Resveratrol Binds to the Sulfonylurea Receptor (SUR) and Induces Apoptosis in a SUR Subtype-specific Manner*

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Sulfonylurea receptors (SURs) constitute the regulatory subunits of ATP-sensitive K⁺ channels (KATP channels). SUR binds nucleotides and synthetic KATP channel modulators, e.g. the antidiabetic sulfonylurea glibenclamide, which acts as a channel blocker. However, knowledge about naturally occurring ligands is very limited. In this study, we show that the plant phenolic compound trans-resveratrol can bind to SUR and displace binding of glibenclamide. Electrophysiological measurements revealed that resveratrol is a blocker of pancreatic SUR1/K̂IR6.2 KATP channels. We further demonstrate that, like glibenclamide, resveratrol induces enhanced apoptosis. This was shown by analyzing different apoptotic parameters (cell detachment, nuclear condensation and fragmentation, and activities of different caspase enzymes). The observed apoptotic effect was specific to cells expressing the SUR1 isoform and was not mediated by the electrical activity of KATP channels, as it was observed in human embryonic kidney 293 cells expressing SUR1 alone. Enhanced susceptibility to resveratrol was not observed in pancreatic β-cells from SUR1 knock-out mice or in cells expressing the isoform SUR2A or SUR2B or the mutant SUR1(M1289T). Resveratrol was much more potent than glibenclamide in inducing SUR1-specific apoptosis. Treatment with etoposide, a classical inducer of apoptosis, did not result in SUR isoform-specific apoptosis. In conclusion, resveratrol is a natural SUR ligand that can induce apoptosis in a SUR isoform-specific manner. Considering the tissue-specific expression patterns of SUR isoforms and the possible effects of SUR mutations on susceptibility to apoptosis, these observations could be important for diabetes and/or cancer research.

Sulfonylurea receptors (SURs)² are members of the ATP-binding cassette protein family (subfamily C). SURs are known to be the important regulatory subunits of ATP-sensitive K⁺ channels (KATP channels). These channels are heteromeric complexes composed of four SUR subunits that surround a central pore formed by four subunits from the K̂IR6.2x family. KATP channels found in various tissues exhibit distinct physiological and pharmacological properties because of the combination of different subunit isoforms (reviewed in Ref. 1). In addition to two nucleotide-binding domains, SUR possesses binding sites for synthetic KATP channel modulators. The binding sites for blockers and openers are different, but they are linked via complex allosteric interactions (2, 3).

Because of their nucleotide sensitivity, KATP channels couple the energy metabolism of a cell to the membrane potential. This is important in the pancreatic β-cell, in which closure of KATP channels triggers insulin secretion via membrane depolarization in response to changes in blood glucose levels (4–6). KATP channel-blocking drugs such as the sulfonylureas and the gli- inides can promote insulin secretion and are used in the treatment of diabetes type 2. Insulin secretion is also modulated and amplified by other pathways within the β-cell that are, in general, KATP channel-independent (7, 8). In addition, the extent of insulin secretion is also influenced by adaptive variation of the total number of β-cells. It has been observed that β-cell mass is often increased in obese non-diabetic patients and is significantly reduced in patients suffering from diabetes type 2 (9). Changes in β-cell mass can be achieved via controlled proliferation or apoptosis, but the molecular mechanisms are unknown (10–13). In several studies, it has been shown that the sulfonylurea compounds glibenclamide and tolbutamide can induce apoptosis in β-cells or clonal β-cell lines under certain conditions (10, 14–18). The mechanisms behind this drug-induced cell death have not been clarified, and its in vivo relevance for long-term treatment with lower, therapeutic concentrations remains to be proven. Using a recombinant cell model, we have shown that expression of the pancreatic isoform SUR1 can render cells more susceptible to glibenclamide-induced apoptosis (19). This effect was not observed in cells expressing the vascular smooth muscle isoform SUR2B or the mutant SUR1(M1289T), in which a single amino acid in the last C-terminal transmembrane helix 17 was exchanged with the corresponding amino acid of SUR2.

The objective of this study was to investigate whether SUR1-specific programmed cell death can be induced by more potent apoptotic agents than glibenclamide. We hypothesized that trans-resveratrol (trans-3,5,4’-trihydroxystilbene) is a ligand of SUR able to induce SUR1-specific apoptosis similar to glibenclamide. In addition to several other physiological effects, resveratrol has been shown to trigger apoptosis under certain con-
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ditions, depending on cell type and concentration. Resveratrol is present in many plants and plant products, e.g. in grapes, nuts, and wines, and as a phytoalexin, it is produced in large amounts in response to stress factors such as fungal infection (for review, see Refs. 20–22). Resveratrol is structurally similar to DIDS, a synthetic K<sub>ATP</sub> channel modulator that binds to SUR (23–25). Resveratrol is supposed to interact with several ATP-binding cassette proteins, including BCRP (breast cancer resistance protein) (26), CFTR (cystic fibrosis transmembrane conductance regulator) (27), and MRP2 (multidrug resistance-associated protein 2) (28, 29), and with several different fungal ATP-binding cassette transporters (30–32). Apart from the nucleotides, knowledge about naturally occurring ligands of SUR is, however, very limited. It has been observed that small phosphoproteins termed endosulfines at micromolar concentrations act as natural equivalents to the sulfonlylurea compounds (33) and that certain lipids can interfere with binding of synthetic channel modulators at high concentrations (34).

