

Identification of a novel proapoptotic function of resveratrol in fat cells: SIRT1-independent sensitization to TRAIL-induced apoptosis

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ABSTRACT The phytochemical resveratrol has recently gained attention for its protection against metabolic disease and for extension of life span, which have been linked to its metabolic effects and SIRT1 activation in fat cells. However, little is known about the effect of resveratrol on fat cell apoptosis. Here, we identify a novel, SIRT1-independent mechanism by which resveratrol regulates fat cell numbers. We demonstrate for the first time that resveratrol enhances TNF-related apoptosis-inducing ligand (TRAIL)- or CD95-induced apoptosis of human preadipocytes in a highly synergistic manner (EC₅₀ at 72 h: resveratrol, >300 μM; TRAIL, >100 ng/ml; combination: 30 μM resveratrol and 10 ng/ml TRAIL, combination index 0.4). Similar results in primary human preadipocytes prepared from subcutaneous white adipose tissue and mature human adipocytes underline the relevance to human physiology. Mechanistic studies reveal that resveratrol inhibits PI3K-driven phosphorylation of Akt, leading to increased Bax activation, loss of mitochondrial membrane potential, cytochrome *c* release, and caspase-dependent apoptosis. The synergistic interaction of resveratrol and TRAIL depends on the intrinsic apoptosis pathway and caspases, since Bcl-2 overexpression and the caspase inhibitor zVAD.fmk inhibit apoptosis, whereas knockdown of SIRT1 by RNA interference has no effect. The discovery of this novel activity of resveratrol significantly advances the knowledge of fat tissue regulation by resveratrol and has important implications for its use in metabolic and age-related diseases.—Mader, I., Wabitsch, M., Debatin, K.-M., Fischer-Posovszky, P., Fulda, S. Identification of a novel proapoptotic function of resveratrol in fat cells: SIRT1-independent sensitization to TRAIL-induced apoptosis. *FASEB J.* 24, 1997–2009 (2010). www.fasebj.org

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OBESITY IS A MAJOR HEALTH PROBLEM in developed countries, with steadily increasing numbers (1). Since the aberrant increase in body fat alters adipose tissue metabolic and endocrine functions, obesity is now

considered to be a state of chronic inflammation (2). Accordingly, adiposity results in an increased release of fatty acids, hormones, and proinflammatory molecules such as tumor necrosis factor (TNF)-α from fat cells that contribute to obesity-related disorders, *e.g.*, cardiovascular diseases, insulin resistance, and type 2 diabetes mellitus (1). In addition, the secretion of chemokines and cytokines from fat cells triggers macrophage accumulation and activation in adipose tissue (2–4). On activation, macrophages secrete various cytokines, including TNF-α (2, 5), and also up-regulate other death-receptor ligands, such as TNF-related apoptosis-inducing ligand (TRAIL) on their surface (6).

It is well established that weight loss in overweight and obese patients will lead to a significant amelioration of obesity-associated dysfunctions (1). The size of white adipose tissue is also considered as a key factor influencing life span in mammals (7). For example, mice with decreased white adipose tissue due to the lack of the insulin receptor in adipose tissue show increased longevity (8). Accordingly, calorie restriction has been shown to lead not only to a reduction of total body fat mass from white adipose tissue and increased insulin sensitivity, but also to an extended life span (7). However, the regulatory pathways that mediate these health-beneficial effects of calorie restriction have only partly been unraveled. Recently, SIRT1, the mammalian homologue of yeast Sir2, was identified as a key mediator that links calorie restriction and longevity in mammals (9). SIRT1 is up-regulated on starvation and promotes fat mobilization of white adipose tissue *via* repression of the transcription factor PPARγ (9).

Furthermore, small-molecule activators of sirtuins have been identified that, similar to SIRT1, can extend life span in yeast and higher organisms (10, 11). Sirtuins are a conserved family of NAD⁺-dependent deacetylases (class III histone deacetylases) (12). One of these sirtuin-activating compounds is the natural

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product resveratrol (3,4',5-trihydroxystilbene), a polyphenol that is synthesized by plants and is present in many dietary items, including grapes and red wine (13, 14). Recently, resveratrol has been shown to improve energy balance and increase mitochondrial function in mice by activating SIRT1 (13, 15). Resveratrol has previously gained considerable attention because of its beneficial effects as a cardioprotective, cancer chemopreventive, and chemotherapeutic agent (14, 16).

There is increasing evidence that a continuous turnover of fat cells takes place in human white adipose tissue also in adult life (17–20). In principle, two key factors determine white adipose tissue mass: the number of fat cells and their volume, *i.e.*, how much fat each of them stores, the latter being largely regulated by metabolic and endocrine factors (18). A large pool of precursor cells exists in white adipose tissue throughout life that are able to differentiate into adipocytes (18). Therefore, the number of fat cells in the body has to be kept within a certain regulated range in order to maintain adipose tissue homeostasis, *e.g.*, through apoptotic deletion of excess fat cells (19). Thus, apoptosis functions as a homeostatic mechanism by which the total number of fat cells is controlled.

Apoptosis (programmed cell death) is the cell's intrinsic death program that plays a crucial role in the regulation of tissue homeostasis (21). Two key apoptosis pathways exist, *i.e.*, the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway (22). Stimulation of death receptors of the TNF-receptor superfamily, such as CD95 (APO-1/Fas) or the agonistic TRAIL receptors TRAIL-R1 and TRAIL-R2, leads to the recruitment to and activation of caspase-8 at the death-inducing signaling complex (DISC), which, in turn, can cause direct cleavage of downstream effector caspases (23). The mitochondrial pathway is initiated by the release of apoptogenic factors, such as cytochrome *c* or Smac (second mitochondria-derived activator of caspase)/DIABLO from the mitochondrial intermembrane space (24). The release of cytochrome *c* into the cytosol triggers caspase-3 activation through formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex (24).

Under physiological conditions, preadipocytes and adipocytes display low sensitivity to TNF- α , CD95 ligand- and TRAIL-induced apoptosis despite surface expression of the corresponding death receptors (25). However, these cells readily undergo apoptosis in the presence of an inhibitor of biosynthesis (25), indicating that the death-receptor signaling pathway is principally intact.

While resveratrol has been reported to alter the metabolic functions of white adipose tissue and to suppress proliferation of preadipocytes (9, 26–35), little is yet known about the effect of resveratrol on fat cell apoptosis and thus on the control of total fat cell numbers. In cancers, we previously reported that subtoxic concentrations of resveratrol profoundly sensitize cancer cells for death-receptor- and chemotherapy-induced apoptosis (36–38). In the present study, we

therefore investigated whether resveratrol regulates fat cell numbers by modulating apoptosis sensitivity.

MATERIALS AND METHODS

Materials

Cell culture medium, fetal bovine serum, and penicillin/streptomycin were obtained from Life Technologies (Karlsruhe, Germany), TRAIL was obtained from R&D Systems (Wiesbaden, Germany), PI-103 was obtained from Alexis (Grünberg, Germany), and resveratrol and other chemicals were purchased from Sigma Chemical (Taufkirchen, Germany). Anti-CD95 (APO-1) monoclonal antibody was produced as described previously (39). Rosiglitazone (Life Technologies; 49653) was a kind gift from Smith Kline Beecham (London, UK).

Cell culture and preparation of primary human preadipocytes

SGBS preadipocytes were cultured as described previously (40). For serum-free conditions, cells were cultured in serum-free DMEM/Ham's F-12 (1:1) medium, 33 μ M biotin, and 17 μ M pantothenate. Cells were seeded in 12-well plates at a density of 20,000 cells/well, unless stated otherwise. Adipogenic differentiation was induced, according to an established protocol (25). Abdominal subcutaneous white adipose tissue was obtained from healthy females undergoing plastic surgery. The experimental protocols were approved by the ethical committee of Ulm University, and all patients gave their informed consent. Preadipocytes were prepared from adipose tissue samples by collagenase digestion according to an established protocol (41). Cells were seeded in DMEM/Ham's F-12 (1:1) medium containing 10% FBS, 33 μ M biotin, 17 μ M pantothenate, and 1% penicillin/streptomycin.

