

Effect of a β_2 -Adrenoceptor Stimulation on Hyperglycemia-Induced Endothelial Dysfunction

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ABSTRACT

To investigate whether β_2 -adrenoceptors exist on endothelial cells and whether a β_2 -adrenoceptor stimulation might prevent the development of hyperglycemia-induced endothelial dysfunction, porcine aortic endothelial cells (PAECs) were cultured and chronically exposed to either 5 mM D-glucose ("normoglycemia") or 20 mM D-glucose ("hyperglycemia"), with or without 100 nM salbutamol in absence or presence of β_2 -adrenoceptor antagonist ICI 118,551 [1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)-amino]-2-butanol] or β_1 -antagonist metoprolol. For osmotic control, PAECs were exposed to 15 mM L-glucose. We measured nitric oxide release using the met-hemoglobin assay and assessed β -adrenoceptor density and subtypes by radioligand binding. Furthermore, we determined intracellular NADH and NADPH using high-performance liquid chromatography. High D-glucose concentrations but not L-glucose led to significantly re-

duced basal and stimulated nitric oxide release. Chronic salbutamol treatment significantly antagonized the impairment of the nitric oxide response, which was inhibited by ICI 118,551 but not by metoprolol. The number of giant cells was significantly increased in hyperglycemia, which could be prevented by salbutamol. Binding of the radioligand (-)-[¹²⁵I]iodocyanopindolol revealed a total β -adrenoceptor density of 29.8 ± 3.7 (normoglycemic) and 30.3 ± 3.6 (hyperglycemic) fmol/mg protein. Displacement by ICI 118,551 revealed β -adrenoceptor subtype distribution with 30.3 ± 4.4 (normoglycemic) and $29.1 \pm 3.8\%$ β_2 -adrenoceptors. NADH production increased in hyperglycemia, which was completely prevented by salbutamol. We conclude that hyperglycemia in PAEC induces endothelial dysfunction with impaired nitric oxide release and that this can be prevented by β_2 -adrenoceptor stimulation.

An intriguing problem in antidiabetic therapy is the development of generalized angiopathy and concomitant hypertension in diabetes mellitus. The nature of the underlying processes is complex and not yet fully understood. Several lines of evidence suggest that vascular endothelial function is disturbed in diabetes (Fortes et al., 1983; Cagliero et al., 1991; Sank et al., 1991; Hein and King, 1996; Magill and Dananberg, 1996; Donnelly et al., 2000). In isolated mesenteric beds of Wistar rats with 6 months diabetes, a reduction in endothelium-dependent relaxation was found (Olbrich et al., 1996, 1999). Investigations on the influence of high glucose concentrations on cultured porcine aortic endothelial cells (PAECs) under chronic exposure during one entire cell culture passage indicated hyperglycemia-induced endothe-

lial dysfunction as revealed by reduced endothelial nitric oxide (NO) production or release (Olbrich et al., 1999; Dhein et al., 2003), severe changes in endothelial cell structure, e.g., an increasing incidence of giant cells with increased cytoplasmic and total cell area and decreased NADPH-diaphorase activity (Salameh et al., 1997) as well as impaired intracellular calcium handling and diminished calcium signals (Kamata et al., 1995; Salameh and Dhein, 1998).

An interesting recent finding was that the development of endothelial dysfunction could be antagonized by celiprolol (Olbrich et al., 1999), a selective β_1 -adrenoceptor antagonist with additional β_2 -partial agonistic activity (Dhein et al., 1992). This protective effect was specific for celiprolol and was not seen with the β_1 -selective adrenoceptor-antagonist metoprolol lacking a β_2 -agonistic effect (Olbrich et al., 1999). Thus, the protective effect might be due to β_2 -adrenoceptor stimulation. In consequence, we supposed that β_2 -adrenoceptor stimulation might exert protective effects against hyperglycemia-induced endothelial dysfunction. In further support

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ABBREVIATIONS: PAEC, porcine aortic endothelial cell; NO, nitric oxide; PKA, protein kinase A; ROS, reactive oxygen species; eNOS, endothelial nitric-oxide synthase; Oxy-Hb, oxyhemoglobin; ICYP, (-)-[¹²⁵I]iodocyanopindolol; HPLC, high-performance liquid chromatography; NBT, nitro blue tetrazolium; PKC, protein kinase C; CGP12177, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one; ICI 118,551, 1-[2,2-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)-amino]-2-butanol.

of this hypothesis, Ferro et al. (1999) showed in human umbilical vein endothelial cells that β_2 -adrenoceptor stimulation enhances nitric oxide release via activation of the cAMP/PKA pathway. The reduced NO release in hyperglycemia has been hypothesized to be at least partially due to enhanced production of reactive oxygen species (ROS) (Tessarum, 1994) due to enhanced glucose flux through glycolysis and ROS generation in the mitochondrion (Nishikawa et al., 2000; Du et al., 2001). This enhanced glucose flux should result in increased reduced form of nicotinamide adenine dinucleotide (NADH) and pyruvate generation and in consequence, enhanced electron transport in the mitochondrion and ROS production (Nishikawa et al., 2000). Because β_2 -adrenoceptor stimulation might interfere with glucose and pyruvate metabolism, theoretically this could influence NADH generation. Moreover, it was hypothesized that eventually a shortage in NADPH supply via the activation of poly(ADP-ribose) polymerase (an NADPH-consuming process, which can be activated in diabetes mellitus) might reduce endothelial nitric-oxide synthase (eNOS) function, because NADPH is essential for eNOS function (Soriano et al., 2001). Thus, a change in either NADH or NADPH might contribute to endothelial dysfunction and might be influenced by β_2 -adrenoceptor stimulation.

Other factors such as activation of protein kinase C (PKC) (Hempel et al., 1997), advanced glycation end products (Nakamura et al., 1993), changes in the polyol pathway (Cameron and Cotter, 1992), and altered arginine utilization (Pieper and Peltier, 1995) are also known to contribute to endothelial dysfunction in hyperglycemia. Moreover, it should be kept in mind that non-NO factors, such as prostacyclin and other eicosanoids may also be involved in endothelial dysfunction. However, to investigate the background of our previous in vivo observation of prevention of impairment of NO release in diabetes mellitus and hyperglycemia by celiprolol and not metoprolol, indicating a possible role for β_2 -adrenoceptors (Olbrich et al., 1999), our present study was focused on the role of β_2 -adrenoceptor stimulation in the NO release in hyperglycemia.

Thus, the aim of the present study was 1) to investigate whether β -adrenoceptor-subtypes can be identified in confluent porcine aortic endothelial cells cultured under normal and high glucose conditions; 2) whether these subtypes (β_1 - or β_2 -adrenoceptors) might be altered in hyperglycemia; 3) whether β_2 -adrenoceptor stimulation by salbutamol, a widely used β_2 -adrenoceptor agonist, can antagonize hyperglycemia-induced endothelial dysfunction as characterized by impaired nitric oxide release; and 4) whether β_2 -adrenoceptor stimulation induces intracellular changes of reduced nicotinamide adenine dinucleotide phosphates levels.

Materials and Methods

All experiments were performed according to the ethical rules of the Council for International Organization of Medical Science and the German laws for animal welfare.

