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Inhibition of Death-Receptor Mediated Apoptosis in Human Adipocytes by the Insulin-Like Growth Factor I (IGF-I)/IGF-I Receptor Autocrine Circuit

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Adipose tissue mass is reflected by the volume and the number of adipocytes and is subject to homeostatic regulation involving cell death mechanisms. In this study we have investigated the mechanisms of apoptosis in human preadipocytes and adipocytes that may play a role in the regulation of adipose tissue mass. We found that death receptors (CD95, TNF-related apoptosis-inducing ligand receptors 1 and 2, and TNF receptor 1) are expressed in human fat cells and that apoptosis can be induced by specific ligands. Sensitivity to apoptosis could be stimulated by an inhibitor of biosynthesis. In addition, inhibition of auto-/paracrine action of IGF-I dramatically sensitizes human adipocytes for death ligand-induced apoptosis. Phosphoinositide 3-kinase and, to a weaker extent, p38 MAPK are involved in IGF-I-mediated survival. IGF-I protects human fat cells from apoptosis by maintaining the expression of antiapoptotic proteins, Bcl-x_L and Fas-associated death domain-like IL-1-converting enzyme inhibitory protein. In conclusion, we identified mechanisms of apoptosis induction in human fat cells. We furthermore demonstrate that human fat cells protect themselves from apoptosis by IGF-I in an auto-/paracrine manner. (*Endocrinology* 145: 1849–1859, 2004)

IN ADDITION To its primary function of energy storage, white adipose tissue plays significant roles in metabolic regulation and overall energy homeostasis, and in part regulates satiety and insulin sensitivity (1). Adipose tissue mass is determined by competing processes regulating both the volume and the number of adipocytes. Increases in adipose tissue mass can result from an increase of the volume of adipocytes. Furthermore, adipocyte number may increase due to proliferation and differentiation of adipocyte precursor cells, which are found in large amounts in adipose tissue (2). Alternately, a reduction in fat tissue mass generally involves the loss of stored lipids by lipolytic processes. A reduction of the number of adipocytes may also be seen, especially in conditions where large amounts of fat are lost (3, 4).

There is now growing evidence that decreases in adipose tissue mass in humans could result from a loss of fat cells through programmed cell death (5–12). Fat cell apoptosis was demonstrated in patients with tumor cachexia (6) and in human immunodeficiency virus (HIV) patients during treatment with protease inhibitors (9). Patients with acquired forms of lipodystrophy (Lawrence syndrome and Barraquer-Simons syndrome) show an immunologically mediated loss of fat cells, probably by apoptosis (12). Studies in rodents demonstrate that induced weight loss, such as starvation, streptozotocin-induced diabetes, or intracerebroventricular administration of leptin, results in apoptosis of fat cells (13–16). In 3T3-L1 cells, apoptosis may be induced by serum deprivation or exposure to TNFα and HIV protease inhibitors (17–19).

To date, general mechanisms for apoptosis induction in human adipocytes have not been intensively investigated. The identification of such mechanisms as well as factors modulating apoptosis sensitivity in human fat cells would be important, however, for understanding the pathogenesis of fat cell loss in different clinical conditions. In the present study, therefore, we studied death receptor-mediated apoptosis in human fat cells using the SGBS cell strain (20) as well as primary cells obtained from sc adipose tissue from healthy patients.

Materials and Methods

Adipose tissue samples

Subcutaneous adipose tissue was obtained from 12 healthy, female patients (mean age, 44.2 ± 4.9 yr; mean body mass index, 32.2 ± 3.7 kg/m^2) undergoing abdominal reduction. The experimental protocols were approved by the ethical committee of University of Ulm, and all patients gave informed consent to the procedure.

Cell culture

Preadipocytes were prepared from adipose tissue samples by collagenase digestion according to an established protocol (21, 21). Cells were seeded in DMEM/Ham's F-12 (1:1) containing 10% fetal bovine serum (FBS), 33 μM biotin, 17 μM pantothenate, and antibiotics. After 12 h, monolayers were washed three times with PBS to remove nonattached cells. Cultures were thereafter used for experiments, or adipogenic dif-

Abbreviations: CHX, Cycloheximide; DAPI, 4′,6′-diamidino-2-phenyindol; FBS, fetal bovine serum; FLIP, Fas-associated death domain-like IL-1-converting enzyme inhibitory protein; HIV, human immunodeficiency virus; IGF-IR, IGF-I receptor; MEK, MAPK kinase; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; TNFR, TNF receptor; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TNF-related apoptosis-inducing ligand receptor.

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ferentiation was induced as described below. SGBS preadipocytes were cultured in DMEM/Ham's F-12 (1:1) containing 33 μM biotin, 17 μM pantothenate, antibiotics (serum-free, basal medium), and 10% FBS.

In SGBS cells, adipogenic differentiation was induced after reaching near confluence. In human preadipocytes, adipogenic differentiation was induced 12 h after preparation. Cells were washed three times with PBS and cultured in serum-free, basal medium supplemented with 10 μg/ml iron-poor transferrin, 10 nM insulin, 200 pM T_3, and 1 μM cortisol. For the first 4 d, 2 μM rosiglitazone (BRL 49653), 250 μM isobutylmethylxanthine, and 25 nM dexamethasone were added. The medium was changed every 4 d. Morphologically differentiated adipocytes were obtained after 14 d. The number of differentiated cells was estimated in the monolayers by direct counting using a net micrometer, and cultures were used for experiments when the differentiation rate was 85% or greater.

Detection of CD95 and CD120a expression

For flow cytometric analysis, preadipocytes grown in 12-well plates were detached using accutase (PAA Laboratories, Linz, Austria) and washed once with PBS. Cells were incubated in the dark for 30 min at 4 C with PE-conjugated anti-CD95 (Dako, Hamburg, Germany), anti-CD120a [TNF receptor (TNFR1); Dako], or IgG1 control antibody (Dako). After washing, cells were analyzed by flow cytometry. Adipocytes were stained as monolayers in 12-well plates while still adherent. Cells were incubated in the dark for 30 min at 4 C with Ab as described before. After washing, adipocytes were carefully detached using accutase and directly analyzed by flow cytometry.

RT-PCR

Total RNA was prepared from preadipocytes and adipocytes using TRIzol (Life Technologies, Inc./BRL, Karlsruhe, Germany). RT-PCR was performed using the Gene Amplification RNA-PCR kit (PerkinElmer Life Sciences, Zaventem, Belgium) according to the manufacturer's instructions with the following modifications: annealing temperature and cycle number were 57 C and 27 for TNF-related apoptosis-inducing ligand (TRAIL) receptor 1 (TRAIL-R1), 61 C and 27 for TRAIL-R2, and 58 C and 28 for β-actin. Primers for TRAIL-R1 were 5′-TGA GCC GAT GCA ACA ACA GAC AAT-3′ and 5′-CCT CGG CTC CGG GTC CAC AAG A-3′; primers for TRAIL-R2 were 5′-GGC CCC ACA ACA AAA GAG GTC CAG-3′ and 5′-CAG CCC CAG GTC GTT GTG AGC-3′. RT-PCR of IGF-I was performed with primers as described previously (22). β-Actin primers were used for controls (MWG-Biotech, Ebersberg, Germany). Electrophoretically separated PCR products were ethidium bromide-stained, and the fluorescence images were analyzed by an ImageMaster VDS (Pharmacia Biotec, San Francisco, CA). The ratio of the background-corrected integrated ODs of the DNA bands related to β-actin expression was calculated.