Determination of apoptosis and cell viability

Apoptosis was determined by fluorescence-activated cell-sorting (FACScan, Becton Dickinson, Heidelberg, Germany) analysis of DNA fragmentation of propidium iodide-stained nuclei, as described previously (42), or by annexin-V (Roche, Grenzach, Germany), according to the manufacturer's instructions. For *in vitro* differentiated adipocytes, the protocol was modified as described previously (25). Specific apoptosis was calculated using the following formula: (observed apoptosis – spontaneous apoptosis) \times [100/(100 – spontaneous apoptosis)]. Cell viability was determined by MTT assay as described previously (43). Morphological features of apoptosis were analyzed by nuclear staining with 4',6'-diaminido-2-phenylindol (DAPI).

Western blot analysis

Western blot analysis was performed as described previously (44), using the following antibodies: caspase-3, phospho-Bad (Ser-136), Bad, phospho-Akt (Ser-473), phospho-ERK, phospho-S6 ribosomal protein, S6 ribosomal protein (all from Cell Signaling, Beverly, MA, USA), XIAP, Bcl-2, FADD, Akt (all from BD Biosciences, Franklin Lakes, NJ, USA), Bcl-2 (Zymed, South San Francisco, CA, USA), survivin, Bid (both from R&D Systems, Minneapolis, MN, USA), BaxNT, SIRT1 (both from Upstate Biotechnology, Lake Placid, NY), caspase-8, cFLIP (both from Alexis), TRAIL-R2 (Chemicon, Billerica, MA, USA), ERK, β -actin (both from Sigma), and goat-anti-mouse IgG, goat-anti-rabbit IgG, goat-anti-mouse

IgG1, or goat-anti-mouse IgG2b (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) conjugated to horseradish peroxidase. Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany). Representative blots of at ≥ 3 independent experiments are shown unless otherwise indicated.

Bax immunoprecipitation

Bax immunoprecipitation was performed as described previously (45). Briefly, cells were lysed in CHAPS lysis buffer (10 mM HEPES, pH 7.4; 150 mM NaCl; and 1% CHAPS); 2 mg protein was incubated with 16 μ g mouse anti-Bax antibody (6A7, Sigma) overnight at 4°C, followed by the addition of 10 μ l pan-mouse IgG Dynabeads (Invitrogen, Carlsbad, CA, USA), then incubated for 2 h at 4°C, washed with CHAPS lysis buffer, and analyzed by Western blot analysis using BaxNT antibody (Upstate Biotechnology).

Determination of mitochondrial membrane potential, cytochrome *c* release, and TRAIL-receptor staining

Mitochondrial membrane potential and cytochrome *c* release were determined as described previously (46). Briefly, CMXRos (1 μ M; Molecular Probes, Karlsruhe, Germany) was used to measure the mitochondrial transmembrane potential. Cells were incubated for 30 min at 37°C in the presence of the fluorochrome and immediately analyzed by flow cytometry. Cytochrome *c* release was determined in permeabilized cells using mouse anti-cytochrome *c* monoclonal antibody (BD Biosciences). To determine surface receptor expression of TRAIL receptors, cells were incubated with mouse anti-human TRAIL-R1 to -R4 monoclonal antibody (all from ApoTech Corporation, Epalinges, Switzerland) for 30 min at 4°C, washed in PBS containing 1% FCS, incubated with rabbit anti-mouse-F(ab')₂IgG/Biotin (BD Biosciences) for 20 min at 4°C in the dark, washed in PBS containing 1% FCS, incubated with streptavidin-PE (BD Biosciences) for 20 min at 4°C in the dark, and analyzed by flow cytometry.

Transduction

SGBS preadipocytes were seeded at a density of 130,000 cells/25 cm² cell culture flasks for 24 h, followed by overnight incubation with virus-containing supernatant. For Bcl-2 overexpression, cells were transduced with pMSCV vector containing mouse Bcl-2 or empty vector using the packaging cell line PT67 (BD Biosciences). Stable cell lines were selected by 10 μ g/ml blasticidin (Invitrogen). For generation of SIRT1-knockdown cells, SGBS preadipocytes were stably transduced with empty pRETRO-Super vector, vector containing shRNA against SIRT1 (TGAAGTGCCTCAGATATTA), or nonsense control (GATCATGTAGATACGCTCA). Stable cell lines were selected by 0.5 mg/ml puromycin.

TRAIL DISC immunoprecipitation

Cells were incubated for 30 min at 37°C either with Flag-tagged TRAIL (Alexis) (1 μ g/ml) and/or resveratrol (100 μ M) or left untreated. After lysis using a buffer containing 50 mM Tris-HCl, 1% (v/v) Triton-X 100, 150 mM NaCl, and protease inhibitor cocktail (Roche, Mannheim, Germany), 1 μ g/ml Flag-tagged TRAIL was also added to the resveratrol-treated and untreated samples. The TRAIL-receptor-associated DISC was then immunoprecipitated from the lysates using 1.25 μ g/ml mouse-anti Flag M2 antibody (Sigma). Elution of the precipitate was done adding 10 μ l pan-mouse IgG Dynabeads (Invitrogen)

and overnight rotation. On the next day, samples were washed 3 times with washing buffer I [50 mM Tris-HCl, 500 mM NaCl, and 1% (v/v) Igepal CA-630 (Nonidet P-40; Sigma)], and once with washing buffer II (25 mM Tris-HCl). Samples were analyzed by Western blot for expression of TRAIL-R2, cFLIP, FADD, and caspase-8.

Statistics

Statistical significance was assessed by Student's *t* test using Winstat software (R. Fitch Software, Bad Krozingen, Germany). Interaction between resveratrol and TRAIL was analyzed by the combination index (CI) method, based on that described by Chou (47), using CalcuSyn software (Biosoft, Cambridge, UK). CI < 0.9 indicates synergism, 0.9–1.1 additivity, and >1.1 antagonism.

RESULTS

To investigate the effect of resveratrol on apoptosis of human fat cells, we used the human preadipocyte cell strain SGBS (40), as well as primary preadipocytes isolated from subcutaneous white adipose tissue from healthy adults.

Resveratrol sensitizes preadipocytes to death-receptor-induced apoptosis

To explore whether resveratrol alters the susceptibility of preadipocytes to death-receptor stimulation, we treated SGBS cells with varying concentrations of the death-receptor ligand TRAIL in the presence or absence of increasing concentrations of resveratrol. Notably, the addition of resveratrol profoundly enhanced TRAIL-induced apoptosis in a dose-dependent manner, as determined by analysis of DNA fragmentation, whereas even relatively high concentrations of either agent alone (300 μ M resveratrol, 100 ng/ml TRAIL) had only minor effects (Fig. 1A and data not shown). Calculation of combination index demonstrated that the interaction of resveratrol and TRAIL is strongly synergistic (Table 1). Kinetic analysis revealed that resveratrol acted in concert with TRAIL to trigger apoptosis in a time-dependent manner (Fig. 1B). Apoptotic cell death was confirmed by the analysis of phosphatidylserine exposure and by typical morphological features, *e.g.*, nuclear condensation and fragmentation (Fig. 1C, D). Using these parameters as readouts of apoptotic cell death, resveratrol similarly cooperated with TRAIL to trigger apoptosis compared to either agent alone (Fig. 1C, D).

To examine the effect of resveratrol and TRAIL on cell viability, we performed an MTT assay. Notably, resveratrol acted in concert with TRAIL to reduce cell viability (Fig. 1E). We observed that treatment with TRAIL slightly promoted cell viability compared to untreated cells (Fig. 1E), in line with the notion that TRAIL may stimulate survival pathways in certain cell types (48).