Cell Isolation and Culture

In previous investigations, we established a subchronic cell culture model of hyperglycemia-induced endothelial dysfunction (Salameh and Dhein, 1998) using PAECs exposed to hyperglycemia for an entire culture cell passage (3–4 days). For that purpose, PAECs were isolated and cultured in medium 199 with 10%

fetal calf serum as described previously (Salameh et al., 1997; Dhein et al., 2003). Briefly, PAECs were harvested from porcine thoracic aorta by treatment with 1 mg/ml dispase for 15 min at 37°C, rinsed with medium 199 (containing 10% fetal calf serum, 5 mM D-glucose, 2 mM L-glutamine, 30 mM HEPES, 100 mg/l penicillin G, and 100 mg/l streptomycin), and seeded (100,000 cell/cm²) in plastic 9.6-cm² Petri dishes (Nunc, Wiesbaden, Germany) coated with 0.1% gelatin. The cells were kept in a type 2500 E NuAire IR autoflow water-jacketed incubator (Zapf Instruments, Sarstedt, Germany) at 37°C, saturated humidity, and 5% CO₂. After reaching confluence (5–8 days), cells were passaged using trypsin (0.05%) and EGTA (0.02%) and subcultured in 25-cm² plastic flasks (first passage), with a split ratio of 1:3. To achieve sufficient amounts of cells of the same aorta, the cells were again subcultured on 75-cm² plastic flasks with a split ratio 1:5 (second passage). After reaching confluence cells were subcultured another time (third passage) and seeded either on 148-cm² glass petri dishes for the binding studies or on 64-cm² glass petri dishes (Schott-Glas, Mainz, Germany) for NO measurement. At the beginning of the third passage, the cells were submitted to the various treatments (see below). Purity of the cell culture was tested by uptake of 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine-acetylated low-density lipoprotein (Voyta et al., 1984) and, for detecting contaminating smooth muscle cells, by staining of α -smooth muscle actin (Rosenthal and Gotlieb, 1990). The treatment of the different control or experimental groups started at the third passage and lasted until the cells had reached confluence (3–4 days). Medium was changed every 48 h.

Preparation of Endothelial Cells for the Binding Assay

Binding studies were performed according to Brodde et al. (1998) and O'Hara and Brodde (1984) with two experimental groups of cultured PAECs in passage 3 subjected to 5 mM D-glucose ("normoglycemia") or 20 mM D-glucose (high D-glucose, "hyperglycemia"). After reaching confluence in culture passage 3, PAECs were washed twice with ice-cold phosphate-buffered saline, pelleted, and resuspended in ice-cold 154 mM NaCl solution, centrifuged at 1000 rpm, 5 min, 4°C and resuspended in 1 mM KHCO₃ solution. Cells were disrupted by Ultra-turrax and centrifuged at 20,000 rpm (20 min, 4°C). The pellet was resuspended in Tris buffer (10 mM Tris, 154 mM NaCl, 0.55 mM ascorbic acid, pH 7.4), subjected to protein determination according to Bradford (1976) using bovine immunoglobulin G as a standard, and diluted with Tris buffer to equal amounts at a protein concentration of 20 μ g/sample (150 μ l).

Binding Assay

Saturation binding assays were routinely carried out as described previously (Brodde et al., 1998; Dhein et al., 2000) in new disposable polypropylene tubes (Sarstedt, Nürnberg, Germany). Briefly, (-)-[¹²⁵I]iodocyanopindolol (ICYP) and all drugs were prepared in ice-cold incubation buffer (10 mM Tris, 154 mM NaCl, 0.55 mM ascorbic acid, pH 7.4). Aliquots of 20 μ g of protein per sample were incubated at six concentrations of (-)-[¹²⁵I]ICYP ranging from 5 to 200 pM in a total volume of 250 μ l for 90 min at 37°C in a shaking water bath (Brodde et al., 1998; Dhein et al., 2000). Incubation was terminated by adding 10 ml of 10 mM Tris, 154 mM NaCl buffer, pH 7.4, to the entire reaction mixture followed by rapid filtration over Whatman GF/C filters. Each filter was washed with additional 10 ml of buffer and radioactivity of the wet filters was determined in a gamma counter (Beckmann Coulter gamma 4000). Nonspecific ICYP binding was defined as binding to membranes that could not be displaced by a high concentration of the nonselective β -adrenoceptor antagonist (\pm)-CGP12177 (1 μ M). Specific ICYP binding was defined as total binding minus nonspecific binding and was usually 70 to 80% at 50 pM ICYP.

Displacement

To determine the relative amount of β_1 - and β_2 -adrenoceptors competition binding experiments were carried out. Therefore, PAEC membranes were incubated for displacement with ICYP (100 pM = 100,000 cpm; this concentration was used in accordance with previous studies; Dhein et al., 2000; Brodde et al., 1998) and because a saturation of ICYP binding was found in the range of 80 to 100 pM in presence or absence of 21 concentrations (ranging from 10^{-9} to 10^{-4} M) of the highly selective β_2 -adrenoceptor-antagonist ICI 118,551 (Brodde and Michel, 1999). Specific binding was determined as described above.

NO Measurement

To characterize endothelial function, we measured the NO release spectrophotometrically (UV-DU-7500; Beckmann Coulter, Munich, Germany) as described by Feelisch and Noack (1987) and Dhein et al. (2003) under basal conditions and after stimulation with ATP (1 mM) using the methemoglobin assay, based on the rapid oxidation of reduced methemoglobin (Oxy-Hb, oxyhemoglobin, Fe^{2+}) to methemoglobin (Fe^{3+}) by NO. The suitability and specificity of this assay has been demonstrated previously (Kelm et al., 1997). We monitored increasing amounts of methemoglobin versus oxyhemoglobin by means of the difference spectrum. The bioassay was calibrated as described previously (Feelisch and Noack, 1987; Kelm et al., 1997). We found an extinction coefficient ϵ of $39 \text{ mM}^{-1} \text{ cm}^{-1}$, which was nearly identical to that described by Feelisch and Noack (1987). After reaching confluence, PAECs

were washed three times with HEPES buffer (composed of 145 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1.0 mM MgCl_2 , 10.0 mM HEPES, 5 mM D-glucose) at pH 7.4, 37°C ., preincubated with 4 ml of HEPES buffer for 20 min, 37°C ., and supplemented with Oxy-Hb-solution (4 μM). After an equilibration period of 50 min, 1 mM ATP was added and the resulting NO release was recorded for 40 min, cycling time was 10 min, at 37°C ., for each cell culture condition intraindividually. A cell-free Oxy-Hb solution served as control.

To investigate the effect of hyperglycemia with and without concomitant incubation with β_2 -adrenoceptor agonist salbutamol, the following experimental protocols were carried out with PAECs of the same cell line for intraindividual control (i.e., all cells were derived from the same aorta): 5 mM D-glucose alone ($n = 11$) and 20 mM D-glucose (high D-glucose, $n = 11$), 5 mM D-glucose plus 100 nM salbutamol ($n = 5$) and 20 mM D-glucose plus 100 nM salbutamol ($n = 5$), 5 mM D-glucose plus 1000 nM salbutamol ($n = 8$) and 20 mM D-glucose plus 1000 nM salbutamol ($n = 8$).

To determine the possible influence of osmotic pressure, we used the following experimental groups: 5 mM D-glucose alone ($n = 6$), 20 mM D-glucose (high D-glucose, $n = 6$), and 5 mM D-glucose plus 15 mM L-glucose (for osmotic control, $n = 6$).