Induction of apoptosis

Confluent cultures of SGBS cells were used for apoptosis induction. In human primary preadipocytes, apoptosis was induced 12 h after preparation. In primary human, *in vitro* differentiated adipocytes as well as in human, *in vitro* differentiated SGBS adipocytes, apoptosis was induced after 14 d in adipogenic medium. All experiments were performed in triplicate in 12-well plates. For studying the effects of serum deprivation, monolayers were washed three times with PBS and then incubated in basal medium in the presence or absence of 10% FBS. In all other experiments apoptosis was induced in serum-free, basal medium by adding 1 μg/ml α-APO-1 IgG3, an agonistic monoclonal antibody for CD95 (23), 10^{-8} M TNFα (PeproTech, Rocky Hill, NJ), 100 ng/ml TRAIL (PeproTech), and 10 μg/ml cycloheximide (CHX; Sigma-Aldrich Corp., Taufkirchen, Germany). To investigate whether a growth factor protects cells from apoptosis, IGF-I (provided by Novo Nordisk, Måloev, Denmark) was added at the concentrations indicated. For inhibitor treatments, cells were incubated with 50 μM PD98059, 50 μM SB203580, and 100 μM LY294002 in the absence or presence of death ligands. In some experiments an IGF-I receptor (IGF-IR) blocking Ab α-IR3 (24) (Oncogene, Bad Soden, Germany) was added to a final concentration of 10 μg/ml.

Determination of number of adherent cells

The number of adherent cells was determined after aspirating the medium and washing the monolayer with PBS. Cells were trypsinized and then resuspended in medium containing 10% FBS. The number of cells in the medium was determined using a Coulter counter (Coulter, Hialeah, FL) according to the manufacturer's instructions.

Determination of apoptosis

Quantitative determination of apoptosis was performed according to the method described by Nicoletti *et al.* (25). Preadipocytes were harvested at the time points indicated and washed once with PBS. Cells were resuspended in hypotonic fluorochrome solution containing 50 μg/ml propidium iodide, 0.1% sodium citrate, and 0,1% (vol/vol) Triton X-100. After incubation at 4 C for 16 h, the content of hypodiploid DNA was determined by flow cytometry. In all experiments besides growth factor deprivation, the percentage of specific apoptosis was calculated as follows: 100 × [experimental apoptosis (%) − spontaneous apoptosis in medium (%)]/[100% − spontaneous apoptosis in medium (%)].

For *in vitro* differentiated adipocytes the protocol was modified as follows. At the time points indicated, an equal volume of 2-fold concentrated hypotonic fluorochrome solution (100 μg/ml propidium iodide, 0.2% sodium citrate, and 0.2% Triton-X 100) was added to culture dishes containing adipocytes in medium. After lysis at 4 C for 16 h, cells were resuspended and analyzed as described above.

For analysis of DNA fragmentation, cells were harvested and washed once with PBS. Cell pellets were resuspended with lysis buffer [10 mM Tris-HCl, 400 mM NaCl, 2 mM Na_2EDTA (pH 8.2), and 0.75% sodium dodecyl sulfate] containing 2 mg/ml proteinase K (Roche, Mannheim, Germany) and incubated overnight at 37 C. Protein was precipitated with a 0.3× volume of 5 M NaCl at room temperature (1 h) and centrifuged at 3300 × *g*. DNA in the supernatant was precipitated overnight with a 2× volume of ethanol at −20 C. After washing once with 70% ethanol, the pellet was air-dried and dissolved overnight at 37 C in TE buffer (10 mM Tris-HCl and 1 mM Na_2EDTA, pH 7.2) containing 25 μg/ml ribonuclease A. DNA was separated on a 2% agarose gel stained with ethidium bromide and visualized by UV illumination.

4′,6′-Diamidino-2-phenyindol (DAPI)/Nile Red staining

Preadipocytes and adipocytes grown on chamberslides (BD Biosciences, Heidelberg, Germany) were used for staining nuclei and lipid droplets to show morphological signs of apoptosis. After induction of apoptosis for 24 h, monolayers were washed twice with PBS and then fixed for 10 min at room temperature with 2% paraformaldehyde. Nile Red (Sigma-Aldrich Corp.) was added to a final concentration of 0.5 μg/ml. After removing staining solution, slides were mounted in Mowiol (Calbiochem, Bad Soden, Germany) containing 0.2 μg/ml DAPI (Sigma-Aldrich Corp.). Slides were viewed at room temperature using a 490-nm filter on an Olympus AX70 fluorescence microscope (Olympus Optical Co., Hamburg, Germany). Images were captured with a CC12 digital camera (Soft Imaging System, Munster, Germany) using AnalySIS 3.1 software (Soft Imaging System).

Western blotting and immunoprecipitation

Preadipocytes and adipocytes grown in 75-cm^2 flasks were stimulated with 50 μM PD98059, 50 μM SB203580, or 100 μM LY294002 for 6, 24, and 48 h. Cells were lysed for 15 min at 4 C in lysis buffer [30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton-X 100, 10% glycerol, 1 μM dithiothreitol, and a mixture of proteinase inhibitors (Roche)], followed by high speed centrifugation. Fifty micrograms of lysate were separated on a 10–20% gradient SDS-PAGE and electroblotted onto a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Braunschweig, Germany). Membranes were blocked for 1 h in PBS supplemented with 5% milk powder and 0.1% Tween 20. Membranes were stained for 2 h (mouse monoclonal antibodies) or overnight (rabbit and goat polyclonal IgG) with the primary antibody, followed by 1-h incubation with the horseradish peroxidase-conjugated second antibody, and detection was performed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany). In some experiments, the ratio of the background corrected integrated ODs of the protein bands

related to α-tubulin expression was calculated. Comparisons between medium control and different treatments were made using a *t* test.

The following antibodies were used: Bcl-x (clone 2H12, BD Transduction Laboratories, Germany) and α-tubulin (Ab-1, Oncogene, Bad Soden, Germany). Flip monoclonal antibody was provided by P. Krammer (Heidelberg, Germany), IGF1R monoclonal antibody was provided by K. Siddle (Cambridge, UK). Horseradish peroxidase-conjugated goat antimouse IgG and goat antirabbit IgG were obtained from Santa Cruz Biotechnology, Inc., Europe (Heidelberg, Germany).

For immunoprecipitation, SGBS preadipocytes and adipocytes were lysed in lysis buffer as described above for 15 min at 4 C, followed by high speed centrifugation. Five hundred microliters of lysate in 1 ml lysis buffer were incubated overnight at 4 C with 20 μl protein A/G-Plus/Sepharose (Santa Cruz Biotechnology, Inc., Europe) and 2 μg/ml of a specific antibody against either the IGF-IR (clone 2C8, RDI, Flanders, The Netherlands) or the insulin receptor (clone 83-14, Neomarkers, Fremont, CA), respectively. Sepharose beads were washed three times with lysis buffer and resuspended in sodium dodecyl sulfate-reducing sample buffer. After boiling for 5 min at 95 C, samples were separated by 4–20% gradient SDS-PAGE, followed by Western blotting as described above.

Measurement of IGF-I concentrations in culture medium

IGF-I concentrations in medium were measured by a sensitive and specific RIA (26). The sensitivity of the assay was 0.03 ng/ml; the intraassay variability was 1.6%, and the interassay variability was 6.4%.

Results

Human SGBS preadipocytes show low sensitivity to apoptosis induced by serum deprivation

SGBS preadipocytes were grown in basal medium in the presence or absence of 10% FBS (Table 1). When maintained under serum-containing conditions, preadipocytes started to proliferate. Under serum-free conditions, the number of adherent, viable cells remained constant for 2 d. After 3 d in serum-free medium a slight increase in the number of cells was detected. In parallel, the percentage of apoptotic cells was determined by measuring the content of hypodiploid DNA (Table 1). From the beginning, a basal rate of approximately 3% apoptosis was detectable in all cultures. During the first 3 d, the rate of apoptosis did not further increase in the presence or in the absence of 10% FBS. After 3 d, the rate of apoptosis was slightly increased in preadipocytes grown in serum-free medium, reaching approximately 13% on d 7. However, the rate of apoptosis was higher in preadipocytes grown in serum-containing medium (~24%), probably due to the fact that the cells had reached a very high density, and medium was not changed during the entire incubation period.