To verify that resveratrol is a ligand of SUR and to compare its binding properties with those of glibenclamide, we performed competition binding assays with membranes from recombinant human embryonic kidney (HEK) 293 cells expressing different SUR forms. In these experiments, [<sup>3</sup>H]glibenclamide was used as the radioligand and resveratrol as the competitor. Electrophysiological studies on single-cells were performed by assessing different apoptotic parameters (cell death programs, we also determined the activities of key caspase-3 activity). Because of the variety of different apoptotic cell death programs, we also determined the activities of key caspase enzymes (caspase-8, -9, and -12) to identify the possible induction of a specific pathway. The apoptotic effect of resveratrol was compared with that of etoposide, the action of which is not affected by SUR1 expression (19). In this context, the potential value of resveratrol as a tool for exploring a possible new mechanism for β-cell apoptosis is discussed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HEK293 cell lines were stably transfected with the pcDNA3.1 expression vector (Invitrogen, Karlsruhe, Germany) containing the coding sequence of SUR1 (GenBank<sup>™</sup> accession number X97279), SUR1(S1238Y), SUR1(M1289T), SUR2A (accession number D86037), SUR2B (accession number D86038), or SUR2B(Y1260S), or they were transfected with the empty pcDNA3.1 expression vector (Invitrogen). The mutants had been constructed from SUR1 or SUR2B cDNA using the QuikChange mutagenesis system (Stratagene, Amsterdam), and the different cell lines were isolated and cultured according to Hambrock et al. (19). Transient cotransfections of HEK cells were performed as described (19). The cell lines HIT-T15 and RIN-m5F (expressing SUR1/K<sub>Ir6.2</sub>) and A-10 (expressing SUR2B/K<sub>Ir6.2</sub>) channels were purchased from American Type Culture Collection (Manassas, VA). These cells were maintained in RPMI 1640 medium containing l-glutamine (HIT-T15 and RIN-m5F cells) or in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose and l-glutamine (A-10 cells) in the presence of 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. The cells were routinely grown at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> and passaged once a week after trypsin/EDTA detachment.

**Preparation of Pancreatic Islets**—Experiments were performed on pancreatic islets of Langerhans isolated from wild-type C57BL/6 mice (Charles River GmbH, Sulzfeld, Germany) or from SUR1 knock-out (SUR1KO) mice (35). Islets were obtained from male or female mice (age, 20–25 weeks; and weight, 25–35 g) by collagenase IV digestion of the pancreas. After microscopic selection, the islets were cultured in RPMI 1640 medium containing 5 mM glucose, 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Treatment with Resveratrol or Etoposide**—At 60–80% confluence (HEK293 cell lines) or 50–70% confluence (HIT-T15, RIN-m5F, and A-10 cells), cells were treated with resveratrol or etoposide by addition of 10 or 20 μl, respectively, of a stock solution (100 mM resveratrol in Me<sub>2</sub>SO or ethanol or 50 mM etoposide in Me<sub>2</sub>SO) to 10 ml of culture medium. In control experiments, 10 or 20 μl, respectively, of solvent was added. Transient transfection of HEK cells or isolation of islets was performed 24 h before treatment. Drug exposure was carried out in at least two culture dishes per single experiment.

**Quantification of Cell Detachment**—The numbers of adherent and detached cells were determined separately with a CASY TT analyzer system (Schräfe System GmbH, Reutlingen, Germany) according to Hambrock et al. (19). Briefly, three 100-μl aliquots from the supernatant of each culture dish were withdrawn for measurement. The adherent cells were then rinsed off with CASY electrolyte solution; the cell suspension was diluted, if necessary; and again, 100-μl aliquots were analyzed in triplicate.

**Determination of Apoptotic Nuclei**—Condensed and fragmented apoptotic nuclei of adherent or detached cells were visualized by Hoechst 33258 (bisbenzimide) staining after paraformaldehyde fixation (3% in phosphate-buffered saline). DNA fragmentation was determined after agarose gel electrophoresis of cytoplasmic DNA isolated from combined adherent and detached cells. Both methods were performed according to Hambrock et al. (19). To quantify the degree of condensation and fragmentation, the number of total and apoptotic cells (adherent HEK, HIT-T15, or RIN-m5F cells or islet cells) was determined per 50 μM aliquots per 50 μM aliquots of each culture dish were withdrawn for measurement. The adherent cells were then rinsed off with CASY electrolyte solution; the cell suspension was diluted, if necessary; and again, 100-μl aliquots were analyzed in triplicate.

**Determination of the Activities of Different Caspase Enzymes**—The activities of different caspase enzymes were determined by cleavage of specific peptide substrates (DEVD, IETD, LEHD, or ATAD) conjugated to the fluorophore 7-amino-4-trifluoromethylcoumarin (AFC) and were monitored by fluorescence emission at 510 nm (excitation at 390 nm) with a Wallac 1420 VICTOR<sup>2</sup> multilabel reader (PerkinElmer Life Sciences, Rodgau-Jügesheim, Germany). Caspase activities were calcu-
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lateral as increase in fluorescence per min and normalized to the protein content of each sample. Despite the high substrate specificity of the different caspase isoenzymes, cleavage of the respective peptide substrates by other enzymes with lower affinity cannot be completely excluded. Hence, turnover of a specific substrate is, strictly speaking, due to the activities of “caspase isofrom–like enzymes.” For determination of caspase-3 (DEVD-caspase), caspase-8 (IETD-caspase), and caspase-9 (LEHD-caspase) activities, cells were prepared and analyzed as described by Hambrock et al. (19) and measured at room temperature in 20-min intervals over a total period of 160 min. Caspase-12 (ATAD-caspase) activity was determined using a caspase-12 fluorometric assay kit (MBL International Corp., Woburn, MA) according to the manufacturer’s instructions at 37 °C in 30-min intervals over a total period of 120 min. The protein concentrations employed in the assays were 0.5–1.0 mg/ml (caspase-3), 0.7–1.5 mg/ml (caspase-8), and 1.3–2.1 mg/ml (caspase-9 and caspase-12).

