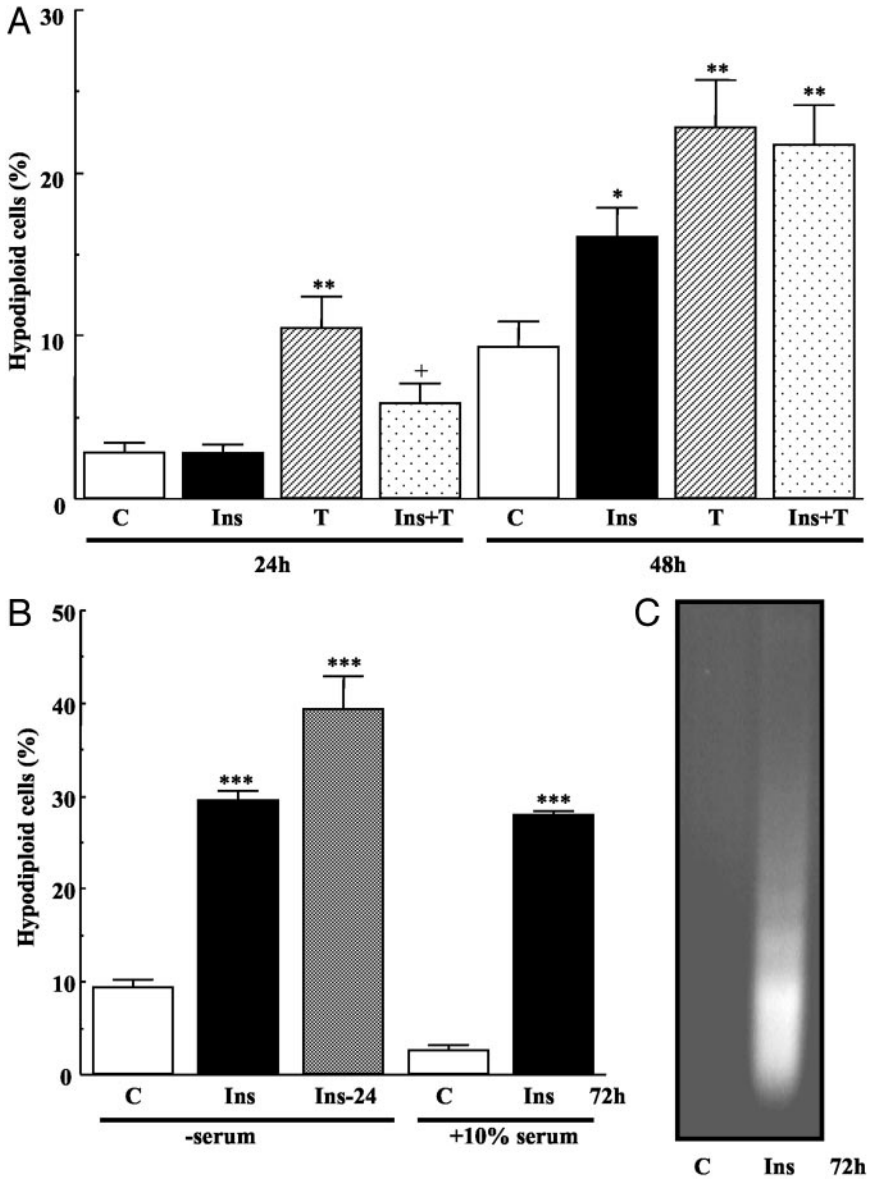


FIG. 1. Long-term treatment with insulin induces a process of apoptosis in brown adipocytes. Cells were maintained without serum, either untreated (C), treated with 10 ng/ml TNF-$\alpha$ (T), 20 nM insulin (Ins), or with both signals for the indicated time periods (24, 48, or 72 h). Insulin was either added at time 0 and after 48 h (for Ins 72 h) or daily (Ins-24). When indicated, cells were maintained with 10% serum. A and B, Histograms showing the percentage of cells with DNA content lower than 2C (hypodiploid cells). Results are means ± SEM of three independent experiments. Statistical analysis was carried out using Student's $t$ test by comparison either with control cells (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$) or with cells treated with TNF-$\alpha$ ($^+$, $P < 0.05$). C, Representative agarose electrophoresis of DNA from the extranuclear fraction from control cells or cells treated with insulin for 72 h, showing DNA ladder.

whereas treatment for 24 h did not (Fig. 1A). Based on these results, we have studied the effect of insulin on brown adipocytes survival at 72 h. After 72 h of treatment, insulin induced a significant increase in the number of apoptotic cells, either in serum-starved cells or in cells maintained in a medium with 10% serum (Fig. 1B). Moreover, addition of insulin daily induced a slightly higher increase in apoptosis than when it is added at time 0 and after 48 h. In addition, insulin was able to induce DNA laddering after 72 h (Fig. 1C), confirming that insulin was inducing a process of apoptosis in brown adipocytes after long-term treatment and that the continuos presence of insulin is necessary.

### *Conditioned medium from long-term insulin-treated brown adipocytes induces an early apoptotic process*

Because the proapoptotic effect of insulin appeared only after a long-term treatment, we thought about the possibility that insulin could induce the synthesis and release of proapoptotic factor(s) to the medium, which could be involved in the induction of apoptosis. To check this hypothesis, the effect of this conditioned medium on new freshly isolated cells maintained in culture was studied.