TABLE 1. Percentage of apoptotic cells in SGBS preadipocytes

	SGBS[a]			
	No. of adherent cells (×1000)		% of apoptotic cells	
	+ FBS	− FBS	+ FBS	− FBS
d 0	14.8 ± 2.4		3.0 ± 1.4	
d 1	20.0 ± 1.4	14.2 ± 2.4	6.7 ± 0.9	7.4 ± 2.0
d 2	42.1 ± 3.1	14.6 ± 2.1	3.8 ± 0.6	3.2 ± 0.6
d 3	60.7 ± 4.1	17.8 ± 1.4	8.3 ± 1.9	3.1 ± 0.4
d 7	71.1 ± 1.5	18.5 ± 1.4	23.7 ± 3.2	12.9 ± 1.3

[a] SGBS preadipocytes were incubated in basal medium in the absence or presence of 10% FBS; after the time points indicated (days), percentage of apoptotic cells was determined by measuring the content of hypodiploid DNA.

Expression of death receptors in human preadipocytes and adipocytes

We next studied whether apoptosis could be induced via death receptor signaling in human preadipocytes and adipocytes. Flow cytometry revealed surface expression of CD95 and TNFR1 (CD120a) on SGBS preadipocytes as well as adipocytes (Fig. 1, A and B). The expression of CD95 was markedly lower in preadipocytes compared with adipocytes. As we were not able to obtain specific antibodies against TRAIL-R, the expression of TRAIL-R was examined by RT-PCR. TRAIL-R1 and TRAIL-R2 were expressed in SGBS preadipocytes as well as in SGBS adipocytes. Densitometric analysis revealed that the expression of both receptors was slightly higher in adipocytes (Fig. 1C).

SGBS preadipocytes and adipocytes are resistant to death ligand-induced apoptosis, but can be sensitized by an inhibitor of biosynthesis

To examine death ligand-induced apoptosis, SGBS preadipocytes were treated with cross-linking CD95 antibody

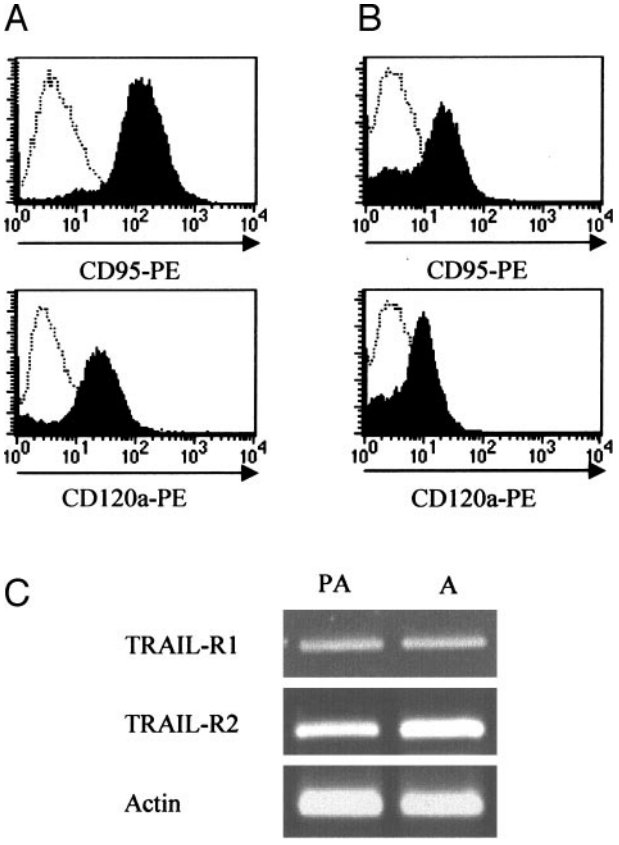

FIG. 1. Expression of death receptors in SGBS preadipocytes and adipocytes. A, Surface expression of CD95 and TNFR1 (CD120a; ■) in SGBS preadipocytes was assessed by FACS analysis. IgG1 antibodies were used as a control (*dotted lines*). B, Surface expression of CD95 and TNFR1 (CD120a; ■) in SGBS adipocytes was assessed by FACS analysis. IgG1 antibodies were used as a control (*dotted lines*). Representative data from three independent experiments are shown. C, mRNA expression of TRAIL-R1 and TRAIL-R2 in SGBS preadipocytes (PA) and SGBS adipocytes (A) were examined by RT-PCR using sequence-specific primers. Actin served as a control for equal conditions. A representative experiment from three performed is shown.

(α-APO-1), TNFα, and TRAIL for different time periods and subsequently evaluated for apoptosis with flow cytometry measuring DNA fragmentation (Fig. 2A). The presence of death ligands alone showed low rates of specific apoptosis (<10% after 72 h). The addition of CHX, an inhibitor of biosynthesis, markedly increased specific apoptosis in SGBS preadipocytes (about ≥80% after 72 h) in a time-dependent manner. In addition, an inhibitor of RNA synthesis, actinomycin D, sensitized SGBS cells for death receptor-induced apoptosis (data not shown). Treatment with CHX alone did not cause cell death (Fig. 2), and pretreatment with CHX for 24 h did not change the results (data not shown). Apoptosis was induced in a dose-dependent manner by the different death ligands, and maximal effects were obtained with the concentrations used here (data not shown). In parallel, apoptosis induction with death ligands was performed and measured in SGBS adipocytes as described above (Fig. 2B). Stimulation with death ligands alone led to low rates of specific apoptosis (<10% after 72 h), whereas costimulation with CHX clearly sensitized SGBS adipocytes to death ligand-induced apoptosis (~60% after 72 h). However, SGBS preadipocytes were more susceptible to sensitization with CHX compared with adipocytes (approximately ≥80% *vs.* 60% after 72 h).

Further evidence of apoptosis in SGBS preadipocytes and adipocytes

To further demonstrate that cell death occurs via apoptosis, SGBS preadipocytes and adipocytes were treated for 24 h with either serum-free, basal medium alone or α-APO-1 and CHX. Staining with DAPI revealed normal nuclear morphology after incubation in medium alone, whereas nuclear chromatin condensation and fragmentation appeared after induction of apoptosis (Fig. 2C). Additional staining of lipid droplets with Nile Red demonstrates clearly that nuclear fragmentation also occurs in lipid-filled adipocytes. Some lipid droplets are visible outside of cells, suggesting that cell membranes become leaky in the final stages of apoptosis. Another biochemical hallmark of apoptosis is the fragmentation of genomic DNA visible upon gel electrophoresis as a distinctive ladder pattern (27). In SGBS preadipocytes, DNA fragmentation

