Protective mechanisms of resveratrol against ischemia-reperfusion-induced damage in hearts obtained from Zucker obese rats: the role of GLUT-4 and endothelin

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Lekli I, Szabo G, Juhasz B, Das S, Das M, Varga E, Szendrei L, Gesztesy L, Varadi J, Bak I, Das DK, Tosaki A. Protective mechanisms of resveratrol against ischemia-reperfusion-induced damage in hearts obtained from Zucker obese rats: the role of GLUT-4 and endothelin. Am J Physiol Heart Circ Physiol 294: H859–H866, 2008. First published December 7, 2007; doi:10.1152/ajpheart.01048.2007.—The resveratrol-induced cardiac protection was studied in Zucker obese rats. Rats were divided into five groups: group 1, lean control; group 2, obese control (OC); group 3, obese treated orally with 5 mg·kg⁻¹·day⁻¹ of resveratrol (OR) for 2 wk; group 4, obese rats received 10% glucose solution ad libitum for 3 wk (OG); and group 5, obese rats received 10% glucose for 3 wk and resveratrol (OGR) during the 2nd and 3rd wk. Body weight, serum glucose, and insulin were measured, and then hearts were isolated and subjected to 30 min of ischemia followed by 120 min of reperfusion. Heart rate, coronary flow, aortic flow, developed pressure, the incidence of reperfusion-induced ventricular fibrillation, and infarct size were measured. Resveratrol reduced body weight and serum glucose in the OR compared with the OC values (414 ± 10 g and 7.08 ± 0.41 mmol/L, respectively, to 378 ± 12 g and 6.11 ± 0.44 mmol/L), but insulin levels were unchanged. The same results were obtained for the OG vs. OGR group. Resveratrol improved postischemic cardiac function in the presence or absence of glucose intake compared with the resveratrol-free group. The incidence of ventricular fibrillation and infarct size was reduced by 83 and 20% in the OR group, and 67 and 16% in the OGR group, compared with the OC and OG groups, respectively. Resveratrol increased GLUT-4 expression and reduced endothelin expression and cardiac apoptosis in ischemic-reperfused hearts in the presence or absence of glucose intake. Thus the protective effect of resveratrol could be related to its direct effects on the heart.

heart; ischemia-reperfusion; diabetes; rat

In the past three decades, an explosive increase in the number of people diagnosed with diabetes was seen worldwide (7, 48). Diabetes is a major risk factor for the development of cardiovascular diseases, which accounts largely for the high morbidity and mortality in diabetic populations (3, 17, 33). Type 2 diabetes is a multifactorial disease that shows heterogeneity in several respects in various populations (21). The underlying mechanisms responsible for the development of diabetes are a complex interplay of various and potential factors, such as increased thrombotic potential (29), endothelial dysfunction (19), and increased oxidative stress (1). In experimental studies, feeding a carbohydrate-enriched diet to normal or obese Zucker rats has been shown to induce insulin resistance and hyperinsulinemia, associated with an elevation in blood pressure (22), and development of vascular endothelial cell damages (35). It has also been shown that resveratrol improves vascular endothelial and postischemic cardiac function in various experimental models (11, 23, 27, 34), which suggests that this agent may change the outcome of myocardial infarction in the diabetic state. However, although resveratrol can protect the myocardium of nondiabetic subjects, there are currently no or little data available about the ability of resveratrol to protect the heart in genetically modified Zucker obese diabetic rats in the presence or absence of glucose intake. Glucose-fed Zucker obese rats provide a model of dietary-induced insulin resistance, which can be used to assess the pathological mechanisms of cardiovascular damages associated with metabolic syndrome in the ischemic myocardium. Therefore, the present study was undertaken to determine the effects of 14 days of treatment with 5 mg/kg of resveratrol on postischemic cardiac function, infarct size, and arrhythmias after ischemia, followed by reperfusion in Zucker obese rats subjected to glucose intake for 3 wk, and to study the mechanism of the cardioprotective effect of resveratrol, with the hypothesis being that the presence of resveratrol would still confer a cardiac protection against ischemia-reperfusion-induced damage in the presence of glucose intake in Zucker obese rats. In addition, we investigated the effect of resveratrol related to endothelin (ET) and GLUT-4 signalings and apoptosis as potential mechanisms in ischemia-reperfusion-induced injury in hearts obtained from Zucker obese rats.

