The Phytostilbene Resveratrol Induces Apoptosis in INS-1E Rat Insulinoma Cells

Cornelia Bortolotti^{1*}, Thomas Kunit^{1*}, Angelika Moder¹, Clemens Hufnagl¹, Sabine Schmidt¹, Arnulf Hartl¹, Christian Langelueddecke¹, Johannes Fürst², John P. Geibel³, Markus Ritter¹ and Martin Jakab¹

¹Institute of Physiology and Pathophysiology, Paracelsus Medical University Salzburg, Salzburg, ²Division of Physiology, Department of Physiology and Medical Physics, Innsbruck Medical University, Innsbruck, ³Department of Surgery and Department of Cellular and Molecular Physiology, Yale University, School of Medicine, New Haven, *CB and TK contributed equally to this work

Key Words

Resveratrol • Beta-cell • Insulin • Proliferation • Apoptosis • Akt • Caspase

Abstract

We investigated the effect of resveratrol on proliferation and induction of apoptosis of INS-1E rat insulinoma cells by cell counting, crystal violet staining, flow cytometry and immunoblotting. Resveratrol treatment of INS-1E cells at concentrations $\geq 50 \mu\text{M}$ resulted in a dose-dependent inhibition of cell proliferation, accumulation of the cells in the S and G0/G1 phase and a significant increase of the percentage of apoptotic cells. This was paralleled by an increase of cell granularity, apoptotic volume decrease (AVD), exposure of phosphatidylserine at the outer leaflet of the plasma membrane, an increase of the 7-AAD signal and caspase activation. The AMP-kinase (AMPK) inhibitor compound C ($10 \mu\text{M}$) significantly inhibited cell proliferation and induced caspase activation within 48 hours but this effect was not modified by resveratrol suggesting that AMPK is not a major target involved in mediating the proapoptotic effect of resveratrol in INS-1E cells. Immunoblotting revealed

a significant inhibition of Akt (PKB) phosphorylation by $100 \mu\text{M}$ resveratrol within 1 hour. Addition of insulin ($10 \mu\text{M}$) to the culture medium strongly enhanced basal Akt phosphorylation. This enhancement was significantly attenuated by 50 and $100 \mu\text{M}$ resveratrol. We conclude that the antiproliferative/proapoptotic effect of resveratrol on INS-1E cells is due to negative interference with Akt signaling and most likely disruption of auto/paracrine insulin signaling.

Copyright © 2009 S. Karger AG, Basel

Introduction

The phytoalexin *trans*-resveratrol, which is synthesised by plant tissues in response to fungal infections or exposure to UV light [1], has been shown to prevent or attenuate the progression of metabolic disorders, cardiovascular diseases or ischemic injuries and can enhance stress resistance. The drug is known for its antioxidant activity, can prevent platelet aggregation and tumor angiogenesis and has been shown to induce cell cycle arrest and apoptosis in primary tumor cells and several tumor cell lines [2-4]. The substance interferes with distinct cellular targets including cyclooxygenases, protein

kinase C, ornithinecarboxylase and sirtuin deacetylases (Sir2/SIRT1). Sir2/SIRT1 activation has been intensively investigated since it seems to underlie the potency of resveratrol to extend the lifespan in lower organisms [4]. The antiproliferative effect of resveratrol is due to interference with signaling cascades regulating cell proliferation/apoptosis like Akt (PKB)- and AMP-activated protein kinase (AMPK) signaling [5-8]. In cultured muscle cells a downregulation of insulin-like pathways by resveratrol was found due to inhibition of phosphoinositide 3-kinase (PI3K) [9].

It has been described in streptozotocin-nicotinamide-induced diabetic rats that resveratrol diminishes renal dysfunction and oxidative stress [10], decreases hyperglycemia and hyperlipidaemia, enhances the tissue responsiveness to insulin and to delay the development of insulin resistance [11, 12]. High plasma concentrations of triglycerides, cholesterol, free fatty acids, insulin and leptin found in the metabolic syndrome model of obese Zucker rats were reduced in animals that received resveratrol [13]. In the light of the beneficial effects of the drug on the metabolic state and complications associated with diabetes it seems conflicting that prolonged exposure to resveratrol for 24 hours induces apoptosis in isolated beta-cells and beta-cell lines (HIT-T15 and RIN-m5F), as recently shown [14]. In isolated pancreatic islets resveratrol has been shown to reversibly and dose-dependently inhibit insulin secretion in a protein kinase C dependent manner consistent with a repression of the amplifying pathway of insulin release [15-17]. In a recent study we have shown a reversible suppression of glucose-, hypotonicity- and tolbutamide-induced membrane depolarization and electrical activity by resveratrol in INS-1E rat insulinoma cells and a complete inhibition of glucose-induced insulin release within 1 hour, suggesting a block of the triggering pathway of insulin secretion [18].

Insulin and insulin-like growth factor 1 (IGF-1) act as growth factors in beta-cells [19, 20]. Decreased beta-cell mass of insulin-receptor knock-out mice and insulin receptor substrate 2-deficient mice are consistent with an auto/paracrine action of insulin and antiapoptotic effects in beta-cells [21] as are data from MIN6 insulin secreting cells showing that high glucose stimulates proliferation and protects cells against apoptosis, which can be prevented by an anti-insulin antibody or PI3K inhibition [22].