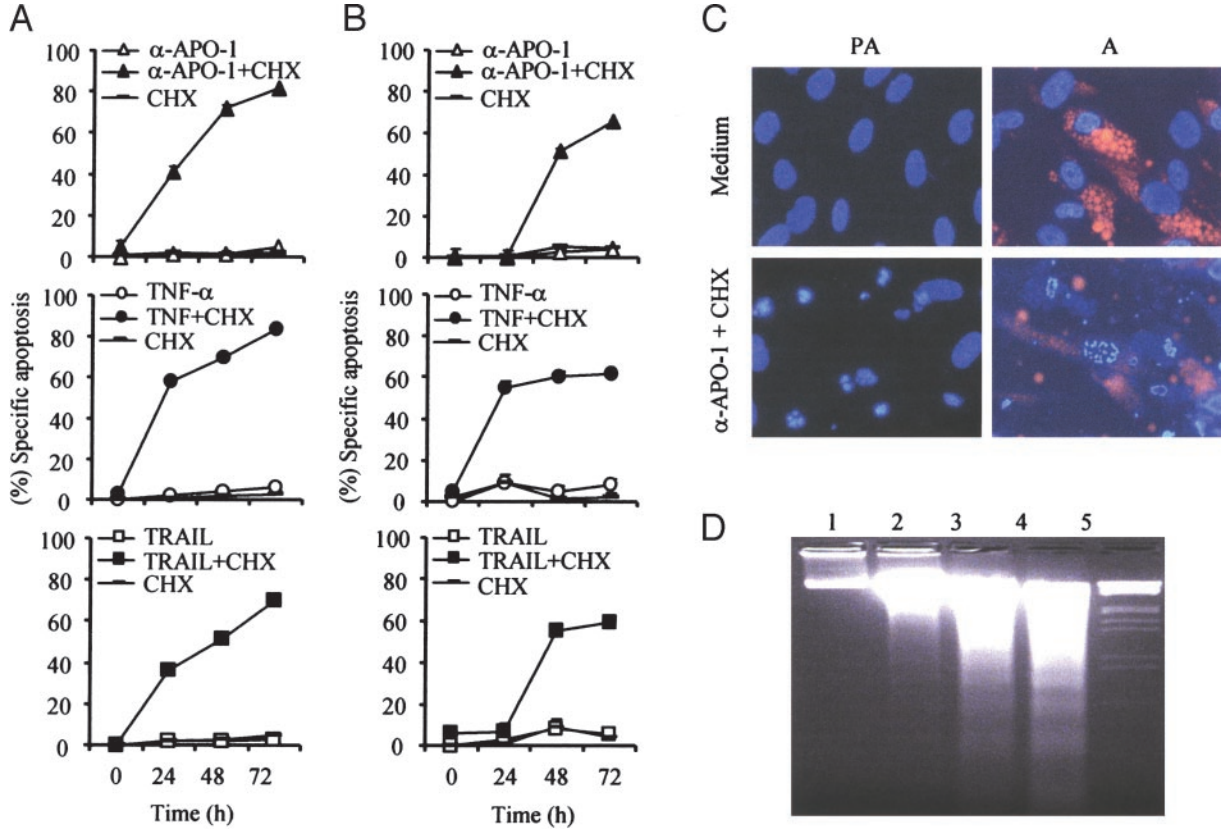

FIG. 2. SGBS preadipocytes and adipocytes can be sensitized for death ligand-induced apoptosis by an inhibitor of protein biosynthesis. A, Subconfluent cultures of SGBS preadipocytes were washed three times with PBS and incubated with 10 μg/ml CHX, 1 μg/ml α-APO-1, 10^{-8} M TNFα, and 100 ng/ml TRAIL. After 24, 48, and 72 h, the content of hypodiploid DNA was determined by flow cytometry. Specific apoptosis was calculated as described in *Materials and Methods*. One representative experiment from three performed is shown. B, SGBS adipocytes were stimulated as described in A. After 24, 48, and 72 h, the content of hypodiploid DNA was determined by flow cytometry. Specific apoptosis was calculated as described in *Materials and Methods*. One representative experiment from three performed is shown. C, SGBS preadipocytes (PA) and adipocytes (A) grown on chamberslides were stimulated with medium alone or with 1 μg/ml α-APO-1 and 10 μg/ml CHX. After 24 h, cells were stained with DAPI and Nile Red and analyzed with a fluorescence microscope. One representative experiment from three performed is shown. D, SGBS preadipocytes were stimulated with medium alone or with 1 μg/ml α-APO-1 and 10 μg/ml CHX. DNA preparation followed after 24, 48, and 72 h. DNA fragmentation is visible after agarose gel electrophoresis. One representative experiment of two performed is shown.

was visible after apoptosis stimulation with α-APO-1 and CHX (Fig. 2D). The typical ladder pattern increased at later time points of stimulation. Comparable results were obtained upon stimulation with TRAIL and CHX and with TNFα and CHX.

The findings that human preadipocytes and adipocytes show only low sensitivity to apoptosis induced by growth factor deprivation and death receptor triggering and that the low sensitivity could be overcome by an inhibitor of protein synthesis (Table 1 and Fig. 2) led us to the suggestion that there might be a factor produced by the cells protecting them from apoptosis. As there are some recent reports describing IGF-I-mediated protection from apoptosis in 3T3-L1 preadipocytes (18, 28, 29) and in brown adipocytes (30), we investigated whether IGF-I could be a factor protecting human fat cells from apoptosis.

SGBS preadipocytes and adipocytes produce IGF-I and express the IGF-IR

Human primary preadipocytes and adipocytes are known to secrete IGF-I *in vitro* (22). In SGBS preadipocytes and adipocytes, IGF-I mRNA expression could be shown by RT-PCR (Fig. 3A). In addition, IGF-I release into the medium was detected by a sensitive RIA; a maximal level of 1.1 ng/ml/48 h (0.14 nM) was measured. The presence of IGF-IR in the cells was shown after specifically precipitating the α-subunit of the IGF-IR and by Western blotting using an antibody against the β-subunit of the IGF-IR (Fig. 3B). No signal could be detected after precipitating the

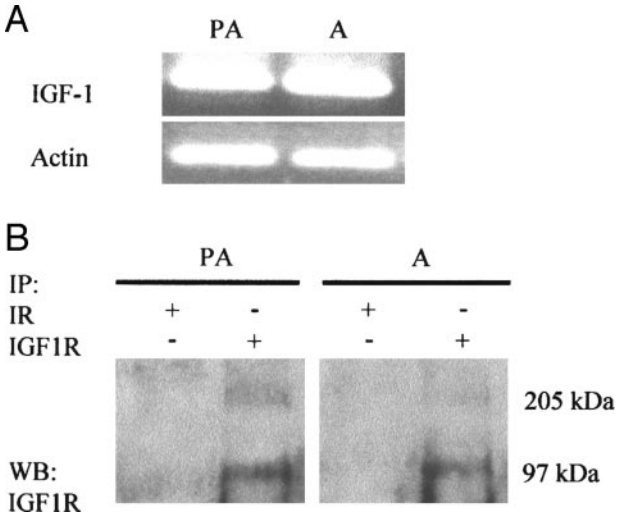

FIG. 3. Expression of IGF-I and IGF-IR in SGBS preadipocytes and adipocytes. A, IGF-I mRNA expression in SGBS preadipocytes (PA) and SGBS adipocytes (A) was examined by RT-PCR using sequence-specific primers. β-Actin served as a control for equal conditions. A representative experiment of three performed is shown. B, SGBS preadipocytes (PA) and adipocytes (A) were lysed, and extracts were incubated with a specific monoclonal antibody to immunoprecipitate IGF-IR as described in *Materials and Methods*. The expression of IGF-IR was determined by Western blot analysis using an IGF-IR monoclonal antibody. The positions of the recognized β-subunit of IGF-IR (97 kDa) and the receptor precursor (205 kDa) are indicated. The specificity of the Ab was controlled by immunoprecipitation of the insulin receptor. One representative experiment of two performed is shown.

α-subunit of the insulin receptor, demonstrating the specificity of the antibodies used. Additional RT-PCR showed IGF-IR mRNA expression (data not shown). Taken together, the expression of both IGF-I and IGF-IR suggests that auto-/paracrine actions in human preadipocytes and adipocytes are possible.