Figure 2A shows that brown adipocytes maintained with conditioned medium from control cells (cells maintained without serum for 72 h) for 24 h or with fresh medium did not present any morphological change. However, brown adipocytes treated with the conditioned medium from cells previously treated with insulin for 72 h lost cellular contacts and many of them detached from the plate after 24 h. All these features resembled the morphological changes occurring in a process of apoptosis. Thus, to further characterize this process, we measured DNA content by flow cytometry after staining with propidium iodide. As shown in Fig. 2B, conditioned medium from cells treated with insulin for 72 h

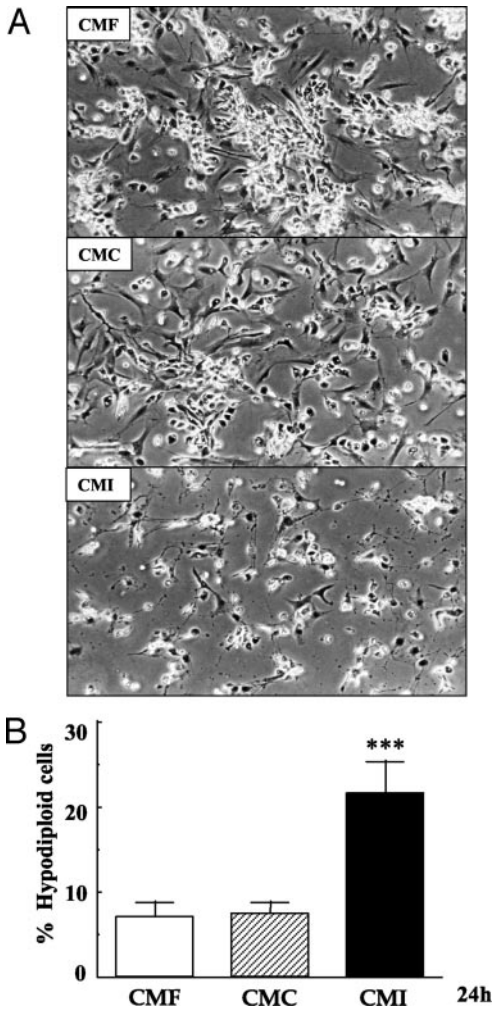


FIG. 2. Conditioned medium from brown adipocytes treated with insulin for long time periods induces an early apoptotic process. Cells were maintained for 24 h with fresh medium without serum (CMF) or conditioned medium from either control cells (CMC) or cells treated with insulin for 72 h (CMI). A, Analysis of cellular morphology by phase-contrast microscopy. B, Histograms showing the percentage of cells with DNA content lower than 2C (hypodiploid cells). Results are means ± SEM of five independent experiments. Statistical analysis was carried out using Student's *t* test by comparison with cells maintained with fresh medium (***, $P < 0.001$).

induced a significant increase in the percentage of cells with DNA content lower than 2C as compared with control cells treated with either fresh medium or conditioned medium from untreated cells.

### *Treatment with PI3K inhibitors or rapamycin prevents long-term insulin-induced apoptosis in brown adipocytes*

Insulin is known to activate different signal transduction pathways, such as ERK, PI3K/Akt, and p70S6K, in different cell types, including brown adipocytes (9, 20–22). Other signaling pathways, such as JNK, p38MAPK, or NF-κB, can be activated by insulin in certain cell types (23–25). Therefore, we have determined the activation of these pathways by insulin in fetal brown adipocytes under our experimental conditions.

Figure 3A shows that insulin treatment for 5 min induced the activation of ERKs, Akt, mTOR, and p70S6K as determined by Western blot analysis using antiphospho-antibodies. However, no activation of JNK, p38MAPK, or NF-κB was detected. Akt, mTOR, and p70S6K were still active after 72 h of treatment with insulin (Fig. 3B), coincident with the initiation of apoptosis, whereas ERK activation was not maintained. In addition, p38 was activated upon 72 h of treatment with insulin, and NF-κB activation was slightly decreased (Fig. 3B).

Because the conditioned medium from insulin-treated cells mimicked the proapoptotic effect of insulin, we wanted to identify the signal transduction pathways involved in this proapoptotic effect. To do this, brown adipocytes were treated with insulin for 72 h, either in the presence or absence of some inhibitors of the signal transduction pathways activated by insulin: PD98059 for MEK/ERK, LY294002 for PI3K/Akt, and rapamycin for mTOR/p70S6K. Then the proapoptotic effect of conditioned mediums obtained under these different treatments was checked.

As shown in Fig. 3C, conditioned medium from cells treated with insulin increased the percentage of apoptotic cells (hypodiploid cells) at 24 h as well as the conditioned medium from cells treated with insulin and the MEK inhibitor, PD98059. However, conditioned medium from cells treated with insulin plus LY294002 or rapamycin did not induce apoptosis. Therefore, it appears that the PI3K/mTOR/p70S6K pathway mediates the proapoptotic effect of the conditioned medium from insulin-treated cells because the PI3K inhibitor and rapamycin, which both block insulin-induced mTOR/p70S6K activation in brown adipocytes (data not shown), prevent this apoptotic effect. Moreover, short-term treatment (5 min) with conditioned medium from cells treated with insulin plus rapamycin (antiapoptotic condition) did not active mTOR and p70S6K, as expected, and prevented the NF-κB activation decrease induced by conditioned medium from insulin-treated cells, whereas Akt was maintained active and ERK and JNK activities remained unchanged (Fig. 3D). Thus, it is clear that mTOR/p70S6K activation is required for cell death, although it cannot be excluded that other pathways that are also differentially modulated under these conditions, such as NF-κB, could participate in the mediation of apoptosis.

The PI3K/mTOR/p70S6K pathway, which seemed to be responsible for the long-term insulin-induced apoptosis, regulates protein synthesis (see reviews in Refs. 26 and 27). Therefore, it is very likely that the proapoptotic effect of insulin depends on protein synthesis regulated by the PI3K/mTOR/p70S6K pathway.

To confirm that the PI3K/mTOR/p70S6K pathway is responsible for the long-term insulin-induced apoptosis, the effect of pretreatment with rapamycin was assessed. Figure 4A shows that long-term insulin treatment induced loss of cellular contacts, and many of them detached from the plate. These morphological changes, characteristic of apoptosis, were not produced in cells pretreated with rapamycin. Similarly, caspase 3 activity was significantly increased by insulin (72 h), and rapamycin pretreatment prevented this activation (Fig. 4B).