Since resveratrol interferes with signaling pathways that regulate beta-cell proliferation/apoptosis and inhibits insulin release from native beta-cells and insulinoma cells,

we performed the present study to determine long term effects of resveratrol (0-72 hours) on proliferation, cell cycle distribution and apoptosis and Akt and AMP kinase signaling in INS-1E rat insulinoma cells.

Materials and Methods

Chemicals and reagents

All salts and chemicals used for the preparation of experimental solutions and cell culture media were p.a. grade. Resveratrol (3,4',5-trihydroxy-*trans*-stilbene), accutase solution, 6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]pyrimidine (compound C), AICA riboside, crystal violet solution and porcine insulin were obtained from Sigma-Aldrich. Stock solutions of resveratrol (10 mM and 100 mM) were prepared in ethanol and stored in aliquots at 4°C. The mouse monoclonal anti-phosphatidylserine antibody clone 1H6 (anti-PS) and fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG₁ were from Upstate Cell Signaling Solutions and SouthernBiotech, respectively. 7-amino-actinomycin D (7-AAD) was obtained from Beckman Coulter. Nuclear isolation medium-4,6-diamidino-2-phenylindole dihydrochloride (NIM-DAPI) and DNA reference calibrator were from NPE systems. The CaspACE™ FITC-VAD-FMK *in situ* marker was obtained from Promega. Rabbit monoclonal Akt (pan) (C67E7) and phospho-Akt (Ser473) (D9E) primary antibodies and the HRP-linked anti-rabbit IgG secondary antibody were from Cell Signaling Technology.

Cell culture

INS-1E cells [23] were cultured in RPMI-1640 medium containing 2.1 mM L-glutamine, 11 mM D-glucose, 23.8 mM NaHCO₃ supplemented with 50 µM β-mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES, 10% fetal calf serum (FCS), 100 µg/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere at 37 °C, 5% CO₂ and 95% air. Except for the experiments shown in Fig. 1B, cells were kept under 11 mM glucose throughout all incubations. Cells were subcultured every third day by trypsin/EDTA treatment. For the measurement of cell proliferation and apoptosis cells were subcultured in 2 ml of RPMI medium into 9.2 cm² tissue culture dishes at a cell density of 2x10⁶ cells/ml. For flow cytometrical measurements cells were detached with accutase or trypsin/EDTA.

Measurement of cell proliferation

INS-1E cells were seeded one day before resveratrol treatment into 9.2 cm² tissue culture dishes each containing 2 ml of RPMI medium. Resveratrol was added to the cells at concentrations of 10, 50 and 100 µM, respectively. Untreated cells or cells treated with the solvent (1% ethanol) served as control. Nine regions were marked on the bottom of each dish. Cells were allowed to adhere and proliferate for ~1 day. Thereafter solvent or resveratrol was added at the respective concentrations and cells were counted immediately (time point 0) as well as 24, 48 and 72 hours after addition, respectively, using a Motic AE 20 microscope. Each cell counting was repeated two

Fig. 1. (A) Concentration-dependent effect of resveratrol (res) at the indicated concentrations or solvent (1% ethanol) on the proliferation of INS-1E rat insulinoma cells. (B) Dependence of cell proliferation on the glucose (gluc) concentration in the cell culture medium. Asterisks denote significant differences to control conditions (ethanol in panel A or 11 mM glucose in panel B). Normalised data.

times by two independent observers (C.B. and T.K.) in a blinded manner. The mean of the two counts in each region was taken as cell count per region and the change in cell number over time was related to the value within an individual region. All experiments were repeated at least 7 times.

Crystal violet cell adhesion assay

The number of adherent cells was determined colorimetrically by a crystal violet test modified from [24]. For the standard curve cells were seeded at a density of 5000, 10000, 20000, 37500, 50000 and 75000 cells/ml per well, respectively, on a 96-well plate. Wells without cells containing crystal violet solution served as blanks. For experiments cells were seeded at a density of 20000 cells/ml. Cells were allowed to adhere for 4 hours and then further incubated for 48 hours in the presence of drugs as indicated. Cells were kept under standard culture conditions in RPMI-1640 medium (11 mM glucose) supplemented with serum throughout the experiment. Crystal violet in the destaining solution was measured photometrically at 570 nm using a Zenyth 3100 multimode reader (Anthos Beckman Coulter). Data are given as cells/ml after 48 hours of incubation.

Flow cytometry

Induction of apoptosis, cell cycle distribution and caspase activity was measured with a Cell Lab QuantaTM SC flow cytometer (Beckman Coulter) equipped with a 488 nm laser, a mercury arc lamp and the appropriate filter sets for excitation

Fig. 2. Cell cycle analysis by NIM-DAPI staining and flow cytometry of INS-1E cells treated for 0, 24, 48 and 72 hours with resveratrol (res) at the indicated concentrations. Cells treated with 1% ethanol for the same time periods served as controls.

and emission as specified below. Calibration for cell diameter/volume measurements was performed by using Flow check beads (Beckman Coulter) with a nominal diameter of 10 μ m. Side scatter (SS) was taken as measure for the cell granularity. For assessment of apoptosis, cell cycle and caspase activation the analysed signals were gated for cells $\geq 7 \mu$ m and $\leq 14 \mu$ m. Experiments were repeated 4 times.