METHODS

All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research, and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication no. 86-23, revised in 1996). The use of animals in our experiments was approved by the Institutional Animal Care and Use Committee of the University of Debrecen, Debrecen, Hungary.

Animals and heart perfusion. Male 23- to 24-wk-old Zucker obese rats fed commercial food pellets were used for all studies. Animals were housed in wire-bottomed cages (three rats in each cage) for 7 days before starting the treatment and experimental studies and throughout the study and were maintained on a 12:12-h light-dark
cycle. In the first series of the study, Zucker obese rats were treated orally with 5 mg·kg⁻¹·day⁻¹ of resveratrol [obese + resveratrol (O+R)] for 2 wk. The results obtained in the O+R group were compared with the resveratrol-free obese control (OC) group. The second group of rats received tap water containing 10% of glucose ad libitum for 3 wk, and, during the 2nd- and 3rd-wk period, rats were orally treated with 5 mg·kg⁻¹·day⁻¹ of resveratrol [obese + glucose + resveratrol (O+G+R group)]. The results obtained in the O+G+R group were compared with the obese + glucose resveratrol-free group (O+G). Resveratrol was dissolved in ethanol and diluted with water (1:10), and 10 ml/kg of final volume were used for oral treatment of rats as a gavage each day.

At the end of each treated period, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg), and then intravenous heparin (5,000 IU) was given. Hearts were removed and perfused with modified Krebs-Henseleit bicarbonate buffer, according to the Langendorff technique for a 10-min washout period at a constant perfusion pressure equivalent to 100 cm of water (10 kPa). During the Langendorff perfusion, the pulmonary vein was cannulated, and the Langendorff preparation was switched to the working mode with 1.7 kPa of preload and 17.0 kPa of afterload for an additional 10-min equilibration perfusion period. The perfusion medium consisted of a modified Krebs-Henseleit bicarbonate buffer (mM concentration: sodium chloride 118, potassium chloride 4.7, calcium chloride 1.7, sodium bicarbonate 25, potassium biphosphate 0.36, magnesium sulfate 1.2, and glucose 10), and, after its oxygenization with 95% of oxygen and 5% of carbon dioxide, pH was 7.4 at 37°C. Our study had two single objectives: the first was whether resveratrol pretreatment could reduce the incidence of reperfusion-induced ventricular fibrillation (VF) and ventricular tachycardia (VT) and infarct size and improve the recovery of postischemic cardiac function. To achieve this, hearts (n = 6 in each group) were subjected to 30-min global ischemia followed by 120 min of reperfusion. The left atrial inflow and aortic outflow lines were clamped during ischemia at a point close to their origins, and reperfusion was initiated by unclamping the atrial inflow and aortic outflow lines.

The second objective of our experiments was to study whether resveratrol treatment could also attenuate the ischemia-reperfusion-induced myocardial damage. Zucker obese rats received 10% of glucose ad libitum for 3 wk, and resveratrol was orally administered in a daily dose of 5 mg/kg during the 2nd and 3rd wk. Then hearts were isolated and subjected to 30 min of ischemia followed by 120 min of reperfusion.

Preselected exclusion criteria for our studies demanded that isolated, working hearts were excluded if 1) ventricular arrhythmias occurred during the period before the induction of cardiac ischemia; and 2) coronary flow (CF) and aortic flow (AF) were <15 and 30 ml/min, respectively, before the initiation of global ischemia.

Indexes measured. An epicardial ECG was recorded by a polygraph throughout the experimental period by two silver electrodes attached directly to the heart. The ECGs were analyzed to determine the incidence of VF. Hearts were considered to be in VF if an irregular undulating baseline was apparent on the ECG. The heart was considered to be in sinus rhythm if normal sinus complexes occurring in a regular rhythm were apparent on the ECG. AF was measured by an inline flow rotameter. CF rate was measured by a timed collection of the coronary effluent that dripped from the heart. Before ischemia and during reperfusion, heart rate (HR), CF, and AF rates were registered. Left ventricular developed pressure (LVDP) was also recorded by the insertion of a catheter into the left ventricle via the left atrium and mitral valve. The hemodynamic parameters were registered by a computer acquisition system (ADInstruments, PowerLab, Castle Hill, Australia).