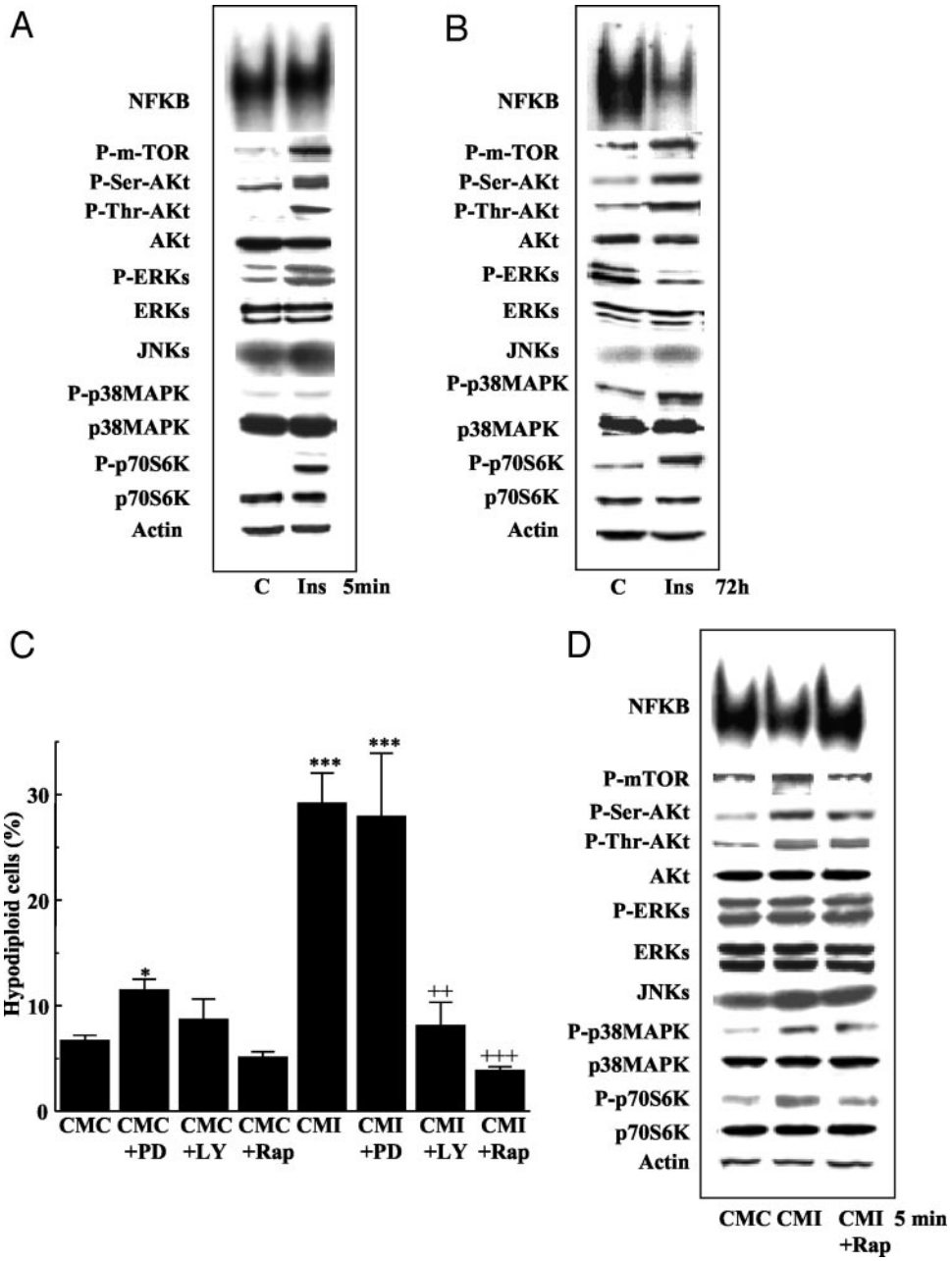


FIG. 3. Conditioned medium from brown adipocytes treated with insulin and either a PI3K inhibitor or rapamycin does not induce apoptosis. Analysis of signal transduction pathways activated by insulin upon either 5 min (A) or 72 h of treatment (B). Serum-starved cells were triggered with 20 nM insulin (Ins) for 5 min or 72 h, or maintained untreated (C) as indicated. Then, the level of the phosphorylated (active) forms of Akt, ERKs, p38MAPK, and p70S6K was determined as well as the activation of JNKs and NF-$\kappa$B, as described in *Materials and Methods*. C, Apoptotic effect of the conditioned medium from cells maintained with insulin for 72 h either in the presence or absence of PD98059 (PD), LY294002 (LY), or rapamycin (Rap). Cells were treated with these conditioned media for 24 h. Histograms show the percentage of hypodiploid cells. Results are means ± SEM of three independent experiments. Statistical analysis was carried out using Student's *t* test by comparison with either cells maintained with control conditioned medium (*, $P < 0.05$; ***, $P < 0.001$) or with conditioned medium from insulin-treated cells ($^{++}$, $P < 0.01$; $^{+++}$, $P < 0.001$). D, Analysis of signal transduction pathways activated upon 5 min of treatment with conditioned medium from either control cells (CMC), cells treated with insulin for 72 h (CMI), or cells treated with insulin plus rapamycin for 72 h (CMI+Rap). Normalization of the blots was done by reprobing them with anti-$\beta$-actin antibody.

*Apoptosis induced by conditioned medium from long-term insulin-treated brown adipocytes involves activation of caspases 8, 9, and 3*

Next, we tried to characterize the mechanism of apoptosis induced by the conditioned medium from long-term insulin-treated cells. We examined Bid cleavage, cytochrome *c* release, and activation of caspases 8, 9, and 3 at different time points.

Figure 5A shows a decrease in the level of Bid proform in brown adipocytes treated with conditioned medium from long-term insulin-treated cells compared with control cells. This decrease begins after 10 h of treatment, being more evident at later time points, and it is probably the consequence of its cleavage by caspase 8. In fact, caspase 8 activity increased after 8 h of treatment under the same conditions and was maintained at least up to 24 h (Fig. 5C). Thus, it is very likely that the active p15 fragment of Bid could be present and could be inserted into the mitochondrial membrane leading to cytochrome *c* release. According to this, a decrease in the mitochondrial cytochrome *c* content was observed (Fig. 5A) at 10, 18, and 24 h after treatment under the same conditions. In addition, an increase in cytosolic cytochrome *c* was clearly detected after 10 h (Fig. 5B). This cytochrome *c* release at 10 h was coincident with an increase in the 35- to 37-kDa active caspase 9 fragments in cells treated with the conditioned medium from long-term insulin-treated cells and with the increase of the active caspase 3 fragment (Fig. 5A). At later time points, active caspase 9 was not detected; however, the proteolytic caspase 3 fragment was present, and its amount increased with time.