Since hormones that are present in the serum may have an effect on TRAIL- and resveratrol-induced apoptosis, we also performed experiments with serum-free

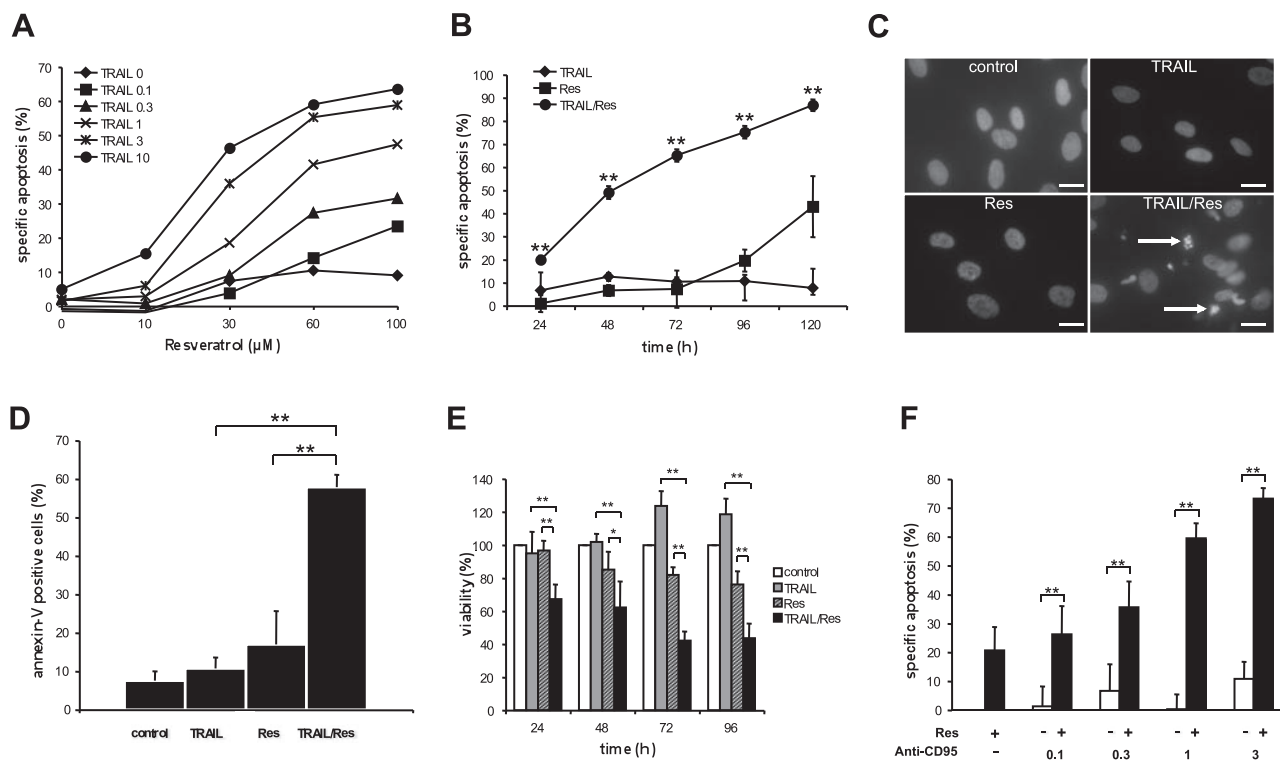


Figure 1. Resveratrol sensitizes human preadipocytes to TRAIL-induced apoptosis. *A, B*) SGBS preadipocytes were treated with indicated concentrations of resveratrol and/or TRAIL (ng/ml) for 72 h (*A*) or with 100 μ M resveratrol and/or 10 ng/ml TRAIL for indicated times (*B*). Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei; specific apoptosis is shown. *C*) SGBS preadipocytes were grown on chamber slides, treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for 24 h, stained with DAPI, and analyzed by fluorescence microscopy. Arrows indicate apoptotic cells. Scale bars = 20 μ M. Images are representative of 3 independent experiments. *D*) SGBS preadipocytes were treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for 72 h, and apoptosis was analyzed by annexin-V staining and flow cytometry. *E*) SGBS preadipocytes were treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for indicated times. Cell viability was measured by MTT assay and is expressed as percentage of untreated cells. *F*) Effect of resveratrol on CD95-induced apoptosis. SGBS preadipocytes were treated for 72 h with 0.1–3 μ g/ml anti-CD95 antibody alone or in combination with 100 μ M resveratrol. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei; specific apoptosis is shown. Data are means \pm sd of 3 independent triplicate experiments (except *A*; means without sd). * P < 0.05; ** P < 0.001.

medium. Similar to the results obtained with medium containing serum, the addition of resveratrol significantly increased TRAIL-induced apoptosis in serum-free medium (Supplemental Fig. 1).

We then extended our studies to CD95 as another death-receptor system to exclude that the “apoptosis-sensitizing” effect of resveratrol was restricted to the death-inducing ligand TRAIL. Notably, resveratrol also significantly enhanced CD95-induced apoptosis in a synergistic fashion (Fig. 1*F*). Together, this set of experiments demonstrates that resveratrol primes human preadipocytes for death-receptor-induced apoptosis.

SIRT1 is dispensable for resveratrol-mediated sensitization to TRAIL-induced apoptosis

To gain insights into the molecular mechanisms that mediate the synergistic action of resveratrol and TRAIL, we examined the involvement of SIRT1, since resveratrol has recently been identified as a SIRT1 activator (10, 11). To this end, we knocked down SIRT1 expression by retroviral short-hairpin RNA (shRNA) vectors. Empty vector and a nonsense RNAi sequence served as con-

trols. Interestingly, silencing of SIRT1 did not interfere with resveratrol- and TRAIL-induced apoptosis, as determined by analysis of DNA fragmentation, despite effective suppression of SIRT1 protein levels (Fig. 2). Assessment of apoptosis by annexin-V staining similarly showed that SIRT1 knockdown had no effect on resveratrol and TRAIL-induced apoptosis (Supplemental Fig. 2). This demonstrates that SIRT1 is dispensable for the resveratrol-mediated sensitization of preadipocytes to TRAIL-induced apoptosis.

Resveratrol enhances TRAIL-induced caspase activation and caspase-dependent apoptosis

Next, we systematically analyzed activation of the extrinsic and intrinsic apoptosis pathways. To this end, we first examined whether resveratrol modulates surface expression of TRAIL receptors. Flow cytometric analysis revealed that treatment with resveratrol did not increase surface levels of TRAIL-R1, -R2, or -R4, while TRAIL-R3 was hardly detectable on SGBS cells (Fig. 3*A*). By comparison, treatment with TRAIL or the combination of TRAIL and resveratrol resulted in a decrease of TRAIL-R1, -R2,

TABLE 1. Synergistic interaction of resveratrol and TRAIL

TRAIL	Resveratrol	CI
0.1	30	0.442
0.1	60	0.506
0.1	100	0.667
0.3	30	0.339
0.3	60	0.383
0.3	100	0.583
1	30	0.294
1	60	0.326
1	100	0.475
3	30	0.277
3	60	0.307
3	100	0.439
10	30	0.427
10	60	0.430
10	100	0.530

CI was calculated as described in Materials and Methods for combined treatment with indicated concentrations of resveratrol and TRAIL. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei after treatment for 72 h. CI < 0.9 indicates synergism; CI = 0.9–1.1 indicates additivity; CI > 1.1 indicates antagonism.

or -R4 (Fig. 3A), which may be explained by rapid internalization of TRAIL receptors on stimulation with TRAIL, as described previously (49).

As the next step in the TRAIL signaling pathway, we assessed formation of the TRAIL DISC on stimulation with TRAIL. The addition of resveratrol did not enhance the recruitment of FADD or caspase-8 to activated TRAIL receptors and also did not substantially alter the recruitment of the long or the short isoforms of cFLIP (Fig. 3B).

Monitoring activation of the caspase cascade by Western blot analysis revealed that the addition of resveratrol enhanced TRAIL-induced cleavage of caspase-8 into p43/p41 intermediate and p18 active fragments as early as 0.25 h, as well as proteolytic turnover of the proenzyme form of caspase-8 (Fig. 4A). In addition, resveratrol increased cleavage of caspase-3 into the active p17 fragment as evident at 1 to 6 h, while treatment with TRAIL alone predominantly resulted in the generation of the intermediate, yet inactive p20 fragment of caspase-3 (Fig. 4A). Caspase-dependent apoptosis was confirmed by the use of the caspase inhibitor zVAD.fmk, which significantly reduced TRAIL- and resveratrol-induced apoptosis (Fig. 4B). Together, this set of experiments demonstrates that resveratrol enhances TRAIL-induced activation of caspases and caspase-dependent apoptosis.