IGF-I protects SGBS preadipocytes and adipocytes from death ligand apoptosis

As shown in Fig. 4, IGF-I inhibited cell death in human preadipocytes and adipocytes induced by α-APO-1, TNFα, and TRAIL in a dose-dependent manner. In preadipocytes (Fig. 4A) IGF-I caused a maximal reduction of apoptosis by approximately 54% after α-APO-1/CHX treatment and by approximately 29% after TNFα/CHX treatment and completely abolished apoptosis induced by TRAIL/CHX treatment. In adipocytes (Fig. 4B), IGF-I caused inhibition of death receptor-triggered apoptosis, which was less pronounced than that in preadipocytes (α-APO-1 plus CHX, ~32%; TNFα plus CHX, ~30%; TRAIL plus CHX, ~61%). A significant influence of IGF-binding proteins on the antiapoptotic effect of IGF-I in our culture system could be excluded. In additional experiments we demonstrated that LR3-IGF-I, an IGF-I analog that does not bind to IGF-binding proteins, showed an inhibitory effect on death receptor-induced apoptosis that was quantitatively comparable to that of IGF-I (data not shown).

Blocking IGF-I signaling sensitizes SGBS preadipocytes and adipocytes for death ligand-induced apoptosis

To further elucidate that IGF-I signaling rescues SGBS cells from apoptosis, preadipocytes and adipocytes were incubated with death ligands together with an antibody specifically neutralizing the IGF-IR (αIR3). We found that blocking IGF-IR sensitized preadipocytes as well as adipocytes for α-APO-1-, TNFα-, and TRAIL-induced apoptosis (Fig. 4C), demonstrating that IGF-IR is involved in an auto-/paracrine regulation of cell survival. Treatment with αIR3 alone also stimulated detectable rates of specific apoptosis in both cell types (~16% in preadipocytes and ~5% in adipocytes). The concentration of αIR3 used in our assays (10 μg/ml) was the minimal concentration needed to ensure specific blocking of IGF-IR as controlled by [^{3}H]thymidine incorporation (data not shown).

Both MAPKs and phosphoinositide 3-kinase (PI3K) are involved in IGF-I-mediated protection from apoptosis

To identify which downstream signals are important for the survival effect of IGF-I, SGBS preadipocytes were incubated with death ligands in combination with specific inhibitors of either p38 MAPK (SB203580) and MAPK kinase 1 (MEK1), the upstream regulator of p42/44 MAPK (PD980059), respectively, or PI3K (LY294002; Fig. 5A). Each inhibitor was used at a concentration that effectively inhibits its respective target as controlled by [^{3}H]thymidine incorporation (data not shown). Inhibitors alone induced low rates of apoptosis (<5%). Blocking p38 MAPK and additional stimulation with α-APO-1 and TRAIL

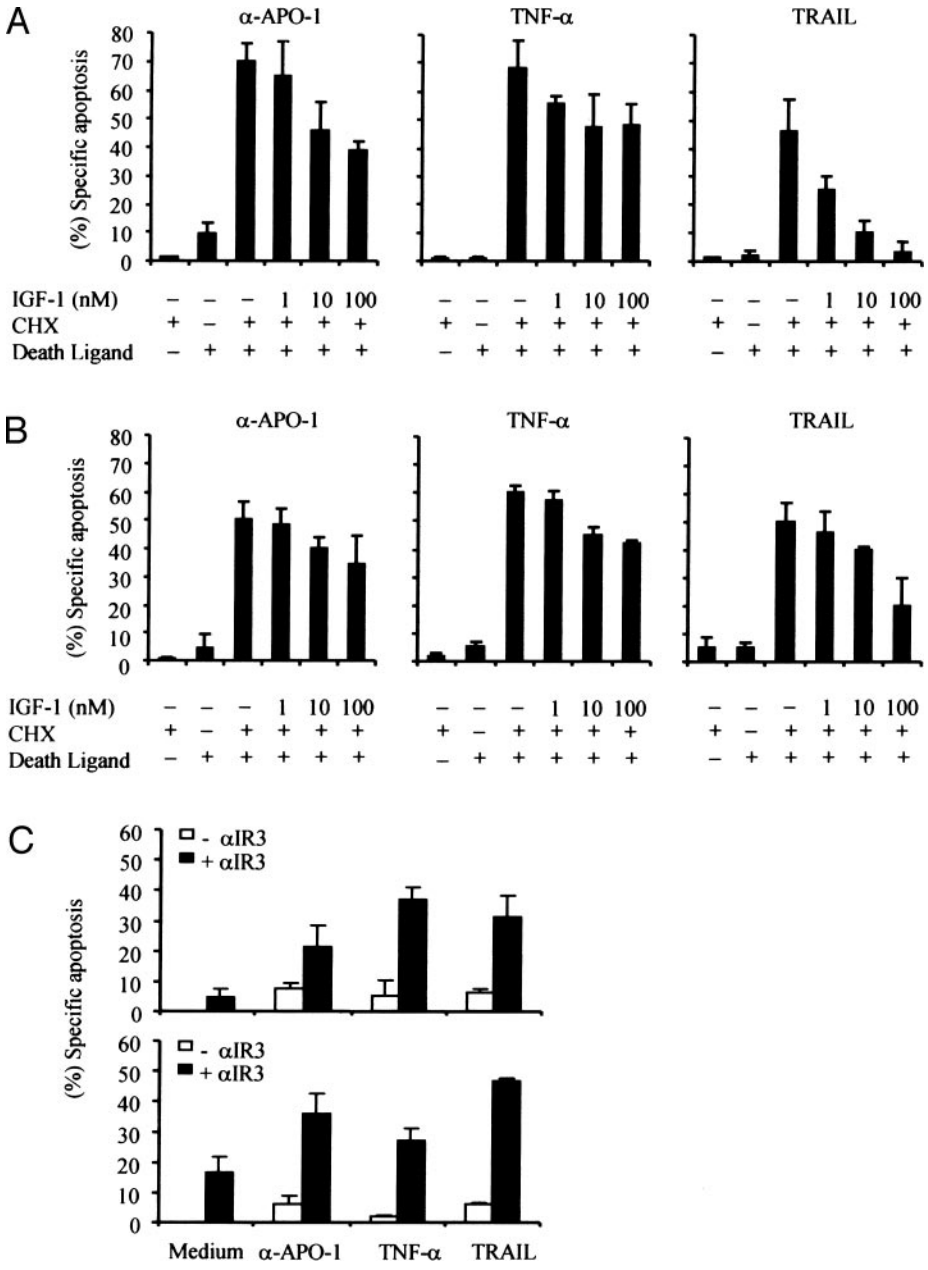

FIG. 4. IGF-I rescues SGBS preadipocytes and adipocytes from death-ligand induced apoptosis. SGBS preadipocytes were incubated with 1 μg/ml α-APO-1, 10^{-8} M TNFα, 100 ng/ml TRAIL, 10 μg/ml CHX, and 1, 10, and 100 nM IGF-I as stated in the figure. After 48 h, the content of hypodiploid DNA was determined by flow cytometry. Specific apoptosis was calculated as described in *Materials and Methods*. One representative experiment of three performed is shown. B, SGBS adipocytes were stimulated as described in A. After 48 h, the content of hypodiploid DNA was determined by flow cytometry. Specific apoptosis was calculated as described in *Materials and Methods*. One representative experiment of three performed is shown. C, SGBS preadipocytes (*upper row*) and adipocytes (*lower row*) were incubated with 1 μg/ml α-APO-1, 10^{-8} M TNFα, and 100 ng/ml TRAIL with or without 10 μg/ml αIR3. After 48 h, the content of hypodiploid DNA was determined by flow cytometry. One representative experiment of three performed is shown.

caused an increase in specific apoptosis by approximately 25% or 30%, respectively. However, inhibition of MEK1 did not sensitize SGBS preadipocytes for α-APO-1- or TRAIL-induced apoptosis. Inhibition of PI3K had a strong effect, increasing specific apoptosis by about 40% (α-APO-1) and about 60% (TRAIL). All of the inhibitors only slightly sensitized SGBS preadipocytes for TNFα-mediated apoptosis (<10%).