To assess β_2 -adrenoceptor subtype specificity, we used 5 mM D-glucose alone ($n = 3$), 20 mM D-glucose (high D-glucose, $n = 53$), 20 mM D-glucose plus 100 nM salbutamol plus 50 nM ICI 118,551 ($n = 53$), 20 mM D-glucose plus 1000 nM salbutamol plus 50 nM of the β_2 -adrenoceptor antagonist ICI 118,551 ($n = 53$), and 20 mM D-

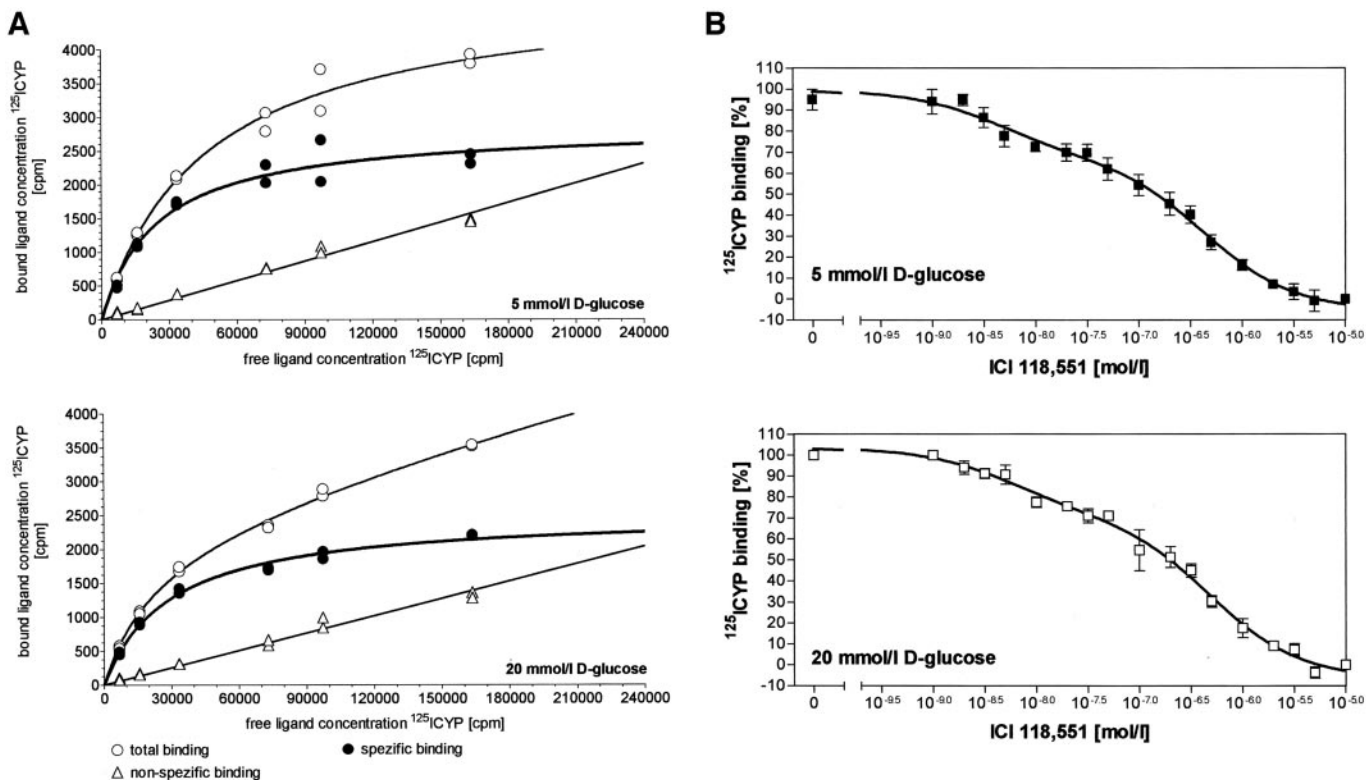


Fig. 1. A, representative saturation plot of specific (—) [^{125}I]ICYP (closed circles) plus total binding (open circles) and nonspecific binding (open triangles) to crude cell membranes from porcine aortic endothelial cells cultured under normo- and hyperglycemic environment in passage 3. Membranes were incubated with various concentrations of ICYP for 90 min at 37°C , and specific binding was determined as described under *Materials and Methods*. Ordinate, ICYP binding for total binding, nonspecific binding, and specific binding (the difference between the other two). Specific binding was figured by a saturation binding curve (1000 cpm equals 0.25 fmol of radiolabeled ligand bound/20 μg of membrane protein). Abscissa, concentration of radioligand (100,000 cpm equals 100 pM). Data are means of duplicate determinations from a single experiment. B, effect on inhibition of (—) [^{125}I]iodocyanopindolol binding by increasing concentrations of the β_2 -adrenoceptor-selective antagonist ICI 118,551 in crude membrane preparations from porcine aortic endothelial cells cultured under normo- (top) and hyperglycemic (bottom) environment in passage 3. Membrane protein (20 μg /250 μl) was incubated with ICYP in the presence of various concentrations of ICI 118,551 and specific binding was performed as described under *Materials and Methods*. Ordinate, ICYP binding in percentage of total binding (=100%); abscissa, molar concentrations of ICI 118,551. Each value is the mean of three experiments (=three different cell lines investigated) carried out in duplicate determination.

TABLE 1

Results of radioligand binding studies

Saturation binding experiments: Radioligand and total β -adrenoceptor number from saturating binding experiments using $(-)[^{125}\text{I}]\text{ICYP}$ in crude membrane preparations from cultured porcine aortic endothelial cells exposed to either normoglycemic or hyperglycemic conditions for passage 3. Data are means \pm S.E.M. of five experiments (five different cell lines), achieved from duplicate determination of each ICYP-concentration applied. Note that K_D and B_{max} are not changed in hyperglycemia. Displacement binding study: Adrenoceptor inhibitor affinity and fraction from competition binding experiments ($n = 3$ for each group) using $(-)[^{125}\text{I}]\text{ICYP}$ as radioligand and ICI 118,551 (10^{-10} to 10^{-3} M) as inhibitor in crude membrane preparations from porcine aortic endothelial cells exposed to either normoglycemia or hyperglycemia for complete cell culture passage 3. K_D values for ICI 118,551 are given for the high- and the low-affinity state. Note that the affinity of ICI 118,551 and the β_1 : β_2 -adrenoceptor ratio are not changed in hyperglycemia.

	D-Glucose	
	5 mM	20 mM
Saturation study		
K_D (pM)	29.75 \pm 3.69	30.32 \pm 3.55
B_{max} (fmol/mg protein)	30.32 \pm 5.7	29.99 \pm 6.44
Displacement study		
K_D high (nM)	4.43 \pm 0.28	5.71 \pm 0.26
K_D low (nM)	441 \pm 11	495 \pm 9
β_1 : β_2 -AR-ratio (%)	69.67 \pm 4.43:30.33 \pm 4.43	70.78 \pm 3.79:29.13 \pm 3.79

glucose plus 1000 mM salbutamol plus 100 nM of the β_1 -adrenoceptor antagonist metoprolol ($n = 53$).

The specific treatment of the different control or experimental groups started at the third passage and lasted until the cells had reached confluence (3–4 days). Medium was changed every 48 h.