Resveratrol acts in concert with TRAIL to induce mitochondrial perturbations and Bax activation

Next, we explored whether resveratrol alters signaling *via* the mitochondrial pathway during TRAIL-induced apoptosis. To address this question, we determined mitochondrial membrane potential and cytochrome *c* release in SGBS cells treated with TRAIL in the presence and absence of resveratrol. Notably, resveratrol significantly enhanced TRAIL-in-

duced loss of mitochondrial membrane potential and cytochrome *c* release from mitochondria (Fig. 5A, B). Similarly, lower resveratrol concentrations acted in concert with TRAIL to trigger drop of the mitochondrial membrane potential and apoptosis (Supplemental Fig. 3).

To gain insights into the signaling events that are regulated by resveratrol upstream of mitochondria, we monitored expression of key apoptosis regulators. Treatment with resveratrol alone for >6 h resulted in a decrease of both cFLIP isoforms, *i.e.*, cFLIP_L and cFLIP_S, and the combination treatment with resveratrol and TRAIL further decreased both cFLIP isoforms (Fig. 5C). In addition, Bid levels substantially decreased in cells that were exposed to resveratrol and TRAIL for more than 6 h pointing to Bid cleavage (Fig. 5C). As for regulators of postmitochondrial apoptosis, survivin was down-regulated on prolonged treatment with resveratrol, in line with our previous findings (36), while expression levels of XIAP were not altered. Since down-regulation of cFLIP, survivin, and Bid occurred on prolonged treatment with resveratrol and/or TRAIL, these changes may contribute to the second phase of loss of mitochondrial membrane potential (Fig. 5A) and to apoptosis; however, they are likely not primarily responsible for the observed rapid mitochondrial perturbations that started after 0.5 h (Fig. 5A). Treatment with TRAIL alone resulted in increased survivin expression at 24 h and 48 h (Fig. 5C), consistent with the notion that TRAIL can also trigger survival cascades, *e.g.*, the PI3K/Akt pathway, which is

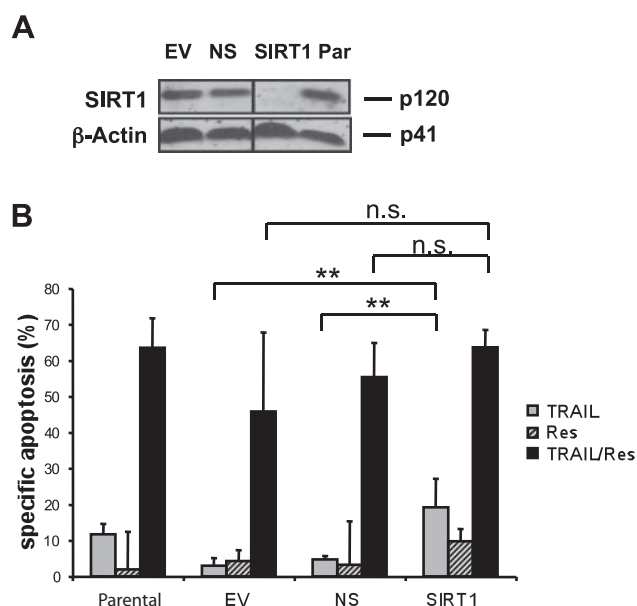


Figure 2. Effect of SIRT1 knockdown on resveratrol-mediated sensitization to TRAIL-induced apoptosis. A) SGBS preadipocytes were stably transduced with empty vector (EV) or a vector containing nonsense (NS) or SIRT1 shRNA. Efficiency of SIRT1 knockdown was monitored by Western blot. Parental (Par) or transduced cells were treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for 72 h. B) Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei; specific apoptosis is shown. Data are means \pm SD of 3 independent triplicate experiments, ** P < 0.001; n.s., not significant.

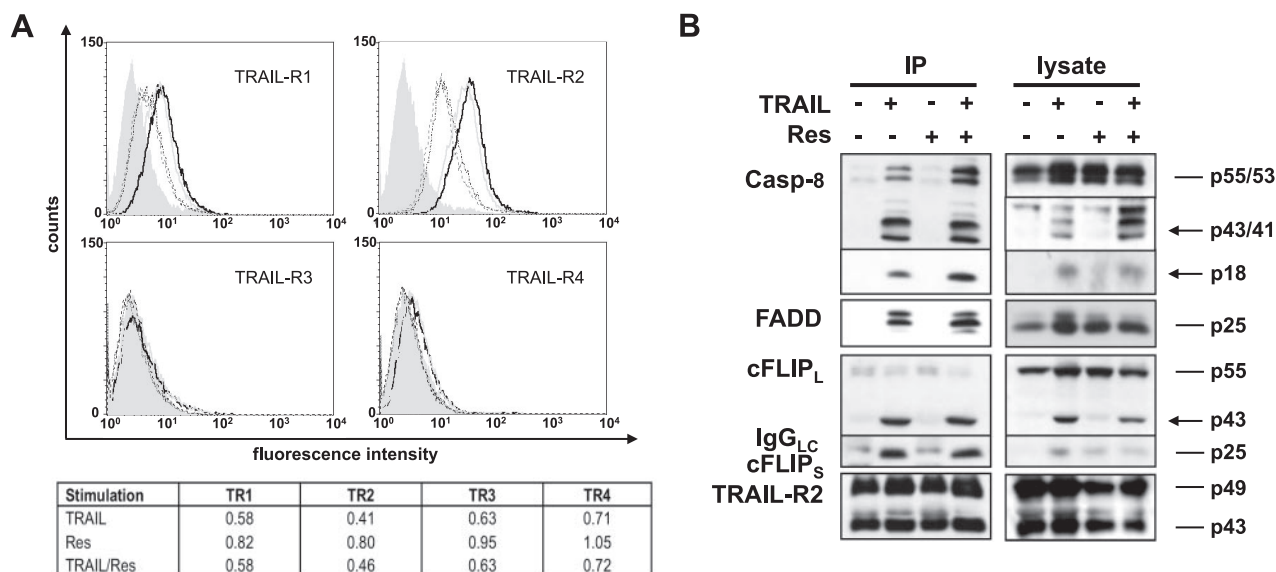


Figure 3. Effect of resveratrol on TRAIL-receptor expression and TRAIL DISC formation. *A*) Surface expression of TRAIL receptors TRAIL-R1 to R4 on SGBS cells after incubation with medium, 100 μ M resveratrol, and/or 10 ng/ml TRAIL for 60 min was determined by fluorescence-conjugated antibodies and flow cytometry (gray curves, untreated cells stained with isotype control; thick black line, untreated cells stained with anti-TRAIL-R1 to R-4 antibodies; thin gray dotted line, cells treated with 10 ng/ml TRAIL and stained with anti-TRAIL-R1 to R-4 antibodies; thick gray line, cells treated with resveratrol 100 μ M and stained with anti-TRAIL-R1 to R-4 antibodies; thin black dotted line, cells treated with the combination of TRAIL and resveratrol and stained with anti-TRAIL-R1 to R-4 antibodies). Fold change in mean fluorescence intensity of TRAIL-R1 to R4 (TR1-4) expression on treatment with resveratrol and/or TRAIL compared to untreated cells is shown at bottom. *B*) SGBS preadipocytes were treated with 1 μ g/ml Flag-tagged TRAIL and/or 100 μ M resveratrol for 30 min, and TRAIL-TRAIL-R complexes were immunoprecipitated by use of anti-FLAG antibody coupled to magnetic beads. Precipitated protein complexes were separated by Western blot and analyzed for caspase-8, FADD, cFLIP_L, cFLIP_S, and TRAIL-R2. IgG_{LC}, IgG light chain.

involved in the regulation of survivin expression (50). Searching for signaling events that are regulated by resveratrol upstream of mitochondria, we next examined activation of Bax, a key regulator of mitochondrial outer membrane permeabilization. To this end, we assessed Bax conformational change by immunoprecipitation using an antibody that specifically recognizes the active conformation

of Bax. Interestingly, we detected Bax in its active conformation only in cells that were simultaneously exposed to resveratrol and TRAIL, but not in cells treated with either agent alone (Fig. 5D). These findings demonstrate that resveratrol and TRAIL cooperate to trigger Bax conformational change and mitochondrial outer membrane permeabilization.