Radioisotope Binding Assays—Binding of resveratrol to different SUR forms was determined in heterologous competition experiments using [3H]glibenclamide as the radioligand (SUR1, SUR1(M1289T), and pcDNA controls, 1 nM; SUR1(S1238Y), SUR2B, and pcDNA controls, 4–5 nM; and SUR2B(Y1206S), 3 nM). Resveratrol was dissolved in ethanol and added as the competitor. Membranes prepared from the respective cell lines (SUR1, SUR1(M1289T), and pcDNA controls, 0.05–0.10 mg/ml membrane protein; SUR2B, SUR2B(S1238Y), and pcDNA controls, 0.5–0.7 mg/ml membrane protein; and SUR2B(Y1206S), 0.1–0.2 mg/ml membrane protein) were incubated in the presence of 1 mM MgATP for 15 min at 37 °C, and binding properties were analyzed as described by Hambrock et al. (36). Nonspecific binding of [3H]glibenclamide was determined in the presence of 10 μM unlabeled glibenclamide or in membranes from pcDNA control cells.

Electrophysiological Experiments—Patch-clamp experiments on single pancreatic β-cells were performed using an EPC 9 amplifier and Pulse software (HEKA Elektronik, Lambrecht, Germany). A bath solution of 140 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 2.5 mM CaCl2, 0.5 mM glucose, and 10 mM HEPES (pH 7.4 adjusted with NaOH) was used. The pipette solution consisted of 130 mM KCl, 4 mM MgCl2, 2 mM CaCl2, 10 mM EGTA, 0.65 mM Na2ATP, and 20 mM HEPES (pH 7.15 adjusted with KOH). Whole cell KATP channel currents were recorded at 25–28 °C and measured at a holding potential of −70 mV and during 300-ms pulses to −80 and −60 mV at 15-s intervals. Pipettes were pulled with a DMZ puller (Zeitz Instruments, Munich, Germany) using borosilicate glass (Clark Electromedical Instruments, Pangbourne, UK). They had resistances of 3–5 megaohms when filled with pipette solution. The β-cells were obtained by dispersion of isolated pancreatic islets in Ca2+-free buffer containing 135 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl2, 10 mM HEPES, 3 mM glucose, 1 mM EGTA, 1 mg/ml bovine serum albumin, 100 units/ml penicillin, and 100 μg/ml streptomycin (pH 7.4 adjusted with NaOH). Cells from at least two different cell preparations were used for each series of experiments.

Data Analysis—Data were calculated as means ± S.E. from single experiments. Statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego). For statistical comparisons, two-tailed unpaired Student’s t test and one-way analysis of variance analysis in combination with Tukey’s post hoc test were employed as appropriate.

The equilibrium inhibition curves were analyzed using the logarithmic form of the Hill equation:

\[ y = A \left( 1 + 10^{(m_{x} - pIC_{50})} \right)^{-1} \]

where A denotes the extent of inhibition; \( n_{H} \) is the Hill coefficient; IC50 is the midpoint of the curve, with \( pIC_{50} = -\log IC_{50} \); and x is the concentration of the compound under study, with \( px = -\log x \). The IC50 values were converted into inhibition constants (\( K_{i} \)) by correcting for the presence of the radioligand (L) according to the Cheng-Prusoff equation:

\[ K_{i} = IC_{50}(1 + L/K_{D}^{\infty})^{-1} \]

where \( K_{D}^{\infty} \) is the equilibrium dissociation constant of the radioligand.

Fits of the equation to the data were performed according to the method of least squares using the SigmaPlot program (SPSS, Inc., Chicago, IL). For determination of binding data, the parameters from individual experiments were analyzed and averaged assuming that amplitudes and \( pIC_{50} \) values were normally distributed (37).

Materials—Dulbecco’s modified Eagle’s medium was purchased from Cambrex Bio Science Verviers S.p.r.l. (Verviers, Belgium). All other reagents used for cell culture or molecular biology were obtained from Invitrogen. AFC standards, glibenclamide, Hoechst 33258, and resveratrol were from Sigma (Deisenhofen, Germany). DEVD-AFC, IETD-AFC, and LEHD-AFC caspase substrate were from BIOMOL International LP (Hamburg, Germany). ATAD-AFC caspase substrate was purchased from International Corp., and etoposide was from Calbiochem (Bad Soden, Germany). [3H]Glibenclamide (specific activity, 1.60 TBq (43.3 Ci/mmol)) and Ultima Gold scintillant were purchased from PerkinElmer Life Sciences.

RESULTS

Detachment of HEK293 Cells Expressing Different SUR Forms after Resveratrol Treatment—Fig. 1A compares the effects of resveratrol (100 μM, 24 h of treatment) on HEK293 cells stably transfected with different forms of SUR or on sham-transfected HEK293 control cells (pcDNA control cells). The relatively high concentration of 100 μM was chosen (i) to be able to compare the results of these experiments with those of other experiments performed with the same concentration of glibenclamide (19) or etoposide; (ii) to see clear effects after a short incubation period; and (iii) to avoid masking or falsifying these effects by side effects such as cell aging, depletion of nutrients, and accumulation of toxic metabolites.

Resveratrol-induced cell detachment was enhanced by ~2-fold in cells expressing SUR1 (but not SUR2B or the mutant SUR1(M1289T)) compared with pcDNA control cells. Detachment of cells expressing SUR2B or SUR1(M1289T) tended to be even slightly but not significantly lower than that of pcDNA control cells. No significant differences were observed in cells expressing the SUR2A isoform after resveratrol treatment compared with SUR2B-expressing or pcDNA control cells, and cell detachment of SUR2A-expressing cells was significantly lower than that of SUR1-expressing cells (SUR2A + resveratrol, 217 ± 41% of the pcDNA/solvent control; SUR2B + resveratrol, 203 ± 49%; pcDNA + resveratrol, 284 ± 39%; SUR1, 626 ± 140%; SUR2A + resveratrol versus SUR2B/pcDNA +
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Resveratrol, \( p > 0.05 \); SUR2A + resveratrol versus SUR1 + resveratrol, \( p < 0.01 \). Treatment with etoposide (100 \( \mu M \), 24 h) also resulted in cell detachment; but no significant differences were detected between the tested cell lines, and detachment was not enhanced by SUR1 expression (Fig. 1B).