Measurement of the Reduced Form of Pyridine Nucleotides

To quantify intracellular content of NADH and NADPH high-performance liquid chromatography (HPLC) was performed as described by Kashiwagi et al. (1994) with PAECs subjected to 5 mM D-glucose (normoglycemia) or 20 mM D-glucose (hyperglycemia) without or with 100 nM salbutamol during entire cell culture passage 3. When confluence was reached (3.5 ± 0.5 days), cells were shock-frozen with liquid nitrogen and harvested in a 1.0-ml aliquot of extraction buffer (3 volumes of 10 mM potassium phosphate buffer, pH 8.5, to 7 volumes of ethanol), disrupted by sonication on ice, and dissolved for 30 min at room temperature. Protein determination was performed as described above according to Bradford (1976). After centrifugation (13,000 rpm, -9°C , 15 min) a 20- μl aliquot of the supernatant was subjected to HPLC. HPLC was performed using a Serva DEAE Daltosil 100 anion exchanger column in an HPLC system (Merck-Hitachi, Darmstadt, Germany) equipped with autosampler AS-4000, L6200 A intelligent pump, and F1050 fluorescence detector (all from Merck-Hitachi). The column was equilibrated in 75 mM potassium phosphate buffer containing 20% methanol at pH 7.4. The fluorescence intensity of the effluent was monitored. The retention times of NADH and NADPH were 6.8 and 14.5 min, respectively, in accordance with Kashiwagi et al. (1994).

Histology

Hematoxylin-Eosin Staining. Cells after the various treatments were fixed with formalin (3.7%) and were submitted to hematoxylin-eosin staining following classical histological protocols as described previously (Salameh et al., 1997). The number of giant cells (cells 3–4 times larger than normal endothelial cells) was counted under the microscope at 400 \times magnification within a visual field of 40 mm², arbitrary marked. [x visual fields per experimental series (i.e., y visual fields per cell line)].

NADPH-Diaphorase Staining. NADPH-diaphorase reaction was carried out according to Salameh et al. (1997) and Hope et al. (1991) using 0.5 mM nitro blue tetrazolium (NBT) (with 1 mM β -NADPH, 0.2% Triton X-100, 50 mM Tris, and 75 mM NaCl, pH 8.0) for 20 h at 37 $^\circ\text{C}$. Briefly, confluent monolayers were fixed in 4% paraformaldehyde (30 min, room temperature) and incubated in the staining solution (0.5 mM nitro blue tetrazolium, 1 mM β -NADPH, 0.2% Triton X-100, 50 mM Tris, and 75 mM NaCl, pH 8.0), for 20 h at 37 $^\circ\text{C}$. Thereafter, the preparations were washed three times in phosphate-buffered saline and embedded in Karion F. For quantitative analysis, the histological specimens were viewed through a microscope (Leitz, Wetzlar, Germany) equipped with videocamera

(video 8, CCD-V90E sensitivity, 7 lux; Sony, Tokyo, Japan) and an image analysis system (QuickCapture Board DT 2855; Data Translation Inc., Marlboro, MA; software: JAVA (Jandel video analysis software; Jandel Scientific, Erkrath, Germany) allowing the determination of NADPH-diaphorase activity by evaluating the intensity of the blue color staining for NADPH-diaphorase (reduced nitro blue tetrazolium) of approximately 100 cells within an area of interest in each cell line.

Statistical Analysis

Experimental data given in text, figures, and tables are means \pm S.E.M. of n experiments. Binding assays and displacements were analyzed by the iterative curve-fitting program GraphPad Prism (GraphPad Software Inc., San Diego, CA). The equilibrium constants (K_D) and the maximal number of binding sites (B_{max}) were calculated from plots with linear regression. IC_{50} values for inhibition of binding by the ICI118,551 were calculated from concentration inhibition curves and converted into K_I values according to the equation of Cheng and Prusoff (1973): $K_I = \text{IC}_{50}/(S/K_D + 1)$, with IC_{50} the concentration of a β -adrenergic drug that inhibits specific binding of ICYP by 50%, S the concentration of radioligand present in the assay, and K_D resembling the equilibrium constant for ICYP. Statistical analysis was performed using the F ratio test to measure the goodness of fit of the competition curves for either one or two sites.

For statistical analysis of NO measurement, NADPH-diaphorase staining, number of giant cells, and nucleotide assessment, a multifactorial analysis of variance was performed. If analysis of variance indicated significant differences or significant interactions between disease and treatment, the data were further analyzed with a post hoc Tukey honestly significant difference test corrected for multiple measurements. For the statistical analysis, we used Systat for Windows software, version 5.02 (Systat, Evanston, IL). Differences were considered significant if $p < 0.05$.

Materials

The following materials were obtained from Sigma Chemie (Deisenhofen, Germany): medium 199, HEPES, trypsin, glutamine, gelatin, penicillin G (1650 U/mg), streptomycin, Dulbecco's phosphate-buffered saline (without Ca^{2+} and Mg^{2+}), D-glucose, L-glucose, salbutamol-hemisulfate, metoprolol, hemoglobin, ATP, anti- α -smooth muscle actin (mouse), and anti-mouse IgG fluorescein isothiocyanate-linked antibody. 1,1'-Diocetadecyl-3,3,3'-tetramethyl-indo-carbocyanine-acetylated low-density lipoprotein was purchased from Paesel & Lorei (Frankfurt, Germany). Fetal calf serum was from Invitrogen GmbH (Karlsruhe, Germany), dispase was obtained from Roche Diagnostics (Mannheim, Germany), the cell culture plastic material was from Nunc and IWAKI Glass (Tokyo, Japan), and glass Petri dishes were from Schott (Mainz, Germany). The following chemicals were derived from Merck (Darmstadt, Germany): ascorbic acid, CaCl_2 , KCl, KHCO_3 , MgCl_2 , and NaCl. $(-)[^{125}\text{I}]\text{ICYP}$ (specific activity 2200 Ci/mmol, stock solution 100,000 cpm = 100 pM) was

