RESEARCH ARTICLE

SERUM-BORNE FACTORS IN CANCER PATIENTS WITH ADVANCED CACHEXIA: INFLUENCE ON ADIPOSE CELLS

Fred Haugen¹, Kristin Hollung¹, Anne M. Ramsrud¹, Andreas J. Wensaas¹, Naeem Zahid¹, Svein Dueland², and Christian A. Drevon¹

¹Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway, and ²Department of Oncology, Ulleval University Hospital, Oslo, Norway

Abstract
Background
The clinical syndrome cancer cachexia is recognized by a considerable weight loss being out of proportion to any reduction in energy intake. The underlying mechanisms are not completely known, but the marked weight loss is attributable to depletion of adipose tissue as well as skeletal muscle mass. Enhanced lipolysis in adipocytes, apoptosis of preadipocytes may be important for loss of adipose tissue.

Results
Sera from cachectic cancer patients induced apoptosis in cultured human preadipocytes at a higher rate than sera from non-cachectic cancer patients (control group). There was a tendency towards increased mRNA levels of the pro-apoptotic Bcl-2 gene Bax after incubation of preadipocytes with cachectic sera. Moreover, the mRNA levels of anti-apoptotic Bcl-XL and pro-apoptotic Bcl-XS were increased and decreased, respectively, as compared to incubation with control sera. However, lipolysis was not enhanced in cultured human adipocytes after incubation with sera from cachectic cancer patients as compared to non-cachectic cancer patients.

Methods
Serum samples from cachectic cancer patients (n=8) and non-cachectic cancer patients (n=6) were collected. Human SGBS (Simpson-Golabi-Behmel syndrome) preadipocytes and differentiated adipocytes were incubated in the presence of serum from cachectic and non-cachectic (control) cancer patients. Induction of apoptosis and necrosis was examined by cell staining with Hoechst 342 (HO342) and propidium iodide (PI), respectively. Expression of pro- and anti-apoptotic Bcl-2 genes was measured by quantitative RT-PCR. Lipolysis was monitored by measuring the release of radiolabeled fatty acids.

Conclusion
Our in vitro data suggest that apoptosis of preadipocytes can be increased by serum-borne factors in cancer cachexia. Our in vitro data suggest that apoptosis of preadipocytes can be increased by serum-borne factors in cancer cachexia. Death or survival of preadipocytes may depend on the balance of pro- and anti-apoptotic mediators. Further studies of patients with cancer cachexia will be needed to reveal if the disease involves loss of adipose tissue due to apoptosis of preadipocytes. We could not show that serum-borne factors associated with cachexia have a major impact on lipolysis in cultured human adipocytes.

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Key words: preadipocytes, adipokines, apoptosis

Introduction
Cachexia is characterized by marked weight loss in individuals with systemic conditions like cancers or serious infections. The weight loss is reflected as a reduction in both adipose tissue and skeletal muscle mass, and it cannot be explained by anorexia alone because it is not reversed by supplementation of nutrients (1). This suggests that a significant metabolic component is involved (2). Approximately 50 % of late stage diseased cancer patients develop cachexia and the occurrence is especially high among patients with pancreatic and gastric cancers, where about 83-87 % suffers from cachexia (3).

Adipose tissue includes a heterogeneous population of cells like mature white adipocytes, T-cells, dendritic cells, nerve cells and endothelial cells (4). A stem cell population within the adipose stromal compartment can be differentiated in vitro toward the adipogenic as well as other lineages (5). Adipose tissues can also communicate with other tissues in the body by production and release of several hormones, termed adipokines, that may act locally or systemically to alter many biological processes like energy expenditure and food intake (6). Dur-
Serum-borne factors in cachexia: influence on adipose cells

...ing starvation, the basal metabolic rate is reduced as the body adapts to conserve energy as well as muscle and adipose tissues (7). In contrast, cancer cachexia is associated with enhanced or unchanged energy expenditure and loss of both adipose and muscle tissue (8-11). **Two processes may determine adipose tissue mass in cachexia: reduced adipocyte size (hypotrophy) and decreased adipocyte number (hypoplasia).**

The hydrolysis of triglycerides from adipose tissue can be regulated by a cAMP-mediated process involving both hormone-sensitive lipase (HSL) and lipid droplet surface proteins like perilipin (12). Hypotrophy of adipocytes seems to arise from an increase in lipolysis, rather than a decrease in lipogenesis (13).