Assessment of apoptosis: Cells were subcultured 2 days before treatment with 10, 50 or 100 μ M resveratrol or ethanol (1%; solvent controls). Immediately after starting the treatment (time point 0) as well as 24, 48 and 72 hours later cells were harvested and centrifuged at 300xg for 5 minutes. The supernatant was discarded and the cell pellets were resuspended in a buffer containing (in mM): 150 NaCl, 5 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 HEPES, pH 7.4, 1% bovine serum albumin. 1.5×10^6 cells were subsequently incubated with anti-PS antibody (1:25) for 30-45 minutes at room temperature, washed once and incubated for another 30-45 minutes with FITC-conjugated secondary antibody (1:12.5) and 10 μ l 7-AAD. After staining, cells were washed twice with buffer and resuspended

Fig. 3. Induction of apoptosis by resveratrol (res) in INS-1E cells measured by flow cytometry. (A) Development of the population of non apoptotic (PS-/7-AAD-), early apoptotic (PS+/7-AAD-) and late apoptotic (PS+/7-AAD+) cells over 72 hours. Cells were gated for diameter ($\geq 7 \mu\text{m}$ and $\leq 14 \mu\text{m}$). Particles $<7 \mu\text{m}$ were regarded as debris. (B) Representative plots showing the distribution of non apoptotic (1), early apoptotic (2) and late apoptotic (3) cells under 50 μM resveratrol at the indicated time points.

in a final volume of 400 μl . The samples were analysed within one hour using 488 nm laser excitation. PS negative/7-AAD negative cells were considered as non apoptotic, PS positive/7-AAD negative cells as early apoptotic, PS positive/7-AAD positive cells as late apoptotic. Quadrant regions were set according to the distribution of control cells.

Assessment of cell cycle arrest: After treatment with resveratrol in different concentrations (10, 50 and 100 μM) for 0, 24, 48, 72 hours the cells were washed with PBS, harvested and stained with NIM-DAPI for 10 minutes at room temperature. Thereafter cells were washed once with PBS and analysed. The excitation light from the mercury arc lamp was passed through a 355/37 nm bandpass filter. The emission light was directed towards the photomultiplier tube by a dichroic mirror (cut-off 550 nm) and passed through a 465/30 nm bandpass filter. The instrument was calibrated using trout red blood cells (DNA reference calibrator, NPI systems).

Measurement of caspase activation: Caspase activation was measured using CaspACETM FITC-VAD-FMK *in situ* marker, a fluoroisothiocyanate (FITC)-conjugate of the membrane permeable pan-caspase inhibitor VAD-FMK (Promega). The excitation wavelength was 288 nm, emission was measured at 525 nm. Cells treated for 24 or 48 hours with 10, 50 or 100 μM resveratrol or ethanol (1%; solvent controls) were washed once with PBS, detached from the culture dish with trypsin/EDTA and centrifuged for 5 minutes at 300xg, washed twice with PBS, incubated with caspase inhibitor reagent for 30 minutes at 37 $^{\circ}\text{C}$, washed twice and resuspended to $1\text{--}2 \times 10^6$ cells/ml for measuring caspase activation by flow cytometry.

SDS-PAGE and Immunoblotting

Cells were grown in 100 mm petri dishes for 3 days to ~70% confluency and treated with resveratrol in the absence or presence of insulin for 1 hour at the indicated concentrations. Equal volumes of cell lysates were subjected to SDS-PAGE (4–15% Tris-HCl) and proteins were blotted onto nitrocellulose membranes (PROTRAN BA 85, Schleicher & Schuell). Membranes were incubated over night with primary antibodies at dilutions of 1:1000 and for 2 hours with the secondary antibody (1:2000). Protein bands were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce). Images were acquired using a BioSpectrum AC Imaging System and VisionWorks LS Software (UVP).

Statistics and data presentation

Data are expressed as mean $\pm$ SEM. Statistical comparisons were performed using paired or unpaired student's t-test, where applicable. $P < 0.05$ between groups was considered as statistically significant (* or §, ** and *** denotes $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; ns, not significant). For all series of experiments control measurements using solvent (1% ethanol) were performed. Since in none of the experimental series a significant difference between untreated and ethanol treated samples was found the data obtained from untreated cells were omitted from most graphs for simplicity or only data of untreated cells are shown. Data were analysed and plotted using GraphPad Prism version 5.00 (GraphPad Software, San Diego California, USA) or Igor Pro 6.03 (WaveMetrics, Lake Oswego, USA) for Macintosh.

Results

Effect of resveratrol and glucose concentration on the proliferation of INS-1E rat insulinoma cells

Since resveratrol is known to exert proapoptotic and antiproliferative effects in various cell types, we investigated the influence of the drug (10–100 μ M) on the proliferation of INS-1E cells over 72 hours. Fig. 1A shows that under control conditions (11 mM medium glucose) the cell number increased to $187.36 \pm 12.94\%$ ($n=96$), $302.99 \pm 25.58\%$ ($n=96$) and $463.19 \pm 36.81\%$ ($n=96$) within 24, 48 and 72 hours, respectively. Addition of solvent (ethanol 1%) or 10 μ M resveratrol did not significantly change the growth rate. In the presence of 50 and 100 μ M resveratrol, however, cell growth was dose- and time-dependently inhibited; significant inhibition was evident already after 24 hours at 100 μ M ($112.76 \pm 8.55\%$, $n=55$) and after 72 hours at 50 μ M resveratrol ($311.50 \pm 57.74\%$, $n=64$). The dependence of INS-1E cell proliferation on the medium glucose concentration is shown in Fig. 1B. Whereas proliferation was unaffected by reduction from 11 to 9 or 7 mM, proliferation was significantly attenuated under 5 mM glucose.

