Human Diabetes Is Associated With Hyperreactivity of Vascular Smooth Muscle Cells Due to Altered Subcellular Ca^{2+} Distribution

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Alterations of vascular smooth muscle function have been implicated in the development of vascular complications and circulatory dysfunction in diabetes. However, little is known about changes in smooth muscle contractility and the intracellular mechanisms contributing to altered responsiveness of blood vessels of diabetic patients. Therefore, smooth muscle and endothelial cell function were assessed in 20 patients with diabetes and compared with 41 age-matched control subjects. In rings from uterine arteries, smooth muscle sensitivity to K^+, norepinephrine (NE), and phenylephrine (PE) was enhanced by 1.4-, 2.3-, and 9.7-fold, respectively, and endothelium-dependent relaxation was reduced by 64% in diabetic patients, as compared with control subjects. In addition, in freshly isolated smooth muscle cells from diabetic patients, an increased perinuclear Ca^{2+} signaling to K^+ (30 mmol/l >73%; 60 mmol/l >68%) and NE (300 nmol/l >86%; 10 µmol/l >67%) was found. In contrast, subplasmalemmal Ca^{2+} response, which favors smooth muscle relaxation caused by activation of Ca^{2+}-activated K^+ channels, was reduced by 38% in diabetic patients as compared with control subjects, indicating a significant change in the subcellular Ca^{2+} distribution in vascular smooth muscle cells in diabetic patients. In contrast to the altered Ca^{2+} signaling found in freshly isolated cells from diabetic patients, in cultured smooth muscle cells isolated from control subjects and diabetic patients, no difference in the intracellular Ca^{2+} signaling to stimulation with either K^+ or NE was found. Furthermore, production of superoxide anion ($\cdot O_2^-$) in intact and endothelium-denuded arteries from diabetic patients was increased by 150 and 136%, respectively. Incubation of freshly isolated smooth muscle cells from control subjects with the $\cdot O_2^-$-generating system xanthine oxidase/hypoxanthine mimicked the effect of diabetic patients on subcellular Ca^{2+} distribution in a superoxide dismutase–sensitive manner. We conclude that in diabetic subjects, smooth muscle reactivity is increased because of changes in subcellular Ca^{2+} distribution on cell activation. Increased $\cdot O_2^-$ production may play a crucial role in the alteration of smooth muscle function. *Diabetes* 48:1323–1330, 1999

Diabetes is associated with an increased risk for vascular complications and atherosclerosis (1). So far, most studies are focused on the mechanisms of attenuated endothelium-dependent relaxation in diabetes (2). However, besides alterations in endothelium-dependent relaxation, there is increasing evidence that in diabetes, smooth muscle responsiveness to the relaxing agent nitric oxide is reduced (3), while contractility to norepinephrine (NE) is enhanced (4–7). This increase in the reactivity of the smooth muscle cells is thought to be due to an enhanced intracellular Ca^{2+} signaling of the vascular smooth muscle cells to contractile stimuli (5,8,9). Furthermore, excess production of superoxide anions ($\cdot O_2^-$) in the vasculature under hyperglycemic conditions has been reported (3,10,11), which might result in increased degradation of nitric oxide (12) and, thus, in a reduced endothelium-dependent relaxation (3,13). All the above-cited studies were performed in streptozotocin-treated animals or in vessels/cells under hyperglycemic conditions, while, to the best of our knowledge, in human arteries the effects of diabetes on smooth muscle contractility and Ca^{2+} signaling have not been evaluated. Evidence is provided that the Ca^{2+} concentrations of at least two compartments of the cytoplasm contribute differentially to the regulation of the smooth muscle tone (14–17): *1*) the perinuclear Ca^{2+} concentration ($[Ca^{2+}]_{peri}$), which promotes smooth muscle contraction (18), and *2*) subplasmalemmal Ca^{2+} concentration ($[Ca^{2+}]_{sub}$), which favors smooth muscle relaxation caused by activation of Ca^{2+}-activated K^+ channels (19). The present study was designed to investigate in freshly isolated human arteries whether in human diabetes reactivity of the vascular smooth muscle to KCl and NE/phenylephrine (PE) is altered and whether increased $\cdot O_2^-$ release occurs. Furthermore, perinuclear and subplasmalemmal Ca^{2+} signaling to KCl and NE/PE was assessed in single smooth muscle cells freshly isolated from control subjects and diabetic patients.

RESEARCH DESIGN AND METHODS

Subjects. Uterine arteries were isolated from 24 women with type 1 diabetes and 45 healthy control subjects. All patients were undergoing hysterectomy for uncomplicated uterus myomatosus. This kind of operation ensured a group of healthy control subjects. The study population was very homogenous (i.e., all

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$[Ca^{2+}]_{peri}$, perinuclear Ca^{2+} concentration; $[Ca^{2+}]_{sub}$, subplasmalemmal Ca^{2+} concentration; DMEM, Dulbecco's minimal essential medium; EC_{50}, half-maximal concentration; KHS, Krebs-Henseleit solution; NE, norepinephrine; PE, phenylephrine; $\cdot O_2^-$, superoxide anion; SOD, superoxide dismutase.

TABLE 1
Clinical characteristics of the two groups of subjects

	Control subjects	Diabetic patients
n	45	24
Age (years)	55.5 ± 8.2	60.3 ± 11.0
Systolic blood pressure (mmHg)	134 ± 10	147 ± 20
Diastolic blood pressure (mmHg)	78 ± 11	81 ± 8
HbA_{1c} (%)	5.0 ± 1.0	8.19 ± 1.3*
D-glucose (mg/dl)	93.0 ± 9.6	153.9 ± 24.2*
Cholesterol (mg/dl)	197.0 ± 28.8	216.8 ± 38.9
LDL cholesterol (mg/dl)	116.1 ± 31.7	138.6 ± 73.6
HDL cholesterol (mg/dl)	58.0 ± 19.5	41.0 ± 9.2
Triglycerides (mg/dl)	103.8 ± 41.8	158.3 ± 62.4
Creatinine (mg/dl)	0.81 ± 0.15	0.85 ± 0.15
Glutamic oxalacetic transaminase (U/l)	8.3 ± 1.0	11.0 ± 4.1

Data are means ± SE. *$P < 0.05$.

women were of a similar age and postmenopausal). Clinical particulars of both groups are shown in Table 1. The groups differed only in terms of blood D-glucose concentration and HbA_{1c}. Except for insulin in the diabetic group, no additional drug therapy was used in either group.

Materials. Cell culture chemicals were obtained from Life Technologies (Vienna), and fetal calf serum and donor horse serum was from PAA Laboratories (Linz, Austria). Cell culture plastic ware was from Corning (Vienna). Fura-2/am was purchased from Molecular Probes (Leiden, the Netherlands), and FFP-18 was from Calbiochem (Vienna). Buffer salts were from Merck (Vienna). All other materials were from Sigma (Vienna).

Vessel preparation. Samples of the ascending main branches of the uterine artery were dissected free from connective tissue, and ring segments of a 3-mm diameter and a 2-mm length were used for organ chamber experiments. Wall thickness in each group did not differ, nor were vessels with visible plaques used. To remove the endothelium, air was blown through the intact vessel followed with an insertion of a metal probe into the lumen. Absence of endothelium was confirmed by the lack of relaxation in response to 1 µmol/l A23187.