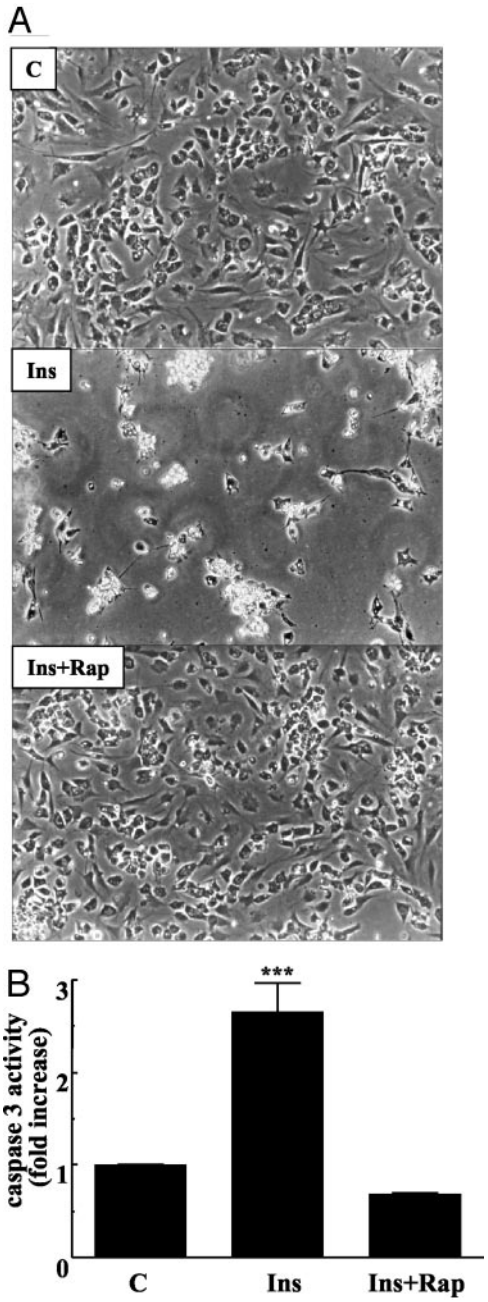


FIG. 4. Inhibition of mTOR/p70S6K with rapamycin blocks insulin-induced apoptosis. Cells were maintained without serum, either untreated (C) or treated with 20 nM insulin (Ins) or with insulin plus rapamycin (Ins+Rap) for 72 h. A, Analysis of cellular morphology by phase-contrast microscopy. B, Caspase 3 activity. Relative values of the specific enzymatic activity expressed as fold increase of control value. Results are means ± SEM of five independent experiments. Statistical analysis was carried out using Student's *t* test by comparison with control value (***, $P < 0.001$).

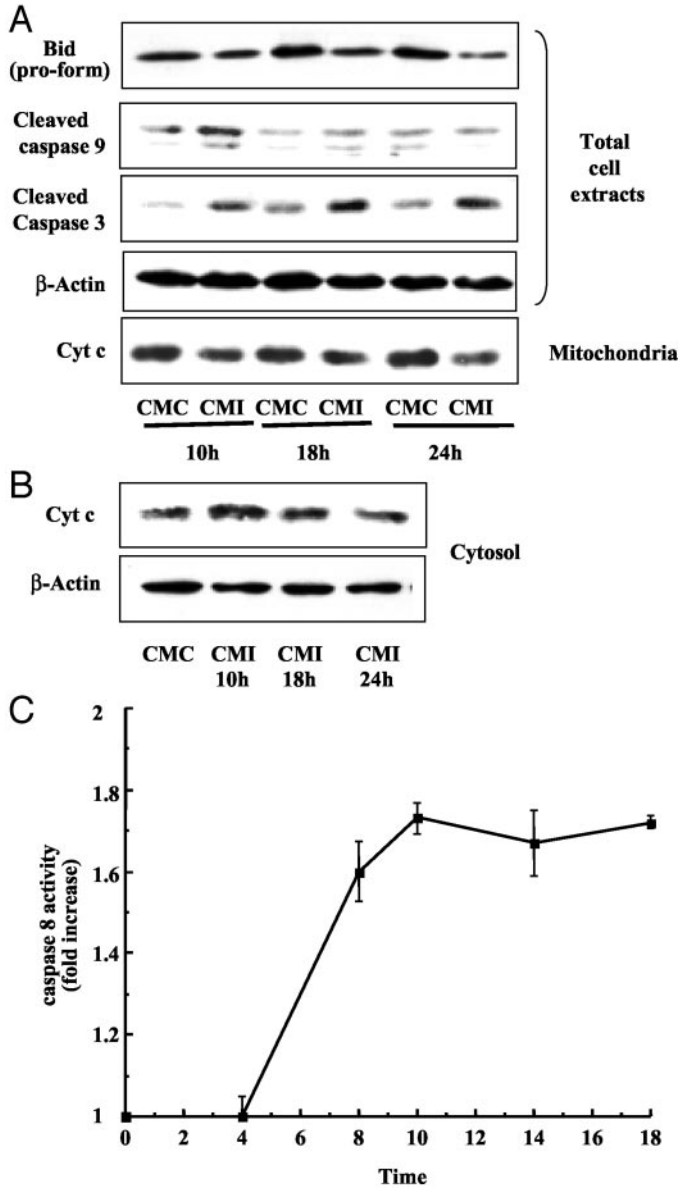


FIG. 5. Time course of caspases activation in the apoptosis induced by conditioned medium from insulin-treated brown adipocytes. Cells were treated with conditioned medium from either control cells (CMC) or cells treated with insulin (CMI) for different time periods as indicated. A, Representative Western blot analysis of Bid proform, cleaved caspases 9 and 3, and cytochrome *c* (Cyt *c*; from mitochondria) levels corresponding to one independent experiment of three. B, Representative Western-blot analysis of cytosolic cytochrome *c*. Normalization was done by reprobing the blots of total and cytosolic extracts with anti-β-actin antibody. An equal amount of mitochondrial proteins in the blots was assured by staining with Ponceau Red (not shown). C, Caspase 8 activity. Relative values of the specific enzymatic activity expressed as fold increase of control value. Results are means ± SEM of three independent experiments.

Inhibition of caspases by the pan-caspase inhibitor, Z-VAD, prevented caspase 8 activation (Fig. 6A), the decrease in Bid level, and the activation of caspases 9 and 3 (Fig. 6B) produced at 10 h. In addition, apoptosis induced by conditioned medium from long-term insulin-treated brown adipocytes was blocked by Z-VAD at 24 h (Fig. 6C).

