

# Resveratrol-induced inhibition of insulin secretion from rat pancreatic islets: evidence for pivotal role of metabolic disturbances

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**Szkudelski T.** Resveratrol-induced inhibition of insulin secretion from rat pancreatic islets: evidence for pivotal role of metabolic disturbances. *Am J Physiol Endocrinol Metab* 293: E901–E907, 2007. First published June 19, 2007; doi:10.1152/ajpendo.00564.2006.—Resveratrol is a stilbene present in different plant species and exerting numerous beneficial effects, including prevention of diabetes and attenuation of some diabetic complications. Its inhibitory effect on insulin secretion was recently documented, but the exact mechanism underlying this action remains unknown. Experiments employing diazoxide and a high concentration of  $K^+$  revealed that, in depolarized pancreatic islets incubated for 90 min with resveratrol (1, 10, and 100  $\mu$ M), insulin secretion stimulated by glucose and leucine was impaired. The attenuation of the insulin secretory response to 6.7 mM glucose was not abrogated by blockade of intracellular estrogen receptors and was found to be accompanied by diminished islet glucose oxidation, enhanced lactate production, and reduced ATP levels. Glucose-induced hyperpolarization of the mitochondrial membrane was also reduced in the presence of resveratrol. Moreover, in depolarized islets incubated with 2.8 mM glucose, activation of protein kinase C or protein kinase A potentiated insulin release; however, under these conditions, resveratrol was ineffective. Further studies also revealed that, under conditions of blocked voltage-dependent calcium channels, the stilbene reduced insulin secretion induced by a combination of glucose with forskolin. These data demonstrate that resveratrol 1) inhibits the amplifying pathway of insulin secretion, 2) exerts an insulin-suppressive effect independently of its estrogenic/anti-estrogenic activity, 3) shifts islet glucose metabolism from mitochondrial oxidation to anaerobic, 4) fails to abrogate insulin release promoted without metabolic events, and 5) does not suppress hormone secretion as a result of the direct inhibition of  $Ca^{2+}$  influx through voltage-dependent calcium channels.

amplifying pathway; glucose oxidation; lactate

RESVERATROL (3,5,4'-TRIHYDROXYSTILBENE) is a naturally occurring diphenol present in more than 70 plant species. The richest sources of this compound are peanuts (21), grapes (26), and red wine (41). Results of many investigations have provided evidence for its beneficial effects in organisms. The anti-cancer (3, 11), antioxidant (33), and cardioprotective (8, 20) properties of resveratrol have been well established recently. Moreover, this stilbene, as a component of red wine, has been suggested to be one of the factors responsible for a reduced incidence of cardiovascular disease in humans regularly consuming moderate amounts of red wine (43).

Results of recent studies demonstrate the beneficial influence of resveratrol on the pancreas. The stilbene administered to rats in a single dose or for a few days alleviated the severity of acute pancreatitis (27, 28, 30, 32, 45). This action is postulated to be related to the inhibition of the activation of nuclear

factor- $\kappa$ B (NF- $\kappa$ B) and decreased expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-8 (IL-8) (30, 32). Furthermore, the reduced damage of the pancreas by reactive oxygen species and diminished pancreatic tissue infiltration of neutrophils in resveratrol-treated rats were also shown (27, 28).

Data from the literature indicate that resveratrol alleviates some diabetic complications and may play a role in prevention of diabetes. Experiments in vitro revealed that this stilbene attenuates adverse intracellular changes evoked by high glucose. These effects are thought to result predominantly from the inhibitory action of resveratrol on high glucose-induced activation of enzymes involved in induction of apoptosis, such as c-Jun NH<sub>2</sub>-terminal kinase and caspase-3, as well as from the attenuation of oxidative stress in high glucose-treated cells (7). The most recent studies demonstrated that, in streptozotocin-induced diabetic rats, resveratrol abated renal dysfunction and oxidative stress (40), exerted hypoglycemic and hypolipidemic action, and reduced insulinemia (44). Its stimulatory effect on glucose uptake by the muscle strips, adipocytes, and hepatocytes isolated from diabetic rats was also shown (44). Some data indicate that resveratrol may reduce insulin resistance via inhibition of the activity of I $\kappa$ B kinase- $\beta$  (IKK $\beta$ ) (19), NF- $\kappa$ B (47), and protein kinase C (42). The inhibitory action of resveratrol on insulin secretion from pancreatic islets of normal rats was also recently reported (46), but the exact mechanism remains unknown. The restriction of exaggerated insulin secretion is considered to be an important factor in prevention of insulin resistance, type 1 and type 2 diabetes mellitus, and some other metabolic disorders (reviewed in Refs. 16, 36). In the present study, the effects of resveratrol on insulin secretion from isolated pancreatic islets were investigated under different stimulatory conditions. Moreover, the influence of this stilbene on metabolic parameters, namely glucose oxidation, lactate formation and ATP levels in pancreatic islets, was determined.