Measurement of infarct size. The myocardium for infarct size determination was perfused, at the end of each experiment, with 25 ml of 1% triphenyl tetrazolium solution in phosphate buffer (Na₂HPO₄ 88 mM, NaH₂PO₄ 1.8 mM) via the side arm of the aortic cannula, and stored at −70°C for later analysis. Frozen myocardium was sliced transversely (38) in a plane perpendicular to the apical-basal axis into 2- to 3-mm-thick sections, weighted, blotted dry, placed in between microscope slides, and scanned on a Hewlett-Packard Scanjet 5p single pass flat bed scanner (Hewlett-Packard, Palo Alto, CA). Using the National Institutes of Health Image 1.16 image processing software, each digitalized image was subjected to equivalent degrees of background subtraction, brightness, and contrast enhancement for improved clarity. The infarct zone in each slice was traced, and the respective area was calculated in terms of pixels (13). The areas were measured by computerized planimetry software, and these areas were multiplied by the weight of each slice, then the results were summed up to obtain the weight of the risk zone (total weight of the left ventricle; mg) and the infarct zone (mg). Infarct size was expressed as the ratio, in percent, of the infarct zone to the risk zone.

Measurements of serum glucose and insulin levels. Blood samples were obtained from rats before the excision and isolation of hearts, and serum glucose levels were measured by a spectrophotometer at a wavelength of 340 nm using standard assay kits (Sigma). Insulin levels were also determined from the same samples using radioimmunoassay kits (LINCO Research, St. Charles, MO) in all untreated, glucose-treated, and resveratrol-treated groups.

Western blots for GLUT-4 and ET-1 signaling. Heart tissues were homogenized and suspended (50 mg/ml) in sample buffer (10 mM HEPES, pH 7.3, 11.5% sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM diisopropyl fluorophosphate, 0.7 mg/ml pepstatin A, 10 mg/ml leupeptin, and 2 mg/ml aprotinin). Proteins were then solubilized with the addition of the same amount of 2X Laemmli solution [9% SDS (wt/vol), 6% mercaptoethanol (vol/vol), 10% glycerol (vol/vol), and a trace amount of bromphenol blue dye in 0.196 M Tris·HCl (pH 6.7)]. The cellular proteins (50-μg protein) were electrophoresed through 10% SDS-PAGE and then transferred to Immobilon-P membranes (Millipore, Billerica, MA) using a semidry transfer system (Bio-Rad, Hercules, CA). Prestained protein standards (Bio-Rad) were run in each gel. The blots were blocked in Tris-buffered saline/Tween 20 (20 mM Tris base, pH 7.6, 137 mM NaCl, and 0.1% Tween 20), supplemented with 5% BSA for 1 h, incubated with 1:1,000 diluted primary rabbit antibodies specifically against GLUT-4 and ET-1 (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, and then incubated with 1:5,000 diluted secondary antibodies of horseradish peroxidase-conjugated anti-rabbit IgG (Boehringer Mannheim, Mannheim, Germany) for 1 h at room temperature. After three washes of 5 min each, blots were treated with enhanced chemiluminescence (Amersham, Piscataway, NJ) reagents, and bands of GLUT-4 and ET-1 were detected by autoradiography for variable lengths of time (15 s to 3 min) with Kodak X-Omat film.