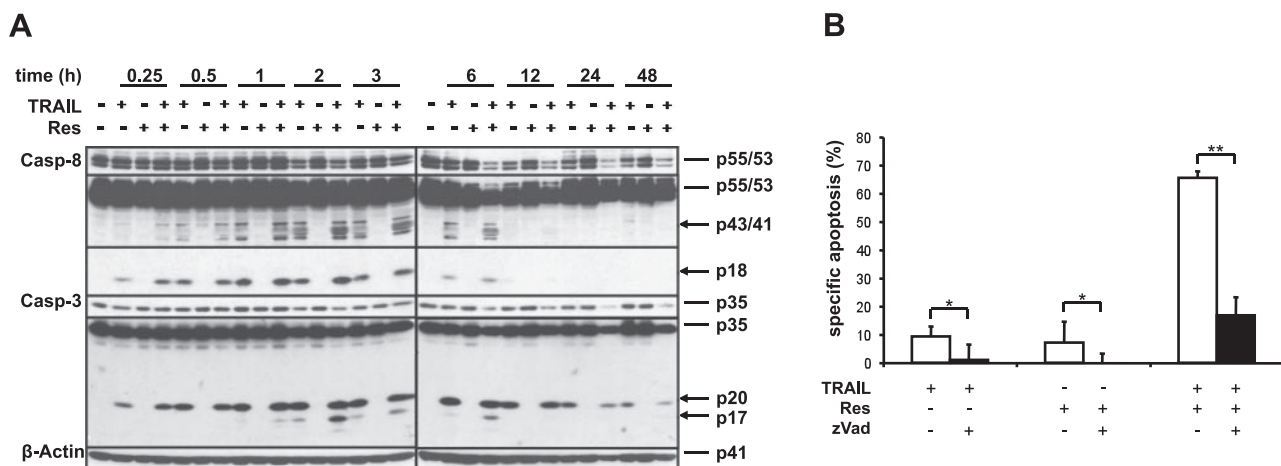


Figure 4. Resveratrol enhances TRAIL-induced caspase activation and caspase-dependent apoptosis. *A*) SGBS preadipocytes were treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for indicated times, and activation of caspase-3 and -8 was analyzed by Western blot analysis. Arrows indicate cleavage fragments. *B*) SGBS preadipocytes were treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for 72 h in the presence or absence of 50 μ M zVAD.fmk. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei; specific apoptosis is shown. Data are means \pm SD of 3 independent triplicate experiments. * P < 0.05; ** P < 0.001.

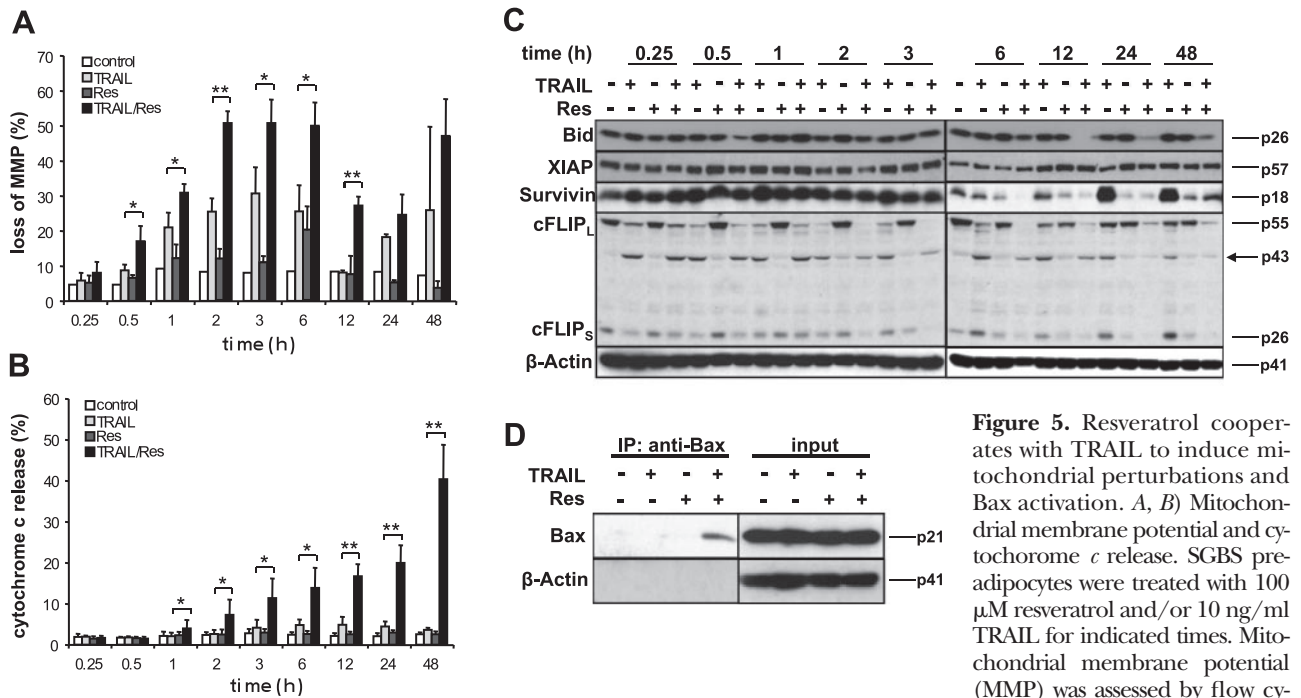


Figure 5. Resveratrol cooperates with TRAIL to induce mitochondrial perturbations and Bax activation. *A, B*) Mitochondrial membrane potential and cytochrome *c* release. SGBS preadipocytes were treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for indicated times, and protein expression of cFLIP_L, cFLIP_S, Bid, XIAP, survivin, and β -actin was assessed by Western blot. *D*) Bax conformational change. SGBS preadipocytes were treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for 1 h, Bax was immunoprecipitated (IP) using an anti-Bax antibody, and detection of Bax was done by Western blot analysis; 5% of protein lysates was used for analysis of input.

cytometry using the fluorescent dye CMXRos. Release of cytochrome *c* from mitochondria was assessed by staining of cytochrome *c* in permeabilized cells and flow cytometry. Data are means \pm SD of 3 independent triplicate experiments. * P < 0.05; ** P < 0.001. *C*) Apoptosis regulatory proteins. SGBS preadipocytes were treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for indicated times, and protein expression of cFLIP_L, cFLIP_S, Bid, XIAP, survivin, and β -actin was assessed by Western blot. *D*) Bax conformational change. SGBS preadipocytes were treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for 1 h, Bax was immunoprecipitated (IP) using an anti-Bax antibody, and detection of Bax was done by Western blot analysis; 5% of protein lysates was used for analysis of input.

Resveratrol inhibits PI3K/Akt signaling, which contributes to the sensitization to TRAIL-induced apoptosis

Searching for molecular events that mediate Bax activation, we asked whether resveratrol modulates PI3K/Akt signaling, since PI3K/Akt signaling has been shown to suppress Bax activation *via* phosphorylation of Bax (51). To address this question, we monitored activation of the PI3K/Akt cascade by Western blot analysis using phosphorylation of Akt, a target of PI3K, and phosphorylation of S6 ribosomal protein, a target of mTOR, as readouts for activation of upstream and downstream components of the pathway. We also assessed phosphorylation of extracellular signal-regulated kinase (ERK), a direct target of mitogen-activated protein kinase (MEK), since interactions between the PI3K/Akt and the Raf/MEK/ERK pathway have been described (22–24). Treatment with resveratrol resulted in rapid and profound inhibition of phosphorylation of Akt and S6 ribosomal protein (Fig. 6A). By comparison, no differences in the phosphorylation status of ERK on treatment with resveratrol and/or TRAIL were observed (Fig. 6A).

To examine whether inhibition of PI3K/Akt signaling can prime SGBS cells to TRAIL-induced Bax activation and apoptosis, we blocked PI3K signaling by pharmacological inhibitors, *i.e.*, LY294002 and PI-103 (52). Inhibition of Akt phosphorylation by LY294002 or PI-103 (Fig. 6B) also significantly increased TRAIL-induced apoptosis in SGBS cells (Fig. 6C). Interestingly,

this induction of apoptosis was accompanied by concomitant activation of Bax and drop of mitochondrial membrane potential in cells that were treated with the combination of LY294002 and TRAIL (Fig. 6D, E), pointing to a link between PI3K inhibition, Bax activation, mitochondrial perturbations, and apoptosis.