Cell culture. For smooth muscle isolation, a 3-cm piece of the artery was cut longitudinally, and the endothelium was removed. The vessel was fixed in a frame and incubated under sterile conditions for 16 h at room temperature in Dulbecco's minimal essential medium (DMEM; pH = 7.4). DMEM contained collagenase (200 U/ml; type II) plus dilutions of essential and nonessential amino acids (0.02 vol/vol; Gibco BRL, Life Technologies), vitamin (0.01 vol/vol; Gibco BRL), donor horse serum (5%), and bovine serum albumin (2 mg/ml). The isolated cells were immediately used for the experiments or were cultured up to passage 2 in Opti-MEM containing 5% fetal calf serum and antibiotics (100 µg/ml streptomycin, 100 U/ml penicillin, and 1.25 µg/ml fungizone) at 37°C under 5% CO_2 atmosphere and 95% humidity. Cell culture purity was tested with smooth muscle α-actin staining.

Organ chamber experiments. Vessel reactivity was assessed according to our previous technique (20). Briefly, arterial ring segments were mounted in an organ bath containing 2 ml of a Krebs-Henseleit solution (KHS) (in mmol/l, 118.4 NaCl, 5.01 KCl, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25.0 $NaHCO_3$; pH adjusted at 7.4), gassed with a mixture of 95% O_2 and 5% CO_2, and maintained at 37°C. Isometric tension was monitored using a force-displacement transducer (Gould and Stratham, Vienna) coupled to a two-channel bridge amplifier, and tension was continuously recorded with a four-channel printer. After mounting the rings, they were allowed to equilibrate for 90 min in the bath medium, while the solution was replaced every 15 min. Before the experiments, vessels were precontracted three times by applying a force of 2*g*. After the vessel reached a stable tone, cumulative concentration response curves were performed.

Separation of glycated proteins from donor sera. To separate the glycated proteins from the donor sera, 2 ml of the sera were mixed with 2 ml Glyko-Gel II B column (*m*-aminophenyl-boronate; Pierce, Vienna) for 10 min under constant shaking. After centrifugation (5 min at 5,000*g*), the nonglycated serum components were found in the supernatant. For cell incubation, the amount of serum added was normalized by means of the total cholesterol levels.

Ca^{2+} measurements. Intracellular Ca^{2+} concentration was examined in freshly isolated and cultured single human smooth muscle cells using a microfluorometric setup described previously (21). Two different Ca^{2+}-sensitive dyes were used to monitor perinuclear free Ca^{2+} concentration ($[Ca^{2+}]_{peri}$; fura-2) and subplasmalemmal Ca^{2+} concentration ($[Ca^{2+}]_{sub}$; FFP-18). In the case of fura-2, cells were loaded with the cell permeable ester (am) of fura-2 by incubating the cells in DMEM containing 2 µmol/l fura-2/acetoxymethyl ester (am). After 45 min at room temperature, cells were centrifuged, washed twice, and resuspended in DMEM. The cell membrane impermeable dye FFP-18 was loaded using the laser stress-wave loading technique described recently (22). Approximately 10^6 cells were suspended in 350 µl phenol red-free DMEM containing 2 µmol/l FFP-18 and transferred into one well of a microtiterplate. After the well has been sealed with a 1.25-mm aluminum foil, laser-induced stress waves were generated by 3–5 shots of an Nd:YAG laser (70 mJ) to the metal foil, focused 0.5 cm behind the foil. Before the experiments, cells were equilibrated for 20 min in the dark at room temperature, centrifuged, and resuspended in DMEM. For fura-2 experiments, cells were transferred into an experiment chamber and, depending on the experimental protocol used, the DMEM was replaced by KHS or nominal Ca^{2+}-free KHS containing 10 µmol/l EGTA by constant superfusion (1 ml/min). For FFP-18 experiments, cells were centrifuged and resuspended in KHS and transferred into a stirred cuvette for experiments. Maximal fluorescence intensities of either fura-2 or FFP-18 to control differences in dye accumulation/distribution were measured after each experiment by the addition of 3 µmol/l ionomycin in the presence of 2.5 mmol/l extracellular Ca^{2+}. In addition, minimal fluorescence and autofluorescence were monitored in the presence of 3 µmol/l ionomycin in nominal Ca^{2+}-free solution containing either 1 mmol/l EGTA or Mn^{2+}. Dye distribution was monitored using digital confocal microscopy as described previously (22,23).

Data acquisition. For fura-2, single-cell Ca^{2+} was recorded using a microfluorometer (22–24), which excited with 360 and 380 nm alternatively. Emission light was detected at 510 nm. Fluorescence intensity for each pair of excitation/emission wavelength was converted to an analog signal (24) and registered on a PC (AxoBASIC 1.0, Axon Instruments, Foster City, CA). For FFP-18, changes in $[Ca^{2+}]_{sub}$ were monitored in suspended cells every 0.25 s at 335- and 364-nm excitation at 490-nm emission in a stirred cuvette using a four-wavelength spectrofluorometer (Hitachi F4500).

Data analysis. Because of the overall failure and the uncertainties in the calibration for intracellular Ca^{2+} concentration (21,24,25), Ca^{2+} concentration in each experiment is expressed as the 360:380 excitation ratio at 510-nm emission (F_{360}:F_{380}; fura-2) or the 335:364 excitation ratio and 490-nm emission (F_{335}:F_{364}; FFP-18). However, to allow comparison with other reports, an in situ calibration procedure as described by Sturek et al. (24) was performed to allow the estimation of absolute concentration of free Ca^{2+} in Fig. 4.

Measurement of $\cdot O_2^-$. The release of $\cdot O_2^-$ was determined photometrically by measuring the superoxide dismutase (SOD)-sensitive reduction of ferrocytochrome-c (11). Briefly, the wet weight of ring segments of the arteries with or without endothelium was determined (~20 mg each). For each donor, rings were incubated in phosphate-buffered solution (in mmol/l, 137 NaCl, 2.7 KCl, 8 Na_2HPO_4, 1.5 KH_2PO_4, and 0.1 EGTA; pH adjusted at 7.4) plus 10 µmol/l ferrocytochrome-c (horse heart type III). The reduction of ferrocytochrome-c was followed at 550 nm for >30 min. To ensure that the observed reduction of the ferrocytochrome-c was due to $\cdot O_2^-$, 500 U/ml SOD was added, and the reduction of ferrocytochrome-c was monitored for a further 30 min. The difference in absorption between the samples without and with SOD was calculated and indicated the SOD-sensitive reduction of ferrocytochrome-c. The concentration of $\cdot O_2^-$ was calculated using the molar extinction coefficient (e = 21,000) (26) of the reduced ferrocytochrome-c.

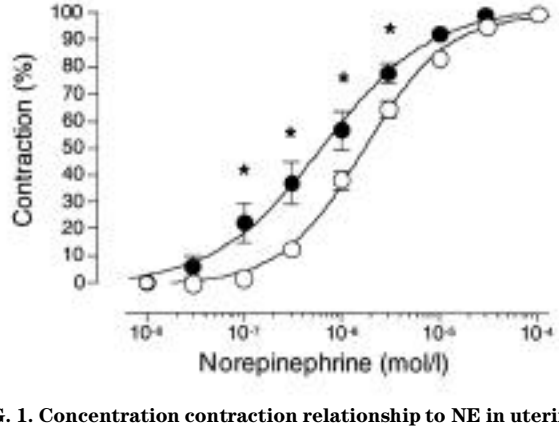

FIG. 1. Concentration contraction relationship to NE in uterine arteries isolated from control subjects (○; n = 13) and diabetic patients (●; n = 8). Changes in smooth muscle tone were isometrically recorded performing a cumulative concentration response curve for NE. Values are means ± SE of contraction achieved, with the indicated concentration of NE calculated in percentage of the maximal contraction to NE (100 µmol/l). *$P < 0.05$ vs. contraction in control subjects.