## MATERIALS AND METHODS

**Materials.** *trans*-Resveratrol, D-glucose, L-leucine, diazoxide, nifedipine, forskolin, antimycin A (from *Streptomyces* sp.), phorbol 12-myristate 13-acetate (PMA), rhodamine-123, Trypan blue solution, thiazolyl blue tetrazolium bromide (MTT), ATP assay kit, bovine serum albumin (fatty acid free), L-lactate dehydrogenase, glycine buffer,  $\beta$ -NAD, and all reagents used to prepare Krebs-Ringer buffer and Hanks' solution were obtained from Sigma (St. Louis, MO). Collagenase P was from Roche Diagnostics (Mannheim, Germany) and dimethyl sulfoxide from ICN Biomedical (Aurora, OH). ICI 182,780 was from Tocris (Ellisville, MO). Liquid scintillation cocktail (OptiPhase) was purchased from Fisher Chemicals (Loughborough,

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Leicester, UK). D-[U-<sup>14</sup>C]glucose (specific activity 250 mCi/mmol) and hyamine hydroxide were from Perkin Elmer (Boston, MA).

**Animals.** Male Wistar rats that weighed 200–280 g were obtained from Brwinow, Poland. The rats were fed ad libitum a standard laboratory diet (Labofeed, Kcynia, Poland) and had free access to tap water. The animals were maintained in cages in an air-conditioned room with a 12:12-h dark-light cycle and a constant temperature of  $21 \pm 1^\circ\text{C}$  and were killed by decapitation. The experiments were performed according to rules accepted by the Local Ethical Commission for Investigation on Animals and were reviewed and approved by the Local Ethical Commission for Investigation on Animals.

**Islet isolation.** Pancreatic islets were isolated by collagenase digestion (25). Hanks' solution (containing, in mM: NaCl 137, KCl 5.36, MgSO<sub>4</sub> 0.81, Na<sub>2</sub>HPO<sub>4</sub> 0.34, KH<sub>2</sub>PO<sub>4</sub> 0.44, CaCl<sub>2</sub> 1.26, NaHCO<sub>3</sub> 4.17) was injected into the common bile duct, and the pancreas was quickly excised. In each experiment, glands obtained from three rats were pooled and cut down with scissors, and islets were incubated with collagenase. Afterward, islets were washed with Hanks' solution and were separated from the remaining exocrine tissue by handpicking under a stereomicroscope. Before the proper experiments, islets were preincubated for 30 min in Krebs-Ringer buffer containing 2.8 mM glucose.

**Insulin secretion.** Insulin secretion from pancreatic islets was measured in static incubation experiments. Groups of five islets were incubated in 1 ml of Krebs-Ringer buffer containing 115 mM NaCl, 24 mM NaHCO<sub>3</sub>, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.5% bovine serum albumin, and the appropriate reagents as indicated in RESULTS and figure legends. In experiments in which the concentration of KCl in the buffer was increased to 30 mM, the concentration of NaCl was diminished to maintain the osmolality. Stock solutions of resveratrol, diazoxide, nifedipine, forskolin, PMA, and ICI 182,780 were prepared in dimethyl sulfoxide. The final concentration of this solvent in the buffer with islets was  $<0.1\%$ . Before use, the buffer was gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>, and its pH was adjusted to 7.4. Islets were incubated for 90 min in a water bath at  $37^\circ\text{C}$  in an atmosphere of O<sub>2</sub>-CO<sub>2</sub> (95%/5%) with gentle shaking.

**Measurement of insulin concentration.** Immediately after the end of islet incubations, aliquots of Krebs-Ringer buffer were sampled and stored ( $-80^\circ\text{C}$ ) for insulin determination. Insulin concentrations in the incubation medium were measured radioimmunologically using kits specific for rat hormone (Linco Research).

**Lactate production.** To determine the effect of resveratrol on lactate production, groups of 200 islets were handpicked under a stereomicroscope and were incubated in 1 ml of Krebs-Ringer buffer (pH 7.4; 0.5% bovine serum albumin) containing 6.7 mM glucose alone or glucose with 1, 10, and 100  $\mu\text{M}$  resveratrol. Incubations were carried out with shaking in a water bath at  $37^\circ\text{C}$  for 90 min. After this time, buffer was sampled and stored ( $-80^\circ\text{C}$ ) until analysis. Lactate concentrations were determined by the measure of NADH generated from NAD<sup>+</sup> in the presence of lactate dehydrogenase (12).

**Glucose oxidation.** Glucose oxidation was determined by measuring the formation of <sup>14</sup>CO<sub>2</sub> from D-[U-<sup>14</sup>C]glucose (35). Groups of 15 islets were incubated in plastic caps in 100  $\mu\text{l}$  of Krebs-Ringer buffer (pH 7.4; 0.5% bovine serum albumin) containing 6.7 mM glucose, 2  $\mu\text{Ci}$  D-[U-<sup>14</sup>C]glucose, and 0, 1, 10, or 100  $\mu\text{M}$  resveratrol. The caps were placed in 20-ml glass scintillation vials and gassed with O<sub>2</sub>-CO<sub>2</sub> (95%/5%), and the vials were capped airtight with rubber membranes. The vials were then placed in a water bath and were incubated with continuous shaking for 90 min at  $37^\circ\text{C}$ . After the end of incubations, 100  $\mu\text{l}$  of 50  $\mu\text{M}$  antimycin A dissolved in 70% ethanol were injected into each cap. Just after this, 250  $\mu\text{l}$  of hyamine hydroxide were added into the scintillation vials followed by an injection of 100  $\mu\text{l}$  of 0.4 M Na<sub>2</sub>HPO<sub>4</sub> (pH = 6.0) into the caps with islets. All of these reagents were added through the rubber membranes. The scintillation vials were then left for 20 h at room temperature to allow <sup>14</sup>CO<sub>2</sub> to be trapped by hyamine hydroxide. Finally, the rubber membranes and the

caps with islets were removed, 10 ml of a scintillation fluid were added to each vial, and after vortexing the radioactivity was measured.