### *Role of oxidative stress in the insulin-induced apoptosis of brown adipocytes: antiapoptotic effect of antioxidants*

To get a further insight into the mechanism of apoptosis after long-term treatment of brown adipocytes with insulin, we analyzed the possibility that oxidative stress could play a role. First, we studied whether antioxidants were able to

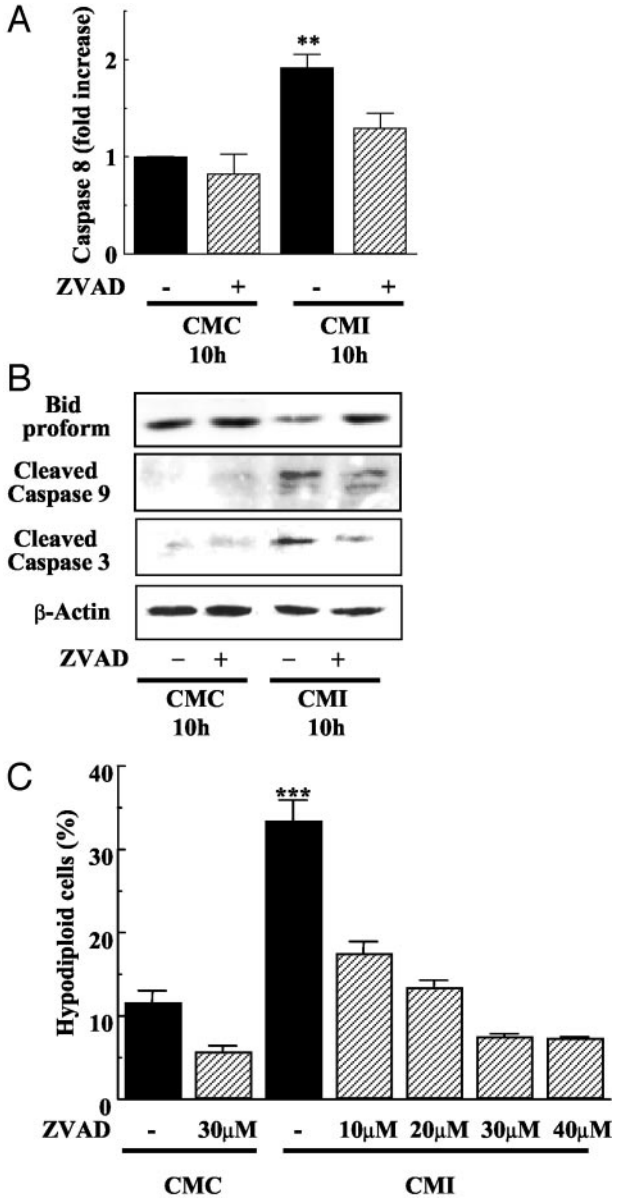


FIG. 6. Z-VAD prevents the activation of the caspases cascade and cell death induced by conditioned medium from insulin-treated brown adipocytes. Cells were treated for 10 h (for A and B) or 24 h (for C) with conditioned medium from either control cells (CMC) or cells treated with insulin (CMI). When indicated, cells were pretreated with pan-caspase inhibitor Z-VAD at 40 $\mu$M (for A and B) or 10–40 $\mu$M (for C). A, Caspase 8 activity. Relative values of the specific enzymatic activity expressed as fold increase of control value (cells treated with the conditioned medium from control cells). Results are means ± SEM of three independent experiments. B, Representative Western blot analysis of Bid proform and cleaved caspase 9 and 3 levels corresponding to one independent experiment of three. Normalization was done by reprobing the blots with anti-$\beta$-actin antibody. C, Histograms showing the percentage of hypodiploid cells. Results are means ± SEM of three independent experiments. A and C, Statistical analysis was carried out using Student's $t$ test by comparison with cells treated with control conditioned medium (**, $P < 0.01$; and ***, $P < 0.001$).

prevent apoptosis induced by insulin. Long-term insulin treatment induced loss of cellular contacts and detachment of cells from the plate (not shown). These morphological changes characteristic of apoptosis were not produced in cells treated with the antioxidants, ascorbate and ascorbate plus superoxide dismutase. Similarly, the insulin-induced increase in the percentage of hypodiploid cells was not produced when these antioxidants were present (Fig. 7A).