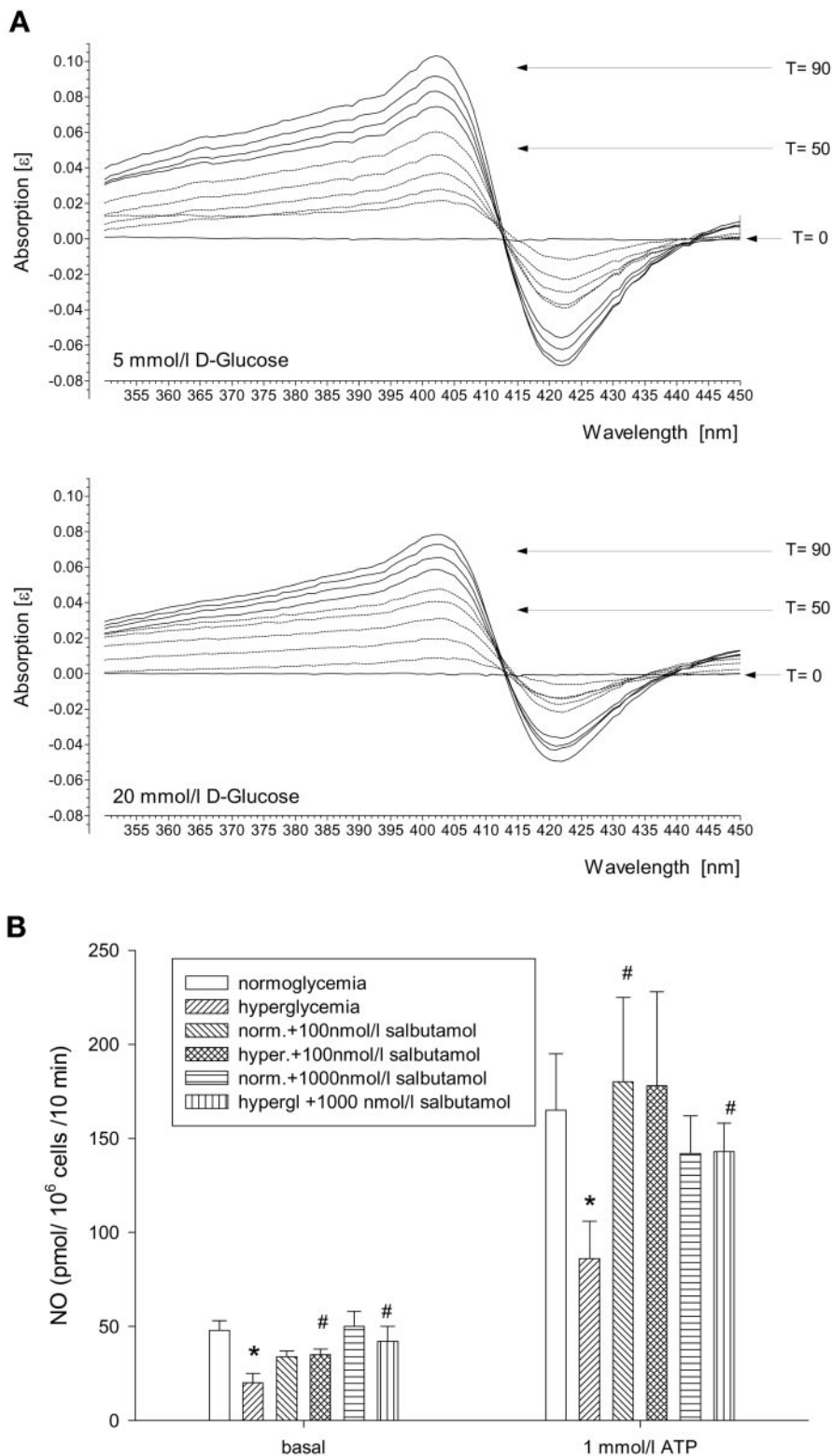


Fig. 2. A, original difference spectrum of oxy-hemoglobin versus increasing amounts of methemoglobin obtained by interaction with NO release from cultured PAECs under normal (top) and hyperglycemic (bottom) conditions over a period of 90 min exhibiting the characteristic absorbance maximum at 401 nm with an isobestic point at 411 nm, a negative absorbance maximum at 421 nm, and straight lines between 401 and 411 nm. (± 1.25 nm tolerance regarding the precision of the instrument). The reaction was carried out in HEPES-buffered solution in the presence of $4.0 \mu\text{M}$ oxyhemoglobin at pH 7.4 and 37°C , as described under *Materials and Methods*. Note the increase of methemoglobin formation evident from increasing absorbance with time and its attenuation in hyperglycemic cells. B, release of NO from normo- and hyperglycemic endothelial cells with or without additional salbutamol treatment at low (100 nM) and high (1000 nM) concentrations under basal conditions (left) and after stimulation with 1 mM ATP (right). All values are given as means \pm S.E.M. Statistical difference to normoglycemia is marked by an asterisk; statistical difference to hyperglycemia is marked by # ($p < 0.05$). All values are given as the means \pm S.E.M. Note that salbutamol prevents from hyperglycemia-induced reduction in basal and stimulated NO release.

supplied by PerkinElmer Life Sciences (Boston, MA), and ICI118,551 hydrochloride was from Sigma/RBI (Natick, MA). All chemicals were of analytic grade and were dissolved in bidistilled water if not stated otherwise.

Results

Binding Studies. In crude PAEC membrane preparations, the properties of ICYP- ^{125}I ICYP binding to β -ad-

renoceptors was investigated by saturation binding experiments. Specific binding of ICYP was monitored with increasing concentrations of free ligands revealing hyperbolic plots in all experiments with a plateau at about 80 pM, indicating that ICYP binding was saturable (Fig. 1A). In PAECs subjected to hyperglycemic conditions for the complete culture passage 3, the total β -adrenoceptor density (30.32 ± 3.55 fmol/mg protein) was not significantly

different from PAECs cultured with normoglycemic environment (29.75 ± 3.69 fmol/mg protein). The K_D values for ICYP as calculated by linear regression from the obtained ligand concentrations bound were highly specific for ICYP (range 15–30 pM; Seyfarth et al., 2000) and revealed no significant changes between normoglycemia and hyperglycemia (Table 1).

In addition, we carried out displacement binding experiments to assess β -adrenoceptor subtype distribution and the relative amount of β_2 -adrenoceptors. ICI 118,551 inhibited ICYP binding with a concentration-inhibition curve that fit significantly better to a two-side model (F-ratio test, $p < 0,001$). The graphical presentation resulted in biphasic displacement curves verifying the coexistence of at least β_1 - and β_2 -adrenoceptors (Fig. 1B). From these curves, it could be calculated that in both normoglycemic and hyperglycemic membrane preparations, approximately 30% of the β -adrenoceptors are of the β_2 -adrenoceptor subtype (Table 1), demonstrating that β -adrenoceptor density and subtypes are not altered by hyperglycemia. K_I values for ICI 118,551 at the high-affinity site (β_2 -adrenoceptor) were 4 to 6 nM ($-\log K_I$ for ICI 118,551 at β_2 -adrenoceptors: 8.3 to 9.2; Brodde and Michel, 1999) and at the low-affinity site were 400 to 500 nM (Table 1), which demonstrates the absence of hyperglycemia-induced differences in ligand binding affinity for the adrenoceptors.

NO Release. Cells reached confluence after 3.5 ± 0.5 days without differences between the groups. Formation of methemoglobin besides decreasing amounts of oxyhemoglobin resulted in a characteristic difference spectrum with an isobestic point at 411 nm (± 1.25 nm tolerance regarding the precision of the instrument) and maximum extinction at 401 nm (± 1.25 nm) as validated by Feelisch and Noack (1987) and Fig. 2A. Under basal conditions using normal cells, there was a slow increase in extinction, as can be seen in Fig. 2A during the first 50 min, indicating increasing formation of methemoglobin and thus of NO. Stimulation with 1 mM ATP led to a further increase in extinction. In cells that were grown under hyperglycemic conditions, however, basal formation of methemoglobin was significantly reduced (Fig. 2, A and B). ATP-stimulated formation of methemoglobin was also clearly diminished (Fig. 2B). We found a significant decrease in basal and ATP-stimulated NO release in hyperglycemia versus normoglycemia ($p < 0.05$; Fig. 2B). Quantitatively, we found basal release of 46.70 ± 7.74 pmol \cdot 1 Mio cell $^{-1} \cdot$ 10 min $^{-1}$ (normoglycemic cells), which was significantly diminished in hyperglycemic cells to 21.46 ± 6.42 pmol \cdot 1 Mio cell $^{-1} \cdot$ 10 min $^{-1}$ ($p < 0.05$) (Fig. 2B). ATP-stimulated NO release was significantly reduced from 166.46 ± 30.91 pmol \cdot 1 Mio cell $^{-1} \cdot$ 10 min $^{-1}$ (normoglycemic cells) to 84.06 ± 12.23 pmol \cdot 1 Mio cell $^{-1} \cdot$ 10 min $^{-1}$ in hyperglycemic cells ($p < 0.05$).