Down-regulation of Fas-associated death domain-like IL-1-converting enzyme inhibitory protein (FLIP) and Bcl-x_L expression

The fact that inhibitors downstream of IGF-I can strongly enhance death receptor-triggered apoptosis suggests that cells are synthesizing intracellular inhibitors of apoptosis. In search of repressors regulated by IGF-I, we monitored the expression levels of various proteins known to modulate death receptor-induced apoptosis. Inhibition of PI3K resulted in down-regulation of FLIP and Bcl-x_L protein levels (Fig. 5B). Densitometric analysis (data not shown) revealed a significant reduction by about 80% in comparison with baseline. In contrast, expression levels of FLIP and Bcl-x_L only slightly decreased by approximately 20% after inhibition of p38 MAPK and MEK1. Alterations in the expression levels of Bcl-2 family members, inhibitor of apoptosis protein family members, and CD95 could not be detected (data not shown).

To demonstrate that the data obtained here with human SGBS preadipocytes and adipocytes represent the situation in human primary cells, we performed additional

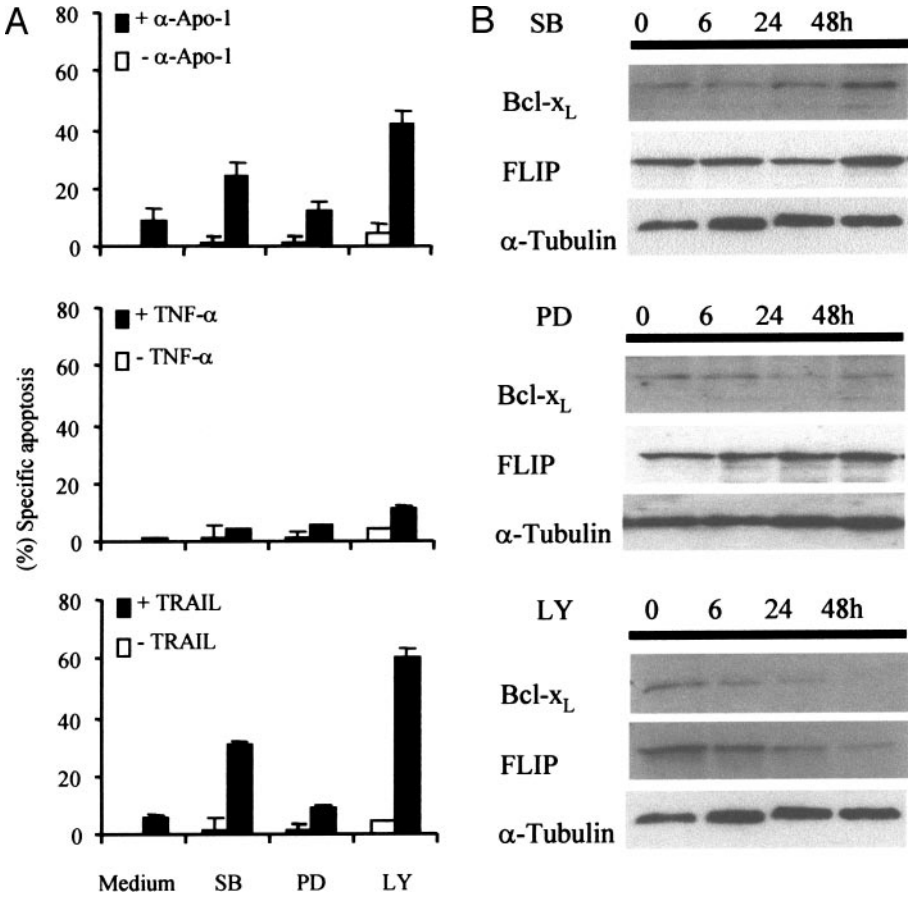

FIG. 5. PI3K and p38 MAPK, but not MEK1, are involved in IGF-I-mediated protection from apoptosis. A, SGBS preadipocytes were incubated with 1 μg/ml α-APO-1, 10^{-8} M TNFα, and 100 ng/ml TRAIL with or without 50 μM PD98059, 50 μM SB203580, and 100 μM LY294002. After 48 h, the content of hypodiploid DNA was determined by flow cytometry. Data are expressed as the mean ± SD (n = 3 independent experiments). B, SGBS preadipocytes were incubated for the indicated time periods with 50 μM PD98059, 50 μM SB203580, and 100 μM LY294002. Cell lysates (50 μg protein/lane) were separated by 10–20% gradient SDS-PAGE. Western blot analysis for FLIP, Bcl-x_L, and α-tubulin was performed using FLIP mAb, Bcl-x_L mAb, and α-tubulin mAb. The expression of α-tubulin was used to control equal protein loading.

TABLE 2. Percentage of apoptotic cells in cultured primary preadipocytes

	% of apoptotic cells							
	Donor 1[a]		Donor 2[a]		Donor 3[a]		Mean response[c]	
	+ FCS[b]	− FCS[b]	+ FCS	− FCS	+ FCS	− FCS	+ FCS	− FCS
d 0	8.0 ± 0.5		12.5 ± 0.3		6.6 ± 0.9		9.0 ± 2.5	
d 1	8.2 ± 0.3	6.7 ± 0.9	12.9 ± 0.8	15.1 ± 1.4	8.0 ± 0.1	7.1 ± 0.4	9.2 ± 1.3	9.7 ± 3.8
d 2	6.4 ± 0.9	5.8 ± 0.1	13.1 ± 0.9	15.7 ± 2.2	8.6 ± 0.9	13.2 ± 1.5	10.4 ± 2.9	11.6 ± 4.2
d 3	6.2 ± 0.4	6.4 ± 1.0	14.2 ± 1.3	15.7 ± 0.2	11.6 ± 1.7	12.1 ± 2.5	9.4 ± 3.4	13.6 ± 6.7
d 7	11.5 ± 0.8	12.8 ± 0.3	14.5 ± 1.2	22.5 ± 3.4	7.8 ± 0.5	10.0 ± 0.3	11.3 ± 2.7	15.6 ± 6.1

[a] Human preadipocytes were prepared from sc adipose tissue from three healthy patients.
[b] Cells were incubated in basal medium in the absence or presence of 10% FBS; after the time points indicated, the percentage of apoptotic cells was determined by measuring the content of hypodiploid DNA.
[c] Mean response obtained in cells of three donors was calculated.

experiments with preadipocytes and *in vitro* differentiated adipocytes obtained from the sc adipose tissue of healthy patients.

Human primary preadipocytes show low sensitivity for apoptosis induction by serum deprivation

Cultured primary preadipocytes showed low sensitivity to apoptosis induced by serum deprivation (Table 2). The mean basal rate of apoptosis 12 h after preparation (d 0) of cells was 9.0 ± 2.5%. The apoptosis rate remained constant during 7 d of incubation in serum-containing medium. Incubation in serum-free medium resulted in a mean increase in apoptosis of approximately 5%.