Effect of resveratrol on cell cycle distribution

To assess a possible effect of resveratrol on the cell cycle distribution, exponentially growing cells treated with resveratrol (10, 50 and 100 μ M) or ethanol were analysed by DAPI staining and flow cytometry immediately after starting incubation as well as 24, 48 and 72 hours thereafter (Fig. 2). Under 100 μ M resveratrol the arrest occurred in the G0/G1 phase with a concomitant reduction of cells in the S- and G2 phase between 24 and 72 hours. Treatment with 50 μ M resveratrol for 24 hours, however, led to an accumulation in the S phase paralleled by a slight, though not significant, reduction of cells in the G0/G1- and G2 phase. Prolonged treatment with 50 μ M resveratrol for 48–72 hours led to an accumulation of cells arrested in the G0/G1 phase and a reduction of cells in the S- and G2 phase. 10 μ M resveratrol did not have any significant effect on the cell cycle distribution.

Resveratrol induces apoptosis in INS-1E cells

To test if the observed cell cycle arrest was paralleled by an induction of apoptosis, we analysed INS-1E cells treated with resveratrol (10, 50 and 100 μ M) for breakdown of phosphatidylserine (PS) asymmetry, loss of cell membrane integrity and altered cell morphology including cell shrinkage, which are prominent signs of

Fig. 4. Effect of resveratrol (res) on INS-1E cell morphology. Development of cell diameter (A) of early apoptotic (PS+/7-AAD-) cells and the side scatter (SS) signal (B) over time in the absence and presence of resveratrol. § indicates significant differences to values measured at time point 0 of untreated and 10 μ M resveratrol-treated cells.

programmed cell death [25–27]. The appearance of PS residues at the outer leaflet of the cell membrane is an early sign of apoptosis. In addition of PS exposure, late apoptotic cells are characterised by a loss of cell membrane integrity and can be discriminated from early apoptotic cells by simultaneous staining with the DNA-specific viability dye 7-AAD. Fig. 3A shows the distribution of non apoptotic (PS-/7-AAD-; upper panel), early apoptotic (PS+/7-AAD-; middle panel) and late apoptotic cells (PS+/7-AAD+; lower panel) between 0 and 72 hours in the absence and presence of resveratrol. Among untreated cells and cells exposed to 10 μ M resveratrol the different cell populations remained unchanged over the whole time course, whereas the percentage of non apoptotic cells progressively decreased over time in the presence of 50 or 100 μ M resveratrol. Simultaneously a significant increase of early apoptotic cells occurred. The percentage of late apoptotic cells (PS+/7-AAD+) was only transiently increased after 24 and 48 hours with no significant changes after 72 hours as compared to untreated cells due to the rapid fragmentation of cells under 50 and 100 μ M resveratrol which then appear as debris (diameter <7 μ m). Fig. 3B shows the distribution of non

Fig. 5. (A) Concentration- and time-dependent effect of resveratrol (res) on caspase activity in INS-1E cells. Y-axis = percentage of caspase positive cells (caspase+ in B). (B) Histograms (counts vs. FITC fluorescence measured at 525 nm) obtained from a representative experiment after 48 hours in the absence (ethanol) and presence of 50 or 100 μ M resveratrol. The medium glucose concentration was 11 mM.

apoptotic, early apoptotic and late apoptotic cells over the time under 50 μ M resveratrol.

Morphology of apoptotic cells

Induction of apoptosis in INS-1E cells exposed to 50 or 100 μ M resveratrol was paralleled by a time- and concentration-dependent reduction of the diameter of early apoptotic (PS+/7-AAD-) cells indicating apoptotic volume decrease (AVD) induced by resveratrol (Fig. 4A). A smaller, though significant, diameter reduction over time was also evident in untreated and solvent-treated cells. Since we observed a similar shrinkage also in untreated, non apoptotic (PS-/7-AAD-) cells (not shown), we assume that this effect is most likely due to the culture conditions. Cell shrinkage under resveratrol was accompanied by a progressive increase of the side scatter (SS) signal (Fig. 4B), which indicates an increase of cell granularity due to membrane blebbing during apoptosis.

Caspase activation by resveratrol

Certain manifestations of apoptosis are associated with caspase activation [28, 29]. Therefore we examined if in INS-1E cells the proapoptotic effect of resveratrol was associated with an increase of caspase activity, which was measured by flow cytometry using a cell permeable fluorescent caspase inhibitor. Resveratrol (100 μ M) led to a significant increase of caspase positive

Fig. 6. Effect of resveratrol on Akt phosphorylation. Cells were treated for 1 hour with 1% ethanol (solvent control), 50 μ M or 100 μ M resveratrol (res) in the absence or presence of 10 μ M porcine insulin (ins) in the cell culture medium. A representative western blot is shown in the upper panel. The lower panel depicts the densitometric analysis (mean $\pm$ SEM) of 3 blots obtained from independent experiments. § indicates significant differences to values obtained in presence of 10 μ M insulin but in absence of resveratrol.