Determination of ET in coronary effluents. ET levels in response to resveratrol were determined in coronary effluents (41) of hearts obtained from Zucker obese rats, subjected to the presence or absence of glucose intake. Coronary effluents (100 ml) were collected in polystyrene containers spiked with EDTA and Triton X-100 to give a final concentration of 5 mM and 0.5% (vol/vol), respectively, in each sample, and then samples were frozen at −70°C until their use. Perfusates were loaded with 3 ml of methanol and 5 ml of water conditioned by Sep-Pak C₁₈ cartridges, and ET was eluted with 2 ml of 60% (vol/vol) acetonitrile in 0.1% (vol/vol) trifluoroacetic acid, yielding a mean final concentration of 60%. Eluates were freeze-dried and kept at −20°C until further radioimmunoassay determination. Residues were re-dissolved in 0.5 ml of assay buffer and concentrated 200 times. ET immunoreactivity was then determined by radioimmunoassay using commercial ET radioimmunoassay kits with cross-reactivity for ET-2 (142%), ET-3 (98%), big ET 1–38, and big ET fragments 22–38 (<1%), with interassay and intra-assay variations of <10 and 5%, respectively (as described by the commercial kit assay).

Determination of cardiomyocyte apoptosis. The formaldehyde-fixed left ventricle was embedded in paraffin, cut into transverse sections (4 μm thick), and deparaffinized with a graded series of H860 CARDIAC PROTECTION WITH RESVERATROL IN ZUCKER OBESE RATS

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xylene and ethanol solutions. Immunohistochemical detection of apoptotic cells was carried out using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) in which residues of digoxigenin-labeled dUTP are catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase, an enzyme that catalyzes a template-independent addition of nucleotide triphosphate to the 3'-OH ends of double- or single-stranded DNA (25). The incorporated nucleotide was incubated with a sheep polyclonal anti-digoxigenin antibody, followed by a FITC-conjugated rabbit anti-sheep IgG as a secondary antibody, as described by the manufacturer (Apop Tag Plus, Oncor, Gaithersburg, MD). The sections (n = 6) were washed in PBS three times, blocked with normal rabbit serum, and incubated with mouse monoclonal antibody recognizing α-sarcromeric actin (Sigma-Aldrich Biotec), followed by staining with tetrarhodamine isothiocyanate-conjugated rabbit anti-mouse IgG (1:200 dilution) (32). For detection of apoptosis in endothelial cells, the sections were first stained with TUNEL (FITC staining). The sections were then incubated with rabbit polyclonal anti-von Willebrand factor (Sigma-Aldrich Biotec), followed by staining with tetrarhodamine isothiocyanate-conjugated goat anti-rabbit IgG as a secondary antibody. The fluorescence staining was viewed with laser confocal microscopy (Fluoview, Olypmus, Tokyo, Japan). For quantitative purposes, the number of TUNEL-positive cardiomyocytes and endothelial cells was counted on ×100 high-power fields (HPF, magnification ×600) from the endocardium through the epicardium of the midportion of the left ventricular free wall in five sections from each heart (18, 37).

**Statistics.** Body weight, serum glucose, insulin levels, infarct size, HR, CF, AF, LVDP, ET-1 and GLUT-4 expression and/or repression, and apoptotic cell death were expressed as mean values ± SE. A two-way analysis of variance was first carried out to test for any differences in mean values between groups. If differences were established, the values of the drug-treated groups were compared with those of the drug-free group by Dunnett’s test. A different procedure, because of the nonparametric distribution, was used for the distribution of discrete variables, such as the incidence of VF. Thus the χ² test was used to compare the incidence of VF between untreated control and treated groups.

**RESULTS**

The results show (Table 1) body weight, serum glucose, and insulin levels in Zucker obese age-matched rats treated with glucose and resveratrol. Thus, in obese rats treated with 5 mg/kg of resveratrol for 2 wk, a significant reduction was observed in body weight and serum glucose from their control values of 414 ± 10 g and 7.08 ± 0.41 mmol/l to 378 ± 12 g (P < 0.05) and 6.11 ± 0.44 mmol/l (P < 0.05), respectively.