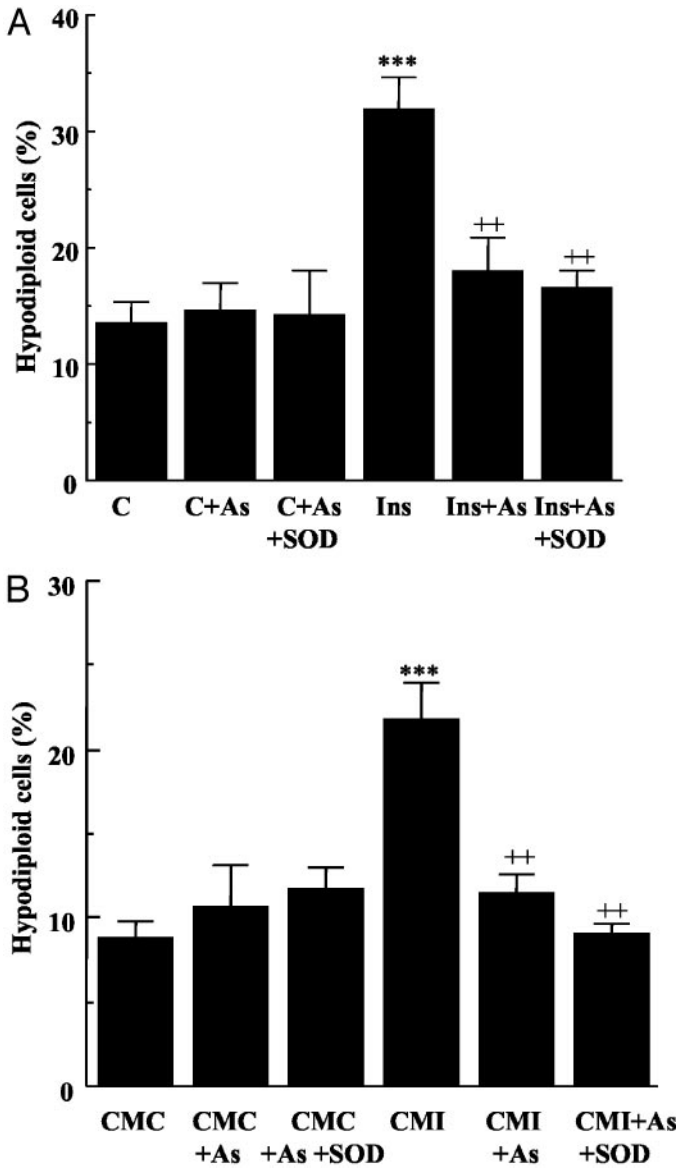


FIG. 7. Apoptotic cell death induced by long-term treatment of brown adipocytes with insulin or by their conditioned medium is blocked by antioxidants. A, Percentage of hypodiploid cells. Cells were maintained untreated (C) or treated with insulin (Ins) for 72 h in the presence or absence of the antioxidants, ascorbic acid (As; 10 mM) or ascorbic acid plus superoxide dismutase (SOD; 60 $\mu$g/ml), as indicated. Results are means ± SEM of four independent experiments. Statistical analysis was carried out using the Student's $t$ test by comparison with control cells (***, $P < 0.001$) or with cells treated with insulin ($^{++}$, $P < 0.01$). B, Percentage of hypodiploid cells. Cells were treated for 24 h with control conditioned medium (CMC) or with conditioned medium from insulin-treated cells (CMI) in the presence or absence of the antioxidants described above. Statistical analysis was carried out using Student's $t$ test by comparison with cells treated with control conditioned medium (***, $P < 0.001$) or with conditioned medium from insulin-treated cells ($^{++}$, $P < 0.01$).

Hence, generation of oxidative stress is involved in the insulin-induced apoptosis of brown adipocytes. Moreover, Fig. 7B shows that the addition of antioxidants also prevented apoptosis induced by conditioned medium from insulin-treated cells, suggesting that the generation of oxidative stress is required to induce this process of apoptosis. This oxidative stress might be created as a consequence of an elevation in the level of ROS and a subsequent imbalance between oxidants and cellular antioxidants such as glutathione. In fact, as shown in Fig. 8A, long-term insulin treatment induced a decrease in the level of GSH. This decrease was also observed in cells maintained with the conditioned medium from insulin-treated brown adipocytes but not in cells cultured with the conditioned medium from brown adipocytes treated with insulin plus rapamycin (Fig. 8B). Moreover, an increase in ROS level was induced by conditioned medium from insulin-treated brown adipocytes but not by conditioned medium from brown adipocytes treated with insulin plus rapamycin (Fig. 8C). The generation of ROS was prevented by treatment with the inhibitor of cellular NADPH oxidases, DPI (Fig. 8D), whereas inhibition of mitochondrial electronic transport chain had no effect (not shown). Therefore, all these data indicate that generation of ROS and the subsequent decrease in GSH level are important mediators of the process of apoptosis induced by insulin. NADPH oxidases appear to play a role in the generation of ROS.

The generation of ROS occurred early during the process of apoptosis (at 8–10 h), raising the possibility that it could be responsible for the activation of caspases 8, 9, and 3. Thus, the effect of antioxidants on Bid cleavage and caspase 8 activation was assessed. As shown in Fig. 8E, the decrease in the level of Bid induced by conditioned medium from insulin-treated brown adipocytes after 10 h was prevented by the addition of antioxidants as well as the activation of caspase 8 (Fig. 8F). Therefore, the increase in ROS is an early event in the cascade of activation of apoptosis, which seems to be responsible for the insulin-induced apoptosis in brown adipocytes. However, the level of UCP-1, -2, and -3 was not modified by treatment with insulin for 72 h (Fig. 8G). In particular, UCP-1 is highly expressed and remains unchanged, so it is unclear that changes in UCP-1 level can account for the insulin-induced ROS generation. However, it cannot be excluded that changes in UCP-1 activity could play a role.

## Discussion

Insulin has been shown to be a survival factor for different cell types (13–15), including adipocytes (11–12). It protects from apoptosis induced by the expression of the oncogene H-ras (H-ras$^{Lys12}$) in brown adipocyte-derived cell lines upon serum deprivation (11). Similarly, insulin protects from apoptosis induced by TNF-$\alpha$ in white adipocytes (12). However, although insulin partially protects from TNF-$\alpha$-induced apoptosis in cultured rat fetal brown adipocytes at 24 h, it does not protect at 48 h (Fig 1). Insulin is also unable to counteract the apoptosis induced by TNF-$\alpha$ plus cycloheximide in cultured brown adipocytes from mice (16). Moreover, we present here a novel function of insulin as a proapoptotic signal for brown adipocytes. We describe a process of apoptosis induced by long-term treatment of brown adipocytes with insulin or by short-term treatment with the conditioned medium from these cells. This process is dependent on the activation of the PI3K/mTOR/p70S6K pathway. The generation of ROS is an early event that appears to mediate activation of caspases 8, 9, and 3 as well as the release of cytochrome *c* from the mitochondria.