Additional experiments were performed to assess the concentration dependence of the resveratrol effect, and the data revealed an obvious correlation between the concentration of resveratrol, time of treatment, and degree of cell detachment (Fig. 2). Differences between SUR1-expressing cells and pcDNA control cells were already seen at concentrations \( \geq 15 \mu M \) after 4 days of resveratrol treatment, and these differences were significant at 25 \( \mu M \) (\( p = 0.0177 \)). The number of detached SUR1-expressing cells after exposure to 20 \( \mu M \) resveratrol for 4 days was comparable with that observed after treatment with 100 \( \mu M \) resveratrol for 24 h.

Nuclear Condensation and DNA Fragmentation in SUR-expressing HEK293 Cells after Resveratrol Treatment—Resveratrol treatment (100 \( \mu M \), 24 h) produced visible changes in nuclei stained with the DNA-binding dye Hoechst 33258; many nuclei of the adherent cells were brightly stained, condensed, and fragmented after exposure to resveratrol, whereas their size, shape, and fluorescence intensity were unchanged after solvent treatment (Fig. 3). These typical signs of apoptosis were very frequent and pronounced in HEK cells expressing SUR1, but occurred only to a small extent in pcDNA control cells or SUR1(M1289T)- and SUR2B-expressing cells (SUR1 + resveratrol, 22.33 \( \pm \) 1.86\% apoptotic nuclei/total number of nuclei; SUR1 + solvent, 1.90 \( \pm \) 0.67\%; SUR1(M1289T) + resveratrol, 6.02 \( \pm \) 0.90\%; SUR1(M1289T) + solvent, 3.18 \( \pm \) 0.77\%; SUR2B + resveratrol, 4.46 \( \pm \) 0.79\%; SUR2B + solvent, 2.53 \( \pm \) 0.62\%; pcDNA + resveratrol, 5.89 \( \pm \) 1.02\%; pcDNA + solvent, 2.10 \( \pm \) 0.53\%; SUR1 + resveratrol versus pcDNA/SUR1(M1289T)/SUR2B + resveratrol, \( p < 0.001 \); SUR1 + resveratrol versus SUR1 + solvent, \( p < 0.001 \); pcDNA/SUR1(M1289T) + resveratrol versus pcDNA/SUR1(M1289T) + solvent, \( p < 0.05 \). In addition, fragmentation of nuclear chromatin, recognized by a characteristic DNA-ladder pattern after agarose gel electrophoresis (fragment length, 400–1500 bp), was observed after resveratrol treatment (data not shown). Etoposide treatment (100 \( \mu M \), 24 h) also produced changes in nuclear morphology; but no differences were observed between the different cell lines, and the degree of nuclear alterations was comparable with that of pcDNA control cells after resveratrol treatment (data not shown).

Changes in the Activities of Caspase Enzymes in SUR-expressing HEK293 Cells after Resveratrol Treatment—The activity of caspase-3 (DEVD-caspase) was significantly elevated among all the resveratrol-treated HEK cell lines in comparison with those after solvent treatment. If resveratrol-induced changes in caspase-3 activity were calculated in the form of “induction factors,” i.e. the ratio of the activity after substance treatment to the activity after solvent treatment, values of 2.14 \( \pm \) 0.67\% (pcDNA), 2.49 \( \pm \) 0.30 (SUR1), and 1.81 \( \pm \) 0.20 (SUR1(M1289T)) were obtained (Fig. 4A). Compared with pcDNA control cells, the activity of caspase-3 enzymes after solvent treatment was slightly higher in SUR1-expressing HEK cells and slightly lower in SUR1 mutant-expressing HEK cells, although these differences were not significant. With induction factors of \( \approx \)1, caspase-8 activity (IETD-caspase) was obviously not induced (induction factors, 1.02 \( \pm \) 0.05 (pcDNA), 1.23 \( \pm \) 0.11 (SUR1), and 0.87 \( \pm \) 0.08 (SUR1(M1289T))) (Fig. 4B). The activity of caspase-9 (LEHD-caspase) was clearly enhanced, however, after resveratrol treatment, with an additional significant increase in SUR1-expressing cells (induction factors, 1.73 \( \pm \) 0.15 (pcDNA), 3.12 \( \pm \) 0.26 (SUR1), and 1.76 \( \pm \) 0.18 (SUR1(M1289T))) (Fig. 4C). The activity of caspase-12 (ATAD-caspase) was generally elevated after resveratrol treatment, but exhibited no significant differences between the cell lines (induction factors, 1.87 \( \pm \) 0.27 (pcDNA), 1.72 \( \pm \) 0.24 (SUR1), and 1.63 \( \pm \) 0.19 (SUR1(M1289T))) (Fig. 4D). The activities of caspase-3 and caspase-9 were also determined for etoposide-treated cells, and no significant differences were detected between the SUR1-expressing cells and the other HEK cell lines (induction factors: caspase-3, 3.23 \( \pm \) 0.14 (pcDNA), 2.89 \( \pm \) 0.60 (SUR1), and 2.25 \( \pm \) 0.18 (SUR1(M1289T)); and caspase-9, 1.17 \( \pm \) 0.09 (pcDNA), 1.36 \( \pm \) 0.06 (SUR1), and 1.54 \( \pm \) 0.10 (SUR1(M1289T)) (\( n = 5–6 \)).
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Influence of Coexpression of SLUR1 and Kir6.2 on Resveratrol-induced Cell Detachment—To determine whether coexpression of the pore-forming $K_{ATP}$ channel subunit, Kir6.2, has a modulatory effect on SUR1-mediated cell detachment, cotransfection experiments were performed in which either equimolar amounts of SUR1- and Kir6.2-encoding plasmids or of SUR1- and pcDNA-encoding plasmids were transiently transfected into HEK293 cells (Fig. 5). Exposure to resveratrol for up to 3 days resulted in marked cell detachment, but there was no significant difference between cells expressing SUR alone and cells coexpressing SUR1 and Kir6.2.