The number of adipocytes present in an organism may be influenced by the adipocyte differentiation process, which generates mature adipocytes from progenitor cells, roughly termed preadipocytes. Depletion of these progenitor cells, occurring by apoptosis or necrosis, may limit the regeneration of adipocytes and result in loss of adipose tissue. The Bcl-2 family is a set of apoptosis-regulatory proteins which act via regulated protein-protein interactions (14,15). The protein family is divided into two groups: the antiapoptotic and proapoptotic family members, based on whether they counteract or promote the apoptotic process. Bcl-2 and Bcl-XL are among the antiapoptotic proteins, preventing mitochondrial release of cytochrome C (16-18), whereas Bax and Bcl-XS are examples of proapoptotic proteins. The different members compete with each other in controlling cytochrome C release from the mitochondria (19). Bax exists in an inactive, cytosolic form that gets inserted into the mitochondrial membrane on a proapoptotic signal and executes its pro-apoptotic activity via release of cytochrome C (20,21). There may be a connection between regulation of expression of certain Bcl-2 family proteins and apoptosis in adipocytes (22,23).

Here, we have investigated the possible role of apoptosis in preadipocytes and lipolysis in mature adipocytes during cancer-induced cachexia.

**Results**

Clinical data from cancer patients with and without cachexia are presented in Table 1. The patients in the cachectic group had lower BMI and serum albumin than the control group, as expected from the inclusion criteria. In addition, serum concentrations of several adipokines were measured (Table 1). Serum concentrations of interleukin-6 (IL-6) were significantly higher in the cachectic patients compared to the controls (Table 1). Adiponectin levels in serum tended to be elevated, whereas leptin levels tended to be lower, when cachectic patients were compared to control patients (Table 1).

**Apoptosis**

A pool of precursor preadipocytes can differentiate and replenish the adipose tissue with mature adipocytes (24). This normally maintains the lipid storing capacity of the adipose tissue during the turnover of adipocytes. It is possible that wasting of adipose tissue seen in cachexia involves apoptosis of preadipocytes.

| Table 1. Clinical variables and adipokine serum concentrations in advanced cancer patients with and without cachexia (control) |
|--------------------------------------------------|------------------|------------------|------------------|------------------|
| Age (years)                                       | Cachexia n = 8   | Control n = 6    | P-value          |
| 70                                                | (42 - 78)        | 69               | (49 - 80)        | 0.95             |
| BMI (kg/m²)                                       | 19.0             | 26.5             | (22.0 - 32.0)    | < 0.01           |
| (15.0 - 20.0)                                     |                  |                  |                  |
| Albumin (mg/mL)                                   | 32               | 42               | (33 - 49)        | 0.01             |
| (25 - 35)                                         |                  |                  |                  |
| Adiponectin (µg/mL)                               | 23.0             | 12.6             | (4.0 - 25.8)     | 0.11             |
| (9.0 - 28.6)                                      |                  |                  |                  |
| Resistin (ng/mL)                                  | 24.3             | 32.4             | (13.6 - 66.2)    | 0.40             |
| (15.9 - 28.1)                                     |                  |                  |                  |
| Leptin (ng/mL)                                    | 2.5              | 13.0             | (2.0 - 22.0)     | 0.09             |
| (0.0 - 14.0)                                      |                  |                  |                  |
| IL-6 (pg/mL)                                      | 29               | 0                | (0 - 50)         | 0.04             |
| (4 - 112)                                         |                  |                  |                  |
| TNF-α (pg/mL)                                     | 1.75             | 1.80             | (1.10 - 2.70)    | 0.85             |
| (0.80 - 3.10)                                     |                  |                  |                  |

Values are medians (minimum value - maximum value). P-values indicate differences between the groups according to the Mann-Whitney U-test. IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha
To test if serum-borne factors might alter the degree of apoptosis of preadipocytes, we incubated cultured SGBS preadipocytes with sera from cancer patients with or without cachexia. Subsequently, we measured the appearance of apoptosis and necrosis using HO342 and PI, respectively. For validation purposes SGBS preadipocytes were incubated with 100 ng/mL TNF-α, and we could distinguish between viable cells (Fig. 1A), apoptotic cells (Fig. 1B), and necrotic cells (Fig. 1C). SGBS preadipocytes were incubated for 72 h with 10% serum from the patients. Sera from cachectic patients induced apoptosis in SGBS preadipocytes at a higher rate than sera from control patients (Fig. 1D). The proportions of necrotic cells were similar after incubation with sera from cachectic and non-cachectic patients (Fig. 1E).