Statistical analysis. The given n values express the number of different patients, whose uterine arteries and freshly isolated smooth muscle cells were tested in 3–4 (contraction studies) and 5–9 replicates (Ca^{2+} experiments). The half-maximal concentration (EC_{50}) values are expressed as mean (95% CI). All other data are means ± SE. Analysis of variance was used for data evaluation, including post hoc verification using Tukey's test. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Contractility of normal and diabetic vessels. Concentration response curves for NE-induced contractions of freshly isolated human uterine arteries were performed (Fig.1). In normal vessels, the EC_{50} of NE for vasoconstriction was 1.69 (1.51–1.88) µmol/l. In diabetic subjects, the EC_{50} for NE to evoke constriction was significantly smaller (0.63 [0.47–0.85] µmol/l; $P < 0.05$) for diabetic patients versus control subjects. Similar results were obtained with PE. In normal vessels, the EC_{50} of PE was 10.69 (9.10–12.55) µmol/l, while in diabetic vessels, the EC_{50} value was reduced to 1.10 (0.72–1.68) µmol/l ($P < 0.05$) for diabetic patients versus control subjects.

Contractility of uterine arteries from control subjects and diabetic patients to increasing extracellular K^+ concentration was also examined (Fig. 2). Compared with the normal vessels (EC_{50} = 33.63 [32.30–35.01] mmol/l), the sensitivity of the vessels to contract to increased extracellular K^+ concentration was increased in diabetic patients (Fig. 2; EC_{50} = 24.67 [23.62–25.77] mmol/l; $P < 0.05$ vs. normal vessels).

In contrast to the sensitivity of the vessel to contract to either KCl, NE, or PE, maximal amount of contraction did not differ between both groups (KCl, control subjects 3.11 ± 0.43 g and diabetic patients 4.96 ± 0.93 g; NE, control subjects 4.56 ± 0.39 g and diabetic patients 5.80 ± 0.85 g; PE, control subjects 5.44 ± 0.72 g and diabetic patients 4.78 ± 1.00 g).

Dilatory properties of normal and diabetic vessels. In addition to the contractile response to NE, PE, and KCl, endothelium-dependent relaxation in response to acetylcholine was tested in arteries from control subjects and diabetic patients (Fig. 3). Acetylcholine-induced relaxation was significantly reduced in the diabetic arteries ($P < 0.007$ vs. control subjects; Fig. 3), while the EC_{50} values for acetylcholine in both groups and were comparable, (control subjects 0.29 [0.04–0.84]; diabetic patients 0.16 [0.02–0.40] µmol/l). In endothelium-denuded vessels, no relaxation in response to 10 µmol/l acetylcholine or 1 µmol/l A23187 was found (data not shown).

Perinuclear Ca^{2+} signaling in single smooth muscle cells freshly isolated from normal and diabetic vessels. Single-cell Ca^{2+} response was monitored in freshly isolated smooth muscle cells, which were loaded with fura-2 and were stimulated in the presence of 2.5 mmol/l extracellular Ca^{2+} with either increased extracellular K^+ concentration (30 and 60 mmol/l; Fig. 4*A*) or 0.3 and 10 µmol/l NE (Fig. 4*C*). In addition, cells were stimulated with 10 µmol/l NE in the nominal absence of extracellular Ca^{2+}, followed by the addition of 2.5 mmol/l Ca^{2+} to stimulated cells (Fig. 4*E*). Compared with the corresponding elevation in $[Ca^{2+}]_{peri}$ in control subjects, in the diabetic patients, increases in $[Ca^{2+}]_{peri}$ to 30 and 60 mmol/l extracellular K^+ were augmented by 73 and 67%, respectively ($P < 0.05$ vs. the effect obtained in control subjects; Fig. 4*B*). In agreement with the enhanced elevation in $[Ca^{2+}]_{peri}$ to increased K^+ concentrations in diabetic patients, the effect of 0.3 and 10 µmol/l NE was greater in diabetic patients, compared with control subjects (Fig. 4*D*). In addition, intracellular Ca^{2+} release evoked by 10 µmol/l NE in nominal Ca^{2+}-free KHS was 75% greater in diabetic patients (Fig. 4*F*). The effect of 2.5 mmol/l Ca^{2+} to elevate $[Ca^{2+}]_{peri}$ in smooth muscle cells prestimulated with 10 µmol/l NE in the nominal absence of extracellular Ca^{2+} was augmented also in diabetic patients (Fig. 4*F*). In agreement with our results with NE, PE-evoked elevation in $[Ca^{2+}]_{peri}$ in the presence and absence of extracellular Ca^{2+} was greater in smooth muscle cells freshly isolated from diabetic patients compared with that from control subjects (data not shown).

In contrast to the elevated increases in $[Ca^{2+}]_{peri}$ in response to KCl, NE, and PE, neither basal $[Ca^{2+}]_{peri}$ (control subjects 0.33 ± 0.01 ratio [F_{360}:F_{380}], n = 13; diabetic patients 0.34 ± 0.01 ratio [F_{360}:F_{380}], n = 8; NS vs. control subjects) nor the effect of 3 µmol/l ionomycin in the presence of 2.5 mmol/l Ca^{2+} differed within both groups (control subjects 0.67 ±

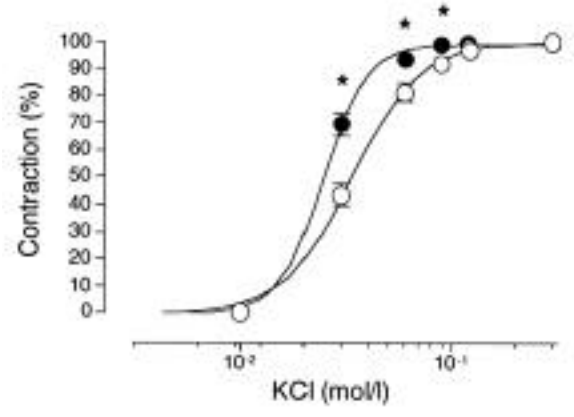

FIG. 2. Concentration contraction relationship to increased extracellular K^+ concentration in uterine arteries isolated from control subjects (○; n = 13) and diabetic patients (●; n = 8). Changes in smooth muscle tone were isometrically recorded, while a cumulative concentration response curve for extracellular K^+ was performed by the addition of KCl. Values are means ± SE of contraction achieved, with the indicated concentration of NE calculated in percentage of the maximal contraction to K^+. *$P < 0.05$ vs. contraction in control subjects.

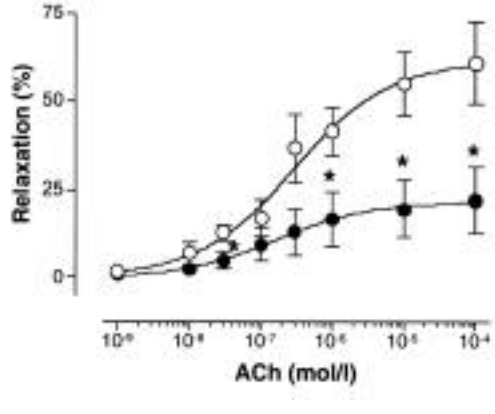

FIG. 3. Cumulative concentration-response curves of the relaxing effects of acetylcholine in uterine arteries from control subjects (○; n = 6) and diabetic patients (●; n = 8). Intact rings were precontracted with 30 mmol/l KCl. Changes in tension to the acetylcholine concentration indicated were measured isometrically and calculated as percentage of the reduction of the KCl-induced tone. Values are means ± SE of relaxation achieved with the acetylcholine concentration indicated. *$P < 0.05$ vs. relaxation in control subjects.