The reduction in basal and ATP-stimulated NO release was significantly antagonized by salbutamol at low (100 nM) and high concentration (1000 nM) (Fig. 2B). The higher concentration of salbutamol had no additional effect on NO release (compared with 100 nM salbutamol). At 100 nM salbutamol, the β_2 -adrenoceptors are known to be occupied, whereas 1000 nM salbutamol is no longer selective, and there is occupancy of β_1 -adrenoceptors as well. To find out whether a β_2 -adrenoceptor agonistic activity of salbutamol might definitively be responsible for the positive effect on the NO

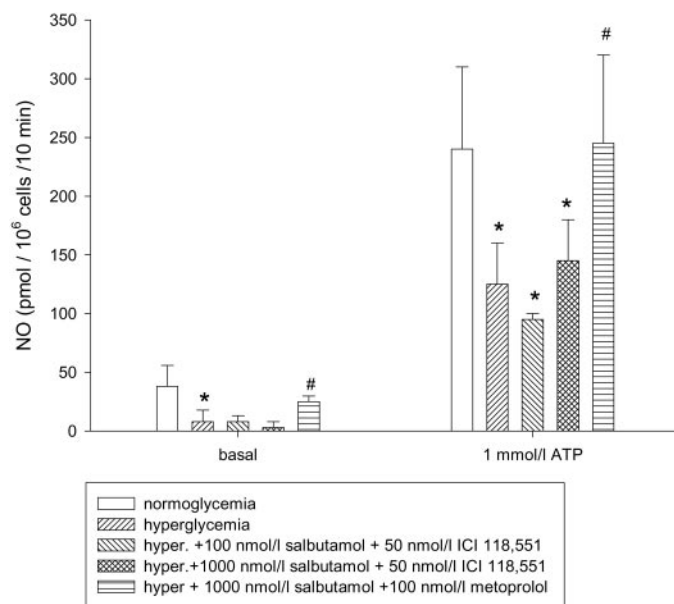


Fig. 3. Basal (left) and ATP-stimulated (right) nitric oxide liberation of porcine aortic endothelial cells cultured under normoglycemic (5 mM D-glucose) and hyperglycemic environment (20 mM D-glucose) with or without salbutamol plus and hyperglycemic cells receiving 100 or 1000 nM salbutamol treatment plus 50 nM selective β_2 -adrenoceptor antagonist ICI 118,551 or 100 nM selective β_1 -adrenoceptor antagonist metoprolol to study the β -adrenoceptor subtype involved in the salbutamol effect. The effect of salbutamol alone is depicted in Fig. 2B. Statistical differences to normoglycemia are indicated by an asterisk; statistical difference to hyperglycemia is marked by # ($p < 0.05$). All values are given as the means \pm S.E.M. of $n = 53$ experiments. Note that selective β_2 -adrenoceptor inhibition leads to inefficiency of salbutamol with the hyperglycemia-induced decrease in NO release, whereas selective β_1 -adrenoceptor inhibition is of no influence on the efficiency of salbutamol treatment.

release, we examined β_2 -adrenoceptor specificity by a combined treatment with salbutamol and the selective β_2 -adrenoceptor antagonist ICI 118,551 or with salbutamol and the selective β_1 -adrenoceptor antagonist metoprolol. Under hyperglycemic conditions, combined treatment with salbutamol (low and high concentration) and 50 nM ICI 118,551 did significantly antagonize the beneficial effect of salbutamol on the NO release (Fig. 3). In contrast, metoprolol did not antagonize the salbutamol effect so that treatment with salbutamol/metoprolol antagonized the glucose effect as efficiently as salbutamol alone (Fig. 3). Thus, the antagonism of the hyperglycemia-induced effect on NO release by salbutamol was found to be due to selective β_2 -adrenoceptor stimulation.

Because it is well known that mammalian cells do not metabolize L-glucose, we investigated the osmotic influence of glucose itself on the NO release. As outlined in Table 2 there was no alteration by 15 mM L-glucose compared with normoglycemia.

TABLE 2

NO release of porcine aortic endothelial cells cultured under normoglycemic conditions or with additional 15 mM L-glucose. All values are given as the means \pm S.E.M. of $n = 6$ experiments.

	D-Glucose	L-Glucose
	5 mM	+15 mM
Basal (pmol \times 1 Mio cell $^{-1} \times$ 10 min $^{-1}$)	53 \pm 12	42 \pm 6
ATP-stimulated (pmol \times 1 Mio cell $^{-1} \times$ 10 min $^{-1}$)	117 \pm 16	119 \pm 20