Fig. 7. Effects of AMPK inhibition by compound C (compC) and addition of excess insulin (ins) to the culture medium on caspase activation measured by flow cytometry (A) and cell proliferation assessed by crystal violet staining (B) under solvent control conditions or under 50 μ M resveratrol. Measurements performed after 48 hours of incubation are shown. Numbers in bars are the number of independent experiments (cell preparations). For the crystal violet assay (B) cells were seeded in 96-well plates at a density of 20000 cells/ml as indicated by the dotted line.

cells already after 24 hours followed by a further increase after 48 hours (Fig. 5A). Under 50 μ M caspase activity was significantly augmented after 48 hours, whereas 10 μ M had no effect. Fig. 5B shows representative histograms obtained after 48 hours from control cells and resveratrol treated cells.

Effect of resveratrol and insulin on Akt phosphorylation

The 3'-phosphoinositide-dependent serine/threonine protein kinase Akt is a prominent mediator of the actions of insulin and insulin-like growth factor 1 (IGF-1) in the maintenance of the beta-cell mass by stimulating proliferation and inhibiting apoptosis [19]. Since resveratrol has been described as a potent inhibitor of phosphoinositide 3-kinase (PI3K) in muscle cells [9], we tested by immunoblotting the effect of resveratrol on the phosphorylation of Akt, a downstream target of PI3K. As shown in Fig. 6, resveratrol (50 and 100 μ M) dose-dependently inhibited Akt phosphorylation within 1 hour. At 100 μ M the phosphorylated protein was reduced by ~70%. In the presence of 10 μ M insulin in the cell culture medium, basal Akt phosphorylation under control conditions (11 mM medium glucose, presence of serum) was ~4-fold increased and the inhibitory effects of increasing resveratrol concentrations were significantly attenuated.

Effect of AMPK inhibition or insulin on caspase activity and INS-1E cell proliferation

To investigate a possible role of AMP-activated protein kinase (AMPK) in resveratrol-mediated apoptosis, we tested the effect of resveratrol on caspase activation and cell proliferation in the absence and presence of the AMPK inhibitor compound C. We chose a concentration of 50 μ M resveratrol and tested its effect after 48 hours (Fig. 5). Similar to 50 μ M resveratrol, compound C

(10 μ M) alone caused a significant increase of caspase positive cells by ~20% and an attenuation of cell proliferation compared to control cells (Fig. 7A and B, respectively). Importantly, simultaneous treatment with compound C and resveratrol did not produce a significant additive effect on caspase activation if compared to the effects of the two substances alone (Fig. 7A). Addition of 10 μ M insulin to the culture medium did neither counteract resveratrol-induced caspase activation nor its antiproliferative effect.

Discussion

We show that long-term exposure of clonal INS-1E rat insulinoma cells to resveratrol $\geq$ 50 μ M leads to a concentration- and time-dependent inhibition of cell proliferation and S or G0/G1 phase cell cycle arrest and induction of apoptosis. This is evident from apoptotic volume decrease (AVD), breakdown of phosphatidylserine (PS) asymmetry, increased cell granularity and caspase activation as are prominent signs of programmed cell death [25, 26, 28-30].

Our results compare to data from other tumour cell lines including hepatoma, glioma, lymphoma cells as well as prostate, breast, promyelocytic HL-60 and colon cancer cells, where resveratrol (tested in wide range of concentrations from 10-300 μ M) has been shown to exert anticancerogenic, antimitogenic and proapoptotic effects through multiple, though cell type specific signaling pathways [31-37]. More generally the resveratrol effects seem to be related to altered expression patterns of cyclins and cyclin-dependent kinases [34, 35, 38]. Other mechanisms involve TRAIL-induced apoptosis associated with survivin depletion [39], induction of p53 and increased Bax levels [35], inhibition of BCL-6 expression with in-

creased expression of p27, p53 and CD69 [40], caspase-3 activation and induction of cytochrome c release from mitochondria with activation of caspase-9 [35, 37, 41]. Recently resveratrol (100 μ M applied over 24 hours) has been found to induce apoptosis in HIT-T15 and RIN-m5F insulinoma cells as well as in native beta-cells by binding to the sulfonylurea receptor SUR1 [14].

To unravel the mechanism underlying resveratrol-induced growth inhibition and apoptosis in INS-1E cells, we studied the effects of the compound on AMP-activated protein kinase (AMPK) and Akt (PKB) signaling pathways, which are involved in beta-cell proliferation and maintenance of beta-cell mass under normal conditions as well as in case of beta-cell dysfunction in type 2 diabetes [19, 20, 42]. Resveratrol is a potent activator of AMPK in neurons, liver tissue and cardiac myocytes [5, 8, 13, 43]. In chemoresistant HT-29 cancer cells and MIN6N8 insulinoma cells AMPK activation by resveratrol exerts proapoptotic effect due to increased ROS production [6, 7]. A similar effect of resveratrol is unlikely in our experimental system since we find that activation of AMPK by AICA riboside does not stimulate apoptosis under control conditions, nor does it significantly add to the resveratrol effect if applied simultaneously (data not shown). However, inhibition of AMPK with compound C strongly promotes caspase activation and inhibits proliferation (Fig. 7), which implies that intact AMPK signaling is essential for INS-1E cell viability. If resveratrol inhibited AMPK, an additive effect of compound C and resveratrol could be expected. A stimulation of AMPK by resveratrol might conversely diminish caspase activation which would rather imply an antiapoptotic effect of the phytoestrogen. Therefore AMPK is probably not a major target of resveratrol involved in stimulating apoptosis in INS-1E cells.