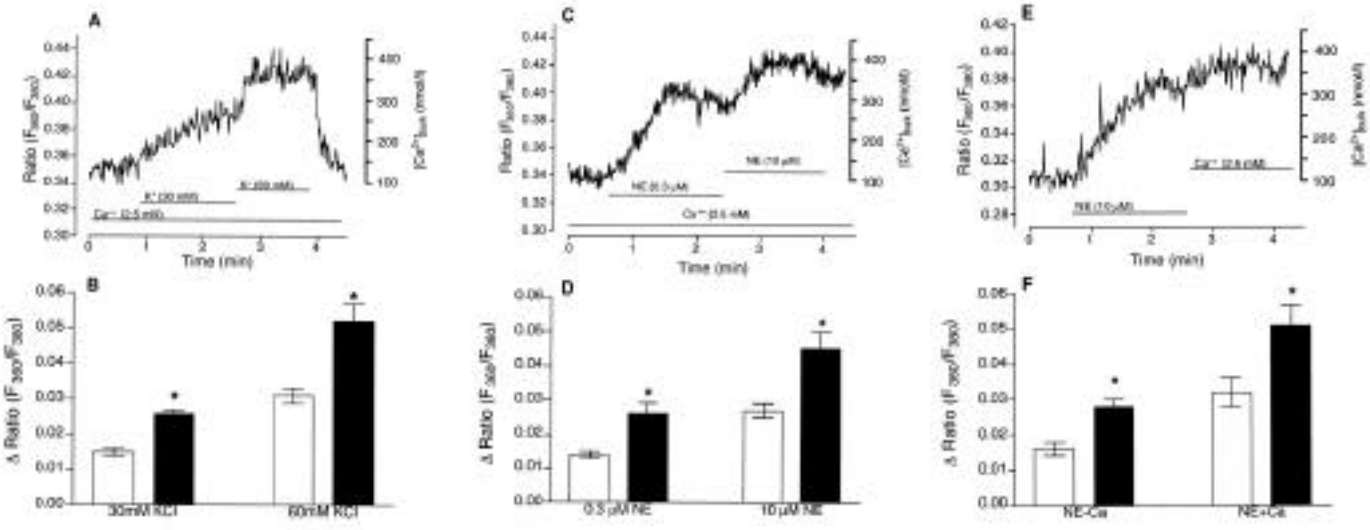

FIG. 4. Measurements of perinuclear Ca^{2+} response in single smooth muscle cells freshly isolated from uterine arteries of control subjects (□; n = 13) and diabetic patients (■; n = 8). Cells were loaded with fura-2/am to monitor changes in $[Ca^{2+}]_{peri}$ in KHS containing 2.5 mmol/l Ca^{2+} (*A* and *C*) and in nominal Ca^{2+}-free KHS (*E*). Experimental protocols are shown (*A*, *C*, and *E*) and the results are given (*B*, *D*, and *E*) as the observed changes of each stimulus (Δ ratio [F_{360}:F_{380}]). The estimated absolute bulk Ca^{2+} concentration ($[Ca^{2+}]_{peri}$) calculated by an in situ calibration (23) is shown (*A*, *C*, and *E*). *P < 0.05 vs. the effect obtained in the control subject group using the same experimental protocol.

0.03 Δ ratio [F_{360}:F_{380}], n = 13; diabetic patients 0.65 ± 0.02 Δ ratio [F_{360}:F_{380}], n = 8; NS vs. control subjects). In addition, minimal fluorescence and autofluorescence were comparable in cells isolated from control subjects and diabetic patients. Distribution of fura-2 tested by digital confocal microscopy (23) did not differ between cells isolated from control subjects or diabetic patients and was found throughout the whole cell (Fig. 5).

Perinuclear Ca^{2+} signaling in cultured single smooth muscle cells derived from normal and diabetic vessels. In contrast to the observed differences in K^+- and NE-induced increases in $[Ca^{2+}]_{peri}$ in the freshly isolated smooth muscle cells from diabetic patients, no differences in K^+- and NE-evoked perinuclear Ca^{2+} signaling were found in cultured single smooth muscle cells (primary culture 4–6 weeks after isolation) isolated from arteries from control subjects and diabetic patients

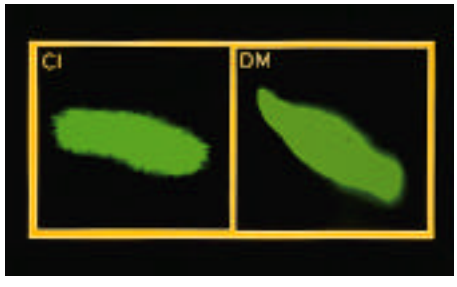

FIG. 5. Two-dimensional distribution of fura-2 in human smooth muscle cells freshly isolated from control subjects (CI) and diabetic patients (DM). Freshly isolated smooth muscle cells were loaded with 2 μmol/l fura-2/am for 45 min at room temperature, washed twice, resuspended in KHS containing 2.5 mmol/l Ca^{2+}, and placed in a chamber with a glass bottom to attach. Fluorescence images were collected using a ×40 objective (numeric aperture 1.3) with an excitation of 380 ± 5 nm and an emission of 510 ± 10 nm (7-s exposure time). Three images were collected at the equator region of each cell with a 0.3-μm interslice distance. Out-of-focus fluorescence was removed by the advanced constrained iterative algorithm (MicroTome; Vaytek, Turnbridge Wells, U.K.) using three interations as described previously (22,23).

(Fig. 6). Identical experiments according to the protocols given in Fig. 4*A*, *C*, and *E* were performed and are demonstrated in Fig. 6*A*–*C*. In agreement with our findings with NE, there was no difference detectable in the elevation of $[Ca^{2+}]_{peri}$ to stimulation with 10 μmol/l PE in cultured cells isolated from control subjects and diabetic patients (data not shown).

To test whether the composition of the sera might contribute to the observed changes in smooth muscle Ca^{2+} signaling, cultured human smooth muscle cells isolated from control subjects were incubated for 24 h in DMEM containing 10% serum isolated from control subjects and diabetic patients, and perinuclear Ca^{2+} signaling to 10 μmol/l PE and 30 mmol/l K^+ were examined. Compared with the Ca^{2+} response in cells incubated with serum from control subjects, in cells incubated with serum from diabetic patients, perinuclear Ca^{2+} signaling to 10 μmol/l PE was increased by 66%, (P < 0.05; Fig. 7). Similar data were obtained using 30 mmol/l extracellular K^+ (data not shown). Despite the elevated increases in $[Ca^{2+}]_{peri}$ to PE and KCl in cells incubated with serum isolated from diabetic patients, maximal values in response to 3 μmol/l ionomycin in the presence of 2.5 mmol/l Ca^{2+} were similar (data not shown). A coincubation with 300 U/ml SOD, while the smooth muscle cells were incubated with sera from control or diabetic patients, reduced the effect of diabetic serum by 32% (data not shown). Moreover, if the sera were cleared from glycated proteins by column chromatography (Glyko-Gel II B), no further differences between the effect of a 24-h treatment with 10% serum from control and diabetic subjects on the effect of 10 μmol/l PE was found (control serum 0.12 ± 0.01 Δ ratio [F_{360}:F_{380}]; diabetic serum 0.13 ± 0.01 Δ ratio [F_{360}:F_{380}], n = 3, NS vs. control subjects).