**Measurement of ATP.** ATP was measured as described previously (13). Isolated islets were incubated in Krebs-Ringer buffer containing 2.8 mM glucose, 6.7 mM glucose, or 6.7 mM glucose with 1, 10, and 100  $\mu\text{M}$  resveratrol at  $37^\circ\text{C}$  for 60 min. After this time, ice-cold trichloroacetic acid was added (final concentration 5%), and tubes were vortexed, left at room temperature for 15 min, and centrifuged. Afterward, 400  $\mu\text{l}$  of the supernatant were mixed with 1.5 ml of diethyl ether, and the upper phase was removed. This step was repeated four times. The obtained extracts were then diluted with the same volume of a buffer (40 mM HEPES, 3 mM MgCl<sub>2</sub>, pH = 7.75) and were frozen until analysis. ATP was determined by a luminometric method with a kit containing firefly luciferase and luciferin.

**Mitochondrial membrane potential.** The effect of resveratrol on glucose-induced changes in the mitochondrial inner membrane potential was measured using rhodamine-123 (10). Pancreatic islets were loaded with 10  $\mu\text{g/ml}$  rhodamine-123 for 20 min at  $37^\circ\text{C}$  in Krebs-Ringer buffer containing 2.8 mM glucose. Islets were then washed with the buffer without dye and were incubated for 15 min with 2.8 mM glucose alone or glucose and 100  $\mu\text{M}$  resveratrol. After this time, islets were placed on a microscope stage and were stabilized for 5 min at  $37^\circ\text{C}$  in the presence of 2.8 mM glucose alone or glucose and resveratrol. Afterward, islets were exposed for 20 min to 20 mM glucose with or without the tested stilbene (100  $\mu\text{M}$ ). Rhodamine-123 fluorescence was excited at 488 nm using the krypton-argon laser of a confocal microscope (Axiovert 200M, LSM 510 META; Carl Zeiss, Jena, Germany), and emitted light was measured using a Plan-Apochromat  $\times 20$  objective and a band-pass 505- to 550-nm filter. Data were normalized to the basal levels of fluorescence obtained before 20 mM glucose.

**MTT assay and islet viability.** MTT assay was based on the method described by Janjic and Wollheim (22). Isolated islets were incubated in Krebs-Ringer buffer containing 6.7 mM glucose alone or glucose and 100  $\mu\text{M}$  resveratrol for 90 min at  $37^\circ\text{C}$ . Afterward, all islets were incubated with MTT (0.5 mg/ml) at  $37^\circ\text{C}$  for 90 min and then incubated with isopropanol at room temperature for an additional 90 min. The absorbance was read at 560 nm.

Islet viability was determined using the dye exclusion assay (1, 18). Isolated islets were incubated in Krebs-Ringer buffer containing 6.7 mM glucose alone or glucose with 100  $\mu\text{M}$  resveratrol at  $37^\circ\text{C}$  for 90 min. Then, islets were resuspended for 3 min in 0.81% NaCl and 0.06% dibasic potassium phosphate containing 0.4% Trypan blue and examined under a microscope (Axiovert 200M, Carl Zeiss).

**Statistical analysis.** The means  $\pm$  SE from three independent experiments in quadruplicate in the case of insulin secretion studies and ATP levels and from five separate experiments concerning lactate production and glucose oxidation were evaluated statistically using analysis of variance and Duncan's multiple range test. In the case of rhodamine-123 fluorescence, means  $\pm$  SE from five independent experiments were evaluated statistically using Student's *t*-test. Differences were considered significant at  $P < 0.05$ .

## RESULTS

**Effects of resveratrol on the amplifying pathway of insulin secretion.** To study the amplifying pathway of insulin secretion, islets were exposed to 6.7 mM glucose or 10 mM leucine in the presence of 250  $\mu\text{M}$  diazoxide to open ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels and 30 mM K<sup>+</sup> to depolarize plasma membrane. It was found that insulin secretion was substantially potentiated in islets incubated with glucose, diazoxide, and high potassium compared with islets incubated with glucose and low potassium, indicating that the insulinotropic effect of this sugar is higher in depolarized islets. A similar effect was observed in the case of leucine. Incubations of pancreatic islets