Immunoblotting revealed that resveratrol significantly reduces the amount of phosphorylated Akt protein in a concentration-dependent manner after 1 hour of incubation (Fig. 6) which depicts the insulin/IGF-1 signaling pathway as a major target of resveratrol in INS-1E cells. Similarly, in INS-1 cells the glucocorticoid dexamethasone has been shown to induce cell death by interfering with IGF-1 signaling [20]. We further show that excess insulin in the culture medium can at least partly overrule the blocking effect of resveratrol on Akt phosphorylation, though the compensatory property of insulin is obviously not sufficient to prevent the antiproliferative and proapoptotic effect (Fig. 7), which points at additional sites of action besides insulin receptor signaling. The massive increase of Akt phosphorylation in the presence

of excess insulin clearly indicates the significance of autocrine/paracrine signaling in beta-cells as previously demonstrated [44]. Addition of insulin to the culture medium or stimulation of insulin release by high concentrations of extracellular K^+ used to depolarize beta-cells has been shown to suppress beta-cell apoptosis induced by glucose deprivation [45].

Glucose is a crucial factor for the survival of beta-cells [46]. Consistently we find that the proliferation rate of INS-1E cells is significantly reduced by lowering the medium glucose concentration (Fig. 1B). Under the given standard culture conditions for INS-1E cells [23], which are commonly used also for other beta-cell lines as well as primary beta-cells [14, 20] (i.e., RPMI 1640 medium containing 11 mM glucose, supplemented with 5-10% FCS), the cells are obviously metabolically adapted to hyperglycemic conditions and the setpoint for growth arrest appears to be shifted to higher glucose concentrations. Since we observed growth arrest also in serum free medium even under 11 mM glucose (data not shown), we used standard culture conditions throughout all incubations to avoid an overestimation of the resveratrol effect due to omission of glucose and/or serum. In MIN6 clonal beta-cells glucose was shown to stimulate proliferation and to protect cells against apoptosis [22]. This effect was prevented by an anti-insulin antibody, PI3K inhibition or by the L-type Ca^{2+} channel blocker nifedipine. Importantly, we previously demonstrated that in INS-1E cells resveratrol inhibits stimulus-secretion-coupling by blocking L- and T-type Ca^{2+} channels and glucose/swelling-dependent Cl^- channels [18] so that it is feasible that the proapoptotic effect of resveratrol is the result of an indirect inhibition of insulin receptor signaling by a suppression of insulin release and a direct inhibition of promitotic/antiapoptotic Akt signaling. Insulin receptor expression in INS-1E cells was confirmed by reverse transcriptase and real-time PCR and remained unaltered within 48 hours in resveratrol treated cells (data not shown). However, at that point we cannot rule out decreased insulin receptor expression and/or degradation at later points along the timeline, which might accelerate apoptosis.

Cell shrinkage during programmed cell death, referred to as apoptotic volume decrease (AVD), is accomplished by the activation of Cl^- and K^+ channels and inhibition of these ion extrusion pathways can prevent apoptosis [27, 47, 48]. In our previous work we found that resveratrol, besides blocking glucose/swelling-dependent Cl^- channels, also inhibits K_{ATP} channels [18]. The fact that this inhibition does

obviously not prevent AVD implies further ion extrusion pathways to be involved in AVD in INS-1E cells.

Although resveratrol has the property to reduce insulin release *in vitro* [15-17] as well as *in vivo* [11], and long term exposure induces apoptosis in other beta-cell lines (HIT-T15 and RIN-m5F) and primary beta-cells [14], no *in vivo* data regarding antiproliferative and proapoptotic effects of the drug in the pancreas are so far available. Therefore *in vivo* studies will be necessary to validate the significance of our findings and the data of others demonstrating direct effects of resveratrol in beta-cell lines and primary beta-cells. To that point it seems that the beneficial effects of the drug found in animal models (hypoglycemic and hypolipidemic effects due to increased insulin responsiveness of hepatocytes, adipocytes and skeletal muscle, stimulated hepatic glycogen synthesis, delayed the onset of insulin resistance, diminished renal dysfunction) [10, 11] outweigh possible adverse effects. This is probably due to the fact that the concentrations of resveratrol tested in most *in vitro* studies (10-100 μ M) are unlikely to be achieved *in vivo* upon oral or intravenous administration due to the rapid metabolism of the compound, so that plasma concentrations of untransformed resveratrol are fairly low (peak concentrations of $\sim$ 2.4 nM unconjugated and $\sim$ 180 nM total resveratrol assuming a daily consumption of $\sim$ 375 ml red wine, the richest source of resveratrol) [4]. However,

enterohepatic recirculation, binding to plasma proteins and an up to $\sim$ 30-fold accumulation of unconjugated resveratrol over plasma concentrations in certain organs like the liver and kidneys may delay the elimination of the drug from the body and bring about prolonged beneficial effects [4, 49, 50], which make the compound a promising therapeutic tool. Yet, given the multiple cellular target sites of resveratrol which can mediate beneficial or adverse effects, synthetic resveratrol analogues which are effective at lower concentrations than the naturally occurring drug will have to be carefully tested for potential harmful side effects.

We conclude that the antiproliferative/proapoptotic effect of resveratrol in the beta-cell line INS-1E is due to direct negative interference with antiapoptotic Akt signaling, which might be aggravated by a disruption of auto/paracrine insulin signaling as a possible consequence of inhibited insulin release.