Subplasmalemmal Ca^{2+} signaling in smooth muscle cells freshly isolated from normal and diabetic vessels. In contrast to our findings on $[Ca^{2+}]_{peri}$ signaling (Fig. 4), the effect of 10 μmol/l NE to elevate $[Ca^{2+}]_{sub}$ in smooth muscle cells in the presence of extracellular Ca^{2+} was reduced by 38% in smooth muscle cells freshly isolated from diabetic patients, com-

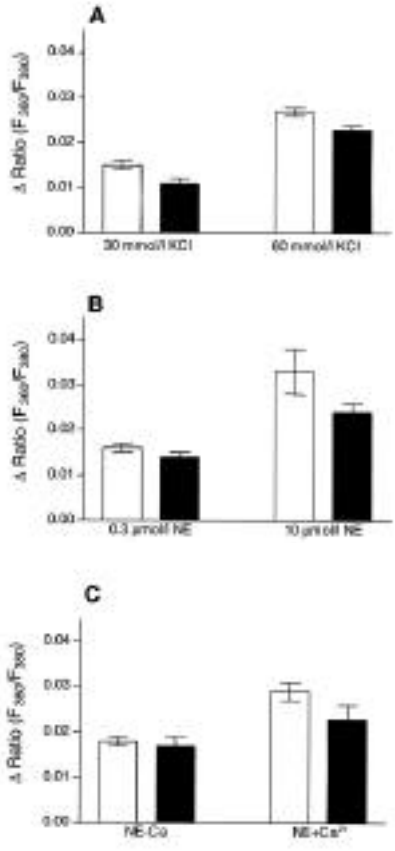

FIG. 6. Measurements of perinuclear Ca^{2+} response in single cultured smooth muscle cells originated from uterine arteries of control subjects (□; n = 23–63) and diabetic patients (■; n = 12–33). Cells were loaded with fura-2/am to monitor changes in perinuclear Ca^{2+} concentration in KHS containing 2.5 mmol/l Ca^{2+} (*A* and *B*) and in nominal Ca^{2+}-free KHS (*C*). Experimental protocols for Fig. *A–C* were identical to those for Fig. 4*A*, *C*, and *E*. Changes in $[Ca^{2+}]_{peri}$ to each stimulus is expressed as the Δ ratio (F_{360}:F_{380}).

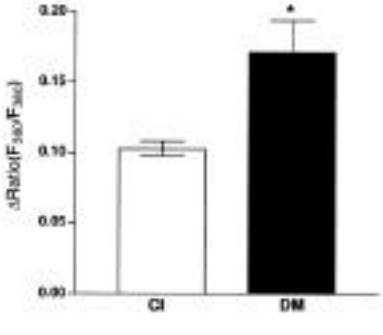

FIG. 7. Perinuclear Ca^{2+} signaling to NE in cultured smooth muscle cells incubated for 24 h in DMEM containing 10% serum isolated from control subjects (CI) and diabetic patients (DM). Cultured smooth muscle cells were incubated with sera from seven control subjects and seven diabetic patients. Cells were loaded with fura-2. In KHS containing 2.5 mmol/l Ca^{2+} changes, cells were stimulated with 10 µmol/l PE. Maximal changes in $[Ca^{2+}]_{peri}$ are given as the Δ values of the ratio (F_{360}:F_{380}). Columns represent the mean of seven experimental series using sera from different control subjects and diabetic patients (seven each) in which the effect of PE was tested five to nine times. *P < 0.05 vs. the effect obtained in cells incubated with sera from control subjects.

pared with that from control subjects (Fig. 8). As previously reported, FFP-18 was found exclusively in the cell membrane (23) without any differences within cells either from control subjects or diabetic patients (data not shown). In cultured smooth muscle cells (primary culture 4–6 weeks after isolation) isolated from control subjects and diabetic patients, no difference in NE-evoked (10 µmol/l) elevation of $[Ca^{2+}]_{sub}$ could be found (data not shown).

In contrast to the decreased elevation in $[Ca^{2+}]_{sub}$ in response to NE, the effect of 3 µmol/l ionomycin in the presence of 2.5 mmol/l Ca^{2+} on $[Ca^{2+}]_{sub}$ did not differ within both groups (control subjects 0.42 ± 0.05 Δ ratio [F_{335}:F_{364}], n = 13; diabetic patients 0.45 ± 0.06 Δ ratio [F_{335}:F_{364}], n = 8; NS vs. control subjects).

In cultured smooth muscle cells (primary culture 4–6 weeks after isolation) isolated from control subjects and diabetic patients, no difference in NE-evoked (10 µmol/l) elevation of $[Ca^{2+}]_{sub}$ could be found (control subjects 0.14 ± 0.02 Δ ratio [F_{335}:F_{364}], n = 4; diabetic patients 0.15 ± 0.03 Δ ratio [F_{335}:F_{364}], n = 4; NS vs. control subjects).

$\cdot O_2^-$ generation in normal and diabetic vessels with and without endothelium. Release of $\cdot O_2^-$ estimated by measuring the SOD-sensitive reduction of ferrocytochrome-c was determined in uterine arteries derived from control subjects and diabetic patients. Strikingly, release of $\cdot O_2^-$ was enhanced by 150% in diabetic patients (Fig. 9*A*). Removal of the endothelium did not affect increased release of $\cdot O_2^-$ in diabetic vessels (Fig. 9*B*).

Effect of the $\cdot O_2^-$-generating system xanthine oxidase/hypoxanthine on the Ca^{2+} signaling in human smooth muscle cells. To evaluate whether the increased production of $\cdot O_2^-$ might contribute to the observed changes in smooth muscle Ca^{2+} signaling, freshly isolated smooth muscle cells from control subjects were incubated with a mixture of 300 µU xanthine oxidase and 1 mmol/l hypoxanthine that produces approximately the same amount of $\cdot O_2^-$ (i.e., 4.51 ± 0.37 µmol/10 min) that was measured in the diabetic vessels (Fig. 9*A*). Incubation of smooth muscle cells with xanthine oxidase/hypoxanthine for 1, 2, and 4 h in DMEM yielded increased elevation in $[Ca^{2+}]_{peri}$ to KCl and NE. After a 4-h treatment with xanthine oxidase/hypoxanthine, elevation in $[Ca^{2+}]_{peri}$ in response to 30 mmol/l extracellular K^+ and 10 µmol/l NE was enhanced by 88 and 81%, respectively (Fig. 10*A*). The effect of xanthine oxidase/hypoxanthine on

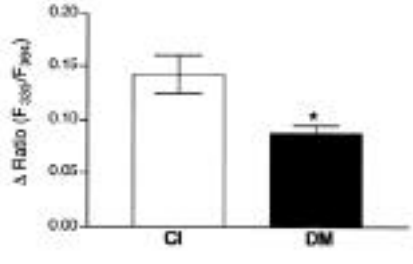

FIG. 8. Subplasmalemmal Ca^{2+} signaling to NE in smooth muscle cells freshly isolated from uterine arteries obtained from control subjects (CI; n = 13) and diabetic patients (DM; n = 8). Cells were loaded with FFP-18 using the laser pulse wave loading technique described in METHODS. In KHS containing 2.5 mmol/l Ca^{2+}, cells were stimulated with 10 µmol/l NE, and $[Ca^{2+}]_{sub}$ was monitored at 335- and 364-nm excitation and 490-nm emission. Maximal changes in $[Ca^{2+}]_{sub}$ are given as the Δ values of the ratio (F_{335}:F_{364}). *P < 0.05 vs. the effect obtained in cells isolated from control subjects.

smooth muscle Ca^{2+} signaling was abolished by a coincubation with 500 U/ml SOD during the xanthine oxidase/hypoxanthine exposure (Fig. 10*A*). In contrast to xanthine oxidase/hypoxanthine, 10 µmol/l hydrogen peroxide did not mimic the effect of diabetes on smooth muscle Ca^{2+} signaling to K^+ and NE (data not shown).