The generation of oxidative stress appears to be the main mediator of this process of apoptosis. The role of ROS in cell death was first shown for the TNF-$\alpha$-induced apoptosis (28–29), and their contribution to the activation of the execution machinery necessary for apoptosis has been extensively demonstrated for many processes of apoptosis, such as those induced by TGF-$\beta$ (18–19), Fas (30), p53 (31), or ischemia (32–33). It has even been recently shown that ROS are required for the oxidation of externalized phosphatidylserine necessary for macrophage clearance of apoptotic cells (34). In brown adipocytes stimulated with the conditioned medium from insulin-treated cells, the generation of ROS is a very early event, coincident with the initial activation of caspase 8, and antioxidants blocked this activation. Thus, ROS might mediate this activation. However, it is unclear the mechanism responsible for this activation. Several mechanisms have been described for the proapoptotic effects of ROS, which are different depending on the cell system and the apoptotic stimulus. Some of them, involves changes in the level of proteins from the Bcl-2 family such as Bcl-$x_L$ (18–19) or Bax or in their subcellular localization (35). For example, in the process of apoptosis induced by TGF-$\beta$ in fetal hepatocytes, ROS induce a decrease in the expression of Bcl-$x_L$ mRNA and protein, which leads to the release of cytochrome c from mitochondria and the subsequent activation of caspases 9 and 3 (18–19). Then, caspase 3 might activate caspase 8 creating a loop of caspases activation (36). However, we have been unable to detect either changes in the level of Bcl-$x_L$, Bcl-2 or Bax or in their subcellular localization (not shown). Hence, the mechanism activating caspase 8 by ROS might be different and could be related with modifications of protein components of the mitochondrial membranes or of polyunsaturated fatty acids (see review in Ref. 35). In membranes, ROS can affect cysteine residues, leading to intramolecular cross-linkings and generation of protein aggregates. ROS can also produce lipid peroxidation or even increase the release of calcium from mitochondria (see review in Ref. 37). Thus, it is possible that either by modifications in proteins, such as those from the Bcl-2 family, or by lipid peroxidation, the permeability of the mitochondrial membranes can be altered, leading to the release of proapoptotic molecules from the mitochondria, such as cytochrome *c* or even caspase 8 (38). In that case, caspase 8 could be activated just because its release to the cytosol or even by a very early activation of caspase 3 as a consequence of cytochrome *c*-dependent caspase 9 activation.

Regarding the origin of ROS, NADPH oxidase might play a role because inhibition of this enzyme with DPI prevented ROS generation. NADPH oxidase, highly expressed in white adipocytes, is also responsible for $H_2O_2$ generation upon short-term insulin treatment of 3T3-L1 adipocytes (39) and human adipocytes (40).