Effect of Resveratrol Treatment on HIT-T15 and RIN-m5F Cells (β-Cell Lines) and A-10 Cells (Vascular Smooth Muscle Cell Line)—Changes in nuclear morphology were assessed using Hoechst 33258 staining after exposure of cells to resveratrol (100 μM, 24 h). Fig. 6 shows that HIT-T15 and RIN-m5F cells, which endogenously express SUR1, exhibited marked signs of apoptosis, whereas the SUR2B-expressing A-10 cells were not visibly affected. To compare the effects of resveratrol on different cell lines, the ratio of apoptosis (the number of apoptotic nuclei/total number of nuclei in a randomly chosen area of $50 \times 50 \mu m^2$) after resveratrol treatment to apoptosis after solvent treatment was determined (HIT-T15 cells, 3.36; RIN-m5F cells, 16.82; and A-10 cells, 1.34). In contrast, etoposide (100 μM, 24 h) produced visible effects in all the cell lines (HIT-T15 cells, 2.09; RIN-m5F cells, 1.96; and A-10 cells, 3.62) (Fig. 6, G–I). In addition, the activity of caspase-3 was measured in RIN-m5F and A-10 cells ($n = 4 – 6$). Caspase-3 activity in RIN-m5F cells after resveratrol treatment (induction factor, 5.37 ± 0.75) was slightly higher than after etoposide treatment (induction factor, 4.01 ± 0.18). In contrast, caspase-3 activity in A-10 cells was lower after resveratrol treatment (induction factor, 5.03 ± 1.43) than after etoposide treatment (induction factor, 8.25 ± 0.77).

Effect of Resveratrol Treatment on Pancreatic Islets from Wild-type Versus SUR1KO Mice—Intact islets of Langerhans were isolated from wild-type or SUR1KO mice and incubated with 100 μM resveratrol for 24 h (Fig. 7). In wild-type islets, many nuclei were condensed or already fragmented, and the number of apoptotic nuclei/total number of nuclei (15.41 ± 1.75%) was significantly higher compared with that in solvent-treated islets (2.03 ± 0.58%, $p < 0.001$). However, when pancreatic islets from SUR1KO mice were treated with resveratrol, no such alterations in islet morphology were observed (2.28 ± 0.67% apoptotic nuclei/total number of nuclei). When islets from normal or knock-out mice were treated only with solvent, no visible apoptotic changes were observed (2.03 ± 0.58 and

FIGURE 2. Effect of different resveratrol concentrations on detachment of SUR1-expressing and pcDNA control cells. The total number of cells in the supernatant was determined after treatment of SUR1-expressing or pcDNA control cells with different resveratrol (RSV) concentrations or with Me₂SO solvent (solv.) for 4 days. Data are the means from five series of experiments. At concentrations ≥35 μM (SUR1) or ≥50 μM (pcDNA), almost all cells had detached themselves, and no vital adherent cells were observed. Significant differences were detected in the case of the following comparisons: SUR1 + 25 μM resveratrol versus pcDNA + 25 μM resveratrol, $p < 0.05$; SUR1 + solvent versus SUR1 + 20 μM resveratrol, $p < 0.05$; SUR1 + solvent versus SUR1 + 25/35/50 μM resveratrol; $p < 0.001$; pcDNA + solvent versus pcDNA + 25/50 μM resveratrol, $p < 0.05$; and pcDNA + solvent versus pcDNA + 35 μM resveratrol, $p < 0.001$.

FIGURE 3. Effect of resveratrol treatment on nuclear morphology. After 24 h of treatment with 100 μM resveratrol (RSV) or Me₂SO solvent (solv.), pcDNA control cells and SUR1- or SUR1(M1289T)-expressing HEK293 cells were stained with Hoechst 33258. Intact nuclei are large and faintly stained, and apoptotic nuclei are brightly stained, condensed, and fragmented. The results for the adherent cells are shown. Nuclei from (former) cells in the supernatant were apoptotic to 100% (not shown). The results for SUR2B-expressing HEK cells (not shown) are the same as those for pcDNA control and SUR1(M1289T)-expressing cells.
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2.27 ± 0.47% apoptotic nuclei/total number of nuclei, respectively), which was in agreement with the observations of Seghers et al. (35) in their first characterization of SUR1KO mice.

Binding of Resveratrol to Different SUR Isoforms or Mutants—

In binding assays with [3H]glibenclamide used as the radioligand, resveratrol specifically bound to SUR, whereas no specific binding to membranes from pcDNA control cells was detected.

In the case of SUR1, resveratrol displaced glibenclamide binding to nearly 100% (extent of inhibition, 97 ± 3%), with an inhibitory constant (K_i) of 80 ± 15 μM (Fig. 8). The resveratrol binding properties of SUR1 were not significantly different from those determined for the mutant SUR1(M1289T) (K_i = 99 ± 21 μM; extent of inhibition, 91 ± 9%). The inhibitory constants and the extent of inhibition were calculated from monophasic fits of the inhibition binding curves as described under "Experimental Procedures" with Hill coefficients (n_H) set to 1. Resveratrol also specifically bound to SUR2B and was able to displace [3H]glibenclamide binding to ~40%, with K_i = 20–60 μM. Because of the low affinity of SUR2B for [3H]glibenclamide, however, a precise determination of binding parameters for resveratrol was not appropriate for SUR2B in this kind of assay.