In contrast to the enhanced perinuclear Ca^{2+} signaling in cultured smooth muscle cells treated with xanthine oxidase/hypoxanthine, subplasmalemmal Ca^{2+} elevation to 10 µmol/l NE was reduced in cells incubated with xanthine oxidase/hypoxanthine for 4 h in DMEM (Fig. 10*B*).

Maximal increases in $[Ca^{2+}]_{peri}$ and $[Ca^{2+}]_{sub}$ to 3 µmol/l ionomycin in the presence of 2.5 mmol/l Ca^{2+} were not altered by the preincubation with xanthine oxidase and hypoxanthine (data not shown).

DISCUSSION

The present study indicates that in human diabetes, besides a reduced endothelium-dependent relaxation, the sensitivity of arterial smooth muscle cells to contractile stimuli is increased. These findings are in agreement with reports in animals (7,27–31). The observed increase in vessel contractility in diabetes was found for receptor-dependent (i.e., the α_1 agonists NE and PE) and receptor-independent (i.e., increased extracellular K^+ concentrations) stimuli, indicating that in diabetes, changes in intracellular mechanisms common for α agonist- and K^+-evoked contraction may occur. In contrast to the increased sensitivity to vasoconstrictor stimuli, the sensitivity to relax in response to acetylcholine was not altered in diabetic patients, while the maximal vasodilatation was decreased. These data might indicate that, in contrast to the smooth muscle cells, diabetes might not affect sensitivity of the acetylcholine-induced signal transduction in the endothelium, while diabetes appears to attenuate synthesis/bioactivity of endothelium-derived relaxing factor(s). These findings are in line with previous reports, which demonstrate that the reduced endothelium-dependent relaxation under diabetic conditions might be due to an enhanced degradation of nitric oxide by increased generation of free radicals (3).

In vascular smooth muscle cells, a localized subplasmalemmal Ca^{2+} signaling, which is insulated from the perinuclear space, has been demonstrated to contribute to smooth muscle relaxation by stimulating Ca^{2+}-activated K^+ channels, while an increase in $[Ca^{2+}]_{peri}$ favors smooth muscle contraction (14–17). While the distribution of fura-2 does not allow measurements of subplasmalemmal Ca^{2+} signaling (22,23), the laser stress-wave loaded fura-2 derivate FFP-18 (32) has been shown to be localized exclusively in the cell membrane (23) and, thus it monitors exclusively subplasmalemmal Ca^{2+} concentration (22,23). We found enhanced increases in $[Ca^{2+}]_{peri}$ in response to increased extracellular K^+ concentration and NE. These data are in agreement with reports in diabetic rats (9,33), where an augmented perinuclear Ca^{2+} signaling to K^+ and NE have been demonstrated.

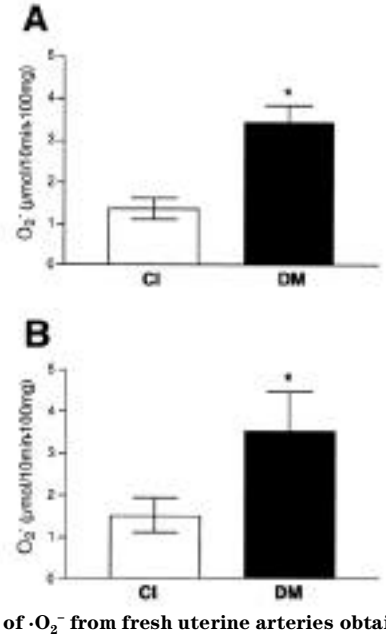

FIG. 9. Release of $\cdot O_2^-$ from fresh uterine arteries obtained from control subjects (CI; n = 13) and diabetic patients (DM; n = 8) with (*A*) and without (*B*) endothelium. $\cdot O_2^-$ release was monitored photometrically by the reduction of ferrocytochrome-c as described in METHODS. Endothelium was removed by air followed by an insertion of a metal probe into the lumen. *P < 0.05 vs. $\cdot O_2^-$ production measured in arteries of control subjects.

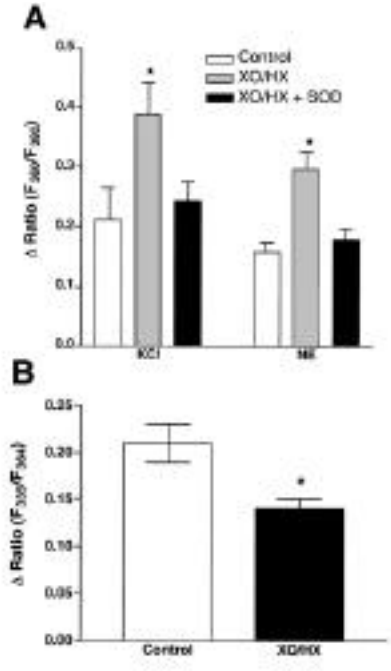

FIG. 10. Effect of an exposure to $\cdot O_2^-$ on the perinuclear (*A*) and subplasmalemmal (*B*) Ca^{2+} signaling in freshly isolated smooth muscle cells. Cells were freshly isolated from uterine arteries obtained from control subjects and exposed for 4 h in DMEM. *A*: Perinuclear Ca^{2+} signaling in single smooth muscle cells in response to increased extracellular K^+ and NE. Cells from human uterine artery were incubated in DMEM (□; n = 22), in DMEM containing 300 µU/ml xanthine oxidase and 1 mmol/l hypoxanthine (▒; n = 24), or in DMEM containing 300 µU/ml xanthine oxidase, 1 mmol/l hypoxanthine, and 500 U/ml SOD (■; n = 24). After the cells have been loaded with fura-2/am, changes in $[Ca^{2+}]_{peri}$ to 30 mmol/l KCl and 10 µmol/l NE were measured in KHS containing 2.5 mmol/l Ca^{2+}. *P < 0.05 vs. cells in DMEM. *B*: Subplasmalemmal Ca^{2+} signaling to NE in smooth muscle cells freshly isolated from uterine arteries obtained from control subjects after a 4-h incubation in DMEM without (□; n = 10) and with (■; n = 10) 300 µU/ml xanthine oxidase and 1 mmol/l hypoxanthine. Cells were loaded with FFP-18 using the laser pulse wave loading technique described in METHODS. In KHS containing 2.5 mmol/l Ca^{2+} cells were stimulated with 10 µmol/l NE, and $[Ca^{2+}]_{sub}$ was monitored at 335- and 364-nm excitation and 490-nm emission. Maximal changes in $[Ca^{2+}]_{sub}$ are given as the Δ values of the ratio (F_{335}:F_{364}). *P < 0.05 vs. the effect obtained in cells isolated from control subjects.

Moreover, our data presented here provide evidence that diabetes is associated with an enhanced autacoid-evoked intracellular Ca^{2+} release and Ca^{2+} entry in human artery smooth muscle cells, indicated by the increased response to NE in the nominal absence of extracellular Ca^{2+} and addition of Ca^{2+} to prestimulated cells (Fig. 4*C*). These findings are consistent with the reports of Abebe et al. (27), who reported enhanced vasoconstriction in diabetic rats in the absence of extracellular Ca^{2+}. In contrast to the observed differences in perinuclear Ca^{2+} signaling to K^+ and NE in smooth muscle freshly isolated from diabetic patients, intracellular Ca^{2+} signaling to K^+ and NE was similar in cultured smooth muscle cells from these patients, compared with that obtained in cultured smooth muscle cells from control subjects. These data, and our findings that incubation of the cultured cells with serum from diabetic patients mimicked the situation in freshly isolated smooth muscle cells from these patients, are consistent with the common hypothesis that in diabetes, changes in serum composition (hyperglycemia, glycated proteins) are the initiators for vascular cell dysfunction.