**Table 1. Effects of resveratrol and glucose intake on body weight, serum glucose, and insulin levels in Zucker obese rats**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body Weight, g</th>
<th>Serum Glucose, mmol/l</th>
<th>Insulin, mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean control</td>
<td>291 ± 7</td>
<td>5.2 ± 0.24</td>
<td>0.60 ± 0.04</td>
</tr>
<tr>
<td>Zucker obese control</td>
<td>414 ± 10</td>
<td>7.08 ± 0.41</td>
<td>4.08 ± 0.37</td>
</tr>
<tr>
<td>Zucker obese + 5 mg/kg resveratrol</td>
<td>378 ± 12*</td>
<td>6.11 ± 0.44*</td>
<td>4.58 ± 0.48</td>
</tr>
<tr>
<td>Zucker obese + 10% glucose</td>
<td>504 ± 16*</td>
<td>9.02 ± 1.20*</td>
<td>4.81 ± 0.36</td>
</tr>
<tr>
<td>Zucker obese + 10% glucose + 5 mg/kg resveratrol</td>
<td>428 ± 11†</td>
<td>7.21 ± 0.51†</td>
<td>4.72 ± 0.52</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 in each group. Comparisons were made to the *Zucker obese control values and †Zucker obese rats treated with 10% glucose for 3 wk; P < 0.05.

However, insulin levels were not changed in the resveratrol-treated group (Table 1).

In Zucker obese rats that obtained 10% of glucose ad libitum for 3 wk, a significant increase in body weight and serum glucose levels (P < 0.05) was detected (Table 1) compared with the glucose-free Zucker obese group. In another group, Zucker obese rats obtained 10% of glucose for 3 wk, and, during the 2nd and 3rd wk, rats also received orally 5 mg·kg⁻¹·day⁻¹ of resveratrol. Thus a significant reduction was observed in the body weight and serum glucose level from 504 ± 16 g and 9.02 ± 1.20 mmol/l (in the glucose-treated group) to 428 ± 11 g (P < 0.05) and 7.21 ± 0.51 mmol/l (P < 0.05) in the resveratrol-treated group, respectively. However, a change in serum insulin levels was not detected in any of resveratrol-treated groups (Table 1).

Table 2 shows the recovery of postischemic cardiac function in isolated hearts subjected to 30-min ischemia, followed by 120 min of reperfusion, obtained from age-matched Zucker obese rats treated with 5 mg/kg (for 2 wk) of resveratrol, in the presence or absence of 10% glucose. The results (Table 2) clearly show that postischemic recovery of CF, AF, and LVDP was significantly improved in the resveratrol-treated groups in the presence or absence of glucose intake compared with the resveratrol-free group. Thus, for instance, after 60 and 120 min of reperfusion, AF (Table 2) was significantly increased from their obese control values of 5.2 ± 0.4 and 5.1 ± 0.6 ml/min to 7.9 ± 0.6 (P < 0.05) and 7.8 ± 1.0 ml/min (P < 0.05) in the obese rats treated with 5 mg/kg of resveratrol, respectively. The same improvement in CF and LVDP was obtained (Table 2) in the obese rats treated with 5 mg/kg of resveratrol in the presence of 10% of glucose intake. However, HR was not significantly changed in hearts subjected to ischemia and reperfusion, in either the presence or absence of glucose intake (Table 2) in the resveratrol-treated groups.

The reduction in the incidence of reperfusion-induced VF in the resveratrol-treated groups is probably related to the recovery of postischemic cardiac function (Table 2) and the beneficial effects of resveratrol on the ischemic myocardium. Thus the incidence of reperfusion-induced VF was significantly reduced in rats treated with 5 mg/kg of resveratrol from its obese drug-free control value of 100% (Fig. 1) to 17% (P < 0.05) and 33% (P < 0.05) in the presence or absence of glucose intake, respectively.

Figure 2 shows the protective effect of resveratrol on the infarct size in ischemic-reperfused myocardium. Thus resveratrol significantly reduced the infarct size from 41 ± 6% (in the OC group) and 42 ± 7% (in the O+G group) to 21 ± 5% (P < 0.05, in the O+R group) and 26 ± 6% (P < 0.05, in the O+G+R group), respectively.