Acknowledgements

We thank Prof. Claes B. Wollheim for providing the INS-1E cells. The technical assistance of E. Iglseder and A. Aschauer is gratefully acknowledged. Supported by the Austrian Science Foundation (FWF) grant no. P17119-B05 to J. F.

References

- 1 Fremont L: Biological effects of resveratrol. *Life Sci* 2000;66:663-673.
- 2 Gusman J, Malonne H, Atassi G: A reappraisal of the potential chemopreventive and chemotherapeutic properties of resveratrol. *Carcinogenesis* 2001;22:1111-1117.
- 3 Fulda S, Debatin KM: Resveratrol modulation of signal transduction in apoptosis and cell survival: a mini-review. *Cancer Detect Prev* 2006;30:217-223.
- 4 Baur JA, Sinclair DA: Therapeutic potential of resveratrol: the *in vivo* evidence. *Nat Rev Drug Discov Nature reviews* 2006;5:493-506.
- 5 Dasgupta B, Milbrandt J: Resveratrol stimulates AMP kinase activity in neurons. *Proc Natl Acad Sci* 2007;104:7217-7222.
- 6 Kim WH, Lee JW, Suh YH, Lee HJ, Lee SH, Oh YK, Gao B, Jung MH: AICAR potentiates ROS production induced by chronic high glucose: roles of AMPK in pancreatic beta-cell apoptosis. *Cell Signal* 2007;19:791-805.
- 7 Hwang JT, Kwak DW, Lin SK, Kim HM, Kim YM, Park OJ: Resveratrol induces apoptosis in chemoresistant cancer cells via modulation of AMPK signaling pathway. *Ann N Y Acad Sci* 2007;1095:441-448.
- 8 Shang J, Chen LL, Xiao FX, Sun H, Ding HC, Xiao H: Resveratrol improves non-alcoholic fatty liver disease by activating AMP-activated protein kinase. *Acta Pharmacol Sin* 2008;29:698-706.
- 9 Frojdo S, Cozzone D, Vidal H, Pirolo L: Resveratrol is a class IA phosphoinositide 3-kinase inhibitor. *Biochem J* 2007;406:511-518.
- 10 Sharma S, Anjaneyulu M, Kulkarni SK, Chopra K: Resveratrol, a polyphenolic phytoalexin, attenuates diabetic nephropathy in rats. *Pharmacology* 2006;76:69-75.
- 11 Su HC, Hung LM, Chen JK: Resveratrol, a red wine antioxidant, possesses an insulin-like effect in streptozotocin-induced diabetic rats. *Am J Physiol Endocrinol Metab* 2006;290:E1339-46.
- 12 Palsamy P, Subramanian S: Resveratrol, a natural phytoalexin, normalizes hyperglycemia in streptozotocin-nicotinamide induced experimental diabetic rats. *Biomed Pharmacother* 2008;62:598-605.
- 13 Rivera L, Moron R, Zarzuelo A, Galisteo M: Long-term resveratrol administration reduces metabolic disturbances and lowers blood pressure in obese Zucker rats. *Biochem Pharmacol* 2009;77:1053-1063.