In contrast to the increase in perinuclear Ca^{2+} signaling in diabetic patients, subplasmalemmal Ca^{2+} response was reduced in freshly isolated smooth muscle cells from diabetic patients. Thus, our data indicate changes in the Ca^{2+} signaling in the two major regulatory sites for smooth muscle tone in diabetes. Remarkably, while autacoid-induced elevation of the $[Ca^{2+}]_{peri}$, which triggers smooth muscle contraction to K^+ and NE, is increased in diabetic smooth muscle cells, subplasmalemmal Ca^{2+} response was reduced. Focal increases in the $[Ca^{2+}]_{sub}$ have been reported to trigger relaxation by activation of Ca^{2+}-activated K^+ channels and are thought to constitute a negative feedback for smooth muscle contraction (14–18). The attenuated elevation of $[Ca^{2+}]_{sub}$ on cell activation found in diabetic patients may result in a reduced activation of Ca^{2+}-activated K^+ channels, which would mediate less inhibitory control for contraction. Thus, our data on the changes in Ca^{2+} signaling in smooth muscle cells suggest that in diabetes, the observed increased blood vessel contractility might, at least in part, be due to the increased perinuclear Ca^{2+} signaling and reduced subplasmalemmal Ca^{2+} response on cell stimulation.

Recent data indicate that in experimental hyperglycemia (3,11,34), increased vascular $\cdot O_2^-$ production occurs, which is thought to derive from membrane-bound NADH/NADPH oxidase on endothelial and smooth muscle cells (35–37). Consistent with these reports, we have found increased $\cdot O_2^-$ production in vessels from diabetic patients. Our findings, that the removal of the endothelium did not reduce $\cdot O_2^-$ release from the diabetic vessels, is in agreement with that in cholesterol-fed and atherosclerotic rabbits, where smooth muscle-derived $\cdot O_2^-$ has been shown to attenuate endothelium-dependent relaxation (38,39). Nevertheless, unlike the report of Ohara et al. (40) in hypercholesterolemic vessels, removal of the endothelium did not normalize increased $\cdot O_2^-$ production of the vessels of diabetic patients. On the basis of these data and our findings, one might speculate that in human uterine arteries during diabetes, it is unlikely that the vascular endothelium is the main origin of increased $\cdot O_2^-$ production.

Antioxidants, such as vitamin C, vitamin E, and dimethylthiourea, have been described to normalize altered blood vessel function in diabetes (12,13,41), thus indicating that the increased appearance of oxygen radicals is involved in vascular dysfunction during these diseases. This conclusion is further supported by our findings that preincubation of normal smooth muscle cells with the $\cdot O_2^-$-generating mixture xanthine oxidase/hypoxanthine mimicked the effect of diabetes and hypercholesterolemia on smooth muscle Ca^{2+} signaling in an SOD-sensitive manner. Recently, we have reported that in endothelial cells, $\cdot O_2^-$ augments Ca^{2+} signaling, possibly by stimulation of tyrosine kinase (42). Because tyrosine kinase has been shown to be involved in smooth muscle contractility (43,44), it needs to be investigated whether, besides the augmented elevation in $[Ca^{2+}]_{peri}$, $\cdot O_2^-$-mediated tyrosine kinase activity contributes to the enhanced contractility of smooth muscle cells in diabetes.

In contrast to the observed differences in perinuclear Ca^{2+} signaling to K^+ and NE in smooth muscle freshly isolated from diabetic patients, perinuclear and subplasmalemmal Ca^{2+} signaling to K^+/NE were similar in cultured smooth muscle cells from these patients, compared with that obtained in cultured smooth muscle cells from control subjects. These data may indicate that the primary differences found in the smooth muscle Ca^{2+} signaling might not be due to genetic differences of the smooth muscle cells per se. On the other hand, it is possible that the phenotypic changes of smooth muscle cells during cell culture (contractile vs. proliferating cell type) are responsible for the normalization of Ca^{2+} signaling. However, although we cannot rule out this possibility, our findings that incubation of the cultured cells with serum from diabetic patients mimicked the situation in freshly isolated smooth muscle cells from these patients favor the common hypothesis that in diabetes, changes in serum composition (glycated proteins) are the initiators for vascular cell dysfunction. This hypothesis is further supported by our findings that separation of glycated proteins by column chromatography (Glyko-Gel II B) (45) ameliorated the effect of diabetic serum on PE-induced Ca^{2+} signaling in cultured smooth muscle cells. However, more detailed experiments are necessary to clarify the exact protein(s) responsible for the observed alterations in smooth muscle Ca^{2+} signaling.

The present findings suggest that alterations in smooth muscle cell function occur in human diabetes. Therefore, in addition to the endothelium, the vascular smooth muscle needs to be considered as a potential source of vascular dysfunction in this disease. The observed changes in smooth muscle reactivity caused by diabetes brings up an important aspect for our understanding of the mechanisms of vascular complications associated with this disease. This might add new strategies for therapeutic intervention against vascular dysfunction, which represent the most common cause of death in diabetes.

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REFERENCES

1. Vogel RA: Coronary risk factors, endothelial function, and atherosclerosis: a review. *Clin Cardiol* 20:426–432, 1997
2. Cohen R: Dysfunction of vascular endothelium in diabetes mellitus. *Circulation* 87 (Suppl. V):V67–V76, 1993