Requirement of the mitochondrial pathway for resveratrol-mediated sensitization to TRAIL-induced apoptosis

To test whether activation of the mitochondrial pathway is indeed required for apoptosis induction by resveratrol and TRAIL, we blocked mitochondrial perturbations by overexpression of Bcl-2. Western blot analysis confirmed strong ectopic expression of Bcl-2 compared to empty vector control or parental cells (Fig. 7A). Notably, overexpression of Bcl-2 almost completely inhibited loss of mitochondrial membrane potential and significantly reduced apoptosis on combined treatment with TRAIL and resveratrol compared to vector control cells (Fig. 7B). This demonstrates that the resveratrol-induced sensitization for TRAIL-induced apoptosis is mediated, at least in part, *via* the mitochondrial pathway.

Resveratrol sensitizes adipocytes and primary human preadipocytes to TRAIL-induced apoptosis

Finally, we extended our experiments to adipocytes, which were generated by *in vitro* differentiation, as

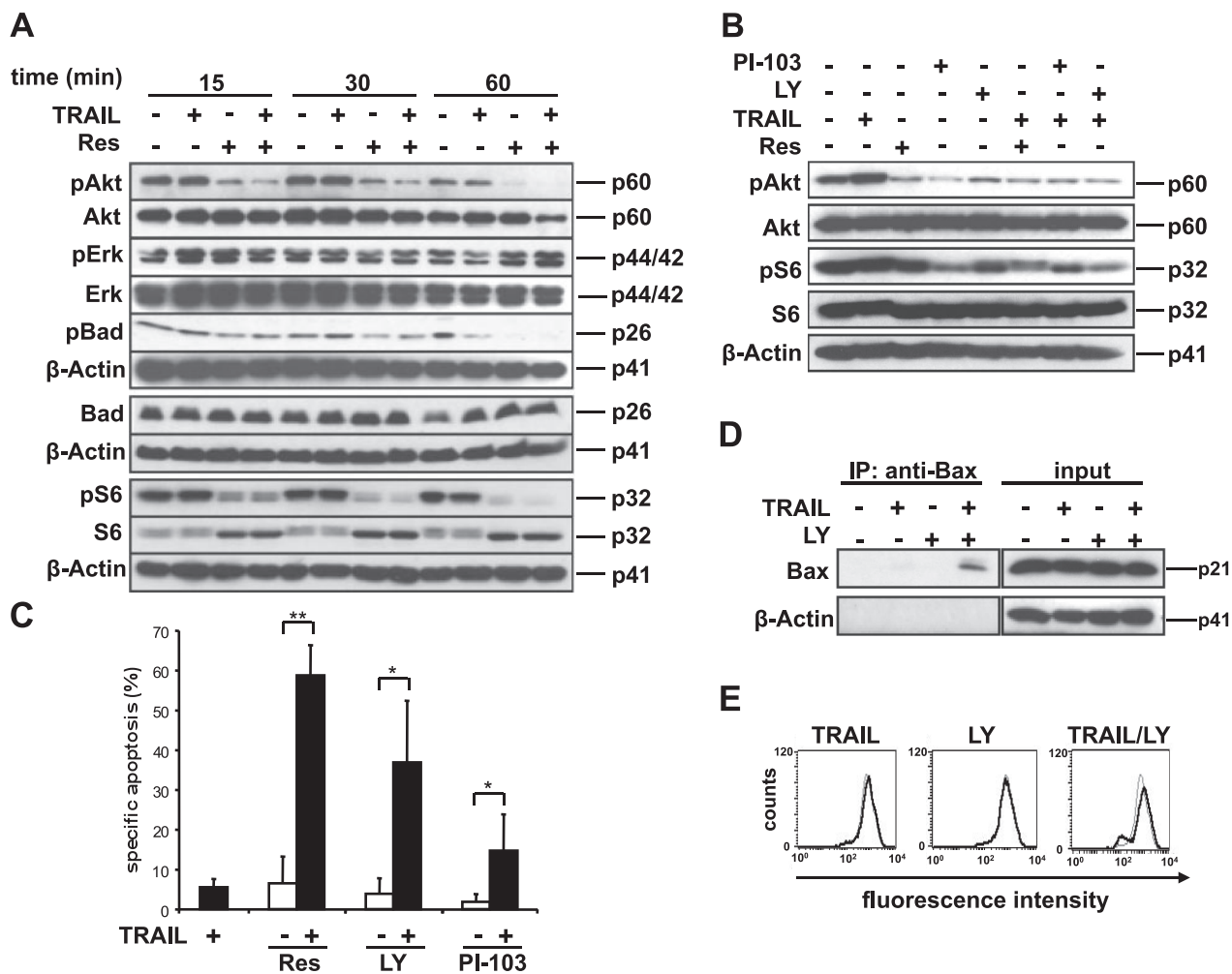


Figure 6. Resveratrol-mediated inhibition of Akt phosphorylation precedes Bax activation. *A*) SGBS preadipocytes were treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for indicated times. Phosphorylation status of Akt, Erk, and S6 ribosomal protein was analyzed by Western blot analysis. *B*) SGBS preadipocytes were treated with 100 μ M resveratrol, 1 μ M PI-103, 40 μ M LY294002, and/or 10 ng/ml TRAIL for 1 h. Phosphorylation status of Akt and S6 ribosomal protein was analyzed by Western blotting. *C*) SGBS preadipocytes were treated with 100 μ M resveratrol, 1 μ M PI-103, 40 μ M LY294002, and/or 10 ng/ml TRAIL for 72 h. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei; specific apoptosis is shown. Data are means \pm SD of 3 independent triplicate experiments. * P < 0.05; ** P < 0.001. *D*) SGBS preadipocytes were treated with 40 μ M LY294002 and/or 10 ng/ml TRAIL for 2 h, Bax was immunoprecipitated (IP) using an anti-Bax antibody, and detection of Bax was done by Western blot analysis; 5% of protein lysates was used for analysis of input. *E*) SGBS preadipocytes were treated with 40 μ M LY294002 and/or 10 ng/ml TRAIL for 6 h, and mitochondrial membrane potential was assessed by flow cytometry using the fluorescent dye CMXRos. Thin line, untreated cells; thick line, cells treated with TRAIL and/or LY294002. A representative experiment of 2 independent triplicate experiments is shown.

described previously (25), and to primary human preadipocytes that were prepared from the subcutaneous white adipose tissue of healthy adults. Similar to preadipocytes, resveratrol significantly increased TRAIL-induced apoptosis in adipocytes (Fig. 8A). Also, resveratrol acted in concert with TRAIL to reduce cell viability of adipocytes (Fig. 8B). Further, resveratrol and TRAIL cooperated to induce apoptosis in primary human preadipocytes, whereas either compound alone exerted no or minimal effects (Fig. 8C). This set of experiments demonstrates that resveratrol acts in concert with TRAIL to induce apoptosis in adipocytes, as well as in primary human preadipocytes, underscoring the relevance of the findings for human physiology.

DISCUSSION

In the present study, we identify a novel proapoptotic function of resveratrol that contributes to resveratrol-mediated reduction of white adipose fat tissue by demonstrating for the first time that resveratrol primes fat cells to death-receptor-induced apoptosis in a SIRT1-independent manner. Resveratrol cooperates with death-receptor ligands such as TRAIL or agonistic CD95 antibody to induce apoptosis in human preadipocytes in a highly synergistic manner. The relevance to human physiology is underscored by similar data obtained in primary human preadipocytes prepared from the subcutaneous white adipose tissue of healthy adults, as well as in mature human adipocytes.