To further assess the interaction of resveratrol with SUR, we also investigated the mutant SUR1(S1238Y). In this mutant, one amino acid within the glibenclamide-binding site is replaced with the corresponding amino acid of SUR2, which leads to reduced glibenclamide affinity of SUR1 (38). The mutation S1238Y abolished displacement of specific [3H]glibenclamide binding by resveratrol within the tested concentration range. (Specific binding of [3H]glibenclamide to this mutant was largely reduced in comparison with SUR1, but with ~25–40% of total binding, it was still detectable at a radioligand concentration of 4–5 nM.) However, the SUR1-specific apoptotic effect of resveratrol was not reduced by the S1238Y mutation (cell detachment: SUR1(S1238Y) + resveratrol, 448 ± 37% of the solvent control; SUR1 + resveratrol, 456 ± 44%; pcDNA + resveratrol, 273 ± 22%; SUR1(S1238Y) + resveratrol versus SUR1 + resveratrol, p > 0.05; SUR1(S1238Y) + resveratrol versus pcDNA + resveratrol, p < 0.05 (n = 5)).

The inverse mutation (Y1206S) of SUR2B, which increases the glibenclamide affinity of this SUR isoform (39), did not improve the ability of resveratrol to displace [3H]glibenclamide binding (K_i = 124 ± 9 μM), but increased the extent of inhibition to 98 ± 2%. The susceptibility of SUR2B(Y1206S)-expressing cells to apoptosis induction was not altered compared with that of SUR2B-expressing cells (cell detachment: SUR2B(Y1206S), 294 ± 57% of the solvent control; SUR2B, 265 ± 28%; pcDNA, 236 ± 48%; SUR2B(Y1206S) + resveratrol versus SUR2B + resveratrol, p > 0.05 (n = 5)).

Effects of Resveratrol on the Pancreatic Channel SUR1/K_ATP—

Electrophysiological experiments were performed with mouse β-cells in the whole cell configuration. 100 μM resveratrol significantly blocked K_ATP currents (current under control conditions, 176.82 ± 19.36 pA; current in the presence of 100 μM resveratrol, 58.64 ± 4.88 pA; and
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Resveratrol, which is known to induce cell death in some cell types under certain conditions, can elicit apoptotic effects that are SUR1-specific. Similar to glibenclamide treatment (19), exposure of SUR1-expressing HEK293 cells to resveratrol causes a marked increase in different apoptotic parameters such as cell detachment, caspase-9 activity, and degree of nuclear fragmentation morphology. This kind of enhanced apoptosis does not occur in sham-transfected HEK cells (pcDNA control cells), which show only a basal level of apoptosis. The enhanced apoptotic effect after resveratrol treatment is also not present in SUR2A- or SUR2B-expressing cells. The SUR1-specific effect of resveratrol is abolished by exchanging a single amino acid in transmembrane helix 17 at position 1289 with the corresponding amino acid of SUR2, as we have already observed for glibenclamide treatment (19). However, the SUR1-mediated effect is not influenced by the mutation S1238Y within the glibenclamide-binding pocket.

**FIGURE 6.** Effect of resveratrol treatment on the nuclear morphology of HIT-T15 and RIN-m5F cells (β-cell lines) and A-10 cells (vascular smooth muscle cell line). The cells from the different cell lines were stained with Hoechst 33258 after 24 h of treatment with Me2SO solvent (solv.; A–C), 100 μM resveratrol (RSV; D–F), or 100 μM etoposide (ETO; G–I). The brightly stained nuclear fragments (D, E, and G–I) are due to treatment with resveratrol or etoposide and have to be distinguished from the small dot-like structures that are generally present within the nuclei of HIT-T15 and RIN-m5F cells. The latter structures are small chromatin clusters, which are typical for β-cells, and they are present independent of the kind of treatment.

**FIGURE 7.** Effect of resveratrol on the nuclear morphology of intact isolated pancreatic islets from wild-type and SUR1KO mice. Isolated islets of Langerhans from normal SUR1-expressing wild-type (wt) C57BL/6 mice or from SUR1KO mice were treated with 100 μM resveratrol (RSV) or Me2SO solvent (solv.) for 24 h and stained with Hoechst 33258. The numerous small dots found in all the nuclei represent tiny clusters of chromatin, which are typical for β-cells and are not identical to the larger nuclear fragments due to apoptotic cell death (see also the legend to Fig. 6).

The current after washout, i.e. removal of resveratrol, 118.95 ± 14.55 pA (n = 11) (Fig. 9). Resveratrol inhibited the current in a reversible manner, but to a lesser extent compared with glibenclamide, which blocked the current almost completely at 10 nM (100 μM resveratrol, 28.96 ± 2.89% of the KATP current under control conditions; and 10 nM glibenclamide: 3.53 ± 0.53% of the control KATP current).

**DISCUSSION**

Regulation of β-cell mass by apoptosis, proliferation, and/or neogenesis of pancreatic β-cells is an important mechanism in controlling pancreatic function (13, 40). Therefore, a better understanding of the mechanisms behind β-cell-specific cell death and knowledge about substances that can elicit this phenomenon are of essential importance. It is known that β-cell death can, for instance, be induced by high levels of glucose or several free fatty acids, oxidative stress, or production of cytokines (12, 13, 41). Strep-tozotocin and alloxan induce β-cell damage via different (at least partially necrotic) mechanisms probably involving generation of reactive oxygen species and/or induction of DNA damage; these substances are often employed to create experimental diabetes models in animals (42). Sulfonylurea drugs such as glibenclamide can also induce apoptosis in β-cells or β-cell lines under certain conditions (10, 14–18). However, the mechanisms behind this effect are not known so far. In a recent study, we demonstrated in a recombinant cell model that expression of SUR1 renders cells more susceptible to glibenclamide-induced apoptosis (19), suggesting that the SUR1 isoform could be specifically involved in variation of β-cell mass and might contribute to regulation of insulin secretion also at this level.