To substantiate the involvement of preadipocyte apoptosis in cachexia we measured the mRNA levels by RT-PCR of several apoptosis-regulatory proteins in the Bcl-2 family. Bax and Bcl-XS are proapoptotic proteins whereas Bcl-2 and Bcl-XL are anti-apoptotic proteins. mRNA$^{\text{Bcl-2}}$ was detected in Jurkat cells but not in the SGBS cells (data not shown). In SGBS preadipocytes incubated for 72 h with cachectic sera there was a tendency towards increased mRNA$^{\text{Bax}}$ levels as compared with cells incubated with

**Figure 1.** Effect of cachectic patient sera on apoptosis and necrosis in SGBS preadipocytes. Microscopic image of viable (A), apoptotic (B) and necrotic (C) SGBS preadipocytes stained with PI and HO342A after incubation with TNFα (8 h; 100 ng/mL). SGBS preadipocytes were incubated with sera (72 h; 10%) from patients with cachexia (n=8) or without cachexia (control; n=6) prior to microscopic analysis of apoptosis (D) and necrosis (E). The charts display the proportion of apoptotic or necrotic cells relative to viable cells; each point represents mean values of two independent cell experiments with serum from one subject. *$P \leq 0.05$ using Mann-Whitney U-test*
control sera (P = 0.16; Fig. 2A). In cells incubated with cachectic sera there was a correlation between mRNA\textsuperscript{Bax} levels and the proportion of apoptotic cells (Fig. 2B). After 72 h incubation with cachectic sera mRNA levels of antiapoptotic Bcl-XL and proapoptotic Bcl-XS were increased and decreased, respectively, as compared to incubation with control sera (Fig. 2D,E).

Prolonged incubation of SGBS preadipocytes with cachectic and control sera (7 days), did not show differences in the proportion of apoptotic and necrotic cells (data not shown). Furthermore, there were no difference in Bax, Bcl-XL and Bcl-XS mRNA levels (data not shown).

**Figure 2.** Effect of cachectic patient sera on expression of genes encoding apoptosis regulatory proteins in SGBS preadipocytes. SGBS preadipocytes were incubated in the presence of 10 % sera from cancer patients with cachexia or without cachexia (control) for 72 h. Then, mRNA levels of apoptosis regulatory proteins were measured by quantitative RT-PCR and normalized to the housekeeping gene G3PDH. Relative levels of mRNA encoding the pro-apoptotic protein Bax were calculated (A). Bax mRNA levels correlated to the proportion of apoptotic cells (Spearman’s rho = 0.07) after SGBS preadipocytes were incubated with sera from patients with cachexia (B). Relative levels of mRNA encoding the anti-apoptotic protein Bcl-XL (C) and the pro-apoptotic protein Bcl-XS (D) were also determined. Each point in the diagram represents the mean value of two separate cell experiments measured in triplicates.

*P ≤ 0.05 using Mann-Whitney U-test
Lipolysis

Mature, lipid-loaded SGBS adipocytes were differentiated in vitro from SGBS preadipocytes. Free fatty acids were released into the culture medium upon induction of lipolysis (Fig. 3A). During cachexia increased lipolytic activity may lead to shrinking of adipocytes and subsequently the adipose tissue as a whole. Sera from cachectic patients did not display increased lipolytic activity but, contrary to our expectations, tended to inhibit lipolysis in the cells (P=0.09; Fig. 3B). In addition, 3T3-L1 cells of murine origin were differentiated into adipocytes and used to assay lipolytic activity. In the 3T3-L1 adipocytes cachectic sera induced lipolysis at a higher rate than sera from control patients (Fig. 3C).

Discussion

Our main finding is that preadipocytes of human origin cultured in the presence of sera from cancer patients with cachexia show increased apoptosis when compared to preadipocytes cultured with sera from control patients. Preadipocytes are considered precursor cells that may differentiate into adipocytes.