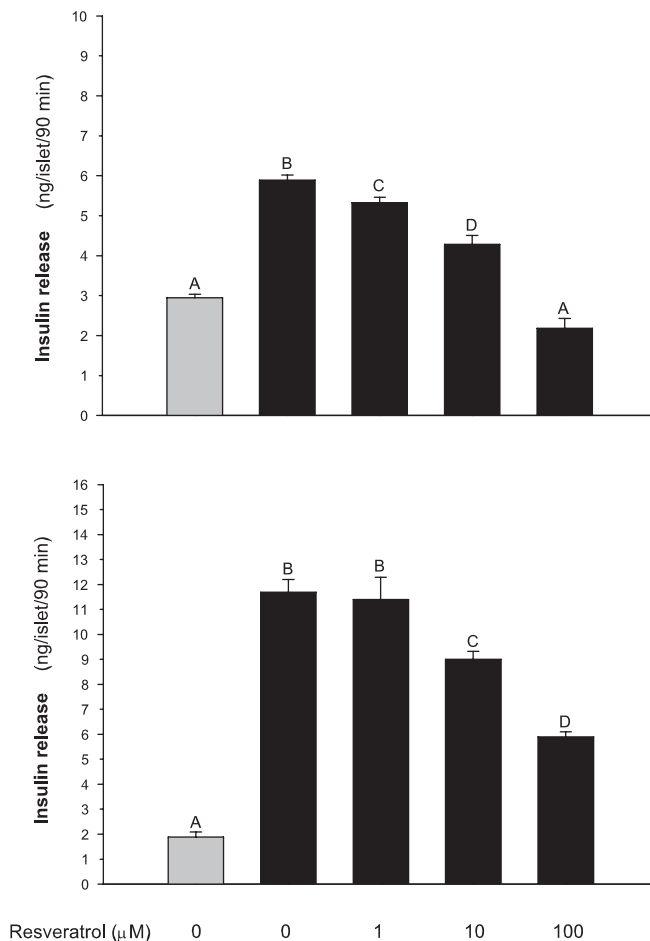


Fig. 1. Effects of resveratrol on the amplifying pathway of insulin secretion in the presence of glucose or leucine. Groups of 5 islets were incubated for 90 min in 1 ml of Krebs-Ringer buffer containing 6.7 mM glucose (*top*) or 10 mM leucine (*bottom*) under classical conditions (grey bars) or in the presence of 250  $\mu$ M diazoxide and 30 mM  $K^+$  (black bars) with or without resveratrol. Values represent means  $\pm$  SE of 12 determinations from 3 separate experiments. Means marked by different letters differ statistically at  $P < 0.05$ .

with glucose, diazoxide, high potassium, and 1, 10, or 100  $\mu$ M resveratrol revealed that the tested compound significantly inhibited the amplifying pathway of insulin secretion ( $P < 0.05$ ). Furthermore, it was demonstrated that leucine-induced insulin secretion studied under conditions of increased  $K^+$  and diazoxide was not affected by 1  $\mu$ M resveratrol but was significantly restricted by 10 and 100  $\mu$ M resveratrol ( $P < 0.05$ ; Fig. 1).

**Effects of resveratrol on metabolic parameters in pancreatic islets.** Incubations of islets with 6.7 mM glucose and 1, 10, and 100  $\mu$ M resveratrol for 90 min resulted in enhanced islet lactate formation compared with islets incubated with glucose alone ( $P < 0.05$ ). This effect was clear-cut at all concentrations of the tested stilbene; however, the increase in lactate output was potentiated particularly in the presence of the highest concentration of resveratrol. Conversely, islet glucose oxidation was substantially reduced by 1, 10, and 100  $\mu$ M resveratrol ( $P < 0.05$ ). The metabolic disturbances elicited by resveratrol allowed the supposition that islet ATP content may be reduced. To verify this assumption, the influence of resveratrol on the glucose-induced rise in ATP was studied. Incubations of

islets for 60 min in Krebs-Ringer buffer containing 6.7 mM glucose increased ATP content by about one-half compared with 2.8 mM glucose (data not shown). This effect of 6.7 mM glucose was significantly attenuated in the presence of 1, 10, and 100  $\mu$ M resveratrol ( $P < 0.05$ ; Fig. 2).

**Effects of resveratrol on mitochondrial membrane potential.** The increase in glucose concentration from 2.8 to 20 mM produced a substantial decrease in rhodamine-123 fluorescence in isolated islets, indicating mitochondrial membrane hyperpolarization. Incubations of islets with 20 mM glucose and 100  $\mu$ M resveratrol revealed that the effect of high glucose on mitochondrial membrane potential was disturbed in the presence of the stilbene. When resveratrol was added to the islets, the glucose-induced decrease in rhodamine-123 fluorescence was significantly blunted ( $P < 0.05$ ; Fig. 3).