Figure 3 shows the effect of resveratrol on the expression or repression of ET-1 (top panel) and GLUT-4 (bottom panel) in isolated hearts subjected to 30 min of ischemia followed by 120 min of reperfusion, obtained from Zucker obese rats in the absence or presence of glucose intake. ET-1 expression was significantly increased in the OC and O+G group compared with the lean control (Fig. 3, top), and resveratrol substantially reduced ET-1 expression compared with the resveratrol-free OC and O+G groups. In contrast to ET-1, the repression of GLUT-4 (Fig. 3, bottom) was detected in isolated hearts obtained from Zucker obese rats in the absence (OC) or presence (O+G) of glucose. When 5 mg/kg of resveratrol were
Table 2. Cardiac function in ischemic-reperfused hearts obtained from Zucker obese rats and treated with resveratrol in the presence or absence of 10% glucose intake.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pre-ischemic</th>
<th>After 60-min Reperfusion</th>
<th>After 120-min Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AF, ml/min</td>
<td>LVDP, kPa</td>
<td>HR, beats/min</td>
</tr>
<tr>
<td>Lean control</td>
<td>25 ± 0.8</td>
<td>18 ± 0.5</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Zucker obese control</td>
<td>24 ± 1.0</td>
<td>17 ± 0.8</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Zucker obese + 5 mg/kg resveratrol</td>
<td>20 ± 0.5</td>
<td>14 ± 0.5</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Zucker obese + 10% glucose</td>
<td>20 ± 0.5</td>
<td>14 ± 0.5</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Zucker obese + 10% glucose + 5 mg/kg resveratrol</td>
<td>20 ± 0.5</td>
<td>14 ± 0.5</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 in each group. *P < 0.05 compared with the ischemic-reperfused O group. †P < 0.05 compared with the drug-free obese ischemic-reperfused control (OC) group.}

In relation to the endothelial signaling in Fig. 3, Fig. 4 shows ET concentrations in coronary effluent in various groups of ischemic-reperfused myocardium. Thus the ET concentration (Fig. 4) was significantly increased from 0.39 ± 0.13 fmol·min⁻¹·g⁻¹ (lean control group) to 1.49 ± 0.25 fmol·min⁻¹·g⁻¹ (OC group) and 1.79 ± 0.35 fmol·min⁻¹·g⁻¹ (O+G group). ET release was significantly attenuated (Fig. 4) in all groups treated with 5 mg/kg of resveratrol. Our results clearly show that attenuation in ET release in resveratrol-treated groups can lead to an elevated cardiac perfusion, which is indicated by an increase in global ischemia followed by 120 min of reperfusion, and the incidence of reperfusion-induced VF was registered. LC, lean control; OC, obese control; O, obese; R, resveratrol; G, glucose (n = 12 in each group). *P < 0.05 compared with the drug-free obese ischemic-reperfused control (OC) group. †P < 0.05 compared with the ischemic-reperfused O+G group.

Fig. 1. Incidence of reperfusion-induced ventricular fibrillation (VF). Rats received 10% of glucose ad libitum in drinking water for 3 wk. During the last 2 wk (2nd and 3rd wk), rats were orally treated with a daily dose of 5 mg/kg of resveratrol, and then hearts were isolated and subjected to 30 min of global ischemia followed by 120 min of reperfusion, and the incidence of reperfusion-induced VF was registered. LC, lean control; OC, obese control; O, obese; R, resveratrol; G, glucose (n = 12 in each group). *P < 0.05 compared with the drug-free obese ischemic-reperfused control (OC) group. †P < 0.05 compared with the ischemic-reperfused O+G group.

Fig. 2. Infarct size was measured in drug-free and resveratrol-treated groups. Rats received 10% of glucose ad libitum in drinking water for 3 wk. During the last 2 wk (2nd and 3rd wk), rats were orally treated with a daily dose of 5 mg/kg of resveratrol. Then hearts were isolated and subjected to 30 min of global ischemia followed by 120 min of reperfusion, and the infarct size was measured. Values are means ± SE; n = 6 in each group. *P < 0.05 compared with the drug-free obese ischemic-reperfused control (OC) group. †P < 0.05 compared with the ischemic-reperfused O+G group.
postischemic CF and AF (Table 2), leading to a better recovery (Table 2) in LVDP (contractility) during reperfusion.