Figure 3. Effect of cachectic patient sera on lipolysis in cultured adipocytes. SGBS preadipocytes were differentiated into adipocytes prior to measurements of lipolysis, estimated as the release of fatty acids into the culture media. Lipolysis was induced in SGBS adipocytes after treatment with various doses of isoprenaline (3-100,000 nM; 4 h) (A). Mature SGBS adipocytes were exposed to sera (3 %) from patients with cachexia and without cachexia (control) for 4 h and the lipolytic activity was measured (B). The 3T3-L1 cell line of murine origin was differentiated into adipocytes prior to incubation with patient sera (3 %; 4 h) and measurements of lipolysis (C).
cytes. Decreased numbers of adipocytes may account for the loss of adipose tissue mass during cachexia. Moreover, a reduction in adipocyte number is hypothesized to occur via preadipocyte and adipocyte apoptosis, and possibly adipocyte dedifferentiation (25). Apoptosis in adipocytes has been observed during cancer cachexia in rabbits and patients (26, 27). Our findings show that also apoptosis of preadipocytes may play a role during cachexia.

Capacities for apoptosis of preadipocytes may vary among different fat depots (28), and SGBS preadipocytes may not reflect the cachectic process in all of them. After treatment with or without cachectic sera, only a small proportion of the SGBS preadipocytes stained with HO342 displayed altered morphology of the nuclear chromatin consistent with apoptosis. Such alterations are associated with late stages of the apoptotic process and it is possible that the methodology used here leads to an underestimation of the number of apoptotic cells.

Whereas apoptosis was enhanced in SGBS preadipocytes incubated with sera from cachectic cancer patients, necrotic cell death was unchanged. Detachment of necrotic cells from the surface of the culture dish may explain this inconsistency. It is also possible that the induction of apoptosis is only starting after 3 days (72 h) of incubation, and that a cumulative effect on the number of necrotic cells occurs at a later time-point. Although sera from the cachetic group tended to increase the number of necrotic preadipocytes after 7 days of incubation, this was still not statistically significant (data not shown). Limitations in our ability to conclude may be due to the relatively small number of patients in our study and variation due to heterogenous types of cancer.

The effect of sera from cachectic cancer patients, i.e. cell death or cell survival, may depend on the balance of pro- and anti-apoptotic mediators. Moreover, the mRNA levels of antiapoptotic Bcl-XL and proapoptotic Bcl-2 were increased and decreased, respectively, as compared to incubation with control sera. This finding was surprising, but may represent a compensatory response. There was a tendency towards increased mRNA levels of proapoptotic Bax after incubation with cachetic sera. Our findings suggest that Bax is a better measure than Bcl-XL for apoptosis in our experimental set-up.

There are several putative mediators of apoptosis which may be present in cachetic sera. Proinflammatory cytokines such as TNF-α and IL-6 have been implicated in adipose atrophy in cachexia (11). Whereas TNF-α induces apoptosis (29), IL-6 has been shown to represent an anti-apoptotic signal (30,31). In accordance with previous studies (32), the cachectic sera used in our present study contained higher levels of IL-6 than the non-cachectic, whereas TNF-α levels were similar. The increased IL-6 levels might represent a compensatory response to increased levels of some unidentified proapoptotic factor.

Also adipokines might affect apoptosis (33). Adiponectin and leptin concentrations tended to be altered in serum from cachectic patients as compared to non-cachectic patients. Because the levels of both these adipokines in serum are influenced by the mass of adipose tissue, we cannot conclude from our study that cachexia per se affects adipokine levels. Further studies of adipokine concentrations in BMI-matched cancer patients are warranted to determine if adipokines may play a role in cancer cachexia.

Conclusion
Factors circulating in the blood of cancer patients with cachexia can increase apoptosis of preadipocytes in vitro.

Methods
Origin of serum samples. Serum was collected from patients for use in biological assays monitoring apoptosis and lipolysis in adipocytes. The patients were recruited at Ullevål University Hospital, Oslo. Inclusion criteria were: histologically diagnosed cancer in lung, kidney, stomach, pancreas, colon or rectum. All patients included had metastatic disease determined by clinical and radiological findings. Serum samples were collected from a group of cancer patients suffering from cachexia (n = 8; 4 female, 4 male) and a group of cancer patients without evident cachexia as controls (n = 6; 1 female, 5 male). The cachectic group of patients included cancers originating in different tissues like pancreas (2), colon (1), ventricle (1), kidney (1) and lung (3). The control group included cancers originating in colon (4), lung (1) and rectum (1). The patients included in the cachectic group had an advanced stage of cachexia development. In this study, cachexia was defined as BMI ≤ 20, and loss of body weight > 5 % over the past 6 months, and serum albumin concentrations ≤ 37 mg/mL. Patients in the control group had BMI > 20, no weight loss the previous 6 months, and serum albumin concentrations > 37 mg/mL. Written informed consent was obtained from all the participants. The study was approved by the regional Ethics Committee.