Human primary preadipocytes and adipocytes show low sensitivity to death ligand-induced apoptosis, but can be sensitized by an inhibitor of biosynthesis

Human primary preadipocytes and adipocytes were treated with α-APO-1, TNFα, and TRAIL, and apoptosis was detected by morphological signs (Fig. 6, A and B) and by measuring hypodiploid DNA by flow cytometry (Fig. 6, C–F). Low rates of specific apoptosis (<10%) were detected in preadipocytes as well as adipocytes after treatment death ligands. Costimulation with CHX strongly increased the rate of apoptosis in both cell types (Fig. 6, C and D). Human primary preadipocytes as well as *in vitro* differentiated adipocytes showed clear morphological

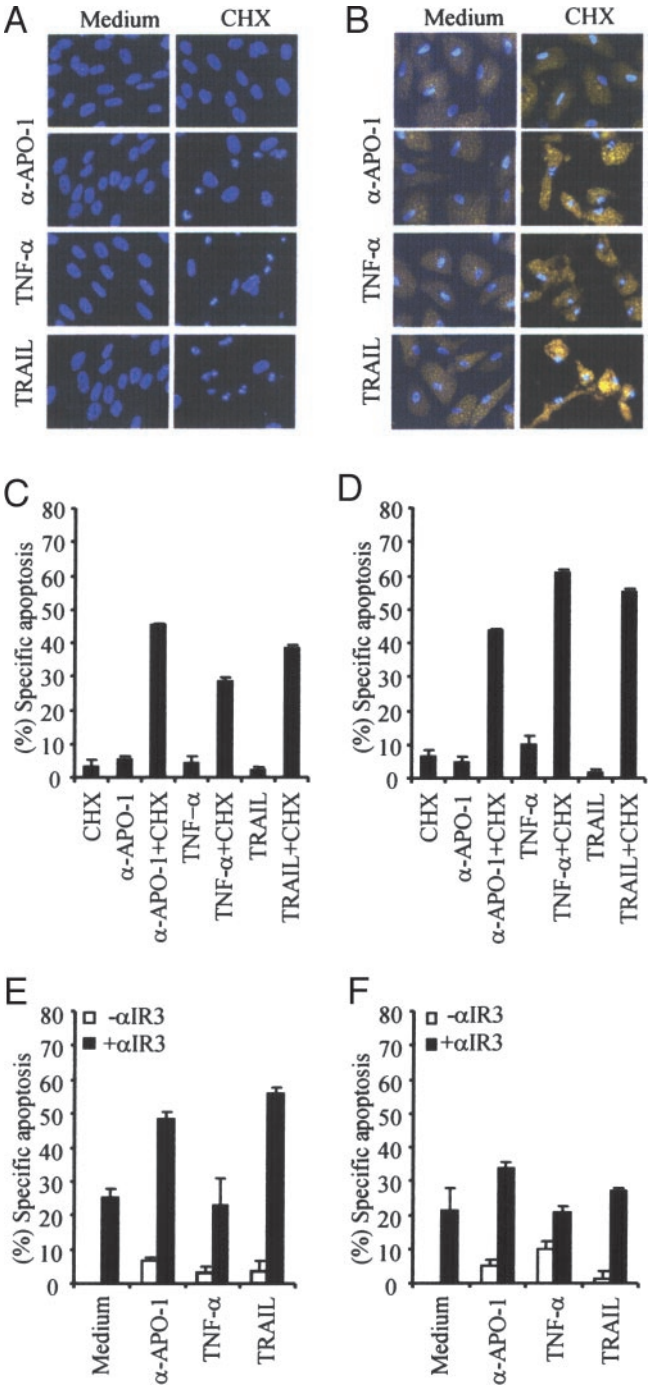

FIG. 6. Apoptosis in human primary preadipocytes and adipocytes. Human primary preadipocytes were derived from sc adipose tissue from healthy donors as described in *Materials and Methods*. Preadipocytes (A) and *in vitro* differentiated adipocytes (B) grown on chamberslides were stimulated with medium alone, 10 μg/ml CHX, 1 μg/ml α-APO-1, 10^{-8} M TNFα, and 100 ng/ml TRAIL. After 24 h, cells were stained with DAPI and Nile Red and analyzed by fluorescence microscope. One representative experiment of three performed is shown. Human primary preadipocytes (C) and *in vitro* differentiated adipocytes (D) were treated with 10 μg/ml CHX, 1 μg/ml α-APO-1, 10^{-8} M TNFα, and 100 ng/ml TRAIL. After 72 h, the content of hypodiploid DNA was determined by flow cytometry. Specific apoptosis was calculated as described in *Materials and Methods*. Human primary preadipocytes (E) and *in vitro* differentiated adipocytes (F) were incubated with 1 μg/ml α-APO-1, 10^{-8} M TNFα, and 100 ng/ml TRAIL with or without 10 μg/ml αIR3. After 72 h, the content of hypodiploid DNA was determined as described above. One representative experiment of three performed is shown.

signs of apoptosis, such as nuclear condensation and fragmentation (Fig. 6, A and B).

IGF-IR signaling is involved in apoptosis protection in human primary preadipocytes and adipocytes

Finally, we demonstrate that the IGF-IR pathway is involved in protection from apoptosis in human primary preadipocytes and adipocytes, as shown in SGBS cells. Specific blocking of the IGF-IR alone by the neutralizing antibody αIR3 caused more than 20% cell death in human primary preadipocytes and adipocytes (Fig. 6, E and F). αIR3 sensitized human primary preadipocytes and adipocytes for α-APO-1 and TRAIL induced apoptosis, suggesting the involvement of IGF-IR in survival mechanisms. Interestingly, blocking the IGF-IR did not further stimulate TNFα-mediated apoptosis. The sensitizing effect of αIR3 to the apoptosis induction by α-APO-1 and TRAIL was more pronounced in preadipocytes than in adipocytes.

Discussion

The concept that adipocyte deletion by apoptosis is a significant contributor to the regulation of adipose tissue mass and to adipose tissue loss during weight reduction is a relatively recent one (7, 31, 32). It is supported by data on adipose tissue cellularity in various clinical conditions (6, 9–12). Recent findings from animal studies strengthen the concept of a possible turnover of fat cells with apoptosis as a homeostatic mechanism controlling fat cell number (13–16, 32–35).

In the present study we have investigated induction of apoptosis via death receptors belonging to the TNFR superfamily in human preadipocytes and adipocytes. The expression of TNFR1, CD95, and TRAIL-R1 and R2 was shown in these cells. Despite receptor expression, cells revealed rather low sensitivity to α-APO-1-, TNFα-, and TRAIL-induced apoptosis, which could be significantly enhanced by an inhibitor of biosynthesis. Morphological features of apoptosis, such as nuclear condensation and fragmentation, were unequivocal not only in preadipocytes, but also in mature, lipid-filled adipocytes. Furthermore, DNA fragmentation was shown by flow cytometry and the typical DNA ladder pattern after gel electrophoresis. These data show that human preadipocytes and adipocytes express functional tools for death receptor-induced apoptosis.

In subsequent studies we showed that IGF-I is able to rescue human preadipocytes and adipocytes from death receptor-triggered apoptosis, whereas blocking the IGF-IR increased the rate of basal apoptosis and sensitized the cells for α-APO-1-, TNFα-, and TRAIL-induced apoptosis. Blocking PI3K and p38 MAPK specifically enhanced CD95- and TRAIL-triggered apoptosis. Inhibition of PI3K resulted in down-regulation of antiapoptotic proteins, Bcl-x_L and FLIP. These results demonstrate that the apoptosis sensitivity of human fat cells can be modulated by specific pathways.

In our studies primary human preadipocytes survived long-term growth factor withdrawal with only a slight increase in specific apoptosis. These data as well as the finding of a low sensitivity of cells to TNFα-induced apoptosis correspond to data reported by other groups (8, 36). Death receptors are expressed on a variety of different cell types, yet many death receptor-expressing cells are resistant to death receptor-triggered apoptosis (37). Maintaining a state of resistance to apoptosis often requires *de novo* protein or RNA synthesis (38). As shown here, inhibition of protein synthesis by CHX rendered human preadipocytes and adipocytes sensitive to CD95-, TNFα-, and TRAIL-triggered apoptosis.

The finding that fat cells show only low sensitivity to apoptosis induced by growth factor deprivation or death receptor triggering, which can be overcome by inhibition of protein biosynthesis suggests the production of a survival factor. Growth factors are generally known to promote survival in many cell types (39), and the IGF-I/IGF-IR system especially is very well investigated (40). IGF-I mediates survival in brown adipocytes (30) and 3T3-L1 preadipocytes (18, 28, 29, 41, 42).