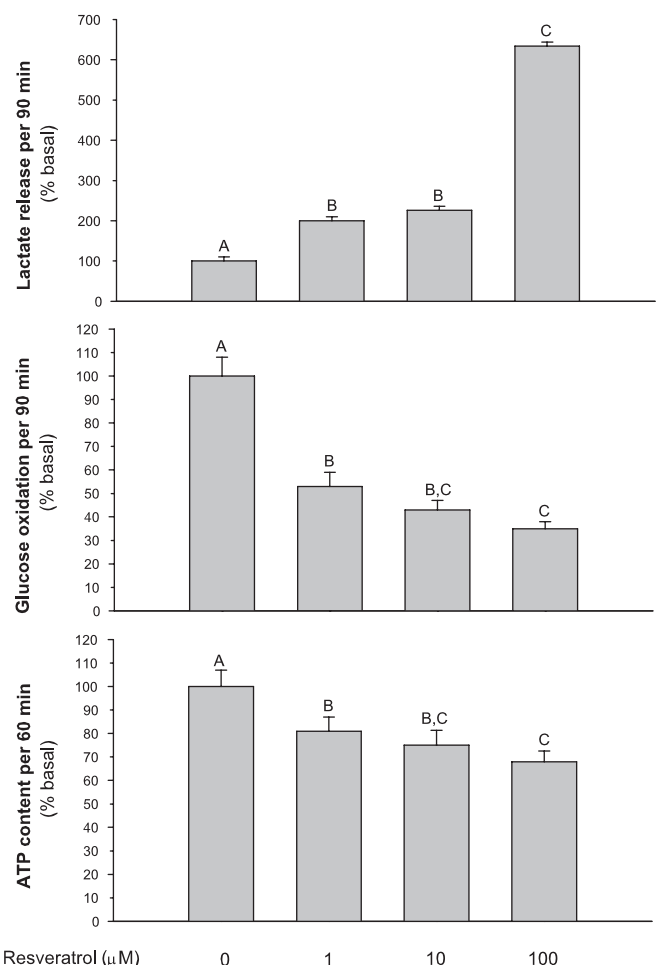


Fig. 2. Metabolic parameters in control and resveratrol-treated islets. To determine the effect of resveratrol on islet lactate production (*top*), groups of 200 islets were incubated for 90 min in 1 ml of Krebs-Ringer buffer containing 6.7 mM glucose with or without the tested compound. To determine the effect of resveratrol on islet glucose oxidation (*middle*), groups of 15 islets were incubated for 90 min in 100  $\mu$ l of Krebs-Ringer buffer containing 6.7 mM glucose and 2  $\mu$ Ci of  $D$ -[ $^{14}$ C]glucose with or without the tested compound. Glucose oxidation was determined by measuring the formation of  $^{14}CO_2$  from  $D$ -[ $^{14}$ C]glucose. Values represent means  $\pm$  SE of 5 separate experiments. To determine the effect of resveratrol on islet ATP content (*bottom*), groups of 5 islets were incubated for 60 min in 0.4 ml of Krebs-Ringer buffer containing 6.7 mM glucose with or without the tested compound. Values represent means  $\pm$  SE of 12 determinations from 3 separate experiments. Means marked by different letters differ statistically at  $P < 0.05$ .



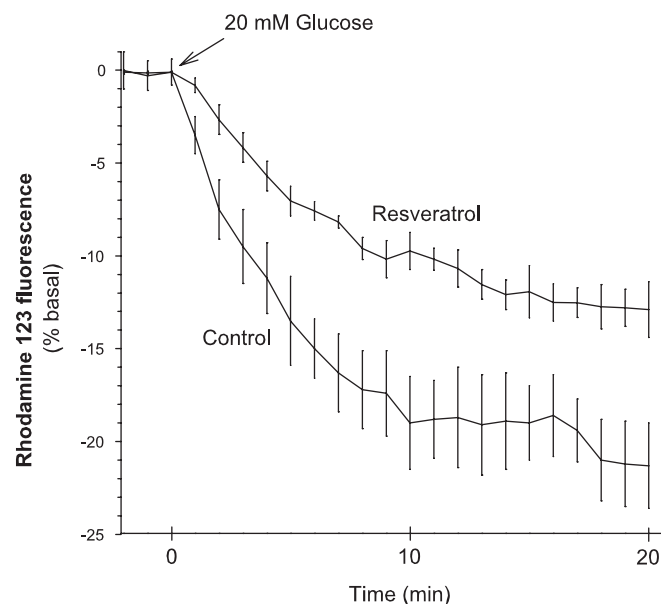


Fig. 3. The effect of resveratrol on mitochondrial membrane hyperpolarization induced by 20 mM glucose. Islets loaded with rhodamine-123 were preincubated with 2.8 mM glucose and then stimulated for 20 min with 20 mM glucose. To determine the effect of resveratrol on glucose-induced changes in mitochondrial membrane potential, islets were preincubated with 2.8 mM glucose and 100  $\mu$ M resveratrol and then incubated in the presence of both high glucose and the tested stilbene. A decrease in rhodamine-123 fluorescence was calculated compared with fluorescence found before stimulation with 20 mM glucose. Data are means  $\pm$  SE from 5 separate experiments.

**Effects of resveratrol on insulin release from depolarized islets stimulated with PMA or forskolin at low glucose concentration.** To test whether resveratrol affects insulin release stimulated without metabolic events in  $\beta$ -cells, islets were incubated with 2.8 mM glucose, 250  $\mu$ M diazoxide, 30 mM  $K^+$ , and 100 nM PMA (an activator of protein kinase C) or 5  $\mu$ M forskolin (an activator of protein kinase A) with or without the tested stilbene. These experiments demonstrated that, in depolarized islets, hormone secretion was dramatically increased when protein kinase C was activated despite a non-stimulatory concentration of glucose. However, under these stimulatory conditions, 1, 10, and 100  $\mu$ M resveratrol failed to affect insulin release. Further studies revealed that depolarized islets incubated with 2.8 mM glucose and exposed to forskolin also responded by increasing release of insulin, although this response was less marked compared with islets incubated with PMA. It was demonstrated that, in depolarized islets, resveratrol did not change insulin release induced by 5  $\mu$ M forskolin in the presence of 2.8 mM glucose (Fig. 4).

**Effects of resveratrol on insulin secretion stimulated by a combination of glucose with forskolin from islets with blocked voltage-dependent calcium channels.** The direct influence of resveratrol on the activity of calcium channels in  $\beta$ -cells may be involved in its insulin-suppressive effect. To verify this assumption, the effect of resveratrol on glucose (6.7 mM)-induced insulin release was studied in the presence of 250  $\mu$ M diazoxide to prevent  $\beta$ -cell membrane depolarization and 5  $\mu$ M nifedipine to block directly voltage-dependent calcium channels. Under these experimental conditions, 6.7 mM glucose failed to stimulate insulin secretion. However, the combination of glucose and 5  $\mu$ M forskolin exerted a clear-cut

insulinotropic effect despite the presence of diazoxide and nifedipine. This insulinotropic effect was significantly diminished by 1, 10, and 100  $\mu$ M resveratrol ( $P < 0.05$ ; Fig. 5).