Materials. Dulbecco's modified Eagle's medium/Nutrient Mix F12 (DMEM/Nutrient Mix F12), RPMI-1640, Parker 199, biotin, DL-pantothenate, penicillin/streptomycin, L-glutamin, human apo-transferrin, human insulin, cortisol, triiodothyronine (T3), dexamethasone, 3-isobutyl-1-methyloxanthine (IBMX), phosphate-buffered saline (PBS), propidium iodide (PI), Hoechst 33342 (HO342), HEPES, bovine serum albumin (BSA),
diethyl pyrocarbonate (DEPC), sodium dodecyl sulphate (SDS) and staurosporine were bought from Sigma Chemicals Co (St.Louis, MO, USA). Fetal calf serum (FCS) was from Gibco BRL (Paisley, UK). Rosiglitazone was a gift from GlaxoSmithKline (Essex, UK) whereas NaHCO3, NaOH, LiCl and chloroform were from Merck (Darmstadt, Germany). Restriction enzymes (EcoRI), ethylene diaminetetraacetic acid (EDTA), Tris, ethidium bromide, RNase inhibitor, isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were obtained from Promega (Madison, WI, USA). Agarose was bought from Bio Whittaker Molecular Applications (Rockland, ME, USA). Dithiothreitol (DTT) was purchased from Bio-Rad Laboratories (Hercules, CA, USA) Bicinchoninic acid (BCA) Protein Assay Reagent was from Pierce (Rockford, IL, USA). Tumor necrosis factor-alpha (TNF-α) was from R&D Systems Inc. (Minneapolis, MN, USA), whereas Genoprep™ mRNA Beads were obtained from Genovision AS (Oslo, Norway). Omniscript™ Reverse Transcriptase kit was bought from Qiagen GmbH (Hilden, Germany), while TOPO™ Cloning Reaction and Transformation kit was from Invitrogen Corp. (Carlsbad, CA, USA). JETquick mini and maxi-prep kits were bought from Genomed GmbH (Bad Oeynhausen, Germany). LightCycler™ Faststart DNA Master SYBR Green I, LightCycler™ Faststart DNA Master Hybridization Probes, LightCycler™ Color Compensation Set, LightCycler™ capillaries and LightCycler™ instrument were supplied from Roche Molecular Biochemicals (Mannheim, Germany). Primers were from Eurogentec (Seraing, Belgium) and labeled hybridization probes were ordered from TIB MOLBIOL (Berlin, Germany).

**Cells.** The SGBS cells were kindly provided by Professor Martin Wabitsch (34). The cells were derived from the stromal cells fraction of subcutaneous adipose tissue of an infant with Simpson-Golabi-Behmel syndrome. During the differentiation process SGBS cells developed a gene expression pattern similar to that found in differentiating human preadipocytes with a characteristic increase in fat cell-specific mRNAs encoding lipoprotein lipase, glycerol-3-phosphate dehydrogenase (GPDH), GLUT4, leptin and others. Differentiated SGBS cells exhibited an increase in glucose uptake upon insulin stimulation and in glycerol release upon catecholamine exposure. SGBS adipocytes were morphologically, biochemically and functionally similar to in vitro differentiated adipocytes from healthy subjects. The SGBS cells were cultured in DMEM/Ham's F12 medium supplemented with 10% heat-inactivated FCS, biotin (8 mg/l), DL-pantothenate (8 mg/l), L-glutamine (2 mmol/l), and streptomycin/penicillin (0.1 mg/ml). SGBS preadipocytes were grown in serum-containing medium in 24-well plates. To induce differentiation to mature adipocytes, confluent preadipocytes were cultured in serum-free basal medium added 20 nmol/l insulin, 0.01 mg/ml apo-transferrin, 0.1 μmol/l cortisol and 200 pmol/l T3 for 14 days. For the first four days of differentiation the medium was supplemented with 25 nmol/l dexamethasone, 500 μmol/l IBMX and 2 μmol/l rosiglitazone. The medium was changed twice a week. During the experiments cells were incubated with DMEM/Ham's F12 medium supplemented with 10% sterile-filtered patient serum. The cell line Jurkat was purchased from Bio Whittaker (Walkersville, MD, USA), and was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, L-glutamine (2 mmol/l), and streptomyein/penicillin (0.1 mg/ml).