Figure 5 shows apoptotic cell death in drug-free and resveratrol-treated groups in the presence or absence of glucose intake. TUNEL-positive nuclei were condensed, representing apoptotic cells (Fig. 5, left). Similar to myocardial infarct size, the numbers of apoptotic cardiomyocytes were significantly reduced (Fig. 5, right) when rats were pretreated by resveratrol and hearts were isolated and subjected to ischemia and reperfusion. Total numbers of cardiomyocytes at ×100 HPF, which covers almost all of the midportion of the left ventricular free wall, were examined for detecting apoptotic cells. The data are expressed in counts/100 HPF and not in percentage of apoptotic cells. For subjects treated with 5 mg/kg of resveratrol, the numbers of cardiomyocyte apoptotic cells were significantly reduced after ischemia-reperfusion in the presence or absence of glucose intake.

DISCUSSION

Increased oxidative stress, including myocardial ischemia-reperfusion, has been implicated in various disease models, such as metabolic syndrome and diabetes. The metabolic syndrome is a complex disease, as defined by the American Heart Association, and consists of hypertension, prothrombotic and proinflammatory states, abdominal obesity, atherogenic dyslipidemia, and insulin resistance. The characteristics of metabolic syndrome are known to be significant risk factors in cardiovascular morbidity, leading to mortality. The metabolic syndrome encompasses Type 2 diabetes, estimating that there will be >300 million cases in the industrialized societies by 2010. The possibility of preventing Type 2 diabetes by different interventions that affect the lifestyle of people at high risk for the development of various cardiovascular diseases is now a subject of a number of clinical and experimental studies. Prevention of cardiovascular complications is a key issue because of the huge premature morbidity and mortality associated with Type 2 diabetes. Diabetic patients have a higher incidence and severity of angina and acute myocardial infarction (31) and have almost twice the rate of mortality compared with nondiabetic subjects (40). In obese and diabetic animal studies, increased ischemic damage has also been reported (20, 31), and normalization of cardiac metabolism in hearts from these animals has been shown to improve the recovery of cardiac function following an episode of ischemia-reperfusion (39).

Glucose transport and glycolytic rates determine the extent of ischemia and the recovery of postischemic function in the myocardium (28, 39, 47). Adipose tissue has been proposed to be the site of insulin resistance and free fatty acid-induced changes in GLUT-4 expression or vesicle trafficking budding or fusion (5, 24). In experiments (9, 14), the impact of raising free fatty acid concentrations on phosphatidylinositol 3-kinase, an important component of insulin signaling cascade in regulating GLUT-4 translocation, was studied. They found that the increase in phosphatidylinositol 3-kinase activity detected in response to insulin stimulation was virtually abolished by lipid
application. Substantial reductions in GLUT-4 levels have been registered in insulin resistance, such as Type 2 diabetes and insulin-deficient states (12, 36). In studies, using cardiac-specific GLUT-4 transporter protein knockout mice showed a reduced glucose uptake and increased myocardial ischemic damage (42). In addition, in cardiac-specific peroxisome proliferator-activated receptor-α (PPAR-α) transgenic mice, the expression of genes involved in fatty acid uptake and oxidation was increased, whereas the repression of genes (GLUT-1 and GLUT-4) involved in glucose transport was increased, a metabolic pathway similar to that of the diabetic myocardium (15). Thus changes in glucose metabolism are definitely determined via the modulation of GLUT-4.

The goal of our study, at least in part, was to determine the importance of GLUT-4 associated with resveratrol treatment and susceptibility of the myocardium obtained from Zucker obese rats to ischemia-reperfusion-induced injury. The results clearly show that resveratrol significantly increased the expression of GLUT-4, which could be an important component in the reduction of apoptotic cell death and the recovery of postischemic cardiac function, because glucose uptake and subsequent glycolysis are the main source of ATP production when oxidative phosphorylation is limited (10). GLUT-4 transporters physiologically reside in plasma membrane vesicles and translocate to the cell membranes by the stimulation of insulin or stress, e.g., ischemia-reperfusion, acting as a main determinant of glycolytic flux. As a consequence, any intervention that inhibits the translocation of GLUT-4 increases the risk of cardiac damage. In our study, Zucker obese rats were subjected to glucose intake to accelerate the development of obesity-induced metabolic and functional changes in the myocardium. Thus the obtained results show that glucose intake resulted in a poor recovery in cardiac function with decreased GLUT-4 expression in obese Zucker rats, and resveratrol interferes with the repression of GLUT-4. In fact, GLUT-4 repression has profound changes in cardiac dysfunction and arrhythmias with accelerated apoptotic cell death. Although it was not the aim of our investigation, it is interesting to note that it is unlikely that all of the observed cardiac protection by resveratrol occurred directly via PPAR-α target, and GLUT-4 gene repression or expression is probably also modulated via PPAR-α-independent transcriptional regulatory pathways linked to alteration in cellular energy metabolism (4, 6).