**Effects of blockade of estrogen receptor on the insulin-suppressive action of resveratrol.** To ascertain whether the inhibitory action of resveratrol on insulin secretion is mediated through estrogen receptors, pancreatic islets were preincubated for 15 min with an estrogen receptor blocker (ICI 182,780; 1  $\mu$ M) and then incubated with the blocker, 6.7 mM glucose, and 1, 10, or 100  $\mu$ M resveratrol. These experiments revealed that inhibition of estrogen receptors with ICI 182,780 did not alleviate the insulin-suppressive action of resveratrol (Fig. 5).

**Effects of resveratrol on mitochondrial integrity and islet viability.** Pancreatic islets preincubated for 90 min with glucose alone or glucose with 100  $\mu$ M resveratrol did not differ in their ability to convert MTT to formazan. The absorbance of isopropanol in both cases was similar. Moreover, there was no difference in Trypan blue uptake between islets preincubated for 90 min with glucose alone or glucose with 100  $\mu$ M resveratrol (data not shown).

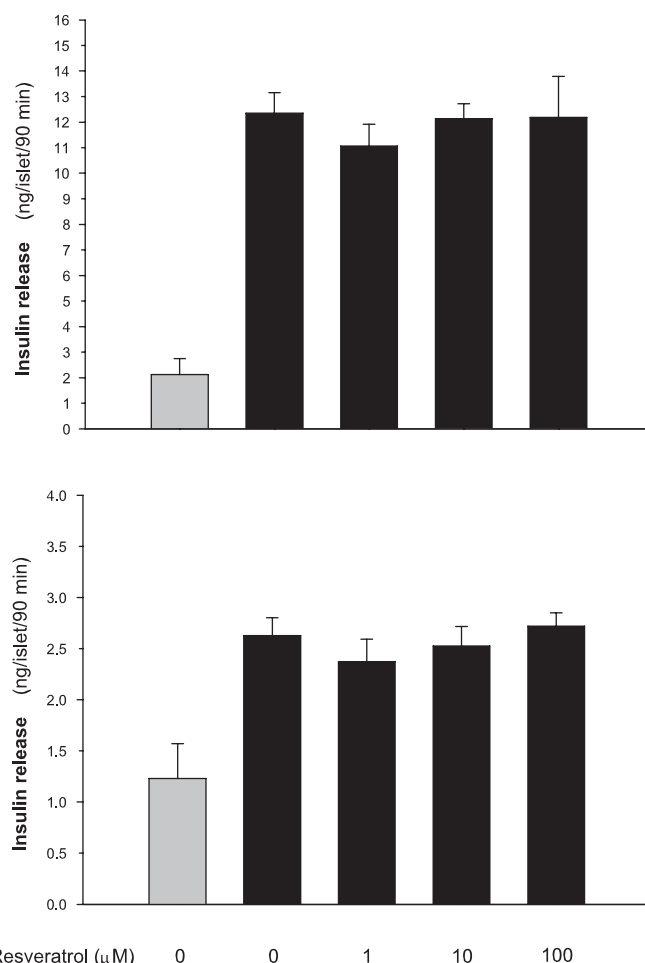


Fig. 4. Effects of resveratrol on insulin release from depolarized islets stimulated with phorbol 12-myristate 13-acetate (PMA; top) or forskolin (bottom) at low glucose concentration. Groups of 5 islets were incubated for 90 min in 1 ml of Krebs-Ringer buffer containing 2.8 mM glucose, 250  $\mu$ M diazoxide, and 30 mM  $K^+$  (grey bars) or 2.8 mM glucose, 250  $\mu$ M diazoxide, 30 mM  $K^+$ , and 5  $\mu$ M forskolin or 100 nM PMA (black bars). Values represent means  $\pm$  SE of 12 determinations from 3 separate experiments.