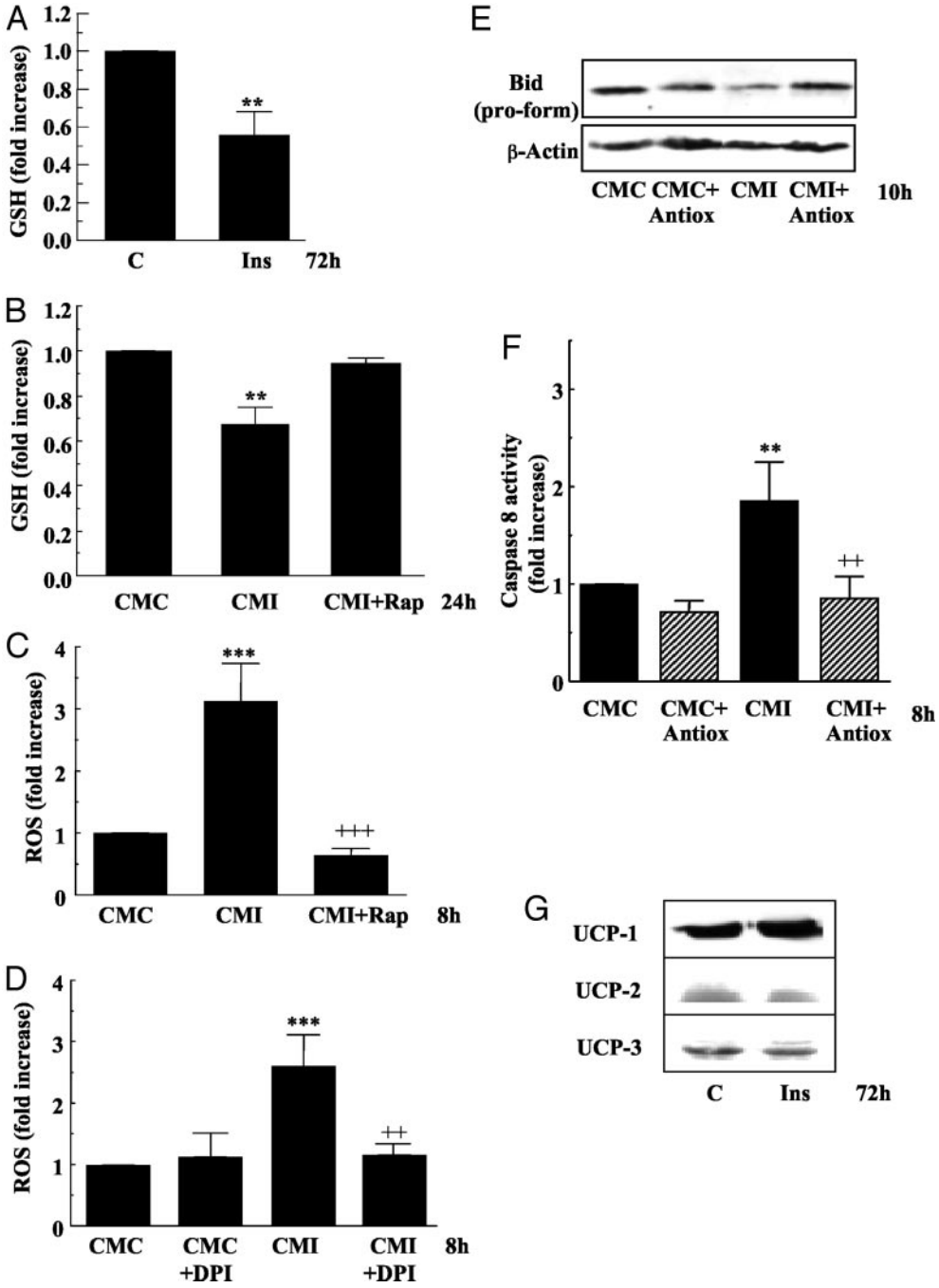


FIG. 8. Role of oxidative stress in the apoptosis induced by long-term treatment with insulin. A, Glutathione (GSH) level in brown adipocytes maintained untreated (C) or treated with insulin (Ins) for 72 h. Results are expressed as fold increase of control value of GSH/$\mu$g protein and are means ± SEM of three independent experiments. Statistical analysis was carried out using Student's *t* test by comparison with control cells (**, $P < 0.01$). B, C, and D, Cells were treated for 24 h (for B) or 8 h (for C and D) with control conditioned medium (CMC) or with conditioned medium from insulin-treated cells (CMI) or from cells treated with insulin plus rapamycin (CMI+Rap), and it was measured. B, glutathione level (GSH/$\mu$g protein); C, ROS level (ROS/$\mu$g protein); and D, the effect of the inhibitor of NADPH oxidase, DPI, on ROS level (ROS/$\mu$g protein). DPI (10 $\mu$M) was added to conditioned mediums 2 h before ROS quantification. Results are expressed as fold increase of control value (cells treated with control conditioned medium) and are means ± SEM of three independent experiments. E and F, Cells were treated for 8 h (for F) or 10 h (for E) with control conditioned medium (CMC) or with conditioned medium from insulin-treated cells (CMI) in the presence or absence of the antioxidants, ascorbic acid and superoxide dismutase (Antiox). E, Representative Western blot analysis of Bid proform. F, Caspase 8 activity. Relative values of the specific enzymatic activity expressed as fold increase of control value (cells treated with the conditioned medium from control cells). Results are means ± SEM of three independent experiments. Statistical analysis was carried out using Student's *t* test by comparison with cells treated with control conditioned medium (**, $P < 0.01$; and ***, $P < 0.001$) or conditioned medium from insulin-treated cells ($^{++}$, $P < 0.01$; and $^{+++}$, $P < 0.001$). G, Representative Western blot analysis of UCP-1, -2, and -3 in mitochondria from brown adipocytes maintained either untreated or treated with insulin for 72 h in the absence of serum. Equal amount of mitochondrial proteins in the blots was assured by staining with Ponceau Red (not shown).

In relation to the possible role of mitochondria in ROS production and/or apoptosis, it is clear that brown adipocytes are cells particularly rich in mitochondria, and this fact is important for the response of cells against apoptotic stimuli. First, mitochondria play a central role in apoptosis because they sequester different proapoptotic factors. In addition, recent data indicate that resistance to apoptosis of tumor cells can be associated with a lower mitochondrial cellular activity as well as with changes in proteins from the Bcl-2 family (41). Moreover, brown fat mitochondria are very peculiar because they present UCP-1, which could play a role in the defense against oxidative stress. UCPs can decrease ROS production by mitochondria, and they can be regulated by ROS. UCP-2 was shown to protect from ROS by inducing mitochondrial uncoupling (42), which would lead to a decrease in ROS. Thus, macrophages from UCP-2-deficient mice generate more ROS than wild-type cells (43). In addition, UCP-2-induced ROS decrease seems to be involved in protection from cell death in cortical neurons from transgenic mice overexpressing UCP-2 (44). Moreover, superoxide was shown to increase mitochondrial proton conductance through activation of UCP-1 in mitochondria from BAT, UCP-2 in mitochondria from kidney, spleen, and pancreatic $\beta$ cells, and UCP-3 in skeletal muscle (45). According to this, skeletal-muscle mitochondria from mice lacking UCP-3 are more coupled and produce more ROS (46). On the other hand, ROS could also induce an increase in the expression of some of the UCPs (see review in Ref. 47). However, the mitochondrial production of ROS in the apoptosis of brown adipocytes induced by insulin appears not to be so relevant because the inhibitor of the respiratory chain, rotenone, did not have any significant effect on ROS production (not shown). In addition, UCP-1 level remains high upon induction of the process, and it is unclear whether changes in UCP-1 level could either account for the insulin-induced ROS generation or could regulate the apoptotic process. However, it cannot be excluded that changes in UCP-1 activity can be produced. Thus, in insulin-treated cells, UCP-1 activity could be lower than in control brown adipocytes because lipogenesis is activated by insulin, and as a consequence, free fatty acids, the activators of UCP-1, would not be available to activate UCP-1. UCP-2 and -3 expression is very low and remains also unchanged, so it is not very likely they can contribute to this process.

On the other hand, this oxidative stress induced by long-term insulin treatment or by the conditioned medium from insulin-treated cells might reflect the presence of a similar mechanism to that observed in the Drosophila and *Caenorhabditis elegans* models, where signaling through the insulin/IGF receptor homologs (InR and DAF-2, respectively) induces oxidative stress, correlated with reduced life span and apoptosis (see review in Ref. 49). The PI3K/Akt pathway and a forkhead transcription factor homolog (DAF-16) are involved (49). Activation of the forkhead transcription factor, Foxo3a, by oxidative stress or by inhibition of PI3K leads to up-regulation of genes involved in DNA repair, apoptosis, cell cycle arrest, and antioxidant response in mammals (50). In addition, phosphorylation and inactivation of Foxo1 induced by insulin through Akt pathway is important for terminal adipocytic differentiation (51–52) and could play a role in other cellular functions of adipocytes. However, our results do not support the presence of a mechanism where Akt and forkhead transcription factors are involved in the insulin-induced cell death. Thus, although the inhibition of the PI3K highly reduces apoptosis of brown adipocytes, the sole inhibition of mTOR/p70S6K by rapamycin is enough to prevent insulin-induced cell death, although Akt activity is maintained high.

The physiological meaning of the proapoptotic effect of insulin in brown adipocytes with long-term treatment is unknown. However, it can be related with its thermogenic function. Different from white adipose tissue, BAT does not accumulate lipids just as a storage depot but also as a source of fatty acids to be oxidized in the mitochondria when BAT thermogenesis is activated to produce heat (1). Thus, it is possible that brown adipocytes treated for a long time with insulin accumulate large amount of lipids that cannot be oxidized in the absence of signals activating thermogenesis. This might cause apoptosis of brown adipocytes to eliminate cells with a high lipid content unable to contribute to thermogenesis.

On the other hand, the apoptosis of brown adipocytes induced by prolonged exposure to insulin, which we describe in this article, might also represent an important mechanism involved in the reduction of BAT function in those situations where insulin is continuously present, such as type 2 diabetes or obesity, although it should be considered that BAT amount in humans is lower than in rodents. In mice, in agreement with our proposal, partial ablation of BAT leads to obesity, insulin resistance, and decreased oxygen consumption (53–54). Moreover, an atrophy of BAT is produced in adult obese animals (55–57), and TNF-$\alpha$-induced apoptosis an important determinant of this reduced BAT function (16). Therefore, although insulin can protect from TNF-$\alpha$-induced apoptosis of brown adipocytes after short-term treatment, the prolonged presence of insulin might have a pathological effect that induces apoptotic cell death and contributes to a loss of BAT mass and function, resulting in a secondary insulin resistance.

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