**Cell staining.** For microscopic analysis of cell viability in SGBS preadipocytes, each well (0.5 ml) was first incubated with 3 μl of PI (0.5 mg/ml) in the dark for 30 min. Then 3 μl of HO342 (1 mg/ml) was added and incubation continued for another 30 min in the dark. DNA staining with PI indicates leaky plasma membranes because this dye cannot cross intact cell membranes. HO342 crosses intact membranes and stains DNA in all cells. PI or HO342 associates with DNA and emit red or blue light, respectively, when exposed to ultraviolet light. The blue color becomes more intense when HO342 associates with condensed DNA found in apoptotic cells as compared to normal cells. At least 200 cells per well were counted manually in a Nikon Eclipse TS 100 fluorescence microscope. The cells were photographed with a Nikon Digital Camera DXM 1200.

**mRNA isolation.** The medium was gently removed from the cells and 100 μl ice-cold RNA lysis/binding buffer (100 mmol/l Tris pH 8.0, 500 mmol/l LiCl, 10 mmol/l EDTA, 1% SDS, 5 mmol/l DTT) was added to each well. The cells were detached by scraping with a pipette tip and the contents of triplicate wells were transferred to an Eppendorf tube and immediately frozen in liquid nitrogen. Cell lysates were stored at –80°C. mRNA was isolated from the cell lysate using Genoprep™ mRNA Beads according to the manufacturer's protocol (Genovision AS, Oslo, Norway). Briefly, 50 μl oligo-dT-beads were prepared, 300 μl frozen cell lysate was defrosted and sonicated for 1-2 s before transfer to the beads and incubation in room temperature for 3-5 min. The beads were washed twice with 250 μl wash solution 1 (10 mmol/l Tris pH 8.0, 150 mmol/l LiCl, 1 mmol/l EDTA, 0.1% SDS), and twice with 250 μl wash solution 2 (10 mmol/l Tris, pH 8.0, 150 mmol/l LiCl, 1 mmol/l EDTA). The mRNA was eluted in 50 μl DEPC water by incubation at 65°C for at least 2 min. The mRNA was stored at –80°C.
**Quantitative RT-PCR.** The RT reactions were performed using Omniscript™ Reverse Transcriptase kit according to the manufacturer’s protocol (Qiagen GmbH, Hilden, Germany). 10 μl of the mRNA was used in each reaction. A LightCycler™ was used in all PCR reactions. It offers kinetic quantification, which is a fast and accurate way for quantification. The SYBR Green I dye, which was used for the genes G3PDH and Bax, binds to minor grooves in double stranded DNA and fluoresces strongly when bound to DNA. In the unbound state the dye has relatively low fluorescence. The intensity of fluorescence from the dye will increase in proportion to the amount of DNA in the PCR. The mRNA_{Bcl-X} is present as a short and a long form. To distinguish these mRNAs fluorescence resonance energy transfer (FRET) hybridization probes were used. One probe (Bcl-X_{s}) was labeled at the 3’-end with fluorescein, which serves as a donor fluorophore. A second probe (Bcl-X_{l}) was labeled at the 5’-end with LightCycler-Red 640, and a third probe (Bcl-X_{S+L}) was labeled with LightCycler-Red 705. Both LightCycler-Red 640 and -705 serve as acceptor dyes. When the donor and acceptor probes recognize adjacent internal sequences in the target gene, the fluorescence signal is generated. The different acceptor probes will fluoresce with different wavelength due to different labeling of the probes, making it possible to quantify both cDNA products in the same reaction. Sequences for primers and probes are described (Table 2). The Bax and G3PDH primers were used at a final concentration of 0.5 μmol/l each, Bcl-X primers 0.75 μmol/l each, and the final concentration of the hybridization probes was 0.3 μmol/l each. The final MgCl₂ concentration was optimized to 3 mmol/l for all reactions. 0.2 μl of the cDNA template from the RT-reaction was added for detection of Bax or G3PDH, whereas 0.3 μl was used for Bcl-X detection. The total reaction volume was adjusted to 20 μl using sterile, PCR grade H₂O. A negative control was always included with the samples. PCR programs for G3PDH and Bax quantification were as follows: 95°C, 10 min followed by 45 cycles of 95°C 10 s, 60°C 7 s, 72°C 10 s. PCR program for Bcl-X quantification was: 95°C, 10 min followed by 45 cycles of 95°C 15 s, 55°C 30 s, 72°C 10 s. Due to cross talk between the detection channels of the LightCycler™ instrument when using differently labelled hybridisation probes, a color compensation file had to be created using the LightCycler™ Color Compensation Set. The Bcl-X and Bax genes were cloned to make standard dilution series for quantification of the respective genes on PCR. The TOPO™ Cloning Reaction and Transformation kit was used according