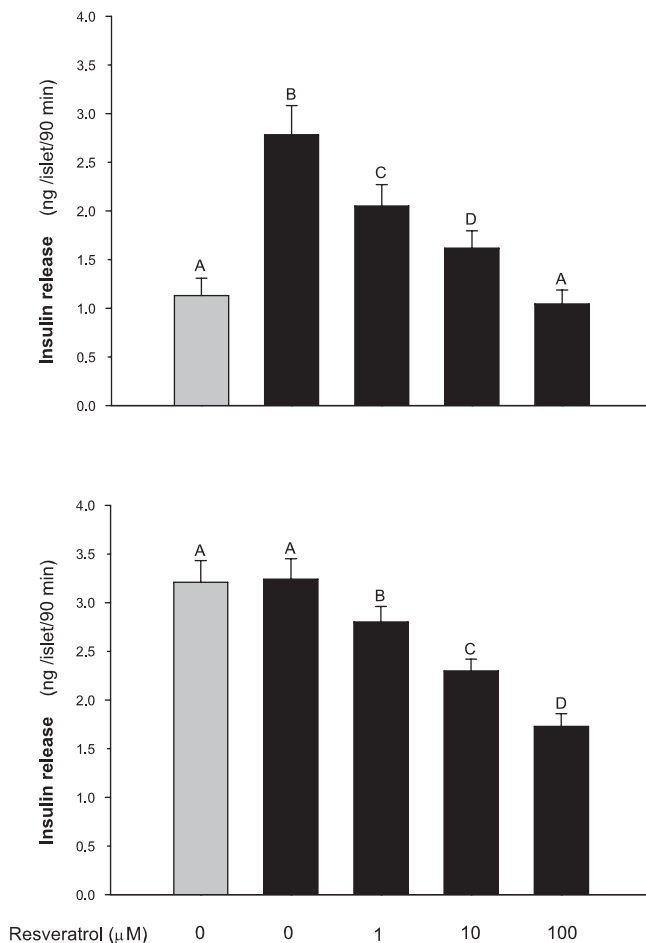


Fig. 5. *Top*: effects of resveratrol on insulin secretion stimulated by a combination of glucose with forskolin from islets with blocked voltage-dependent calcium channels. Groups of 5 islets were incubated for 90 min in 1 ml of Krebs-Ringer buffer containing 6.7 mM glucose, 250 μM diazoxide, and 5 μM nifedipine (grey bar) or 6.7 mM glucose, 250 μM diazoxide, 5 μM nifedipine, and 5 μM forskolin with or without resveratrol (black bars). *Bottom*: effects of blockade of estrogen receptors on the insulin-suppressive action of resveratrol. Groups of 5 islets were preincubated for 15 min in 1 ml of Krebs-Ringer buffer containing 6.7 mM glucose (grey bar) or 6.7 mM glucose and 1 μM ICI 182,780 (black bars) and then incubated for 90 min with these compounds with or without resveratrol. Values represent means  $\pm$  SE of 12 determinations from 3 separate experiments. Means marked by different letters differ statistically at  $P < 0.05$ .

## DISCUSSION

Resveratrol, a diphenolic stilbene present in particularly high concentrations in grapes and red wine, exerts various biological actions. One of the effects is its recently discovered ability to decrease insulin secretion. In pancreatic islets of normal rats, resveratrol inhibited insulin secretion induced by glucose and the mitochondrial fuel leucine with glutamine. The attenuation of the insulin secretory response to glucose was reversible, and the inhibition of protein kinase C activity in  $\beta$ -cells was postulated to be involved (46). This study presents further evidence that resveratrol abates secretion of insulin and provides more information on the mechanisms underlying this action.

Glucose exerts insulinotropic action via a sequence of events involving its transport, metabolism, increase in ATP/ADP ratio, closure of  $K_{ATP}$  channels, membrane depolarization,

opening of voltage-dependent calcium channels, and rise in cytosolic calcium concentration triggering insulin secretion (17). Additional signals, generated in  $\beta$ -cells independently of the closure of  $K_{ATP}$  channels, are necessary to maintain the sustained secretory response. This amplifying pathway of insulin secretion is studied when glucose is unable to close  $K_{ATP}$  channels (13, 14, 17). In pancreatic islets incubated with diazoxide, to open  $K_{ATP}$  channels, and with a high concentration of  $K^+$ , to depolarize plasma membrane, resveratrol restricted glucose-induced insulin release, which demonstrates the inhibitory effect of the drug on the amplifying pathway of insulin secretion. The attenuation of the insulin secretion elicited by resveratrol in the presence of potassium channel opener allows exclusion of the direct influence of the stilbene on  $K_{ATP}$  channel activity as the reason for its insulin-suppressive action. These data are consistent with a previous finding demonstrating that the inhibition of glucose-induced insulin secretion caused by resveratrol is not abrogated as a result of the closure of  $K_{ATP}$  channels in  $\beta$ -cells by sulfonylurea glibenclamide (46).

The inhibitory effect of resveratrol on insulin secretion induced not only by glucose but also by mitochondrial fuels, such as leucine with glutamine under classical conditions (46) and leucine alone in depolarized islets, points to metabolic disturbances in mitochondria. This assumption was supported by the demonstration that resveratrol substantially enhanced islet lactate production in the presence of 6.7 mM glucose. Under physiological conditions, the high activity of mitochondrial glycerol phosphate dehydrogenase and malate-aspartate shuttle and low activities of both lactate dehydrogenase and plasma membrane lactate/monocarboxylate transporter in  $\beta$ -cells ensure efficient mitochondrial metabolism of pyruvate in these cells, whereas formation of lactate is very low (31, 37, 39). However, malfunction of the mitochondrial electron transport system causes accumulation of NADH, inhibition of the tricarboxylic acid cycle, a loss of mitochondrial ATP production, and an increase in lactate generation. The latter effect is suggested to be due to the increased cytoplasmic NADH concentration, which favors formation of lactate from pyruvate (34). In other studies, the direct effect of resveratrol on the respiratory chain was indeed demonstrated. In rat brain and liver preparations, the drug inhibited the activity of ATP synthase (49, 50) and diminished oxygen consumption by mitochondria (50). In the present study, glucose-induced hyperpolarization of the inner mitochondrial membrane was lower in pancreatic islets exposed to resveratrol, indicating reduced metabolic activity of mitochondria. Hyperpolarization of this membrane reflects aerobic glucose metabolism, is related to proton export from the mitochondrial matrix, and is a prerequisite for undisturbed insulin secretion (2).

Further evidence confirming resveratrol-induced metabolic disturbances was a substantial decrease in glucose oxidation in islets exposed to the stilbene. The concomitant increase in lactate formation and decrease in glucose oxidation in the presence of resveratrol demonstrate a shifted metabolism of glucose from mitochondrial oxidation toward anaerobic oxidation. This effect resulted in reduced ATP levels in islets exposed to resveratrol and presumably played a primary role in the insulin-suppressive action of the stilbene.