In this study, we have shown that the naturally occurring phenol trans-resveratrol, which is known to induce cell death in some cell types under certain conditions, can elicit apoptotic effects that are SUR1-specific. Similar to glibenclamide treatment (19), exposure of SUR1-expressing HEK293 cells to resveratrol causes a marked increase in different apoptotic parameters such as cell detachment, caspase-9 activity, and degree of nuclear fragmentation morphology. This kind of enhanced apoptosis does not occur in sham-transfected HEK cells (pcDNA control cells), which show only a basal level of apoptosis. The enhanced apoptotic effect after resveratrol treatment is also not present in SUR2A- or SUR2B-expressing cells. The SUR1-specific effect of resveratrol is abolished by exchanging a single amino acid in transmembrane helix 17 at position 1289 with the corresponding amino acid of SUR2, as we have already observed for glibenclamide treatment (19). However, the SUR1-mediated effect is not influenced by the mutation S1238Y within the glibenclamide-binding pocket.
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FIGURE 8. Binding of resveratrol to SUR1 and SUR1(M1289T). Binding of resveratrol to membranes from HEK cells expressing SUR1 or SUR1(M1289T) was analyzed in heterologous competition experiments using [3H]glibenclamide as the radioligand. The data are expressed as the percentage of specific binding (B) of [3H]glibenclamide and are the means from four independent experiments. The curves were fit according to the equation given under “Experimental Procedures,” with n0 set to 1.

A

RSV 100 μM
GBC 10 nM

200 pA
1 min

100 pA
200 ms

100 pA
200 ms

B

C

KATP current [pA]

KATP current [% of control]

C1
RSV
C2

C1
RSV

GBC

Three different molecular targets, including inhibition of cyclooxygenases 1 and 2, inhibition or stimulation of nitric-oxide synthases, and activation of adenyl cyclase, have been discussed in connection with these different effects (21, 22, 44). The resveratrol concentrations required to achieve most of these different effects in the respective experimental systems have been shown to range from 5 to 100 μM or sometimes even higher (21). However, no specific interaction of resveratrol with SUR has been described until now.

In addition to demonstrating the SUR-specific apoptotic effect of resveratrol, we have confirmed in our radioligand binding assays that resveratrol specifically binds to SUR (SUR1, SUR2B, and SUR1(M1289T)) and is able to displace specific binding of glibenclamide. By showing that, like glibenclamide, resveratrol can act as a blocker of the SUR1/KIR6.2 channel, we have provided further evidence for analogies between both substances. In previous studies, a stimulatory effect of resveratrol on Ca2+-activated or voltage-gated K+ channels was reported, but no evidence for an effect on KATP channels was obtained (45–47). Because the subunit composition of KATP channels might be decisive in this respect, a systematic comparison of channels containing different SUR isoforms could be of future interest.

Although the affinity of resveratrol for SUR1 (Ki = 80 ± 15 μM) is much lower than that of glibenclamide (Ki = 80 ± 15 μM), compared with etoposide, the effects of resveratrol on SUR1-expressing cells are more intensive, whereas the effects of glibenclamide on these cells are smaller. In contrast to resveratrol or glibenclamide, etoposide induces apoptotic processes (cell detachment, enhanced activity of caspase-3, or apoptotic changes in nuclear morphology) that do not differ significantly between the tested cell lines and that are not SUR1-specific. As a further parallel to glibenclamide, resveratrol stimulates SUR1-specific apoptosis in HEK293 cells in the absence of the KIR6.2 KATP channel subunit. This fact and the observation that the extent of apoptosis is not modulated by additional expression of KIR6.2 confirm that complete functional SUR1/KIR6.2 channels are not necessarily required for the apoptotic effect. This provides further evidence for our previous hypothesis that SUR1 might possess an additional function beyond regulating the electrical activity of KATP channels (19).

The SUR subtype-specific effect of resveratrol is not only observed in the recombinant cell model, but is also reflected at the level of cell type- or even tissue-specific effects. This was proven by the detection of typical signs of apoptosis in the SUR1-expressing β-cells lines HIT-T15 and RIN-m5F after exposure to resveratrol and by a different response of the A-10 cell line, which expresses the SUR2B isoform (43). Additional data obtained from pancreatic islets isolated from wild-type or SURIKO mice give further evidence for the correlation between expression of SUR1 and enhanced resveratrol-specific induction of apoptosis. These results completely rule out the possibility that SUR1-specific apoptosis is merely an artifact of recombinant expression in HEK293 cells.

Many beneficial health effects, i.e. antioxidative or anti-inflammatory properties, protection against cardiovascular diseases, and chemopreventive effects, have been attributed to resveratrol. Several different molecular targets, including inhibition of cyclooxygenases 1 and 2, inhibition or stimulation of nitric-oxide synthases, and activation of adenyl cyclase, have been discussed in connection with these different effects (21, 22, 44). The resveratrol concentrations required to achieve most of these different effects in the respective experimental systems have been shown to range from 5 to 100 μM or sometimes even higher (21). However, no specific interaction of resveratrol with SUR has been described until now.