- 14 Hambrock A, de Oliveira Franz CB, Hiller S, Grenz A, Ackermann S, Schulze DU, Drews G, Osswald H: Resveratrol binds to the sulfonyleurea receptor (SUR) and induces apoptosis in a SUR subtype-specific manner. *J Biol Chem* 2007;282:3347-3356.
- 15 Szkudelski T: Resveratrol inhibits insulin secretion from rat pancreatic islets. *Eur J Pharmacol* 2006;552:176-181.
- 16 Szkudelski T: Resveratrol-induced inhibition of insulin secretion from rat pancreatic islets: evidence for pivotal role of metabolic disturbances. *Am J Physiol Endocrinol Metab* 2007;293:E901-7.
- 17 Szkudelski T: The insulin-suppressive effect of resveratrol - An in vitro and in vivo phenomenon. *Life Sci* 2008;82:430-435.
- 18 Jakab M, Lach S, Bacova Z, Langeluddecke C, Strbak V, Schmidt S, Iglseder E, Paulmichl M, Geibel J, Ritter M: Resveratrol Inhibits Electrical Activity and Insulin Release from Insulinoma Cells by Block of Voltage-Gated Ca^{2+} Channels and Swelling-Dependent Cl^{-} Currents. *Cell Physiol Biochem* 2008;22:567-578.
- 19 Tuttle RL, Gill NS, Pugh W, Lee JP, Koeberlein B, Furth EE, Polonsky KS, Naji A, Birnbaum MJ: Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. *Nat Med* 2001;7:1133-1137.
- 20 Avram D, Ranta F, Hennige AM, Berchtold S, Hopp S, Haring HU, Lang F, Ullrich S: IGF-1 protects against dexamethasone-induced cell death in insulin secreting INS-1 cells independent of AKT/PKB phosphorylation. *Cell Physiol Biochem* 2008;21:455-462.
- 21 Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF: Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* 1998;391:900-904.
- 22 Muller D, Jones PM, Persaud SJ: Autocrine anti-apoptotic and proliferative effects of insulin in pancreatic beta-cells. *FEBS Lett* 2006;580:6977-6980.
- 23 Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P: Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology* 2004;145:667-678.
- 24 Reile H, Birnbock H, Bernhardt G, Spruss T, Schonenberger H: Computerized determination of growth kinetic curves and doubling times from cells in microculture. *Anal Biochem* 1990;187:262-267.
- 25 Hoffmann PR, deCathelineau AM, Ogden CA, Leverrier Y, Bratton DL, Daleke DL, Ridley AJ, Fadok VA, Henson PM: Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. *J Cell Biol* 2001;155:649-659.
- 26 Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL: Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *J Biol Chem* 2001;276:1071-1077.
- 27 Lang F, Huber SM, Szabo I, Gulbins E: Plasma membrane ion channels in suicidal cell death. *Arch Biochem Biophys* 2007;462:189-194.
- 28 Cohen GM: Caspases: the executioners of apoptosis. *Biochem J* 1997;326:1-16.
- 29 Hail NJ, Carter BZ, Konopleva M, Andreeff M: Apoptosis effector mechanisms: a requiem performed in different keys. *Apoptosis* 2006;11:889-904.
- 30 Haake AR, Polakowska RR: Cell death by apoptosis in epidermal biology. *J Invest Dermatol* 1993;101:107-112.
- 31 Ragione FD, Cucciolla V, Borriello A, Pietra VD, Racioppi L, Soldati G, Manna C, Galletti P, Zappia V: Resveratrol arrests the cell division cycle at S/G2 phase transition. *Biochem Biophys Res Commun* 1998;250:53-58.
- 32 Hsieh TC, Wu JM: Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines. *Exp Cell Res* 1999;249:109-115.
- 33 Delmas D, Jannin B, Cherkaoui Malki M, Latruffe N: Inhibitory effect of resveratrol on the proliferation of human and rat hepatic derived cell lines. *Oncol Rep* 2000;7:847-852.
- 34 Joe AK, Liu H, Suzui M, Vural ME, Xiao D, Weinstein IB: Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. *Clin Cancer Res* 2002;8:893-903.
- 35 Kim YA, Choi BT, Lee YT, Park DI, Rhee SH, Park KY, Choi YH: Resveratrol inhibits cell proliferation and induces apoptosis of human breast carcinoma MCF-7 cells. *Oncol Rep* 2004;11:441-446.
- 36 Stervbo U, Vang O, Bonnesen C: Time- and concentration-dependent effects of resveratrol in HL-60 and HepG2 cells. *Cell Prolif* 2006;39:479-493.
- 37 Zhang W, Fei Z, Zhen HN, Zhang JN, Zhang X: Resveratrol inhibits cell growth and induces apoptosis of rat C6 glioma cells. *J Neurooncol* 2007;81:231-240.
- 38 Rigolio R, Miloso M, Nicolini G, Villa D, Scuteri A, Simone M, Tredici G: Resveratrol interference with the cell cycle protects human neuroblastoma SH-SY5Y cell from paclitaxel-induced apoptosis. *Neurochem Int* 2005;46:205-211.
- 39 Fulda S, Debatin KM: Sensitization for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by the chemopreventive agent resveratrol. *Cancer Res* 2004;64:337-346.
- 40 Faber AC, Chiles TC: Resveratrol induces apoptosis in transformed follicular lymphoma OCI-LY8 cells: evidence for a novel mechanism involving inhibition of BCL6 signaling. *Int J Oncol* 2006;29:1561-1566.
- 41 Jiang H, Zhang L, Kuo J, Kuo K, Gautam SC, Groc L, Rodriguez AI, Koubi D, Hunter TJ, Corcoran GB, Seidman MD, Levine RA: Resveratrol-induced apoptotic death in human U251 glioma cells. *Mol Cancer Ther* 2005;4:554-561.
- 42 Chang-Chen KJ, Mullur R, Bernal-Mizrachi E: beta-cell failure as a complication of diabetes. *Rev Endocr Metab Disord* 2008.
- 43 Chan AY, Dolinsky VW, Soltys CL, Viollet B, Baksh S, Light PE, Dyck JR: Resveratrol inhibits cardiac hypertrophy via AMP-activated protein kinase and Akt. *J Biol Chem* 2008;283:24194-24201.
- 44 Leibiger IB, Berggren PO: Insulin Signaling in the Pancreatic beta Cell. *Annu Rev Nutr* 2008;28:233-251.
- 45 Navarro-Tableros V, Sanchez-Soto MC, Garcia S, Hiriart M: Autocrine regulation of single pancreatic beta-cell survival. *Diabetes* 2004;53:2018-2023.
- 46 Hoorens A, Van de Castele M, Kloppel G, Pipeleers D: Glucose promotes survival of rat pancreatic beta cells by activating synthesis of proteins which suppress a constitutive apoptotic program. *J Clin Invest* 1996;98:1568-1574.
- 47 Maeno E, Ishizaki Y, Kanaseki T, Hazama A, Okada Y: Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. *Proc Natl Acad Sci U S A* 2000;97:9487-9492.
- 48 Okada Y, Maeno E, Shimizu T, Dezaki K, Wang J, Morishima S: Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). *J Physiol* 2001;532:3-16.
- 49 Jannin B, Menzel M, Berlot JP, Delmas D, Lancon A, Latruffe N: Transport of resveratrol, a cancer chemopreventive agent, to cellular targets: plasmatic protein binding and cell uptake. *Biochem Pharmacol* 2004;68:1113-1118.
- 50 Delmas D, Lancon A, Colin D, Jannin B, Latruffe N: Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer. *Curr Drug Targets* 2006;7:423-442.