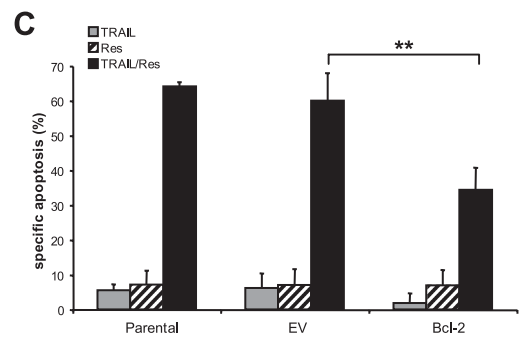
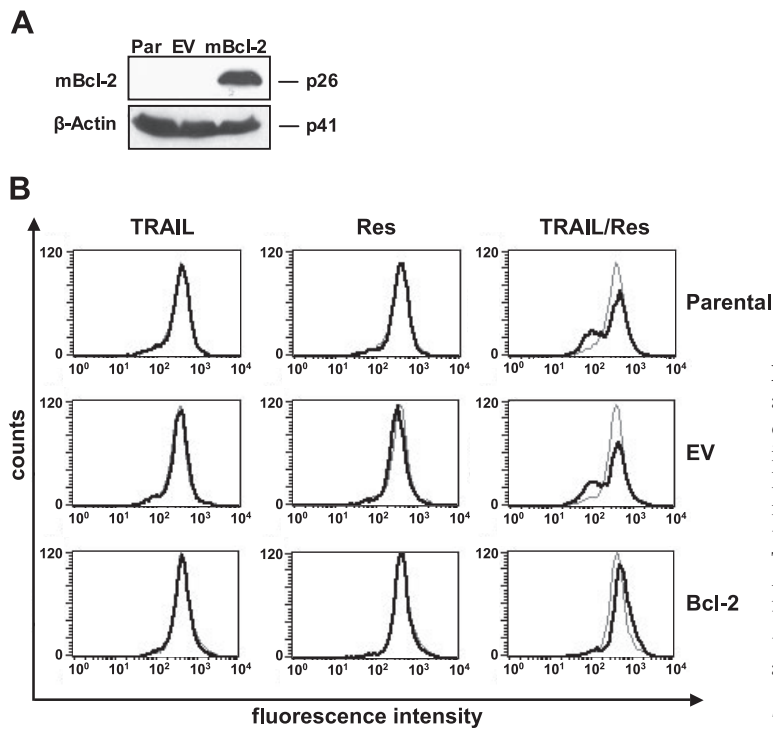


Figure 7. Bcl-2 overexpression reduces resveratrol- and TRAIL-induced apoptosis. SGBS preadipocytes were transduced with pMSCV vector containing mouse Bcl-2 (mBcl-2) or empty vector (EV). Par, parental cells. **A**) mBcl-2 overexpression was monitored by Western blot analysis. **B**) Cells were treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for 6 h, and mitochondrial membrane potential was assessed by flow cytometry using the fluorescent dye CMXRos. Thin line, untreated cells; thick line, cells treated with TRAIL and/or resveratrol. **C**) Cells were treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for 72 h. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei; specific apoptosis is shown. Data are means \pm SD of 3 independent triplicate experiments. ****** $P < 0.001$.

stained nuclei; specific apoptosis is shown. Data are means \pm SD of 3 independent triplicate experiments. ****** $P < 0.001$.

Mechanistic studies reveal that this resveratrol-conferred sensitization to TRAIL is mediated *via* the mitochondrial apoptosis pathway and caspase-dependent apoptosis independent of SIRT1 (Fig. 9). The func-

tional relevance of the mitochondrial pathway for the synergism of resveratrol and TRAIL is shown by ectopic expression of Bcl-2, which significantly reduces resveratrol- and TRAIL-triggered apoptosis. Caspase-depen-

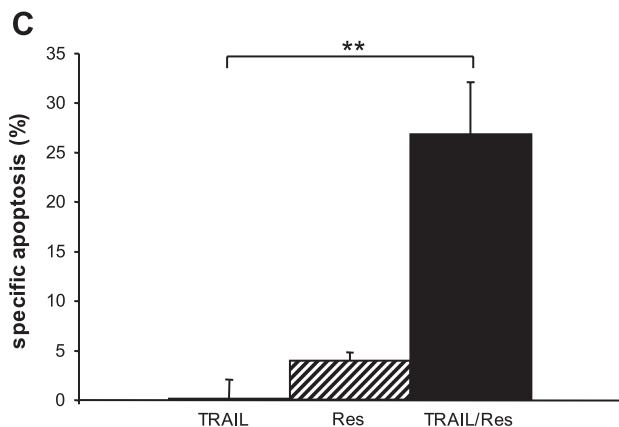
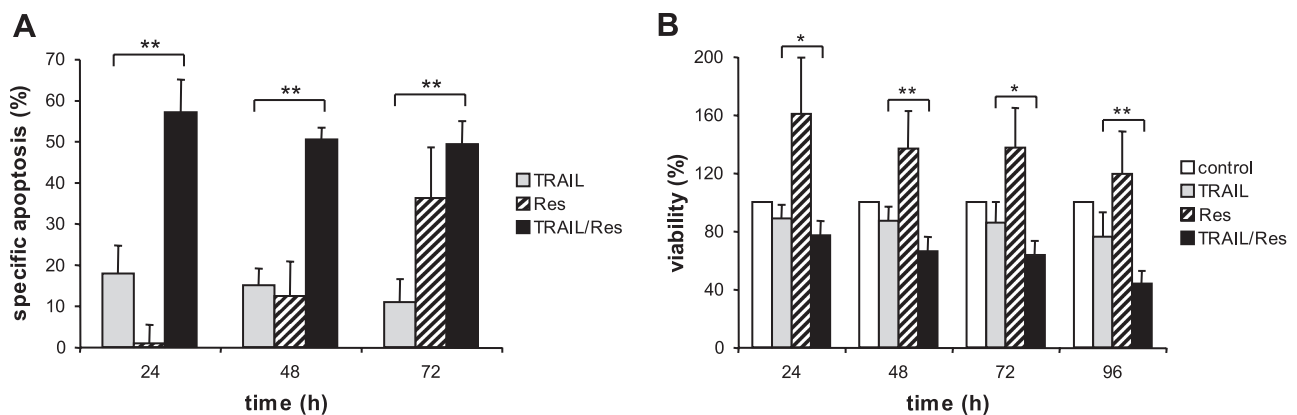


Figure 8. Resveratrol sensitizes adipocytes and human primary preadipocytes for TRAIL-induced apoptosis. **A**, **B**) SGBS preadipocytes were differentiated to adipocytes and then treated with 100 μ M resveratrol and/or 10 ng/ml TRAIL for indicated times. **A**) Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei; specific apoptosis is shown. **B**) Cell viability was determined by MTT assay and is expressed as percentage of untreated controls. **C**) Human primary preadipocytes were prepared from subcutaneous white adipose tissue from healthy donors as described in Materials and Methods. After treatment for 72 h with 100 μ M resveratrol and/or 10 ng/ml TRAIL, apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei; specific apoptosis is shown. Data are means \pm SD of 3 independent triplicate experiments. ***** $P < 0.05$; ****** $P < 0.001$.

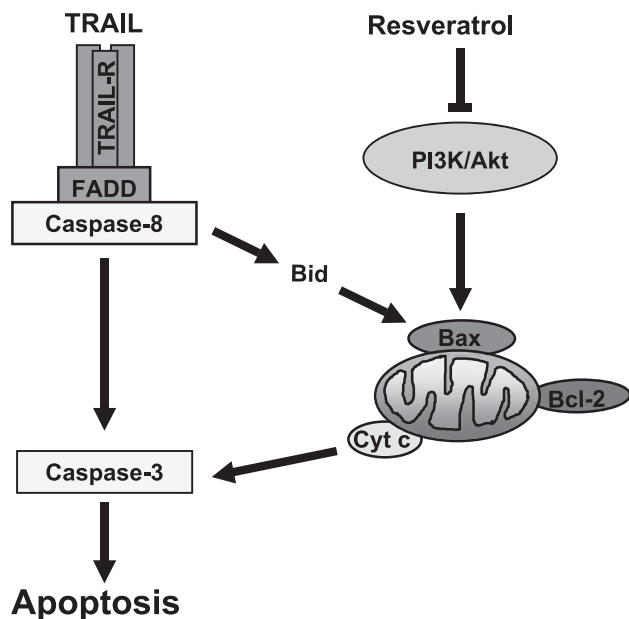


Figure 9. Scheme of synergistic interaction of TRAIL and resveratrol in preadipocytes. Ligation of TRAIL receptors (TRAIL-R) by TRAIL leads to receptor trimerization and recruitment of FADD and caspase-8 to form the DISC, followed by caspase-8 activation. Caspase-8 triggers the first cleavage step of caspase-3 into the yet inactive p20 fragment, as well as cleavage of Bid. Resveratrol inhibits phosphorylation of Akt, relieving the Akt-imposed inhibition of Bax. TRAIL and resveratrol cooperate to activate Bax and to induce loss of mitochondrial membrane potential, cytochrome *c* release, full activation of caspase-3, and apoptosis. See text for more details.