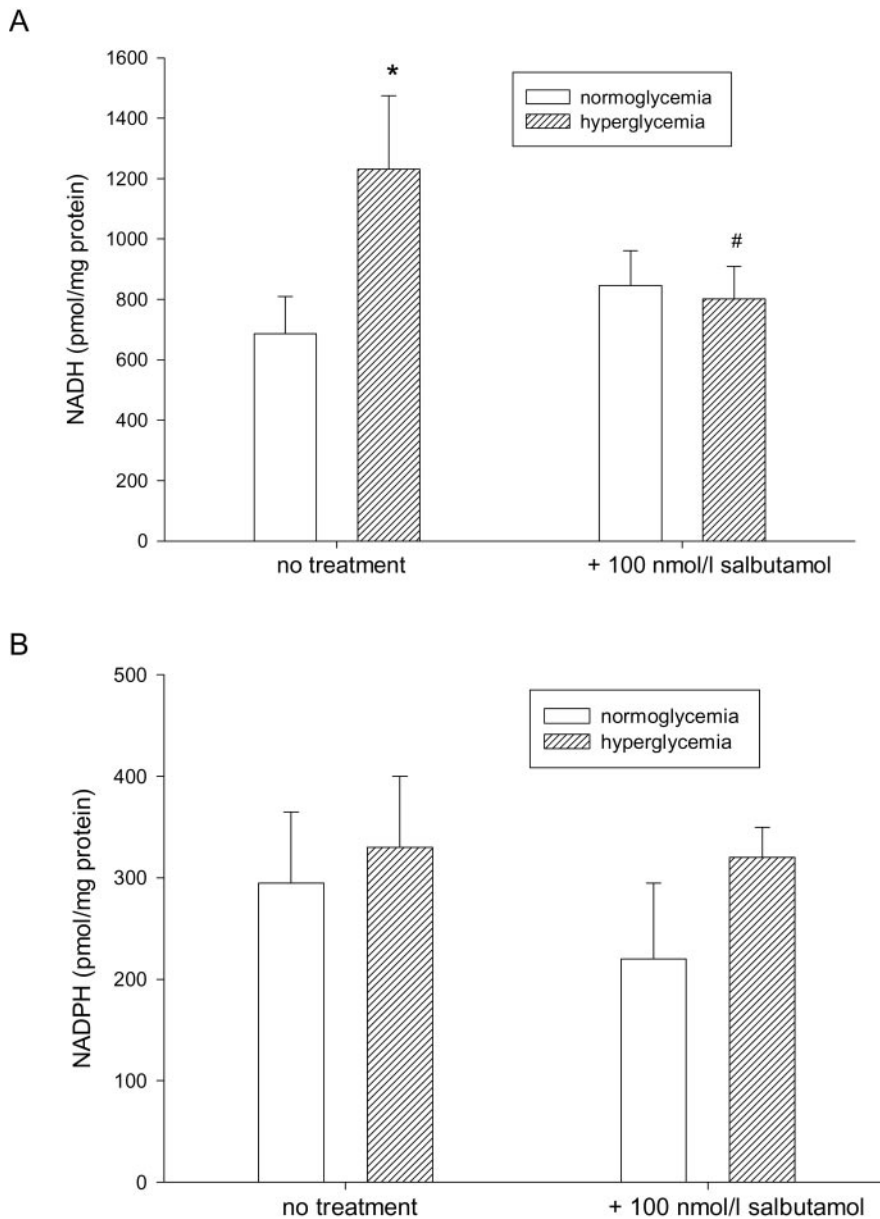


Fig. 4. Formation of intracellular NADH (A) and NADPH (B) in porcine aortic endothelial cells cultured under chronic (one entire cell culture passage), normoglycemic (5 mM D-glucose), and hyperglycemic (20 mM D-glucose) environment without or with additional salbutamol treatment. NADH and NADPH content was measured as described under *Materials and Methods*. The data are expressed as means \pm S.E.M. of $n = 63$ to nine independent experiments with duplicate determination. Statistical difference versus normoglycemia is marked by an asterisk, difference versus hyperglycemia is indicated by # ($p < 0.05$).

Intracellular Reduced Pyridine Nucleotide Concentration. As shown in Fig. 4 we found a marked increase in intracellular NADH content in endothelial cells cultured under hyperglycemic conditions. At basal culture conditions, we found 687.60 ± 122.04 pmol \cdot mg protein $^{-1}$ in normoglycemic cells and 1231.77 ± 244.47 pmol \cdot mg protein $^{-1}$ in hyperglycemic cells ($p < 0.05$). The increase in NADH content in hyperglycemic cells was significantly antagonized by salbutamol (Fig. 4A) and resulted in 846.28 ± 115.36 pmol \cdot mg protein $^{-1}$ in normoglycemic cells and 801.91 ± 106.96 pmol \cdot mg protein $^{-1}$ in hyperglycemic cells.

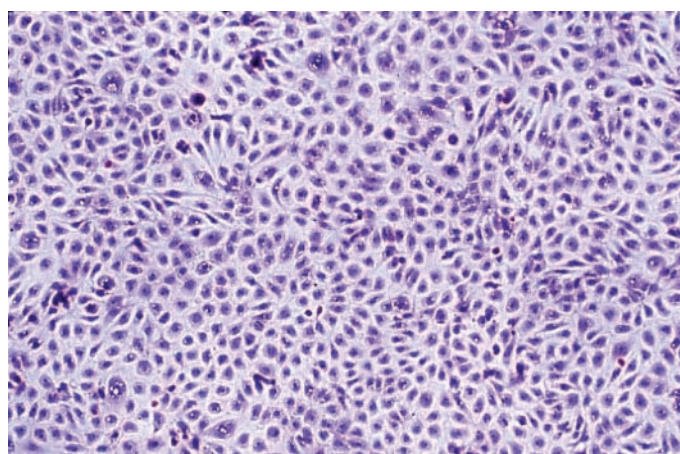
Regarding the intracellular NADPH concentration, we found no differences between normoglycemic and hyperglycemic cells and no influence of salbutamol (Fig. 4B).

Giant Cells. H&E staining revealed that under hyperglycemic conditions there was an increased number of giant cells (Fig. 5). This increase could be completely prevented by salbutamol treatment (Table 3).

NADPH-Diaphorase Activity. We found that the area that stained positive, i.e., the NBT stained area, was decreased in hyperglycemia so that the ratio NBT/area of interest was significantly diminished indicating decreased NADPH-diaphorase activity. This reduction in activity was significantly (but not completely) antagonized by salbutamol treatment (Table 3).

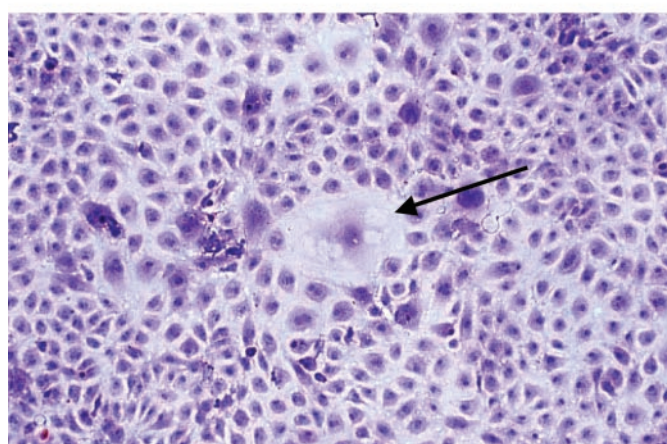
Discussion

Main findings of our study were that 1) porcine aortic endothelial cells express both β_1 - and β_2 -adrenoceptors; 2) density or affinity of β_1 - or β_2 -adrenoceptors is not altered by chronic exposure to high glucose concentrations; 3) NO release is reduced in endothelial cells exposed to high D-glucose concentrations at basal and ATP-stimulated conditions; 4) salbutamol prevents from hyperglycemia-induced impairment of NO release; 5) the effect of salbutamol is specifically



5 mmol/l D-glucose

400x



20 mmol/l D-glucose

400x

Fig. 5. Hematoxylin-eosin staining of porcine aortic endothelial cells under normoglycemic (5 mM glucose; top) and hyperglycemic (20 mM glucose; bottom). Note the occurrence of giant cells under hyperglycemic conditions (arrow).

β_2 -adrenoceptor mediated because it is antagonized by the selective β_2 -adrenoceptor-antagonist ICI 118,551, but not by the selective β_1 -adrenoceptor-antagonist metoprolol; 6) hyperglycemia causes an increase of the intracellular NADH content; 7) this increase in NADH is also antagonized by salbutamol; and 8) hyperglycemia led to an increase in the number of giant cells and to decreased NADPH-diaphorase activity and that these effects were antagonized by salbutamol.

Thus, PAEC developed endothelial dysfunction as characterized by reduced basal and stimulated NO release and morphological changes in response to chronic hyperglycemia. The reduction of NO release was due to hyperglycemia itself, because it was not seen in cells exposed to 15 mM L-glucose, indicating that the enhanced osmotic pressure is not the pathogenetic principle. These results emphasize the pathological importance of glucose itself and they are in accordance with other reports (Salameh et al., 1997; Salameh and Dhein, 1998). The pathogenesis, however, is complex and involves enhanced levels of free radicals (Tsfamariam, 1994; Rösen et al., 1995; Pieper et al., 1997; Du et al., 2000), changes of

the polyol pathway (Cameron and Cotter, 1992; Tesfamariam et al., 1993), glucose-induced activation of PKC isoforms via formation of diacylglycerol (Hempel et al., 1997), advanced glycation end products (Nakamura et al., 1993), peroxy-nitrite-induced activation of poly(ADP-ribose) polymerase (Soriano et al., 2001), and alterations of the arginine-transport/utilization (Pieper and Peltier, 1995; Wu and Meininger, 1995), whereas the NO synthase itself seems to be unaltered (Stockklauser-Farber et al., 2000), at least in BB-rats (Felaco et al., 2001) and human umbilical vein endothelial cells (Mancusi et al., 1996).