We have recently reported the production of IGF-I in cultured human preadipocytes and adipocytes (22). In our present study we could show the expression of IGF-IR in preadipocytes and adipocytes, demonstrating the possibility of an auto-/paracrine action of IGF-I. Neutralizing the IGF-IR and thereby preventing transduction of the survival signal significantly sensitized preadipocytes as well as adipocytes for death receptor-triggered apoptosis. In parallel, we found that IGF-I protected human preadipocytes and adipocytes from death ligand-induced apoptosis. This finding is in line with other reports showing IGF-I-mediated protection from CD95-triggered (43, 44) and TRAIL-induced apoptosis in several cell types (45, 46). However, there are conflicting data in the literature concerning the role of IGF-I in TNFα-induced apoptosis. Some groups identified IGF-I as a survival factor (47, 48), whereas others demonstrated enhancement of TNFα-mediated cell death, including 3T3-L1 preadipocytes (18, 49).

IGF-I action is mediated by its specific tyrosine kinase receptor, which phosphorylates insulin receptor substrates (50). The activation of several kinases ensues, including various MAPKs or PI3K/protein kinase B (PKB/Akt) (50). Recent reports have identified several apoptosis-related proteins as substrates for PKB/Akt, implicating this kinase as an important regulator of the apoptotic process (51). The Ras/MAPK (52) and p38 MAPK have also been implicated in IGF-I-mediated survival signaling (53, 54). However, Peruzzi *et al.* (55) reported that IGF-I-mediated, MAPK-dependent survival signals may predominate only when the PI3K pathway is disabled.

To identify the signaling pathways involved in IGF-I-mediated survival in human preadipocytes and adipocytes, we have coincubated cells with death ligands and inhibitors of PI3K (LY294002), p38 MAPK (SB203580), and MEK1, the upstream regulator of p42/44 MAPK (PD980059), respectively. CD95-triggered as well as TRAIL-induced apoptosis was strongly enhanced by inhibition of PI3K. This finding clearly underlines an important role for the PI3K/Akt pathway in IGF-I-mediated protection from death ligand-induced apoptosis. Very recently, Garofalo *et al.* (56) showed an age-dependent loss of adipose tissue in mice lacking Akt2/PKBβ, further supporting our hypothesis of an IGF-I/IGF-IR autocrine survival circuit in fat cells. A weaker, but still significant, sensitization to apoptosis was shown by inhibition of p38 MAPK, whereas blocking MEK1 did not enhance CD95- or TRAIL-induced apoptosis. The inhibitors used only slightly sensitized preadipocytes for TNFα-induced apoptosis. This intriguing finding suggests that alternate IGF-I-initiated pathways, other than those involving PI3K, p38 MAPK, and p42/44 MAPK, may be responsible for preadipocyte survival upon TNFα stimulation. For example, activation of c-Jun N-terminal kinase by IGF-I has been shown in a breast cancer cell line (57). In addition, IGF-I-mediated activation of c-Jun N-terminal kinase contributes to human T cell survival (58).

Finally, we have investigated whether inhibition of kinases downstream of the IGF-IR influenced the expression of antiapoptotic proteins. We demonstrated that inhibition of PI3K resulted in down-regulation of FLIP and Bcl-x_L. The expression of FLIP as well as Bcl-x_L was not influenced by inhibition of p38 MAPK and MEK1, suggesting that activation of the PI3K pathway is mainly responsible for IGF-I-mediated survival of human fat cells. This is in accordance with results obtained in 3T3-L1 preadipocytes, in which PI3K, but not p38 MAPK, nor MEK1, was required for IGF-I-mediated survival (28).

The receptors of IGF-I and insulin are closely related in structure and function, and the extent of similarities and differences in signaling capacities has not been clearly defined. Like IGF-I, insulin has been reported to mediate survival in several cell types (59–62), although in many of these studies very high concentrations of insulin were used, which could also act via the IGF-IR. In 3T3-L1 preadipocytes and adipocytes, IGF-I and insulin revealed equal potential to inhibit apoptosis induced by serum withdrawal (41). Adipose tissue-selective knockout mice have a low fat mass and showed a polarization in adipocyte size (63). Whether the IGF-I/IGF-IR system plays a role here and whether the rate of apoptosis is increased in adipose tissue of these mice, especially under caloric restriction, has not yet been studied.

Apoptosis is a physiological process by which selected cells can be eliminated from the body. In light of the large pool of precursor cells in human white adipose tissue that are able to differentiate into adipocytes throughout life (2), it is suggested that apoptosis occurs in parallel to the differentiation of new adipocytes. This process might keep the number of fat cells in the body within a certain regulated range (7, 31). Results from animal experiments support this modern concept of a continuous turnover of fat cells in adipose tissue. Recently, it has been shown that treatment of obese Zucker rats with troglitazone results in an increase in the number of small adipocytes and a significant reduction in the number of large adipocytes by apoptosis (64). These results led to the hypothesis that depletion of large adipocytes by apoptosis would result in improvement of the metabolic control seen during treatment with thiazolidinediones (64).

Earlier studies have shown that in rats, starvation results in a reduction of DNA in white fat depots (13). Furthermore, Sprague Dawley rats with streptozotocin-induced diabetes showed a reduction in number of adipocytes that was reversed upon treatment with insulin (14). Treatment of *ob/ob*

mice with the centrally acting substances SKF 38393 and bromocriptine led to weight loss and apoptosis of adipocytes (33). Treatment with a neuropeptide Y antagonist stimulated the apoptosis of white adipocytes in obese rats (34). Intracerebroventricular administration of leptin to rats resulted in dramatic weight loss and an almost complete loss of adipose tissue due to apoptosis of fat cells (15, 32). After this dramatic loss of adipose tissue, rats resiliently returned to control levels associated with an increase in the expression of anti-apoptotic proteins (16). These data show that through as yet unknown mechanisms the central nervous system is not only able to reduce the number of peripheral fat cells by apoptosis, but is probably also able to compensate these reductions in adipose tissue. Further elucidation is required to understand how central signals are translated to the periphery.

It has not yet been shown that apoptosis of fat cells occurs during periods of stable body weight in man. However, recently, apoptosis of adipocytes could be demonstrated in various clinical situations, especially when a fast and extensive loss of fat mass occurs. In adipose tissue of patients with tumor cachexia apoptotic fat cells were detected (6). HIV patients treated with protease inhibitors develop lipodystrophy with a loss of adipose tissue in specific regions due to apoptosis of adipocytes (9–11). In patients with tumor cachexia, TNFα could be a factor inducing apoptosis in fat cells. The mechanism of apoptosis induction by protease inhibitors is less understood (11). Disturbances in local IGF-I production could provide an explanation for the altered apoptosis sensitivity of fat cells after treatment with proteinase inhibitors. In other clinical situations where a regression of fat depots can be observed, such as anorexia nervosa or acquired lipodystrophy syndromes (12), apoptosis of fat cells was also suggested. Earlier studies have demonstrated that prolonged reduction of body weight in adult women reduced the number of adipocytes (4). After a mean weight loss of 30–40 kg, fat cell size was markedly decreased in all adipose tissue depots. In addition, the calculated fat cell number was significantly reduced (3). In the light of our new data, this effect could be explained by low, locally produced IGF-I levels during weight loss. It would be interesting to study whether other states of low IGF-I (*e.g.* GH deficiency) or high IGF-I levels (*e.g.* acromegaly) could influence the IGF-I-dependent survival system in adipose tissue.

In summary, the results obtained in our study show possible mechanisms for induction of apoptosis in human fat cells. Furthermore, they demonstrate that human fat cells are able to potently protect themselves from cell death by the IGF-I/IGF-IR circuit in an auto-/paracrine manner. Factors interacting with the IGF-I/IGF-IR circuit would therefore be able to change the sensitivity of human fat cells for entering into apoptosis.

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