dent apoptosis is confirmed by the demonstration that caspase inhibition also completely blocks resveratrol- and TRAIL-induced apoptosis. Delineation of the signaling pathway shows that resveratrol inhibits PI3K-driven Akt phosphorylation, leading to an increase in Bax activation, loss of mitochondrial membrane potential, cytochrome *c* release, and caspase-3 activation on exposure to TRAIL. Of note, our data reveal that the resveratrol-mediated sensitization for apoptosis occurs independent of SIRT1, whereas we recently found that SIRT1 is required for resveratrol-mediated inhibition of proliferation, adipogenic differentiation, and metabolic and endocrine functions of preadipocytes (unpublished results). Similarly, resveratrol has been reported to mimic calorie restriction in yeast by stimulating the yeast SIRT1 homologue Sir2 (10), to extend life span in metazoans in a Sir2-dependent fashion (10, 11), and to inhibit adipogenic differentiation in a SIRT1-dependent manner (9). Also, resveratrol-mediated protection against metabolic disease and its antiproliferative effect have been linked to its sirtuin-like activity (13, 15, 30). Together, these data indicate that resveratrol controls adipose tissue homeostasis *via* at least two distinct pathways: First, as unraveled in this study, resveratrol regulates fat cell number through apoptotic depletion in a SIRT1-independent fashion *via* potentiation of death-receptor-triggered apoptosis.

Second, resveratrol controls metabolic and endocrine functions of fat cells in a SIRT1-dependent manner.

In principle, two key factors determine white adipose tissue mass: first, the volume of a fat cell, which is largely regulated by metabolic and endocrine factors, and second, the total number of fat cells (17–19). Previous studies on resveratrol and fat cells have focused on its metabolic and endocrine effects. To this end, resveratrol has been reported to inhibit adipogenesis and lipogenesis by blocking the conversion of glucose to lipids and also to directly stimulate lipolysis (9, 26–30, 32, 35). In addition, resveratrol has been shown to decrease PPAR γ expression and its transcriptional activity along with decreased responsiveness to insulin (9, 34), to inhibit NF- κ B-mediated cytokine expression in adipocytes (35) and to reduce leptin secretion from isolated rat adipocytes, leading to reduced food intake (31).

However, much less is known about the effect of resveratrol on cell numbers of adipose tissue, especially when it comes to molecular mechanisms. Recent evidence suggests that resveratrol may have an effect on the total number of fat cells by decreasing adipocyte formation during osteoblast differentiation of mesenchymal stem cells (33). In addition, resveratrol has been described to inhibit pig preadipocyte proliferation and differentiation and to decrease cell viability (26, 28, 30). Although resveratrol has previously been reported to induce apoptosis in adipocytes, the functional relevance of this observation remained unclear, since only data on the relative rather than the absolute increase in apoptosis were provided (26–29). Compared to these earlier reports, our study is the first that identifies a synergistic interaction of resveratrol and death-receptor ligands to trigger massive apoptosis in fat cells and to identify the underlying molecular mechanisms. Notably, this synergism is especially evident at subtoxic concentrations of resveratrol, which only minimally affect viability in the absence of death-receptor ligands such as TRAIL. Thus, the novelty of our study particularly resides in the identification and characterization of a new activity of resveratrol in fat cells: resveratrol at subtoxic doses acts as a sensitizer of fat cells to death-receptor-induced apoptosis. Thus, we introduce a new concept of white adipose tissue regulation by resveratrol.

So far, cooperative interaction of resveratrol and death-receptor ligands has been reported by our group and other investigators in cancer cells and has been linked to suppression of the antiapoptotic protein survivin and enhanced formation of the TRAIL or CD95 DISC (36, 38, 53–58). Thus, the present study is the first to show that resveratrol is also able to prime nonneoplastic cells, such as preadipocytes and adipocytes to apoptosis. In these cells, we observed no substantial alterations of TRAIL or CD95 DISC formation by resveratrol, and down-regulation of survivin expression occurred only after prolonged incubation with resveratrol, whereas both events were reported in cancer cells to mediate apoptosis sensitization by resveratrol (36, 38, 53–58). This points to distinct mecha-

nisms that are responsible for the resveratrol-mediated sensitization for apoptosis in malignant *vs.* nonmalignant cells. The sulfonylurea receptor and integrin $\alpha\beta 3$ have previously been identified as receptor sites for resveratrol (59, 60). Since integrin $\alpha\beta 3$ is expressed on preadipocytes (61), it may present an initiation site for the resveratrol-mediated effect on these cells.

Our findings have several important implications. First, this newly identified function of resveratrol to prime fat cells to death-receptor-induced apoptosis likely contributes to the health beneficial effect of resveratrol in metabolic diseases. By reducing the total number of fat cells through apoptotic depletion, resveratrol diminishes white adipose tissue mass, and thus may be useful in the treatment of obesity-related disorders. Intriguingly, resveratrol has recently also been reported to prevent the development of fatty liver in mice fed a high-calorie diet with 60% calories from fat (13). This implies that resveratrol may simultaneously prevent the development of hepatic steatosis that may otherwise occur in response to the increase in circulating lipids as the result of resveratrol-mediated apoptosis of fat cells. TRAIL may well be present in fat tissue, since TRAIL has been reported to be up-regulated on stimulated macrophages (6), which are recruited to adipose tissue *via* a chemokine and cytokine gradient that is produced by fat cells (2–4). Interestingly, increased serum TRAIL concentrations were reported to correlate with total body fat in healthy adults, with higher levels in obese people (62), suggesting that resveratrol may, in particular, sensitize fat cells of obese rather than lean people to TRAIL-induced apoptosis. Future studies will have to clarify whether sufficiently high TRAIL concentrations are present in fat tissue to trigger apoptosis together with resveratrol. The concentrations of resveratrol that were required for priming fat cells to apoptosis might be achievable in humans, as peak plasma levels of 2.4 or 14 μM after oral administration were reported in humans for resveratrol or its most abundant metabolite, which has been suggested to contribute to the pharmacologic activity of resveratrol (63). In addition, resveratrol analogues with improved potency and bioavailability have also recently been identified (64). Previously, the protective effect of resveratrol against the deleterious effects of a high-calorie diet has been linked to its metabolic effects, *i.e.*, the reduction of insulin-like growth factor-1 (IGF-I) levels, increased AMP-activated protein kinase (AMPK), and peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α) activity and increased mitochondrial number (13). Thus, resveratrol may counteract obesity-associated dysfunctions through both suppression of metabolism, as well as apoptotic depletion of fat cells.

A further corollary of our study is that resveratrol may promote longevity in a SIRT1-independent manner by priming fat cells to death-receptor-induced apoptosis. The primary effect of calorie restriction that eventually leads to an increase in longevity and delays aging has been linked to the reduction of body fat mass of white adipose tissue (7). This decrease in body fat

mass can be achieved by a reduction in the volume of white adipose tissue or alternatively, by a decrease in total cell number (17–19). Genetic evidence supports a key role of SIRT1 in fat mobilization of white adipocytes by repression of PPAR γ (9). Furthermore, small-molecule activators of sirtuins, in particular, resveratrol, have been shown to extend the life span in yeast, as well as metazoans, linking SIRT1 activity to longevity (10, 11). Our data imply that resveratrol may also prolong life span in a SIRT1-independent fashion and thus add a new twist to the effect of resveratrol on longevity by showing that resveratrol in combination with death-receptor ligands depletes preadipocytes and adipocytes irrespective of SIRT1.

In conclusion, the discovery of this novel function of resveratrol, *i.e.*, priming of fat cells for death-receptor-mediated apoptotic depletion, significantly advances the knowledge of fat tissue regulation by resveratrol and has important implications for the future use of resveratrol and its analogues in metabolic disorders and age-related diseases. EJ

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