Compared with glibenclamide, resveratrol is much more potent in inducing apoptosis: equivalent effects are obtained after a shorter time of treatment with resveratrol than with
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3.1 ± 0.8 nM) (36), which is also reflected by its potency to block the pancreatic K_{ATP} channel, resveratrol is much more effective in inducing apoptosis. Therefore, no direct correlation between the affinity of resveratrol/glibenclamide for SUR and its potency in inducing apoptosis exists. This is underlined by the fact that no significant differences in the resveratrol or glibenclamide (36) binding properties of SUR1 and the mutant SUR1(M1289T) could be detected, although the expression of these different SURs is linked to a significantly different susceptibility to resveratrol- or glibenclamide-induced apoptosis. Obviously, the amino acid at position 1289 in TM17, which is essential for binding (36) and the effect (48) of several K_{ATP} channel openers, does not exert a direct effect on binding of the blockers resveratrol and glibenclamide, but probably rather influences other important interactions within the SUR molecule that are essential for eliciting programmed cell death. On the other hand, the ability of resveratrol to displace glibenclamide binding to SUR1 is abolished by the mutation S1238Y within the glibenclamide-binding pocket, which is known to reduce the glibenclamide affinity of SUR1 (38), yet SUR1-specific apoptosis is not influenced by this mutation. Taken together, our data suggest that the resveratrol-binding site might share some parts of the glibenclamide-binding site, although both binding sites are probably not completely identical. However, it is still possible that a mutation in the glibenclamide-binding pocket does not directly affect resveratrol binding, but indirectly influences allosteric interactions between resveratrol and glibenclamide binding. A precise localization of the resveratrol-binding site and a better understanding of the correlation between resveratrol (or glibenclamide) binding and apoptosis induction by this substance should be the subjects of future investigations.

In most of the studies that report an apoptotic effect of sulfonyleureas (10, 14, 17), a mechanism involving blockade of complete functional K_{ATP} channels and depolarization-induced Ca^{2+} influx into the cell is discussed. However, according to Rustenbeck et al. (15), \( \beta \)-cell toxicity is not necessarily linked to the ability of these drugs to act as K_{ATP} channel blockers. This idea is supported by our observation that K_{ir}6.2 is not required for the apoptotic effects of resveratrol or glibenclamide in recombinant HEK cells. At the moment, we cannot exclude the possibility that SUR exerts the apoptotic effect via an association with channel subunits other than K_{ir}6.2. A functional interaction of SUR1 or a SUR-like protein with a novel Ca^{2+}-activated, ATP-sensitive, nonspecific cation channel has been demonstrated, for example, in astrocytes (49). Alternatively, a direct interaction of SUR with other non-channel proteins leading to specific activation of signaling cascades could also well be imagined.

With the specific caspase activity pattern, we can narrow down the spectrum of signal transduction pathways; the specific enhancement of caspase-9 activity in SUR1-expressing cells after exposure to resveratrol provides support for an engagement of the mitochondrial apoptotic pathway. The activity of caspase-8 is generally not altered in all the HEK293 cell lines after resveratrol treatment and consequently, the death receptor pathway is probably not involved here. Enhanced caspase-12 activity is normally linked to endoplasmic reticulum stress, which is often connected with negative effects on \( \beta \)-cell function and viability (12, 41). The increase in caspase-12 activity measured in all the resveratrol-treated HEK cell lines is, however, not SUR1-specific; it might be connected with the basal apoptotic effect of resveratrol, maybe secondary to general disruption of cellular integrity.

In addition to the short-term effects achieved by high concentrations of resveratrol, studying the long-term in vivo effects of lower concentrations would be particularly important. Concerning SUR-mediated apoptosis, it is possible that relevant in vivo effects of resveratrol are already achieved by concentrations lower than those used in our experimental systems because a previous study has shown that resveratrol is more effective in vivo than in a cell culture model (22). In the case of glibenclamide, which is less potent in inducing SUR1-specific apoptosis than resveratrol, it has been shown that significant apoptotic effects in isolated human islets are already achieved after treatment at 1 nM for 4 days (17). Resveratrol is not only part of the human diet (e.g. concentrations in red wine of \( \approx 25 \) \( \mu M \)), but is often ingested as an additional dietary supplement at doses in the 10–20-mg range (21) or even higher. Several studies have focused on the bioavailability and metabolism of resveratrol. Despite often contradictory results, it is commonly assumed that resveratrol is well absorbed and rapidly distributed to various organs (e.g. peak plasma concentrations of up to 2 \( \mu M \) total resveratrol in humans after oral administration of 25 mg/70 kg of body weight) (reviewed in Refs. 21, 22, and 50). Apart from considerations of the physiological relevance of resveratrol as a dietary constituent or as a potential therapeutic agent applied in higher doses in cancer chemoprevention or therapy (50), this compound represents an interesting model substance to provide further insight into the complex regulatory functions of SUR.

In summary, we have shown that trans-resveratrol is a naturally occurring SUR ligand that shows several analogies to the synthetic sulfonyleurea glibenclamide concerning binding properties, ability to block pancreatic K_{ATP} channels, and capacity to induce apoptosis in a SUR subtype-dependent manner. The latter effect does not necessarily require the presence of functional K_{ir}6.2-containing K_{ATP} channels. A SUR1-specific enhancement of apoptosis after exposure to resveratrol is observed not only in recombinant SUR1-expressing cells, but also in native \( \beta \)-cells. This kind of cell type-specific apoptosis could be important in diabetes as well as cancer research, not least because this apoptotic effect can be abolished by a single amino acid mutation in TM17. SUR1-mediated enhancement of apoptosis is observed only with specific substances (i.e. with glibenclamide or resveratrol, but not with etoposide), and it remains to be investigated which structural requirements of a possible SUR ligand are essential for the apoptotic effect. We have shown that a mitochondrial apoptotic pathway is involved in resveratrol-induced apoptosis, but future work has to be done to understand the precise mechanism of apoptosis induction and the exact integration of SUR in the corresponding signal transduction pathway(s).
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