<table>
<thead>
<tr>
<th><strong>Target mRNA</strong></th>
<th><strong>Sequence</strong></th>
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<tr>
<td><strong>G3PDH</strong></td>
<td>Forward primer 5’-TCATCAACGGGAAGCCCATCACCATCTTC-3’</td>
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<tr>
<td><strong>G3PDH</strong></td>
<td>Reverse primer 5’-GTCTTCTGGTGGAGTAATGGCATGACT-3’</td>
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<tr>
<td><strong>Bcl-2</strong></td>
<td>Forward primer 5’-TGCACCTGACGCCCCTC-3’</td>
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<tr>
<td><strong>Bcl-2</strong></td>
<td>Reverse primer 5’-AGACAGCCAGGAAATCAAACAG-3’</td>
</tr>
<tr>
<td><strong>Bax</strong></td>
<td>Forward primer 5’-ACCAAGAAGCTGAGCGAGT-3’</td>
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<tr>
<td><strong>Bax</strong></td>
<td>Reverse primer 5’-ACAAAGATGGTCACG-3’</td>
</tr>
<tr>
<td><strong>Bcl-X</strong></td>
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<tr>
<td><strong>Bcl-X_{S}</strong></td>
<td>FRET probe 5’-LC Red705-3’</td>
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Fluorescence resonance energy transfer (FRET) hybridisation probes; donor fluorophore fluorescein (X); acceptor dyes LightCycler-Red 640 (LCRed640) and LightCycler-Red 705 (LCRed705); phosphate group (p) to block polymerase extension at free 3’-end.
to the manufacturer’s protocol (Invitrogen Corp., Carlsbad, CA, USA). 4 μl freshly made PCR product was used in each cloning reaction, and the incubation time was set to 30 min. OneShot – chemical transformation was utilized, and 50 and 150 μl from each transformation were spread on pre-warmed selective plates (added 40 μl X-Gal and 10 μl IPTG). Plasmid DNA isolation was performed using the JETquick mini- or maxiprep kits according to the manufacturer’s protocol (Genomed GmbH, Bad Oeynhausen, Germany). The plasmids were examined by restriction analysis, using EcoRI as restriction enzyme. The DNA contents of the mini- and maxi-preps of the respective genes were measured spectrophotometrically at 260 nm, and standard dilution series were made for PCR.

**Lipolysis measurements.** During 3 days prior to the experiments SGBS and 3T3-L1 adipocytes were pre-labeled with [1-14C]-D-glucose or [1-14C]-Acetate, respectively. The cells were changed to serum free media 24 h before the incubation with patient sera or isoprenaline. The cells were washed 3 times with preheated PBS, and then given growth media without hormones, but with the addition of fatty acid free BSA (100 μM) and isoprenaline or 3 % serum from cachectic and control patients. After 4 hours incubation the media were removed and the radioactivity released was quantified by counting an aliquot in scintillation fluid using a scintillation counter. Similarly, the cells were lysed and the remaining cell-associated radioactivity was counted. Lipolysis (%) was estimated by calculating (100*released radioactivity/cell-associated radioactivity).

**Statistics.** The SPSS software version 13.0 for Windows was used for the statistical analysis. Non-parametric methods were used due to their resistance to outliers and skewed data distribution. The differences between groups were tested with the non-parametric Mann-Whitney U-test, and P ≤ 0.05 was considered statistically significant. Correlations in the separate groups were explored with the Spearman rank correlation coefficient, and P ≤ 0.10 was considered statistically significant.

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Competing interests

We have no competing interests to declare.

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