Additionally, it is well known that the endothelium plays an important role as a target of a variety of cardiovascular risk factors in a wide range of heart diseases, including atherosclerosis (43), angina (26), and ischemia (45, 46). Vascular endothelial cells participate in cardiovascular regulation by producing several potent vasoactive agents, including the vasoconstrictor molecule ET. A dysfunction in vascular endothelium, identified as increased ET production, has been implicated in the pathophysiology of a number of cardiovascular diseases. Therefore, in the present study, we investigated the effects of resveratrol on ET production and signaling and recovery of postischemic cardiac function in hearts obtained from Zucker obese rats in the presence or absence of glucose intake. Elevated glucose levels are a potent stimulus for the expression and production of ET-1 (44), and, as a consequence of diabetes, the cardiac dysfunction is well documented, and it is also known that ET, especially ET-1, levels are increased in the heart in diabetic subjects (2). Because ET levels are elevated in the myocardium and vasculature in diabetic hearts, its direct toxic effect on cardiac myocytes cannot be questioned. Resveratrol may reduce the expression of ET, leading to the reduction of ET production. It is of interest to note that the effect of resveratrol was not directly connected with various ET receptor blockades in endothelial and myocardial cells, but resveratrol significantly inhibited strain-induced ET mRNA level, protein expression, and promoter activity (30). In additional studies, it was also proven that resveratrol inhibited angiotensin II-induced cell proliferation and ET expression (8), which is related to the phosphorylation of angiotensin II-induced extracellular signal regulated kinase activity. We found that resveratrol significantly reduced ET expression in both OC and O+G intake groups, and the reduction in ET expression was reflected in a direct attenuation of ET production in the resveratrol-treated hearts, in the absence or presence of glucose intake. Thus resveratrol was able to reduce both the

**Fig. 5. Detection of apoptotic cells in various groups.** Left: representative pictures of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay for apoptotic cardiomyocytes corresponding to the numerical values of the right panel. Double immunofluorescence staining for α-sarcoceric actin (specific for cardiomyocyte; red fluorescence) and TUNEL-positive (green fluorescence) were used. The immunohistochemistry and sampling were carried out at the end of the reperfusion period. Data are expressed at counts/100 high-power field. Right: bar graphs show the extent of particular cell death by apoptosis. Aerobe control value is not shown by a bar on the right panel, because no apoptotic cell was detected under this condition. Values are means ± SE; n = 6 in each group. *P < 0.05 compared with the drug-free ischemic-reperfused OC group; †P < 0.05 compared with the ischemic-reperfused O+G group.
ET expression and concentration in the ischemic-reperfused myocardium obtained from Zucker obese rats. The protective effect of resveratrol, at least in part, could also be originating from its well-known antioxidant property against the development of atherosclerosis, because resveratrol is able to modulate lipid and lipoprotein metabolism protecting LDL particles via the inhibition of the peroxidation of membrane lipids (16).

In conclusion, the beneficial effects of resveratrol include an increase in GLUT-4 expression and a reduction in ET expression and production in glucose-feed obese Zucker rats, evidenced by a significant improvement in postischemic cardiac function and a reduction in the incidence of reperfusion-induced arrhythmias. However, it is also interesting to note that resveratrol did not modify insulin production compared with the resveratrol-treated groups, although a significant reduction in body weight and serum glucose was detected. These results suggest that sufficient supplementation of resveratrol could help to prevent, or at least delay, the occurrence and complication of myocardial ischemia-reperfusion-induced damage in the insulin-resistant states.

REFERENCES