To understand how hyperglycemia generates endothelial dysfunction, previous work has shown the generation of ROS in endothelial cells exposed to hyperglycemia as the common element (Nishikawa et al., 2000) linking hyperglycemia-induced damage to reduced eNOS activity by post-translational modification at the Akt phosphorylation site (Du et al., 2001). ROS were detected as superoxide anions that were mainly produced by the proton electrochemical gradient generated within the mitochondrial electron transport chain. Moreover, tricarboxylic acid cycle was determined as the pivotal source of increased ROS generating substrate. During hyperglycemia, enhanced glucose-flux through glycolysis leads to enhanced concentrations of NADH and pyruvate thereby enhancing electron transport in the mitochondrion (Nishikawa et al., 2000), which leads to the generation of ROS (Korshunov et al., 1997; Kwong and Sohal, 1998; Nishikawa et al., 2000). Accordingly, we found that NADH concentrations are enhanced by 85% in hyperglycemic PAECs. Interestingly, this increase in NADH was completely prevented by salbutamol in an ICI 118,551-sensitive manner but was insensitive to the β_1 -adrenoceptor antagonist metoprolol. Because salbutamol is a β_2 -adrenoceptor agonist and ICI 118,551 acts as a β_2 -adrenoceptor antagonist, this indicates that β_2 adrenergic stimulation may interfere with the metabolism of pyruvate and glucose and consequently with the production of NADH.

The existence of a β_2 -adrenoceptor population on PAECs could be demonstrated in our binding study: PAECs express both β_1 - and β_2 -adrenoceptors in a ratio of 70:30% as revealed from the displacement of the β -adrenoceptor ligand ICYP by the β_2 -adrenoceptor antagonist ICI 118,551. The existence of β_2 -adrenoceptors on endothelial cells is in accordance with other studies (Zink et al., 1993). We show here, to our best knowledge for the first time, that hyperglycemia does not alter the density of β -adrenoceptors and does not change the β_1 : β_2 ratio. Hyperglycemia did not significantly alter the B_{max} of β -adrenoceptors, which implicates that the number of β -adrenoceptors was not altered. The finding that K_D for ICYP and K_D (high and low affinity) for ICI 118,551 binding were not affected by hyperglycemia shows that the affinity of the β_1 - and β_2 -adrenoceptors at least for these ligands is not altered in endothelial cells grown under hyperglycemic conditions. Thus, we assume that the binding site of the β -adrenoceptor does not seem to be largely affected by high glucose concentrations. Accordingly, it has been shown, that the β -adrenoceptor in nonendothelial cells may be N-glycosylated, but that this does not alter expression or function (Stiles, 1985; George et al., 1986).

Interestingly, salbutamol did antagonize hyperglycemia-induced impairment of NO release. This effect was evaluated in further experiments and considered as attributable to

TABLE 3

Number of giant cells and NADPH-diaphorase staining results for normoglycemic and hyperglycemic endothelial cells with or without salbutamol treatment

NADPH-diaphorase staining results are given as nitro blue tetrazolium-stained area/area of interest. All values are given as means \pm S.E.M. of $n = 6$ experiments (i.e., six different cell lines).

	D-Glucose	D-Glucose	D-Glucose + Salbutamol	D-Glucose + Salbutamol
Giant cells number	5 mM 7 \pm 2	20 mM 18 \pm 3*	5 mM 6 \pm 3	20 mM 6 \pm 2
NADPH-diaphorase (NBT/AOI)	53 \pm 2	31 \pm 2*	52 \pm 2	41 \pm 2*†

AOI, area of interest.

* Significant changes versus normoglycemia.

† Significant changes versus hyperglycemia ($p > 0.05$).

β_2 -adrenoceptor subtype stimulation, because cocubation of endothelial cells with salbutamol and the selective β_2 -adrenoceptor antagonist ICI 118,551 under hyperglycemic influence did reverse the beneficial effect seen with salbutamol alone. This finding provides further evidence that the β_2 -adrenoceptor is functionally expressed in endothelial cells as also became obvious in our binding experiments. The sensitivity to ICI 118,551 and the lack of effect of metoprolol indicates that the salbutamol effect relies on activation of the β_2 -adrenoceptor pathway. Thus, it can be suggested that cAMP-dependent signaling via protein kinase A may be involved. In support of this idea, it has been reported that β_2 -adrenergic stimulation and cyclic AMP elevation activate the L-arginine/NO system in endothelial cells from human umbilical veins (Ferro et al., 1999).

From these considerations, one may suppose that β_2 -adrenoceptor stimulation antagonizes the hyperglycemia-induced rise in NADH. This should reduce the activity of the mitochondrial electron transport chain and thereby the production of ROS. ROS may inactivate NO by generation of peroxynitrate. Thus, the finding that β_2 -adrenoceptor stimulation antagonized hyperglycemia-induced reduction in NO release and normalized NADH production may indicate reduced ROS generation due to reduced NADH production in response to β_2 -adrenoceptor stimulation, which would result in less inactivation of NO. This would be consistent with our finding of normalized NO release in hyperglycemia cells exposed to salbutamol. According to Nishikawa et al. (2000) mitochondrial ROS production plays a role in hyperglycemia-induced activation of aldose reductase pathway, activation of PKC and generation of advanced glycation end products and thus in the resulting alterations of the cells. In accordance, we found that not only NO release was normalized but also reduced NADPH-diaphorase activity and the number of giant cells.

The finding of unchanged NADPH (which is in good accordance with the observations on the effects of hyperglycemia alone of Asahina et al., 1995) might indicate that NADPH producing processes such as pentosephosphate pathway and NADPH-consuming processes such as poly(ADP-ribose) polymerase activation (Soriano et al., 2001), glutathione-redox cycle, and aldose-reductase pathway are balanced. Thus, a shortage in NADPH supply (NADPH is essential for eNOS activity) does not seem to be of major importance in our model.

However, other factors might also be involved in the action of salbutamol in hyperglycemia. Thus, the hyperglycemia-induced activation of PKC (Xia et al., 1994; Nishikawa et al., 2000) can induce phosphorylation of eNOS at Thr495 and dephosphorylation (via PP2A) at Ser1177, which in turn

leads to deactivation of eNOS (Michell et al., 2001), whereas PKA indirectly leads to phosphorylation at Ser1177 and to dephosphorylation of Thr495, which results in activation of eNOS (Michell et al., 2001). Thus, because β_2 -adrenoceptor stimulation leads to activation of PKA via cAMP, this might counteract the PKC-induced deactivation of eNOS. Future studies will be directed toward this point. Other effects of salbutamol might involve the regulation of phosphofructo kinase, of pyruvate kinase, and of gene expression as regulated by a carbohydrate-responsive element (Yamashita et al., 2001).

However, it should be noted that our data in PAECs cannot be uncritically extended to all aspects of endothelial dysfunction in diabetes mellitus in vivo. Future studies will have to examine the effect of salbutamol in an in vivo model of diabetes mellitus.

We conclude that β_2 -adrenoceptor-stimulation with salbutamol can prevent hyperglycemia-induced impairment of NO release in porcine aortic endothelial cells, that porcine aortic endothelial cells express both β_1 - and β_2 -adrenoceptors, and that density or affinity of β_1 - or β_2 -adrenoceptors is not altered by chronic exposure to high glucose concentrations.

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