These outcomes do not exclude the possibility of other effects (independent of metabolic events) of resveratrol in

$\beta$ -cells contributing to the inhibition of insulin secretion. This was verified at a nonstimulatory concentration of glucose, when islets were exposed to a depolarizing concentration of  $K^+$  and diazoxide (to augment intracellular  $Ca^{2+}$ ) in the presence of PMA, which activates protein kinase C. Activation of this enzyme substantially increases the size of the highly calcium-sensitive pool and, to a lesser extent, the readily releasable pool of secretory vesicles in  $\beta$ -cells (48). Activation of protein kinase C in depolarized islets dramatically enhanced insulin release despite 2.8 mM glucose, but under these conditions, resveratrol was ineffective. The lack of effectiveness could arise from the fact that insulin release was studied at low glucose but also from the presence of PMA, since activation of protein kinase C by PMA was previously found to suppress the inhibitory effect of 1 and 10  $\mu$ M resveratrol on insulin secretion induced by 6.7 mM glucose (46). Therefore, in additional experiments, PMA was replaced by forskolin, which also sensitizes the secretory vesicles to  $Ca^{2+}$  (48) but does not alleviate the inhibitory effect of resveratrol on glucose-induced insulin secretion (46). However, in depolarized islets, insulin release enhanced by activation of protein kinase A was (similar to experiments employing PMA) not affected by resveratrol. These results indicate that resveratrol does not restrict insulin release when this process is promoted independently of metabolic events. Moreover, they support the notion that disturbances in the metabolism of glucose (and the other metabolizable stimuli) are pivotal for the insulin-suppressive effect of the stilbene.

Some studies have reported that resveratrol modifies the activity of certain kinds of  $Ca^{2+}$  channels and thereby affects intracellular  $Ca^{2+}$  levels. Its effects on intracellular  $Ca^{2+}$  may be, however, contradictory depending on the kind of cell. In platelets, the stilbene substantially inhibited calcium influx (9), whereas in vascular smooth muscle cells (6) and in heart valve endothelium (5), the opposite effect was shown. In uncoupling protein-2-overexpressing  $\beta$ -cells, 10  $\mu$ M resveratrol increased intracellular  $Ca^{2+}$  concentrations. However, this observation was made under conditions that differ from those of the present study, since, in uncoupling protein-2-overexpressing  $\beta$ -cells, the insulin secretory response to glucose is substantially abated. Moreover, the rise in intracellular  $Ca^{2+}$  was found in cells incubated without glucose (38). In the present study, the effect of resveratrol on insulin release was investigated when L-type voltage-dependent calcium channels (VDCC) were blocked. In islets with blocked VDCC, insulin secretion elicited by glucose alone is completely blunted but is promoted by glucose and forskolin (24). The insulinotropic effect of the combination of glucose and forskolin, under conditions of blocked VDCC, was found to be restricted by resveratrol, thus suggesting that the insulin-suppressive action of the stilbene does not result from its direct effect on  $Ca^{2+}$  influx through VDCC in  $\beta$ -cells. It is noteworthy that these observations were made in the presence of 6.7 mM glucose, and, as evidenced by Komatsu et al. (24), the insulinotropic action of glucose with forskolin is inhibited by mitochondrial poisons. Therefore, the inhibition of hormone secretion brought about by resveratrol in islets with blocked VDCC may be explained by disturbed mitochondrial metabolism.

Resveratrol is classified as a phytoestrogen because of its ability to bind estrogen receptors- $\alpha$  and - $\beta$  and to act as a mixed agonist/antagonist for these receptors (4). Therefore,

several investigations have focused on its estrogenic or anti-estrogenic properties. It was demonstrated that some effects of this compound are estrogen receptor dependent and may be reversed by the estrogen receptor antagonist ICI 182,780 (23, 29). Results of the present study revealed that blockade of intracellular estrogen receptors with ICI 182,780 failed to attenuate the insulin-suppressive action of resveratrol, which indicates that this action is independent of estrogenic/anti-estrogenic activities of the stilbene.

It can be asserted that the inhibitory effect of resveratrol on insulin secretion is not related to the permanent disturbances of  $\beta$ -cells, as demonstrated using MTT assay and Trypan blue exclusion. This is in agreement with previous observations that, in islets preincubated with resveratrol, glucose-induced insulin secretion was unaffected (46). Moreover, the tested stilbene was found to enhance apoptosis in SUR1-expressing recombinant cells and in native  $\beta$ -cells, but this effect appeared after incubation with 100  $\mu$ M resveratrol for at least 24 h (15).

Recently, Su et al. (44) demonstrated several beneficial insulin-like effects of resveratrol in diabetic rats. However, results of the present study provide evidence that the stilbene is also able to induce metabolic disturbances in  $\beta$ -cells, resulting in restricted secretion of insulin.

In conclusion, results obtained in the present study demonstrate that resveratrol inhibits the amplifying pathway of insulin secretion. A decrease in islet glucose oxidation, an increase in islet lactate formation, and a decrease in ATP content produced by resveratrol indicate that disturbed mitochondrial metabolism of fuel secretagogues is responsible for the insulin-suppressive effect. This was additionally confirmed by the observation that resveratrol attenuated glucose-induced hyperpolarization of the mitochondrial membrane. Moreover, the inhibition of insulin secretion was dependent on the presence of metabolizable stimuli (glucose, leucine) and did not occur when hormone release was induced without metabolic events. The direct influence of resveratrol on  $Ca^{2+}$  influx through voltage-dependent calcium channels seems not to be involved in the inhibition of insulin secretion caused by this drug. It was also evidenced that the observed effects of resveratrol on insulin secretion are independent of its interactions with intracellular estrogen receptors.

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