3. Tesfamariam B, Cohen RA: Free radicals mediate endothelial cell dysfunction caused by elevated glucose. *Am J Physiol* 263:H321–H326, 1992
4. Agrawal DK, McNeill JH: Vascular responses to agonists in rat mesenteric artery from diabetic rats. *Can J Pharmacol* 65:1484–1490, 1987
5. Kamata K, Miyata N, Kasuya Y: Mechanisms of increased responses of the aorta to alpha-adrenoceptor agonists in streptozotocin-induced diabetic rats. *J Pharmacobiodyn* 11:707–713, 1988
6. Kamata K, Miyata N, Kasuya Y: Impairment of endothelium-dependent relaxation and changes on levels of cyclic GMP in aorta from streptozotocin-induced diabetic rats. *Br J Pharmacol* 97:614–618, 1989
7. Taylor PD, Oon BB, Thomas CR, Poston L: Prevention by insulin treatment of endothelial dysfunction but not enhanced noradrenaline-induced contractility in mesenteric resistance arteries from streptozotocin-induced diabetic rat. *Br J Pharmacol* 111:35–41, 1994
8. Sakai Y, Aihara K, Honda H, Inazu M: Calcium mobilization and phosphatidylinositol turnover in vas deferens smooth muscle cells of diabetic rats. *Eur J Pharmacol* 162:475–481, 1989
9. Yoo HJ, Kozaki K, Akishita M, Watanabe M, Eto M, Nagano K, Sudo N, Hashimoto M, Kim S, Yoshizumi M, Toba K, Ouchi Y: Augmented Ca^{2+} influx is involved in the mechanism of enhanced proliferation of cultured vascular smooth muscle cells from spontaneously diabetic Goto-Kakizaki rats. *Atherosclerosis* 131:167–175, 1997
10. Mazière C, Auclair M, Rose-Robert F, Leflon P, Mazière JC: Glucose-enriched medium enhances cell-mediated low-density lipoprotein peroxidation. *FEBS Lett* 363:277–279, 1995
11. Graier WF, Simecek S, Kukovetz WR, Kostner GM: High-D-glucose-induced changes in endothelial Ca^{2+}/EDRF signaling is due to generation of superoxide anions. *Diabetes* 45:1386–1395, 1996
12. Graier WF, Posch K, Wascher TC, Kukovetz WR, Kostner GM: Role of superoxide anions in changes of endothelial vasoactive response during acute hyperglycemia. *Horm Met Res* 29:622–629, 1997
13. Pieper GM, Siebeneich W, Roza AM, Jordan M, Adams MB: Chronic treatment in vivo with dimethylthiourea, a hydroxyl radical scavenger, prevents diabetes-induced endothelial dysfunction. *J Cardiovasc Pharmacol* 28:741–745, 1996
14. van Breemen C, Lukeman S, Leijten P, Yamamoto H, Loutzenhiser R: The role of superficial SR in modulating force development induced by Ca^{2+} entry into artery smooth muscle. *J Cardiovasc Pharmacol* 8:S111–S113, 1986
15. Moore EDW, Etter EF, Philipson KD, Carrington WA, Fogarty KE, Lifshitz LM, Fay FS: Coupling of the Na^+/Ca^{2+} exchanger, Na^+/K^+ pump and sacroplasmic reticulum in smooth muscle. *Nature* 365:657–660, 1993
16. van Breemen C, Chen Q, Laher I: Superficial buffer barrier function of smooth muscle sarcoplasmic reticulum. *TiPS* 16:98–105, 1995
17. Fay FS: Calcium sparks in vascular smooth muscle: relaxation regulators. *Science* 270:588–589, 1995
18. Horowitz A, Menice CB, Laporte R, Morgan KG: Mechanisms of smooth muscle contraction. *Physiol Rev* 76:967–1003, 1996
19. Yamaguchi H, Kajita J, Madison JM: Isoproterenol increases peripheral $[Ca^{2+}]_i$ and decreases inner $[Ca^{2+}]_i$ in single airway smooth muscle cells. *Am J Physiol* 268:C771–C779, 1995
20. Graier WF, Holzmann S, Hoebel BG, Kukovetz WR, Kostner GM: Mechanisms of L-N^G nitroarginine/indomethacin-resistant relaxation in bovine and porcine coronary arteries. *Br J Pharmacol* 119:1177–1186, 1996
21. Graier WF, Simecek S, Sturek M: Cytochrome P450 mono-oxygenase-regulated signaling of endothelial Ca^{2+} entry. *J Physiol Lond* 482:259–274, 1995
22. Graier WF, Paltauf-Doburzynska J, Hill B, Fleischhacker E, Hoebel BG, Kostner GM, Sturek M: Submaximal stimulation of porcine endothelial cells causes focal Ca^{2+} elevation beneath the cell membrane. *J Physiol (Lond)* 506:109–125, 1998
23. Paltauf-Doburzynska J, Posch K, Paltauf G, Graier WF: Stealth ryanodine-sensitive Ca^{2+} release contributes to activity of capacitative Ca^{2+} entry and nitric oxide synthase in bovine endothelial cells. *J Physiol (Lond)* 13: 369–379, 1998
24. Sturek M, Smith P, Stehno-Bittle L: In vitro models of vascular endothelial cell calcium regulation. In *Ion Channels of Vascular Smooth Muscle Cells and Endothelial Cells.* Sperelakis N, Kuriyama H, Eds. Elsevier, New York, 1991, pp 349–364
25. Wang X, Lau F, Li L, Yoshikawa A, VanBreemen C: Acetylcholine-sensitive intracellular Ca^{2+} store in fresh endothelial cells and evidence for ryanodine receptors. *Circ Res* 77:37–42, 1995
26. Steinbrecher UP: Role of superoxide in endothelial-cell modification of low-density lipoproteins. *Biochim Biophys Acta* 959:20–30, 1988
27. White RE, Carrier GO: Vascular contraction induced by activation of membrane calcium ion channels is enhanced in streptozotocin-diabetes. *J Pharmacol Exp Ther* 253:1057–1062, 1990
28. Abebe W, Harris KH, MacLeod KM: Enhanced contractile responses of arteries from diabetic rats to alpha 1-adrenoceptor stimulation in the absence of extracellular calcium. *J Cariovasc Pharmacol* 16:239–248, 1990
29. el Kasher H: Hyperglycemia increased the responsiveness of isolated rabbit's pulmonary arterial rings to serotonin. *Pharmacology* 53:151–159, 1996
30. Pieper GM, Siebeneich W, Dondlinger LA: Short-term oral administration of L-arginine reverses defective endothelium-dependent relaxation and cGMP generation in diabetes. *Eur J Pharmacol* 317:317–320, 1996
31. Pieper GM, Siebeneich W, Moore-Hilton G, Roza AM: Reversal by L-arginine of a dysfunctional arginine/nitric oxide pathway in the endothelium of the genetic diabetic BB rat. *Diabetologia* 40:910–915, 1997
32. Etter EF, Minta A, Poenie M, Fay FS: Near-membrane $[Ca^{2+}]$ transients resolved using the Ca^{2+} indicator FFP18. *Proc Natl Acd Sci U S A* 93: 5368–5373, 1996
33. Tam ESL, Ferguson DG, Bielefeld DR, Lorenz JN, Cohen RM, Pun RYK: Norepinephrine-mediated calcium signaling is altered in vascular smooth muscle of diabetic rat. *Cell Calcium* 21:143–150, 1997
34. Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamuea T, Nyengaard JR, van den Enden M, Kilo C, Tilton RG: Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes* 42:801–813, 1993
35. Griendling K, Ollerenshaw JD, Minieri CA, Alexander RW: Angiotensin II stimulates NADH and NADPH activity in cultured vascular smooth muscle cells. *Circ Res* 74:1141–1148, 1994
36. Mohazzab KM, Kaminski PM, Wolin MS: NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. *Am J Physiol* 266:H2568–H2572, 1994
37. Pagano PJ, Ito Y, Tornheim K, Gallop P, Cohen RA: An NADPH oxidase superoxide generating system in the rabbit aorta. *Am J Physiol* 268:H2274–H2280, 1995
38. Keaney JF, Xu A, Cunningham D, Jackson T, Frei B, Vita JA: Dietary probucol preserves endothelial function in cholesterol-fed rabbits by limiting vascular oxidative stress and superoxide generation. *J Clin Invest* 95:2520–2529, 1995
39. Miller FJ, Gutterman DD, Rios CD, Heistad DD, Davidson BL: Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. *Circ Res* 82:1298–1305, 1998
40. Ohara Y, Peterson TE, Harrison DG: Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest* 91:2546–2551, 1993
41. Graier W, Simecek S, Hoebel B, Wascher T, Dittrich P, Kostner GM: Antioxidants prevent high D-glucose-enhanced endothelial Ca^{2+}/cGMP response by scavenging superoxide anions. *Eur J Pharmacol* 322:113–122, 1997
42. Graier WF, Hoebel BG, Paltauf-Doburzynska J, Kostner GM: Effects of superoxide anions on endothelial Ca^{2+} signaling pathways. *Arterioscler Thromb Vasc Biol* 18:1470–1479, 1998
43. Touyz RM, Schiffrin EL: Tyrosine kinase signaling pathways modulate angiotensin II-induced calcium ($[Ca^{2+}]_i$) transients in vascular smooth muscle cells. *Hypertension* 27:1097–1103, 1996
44. Di Salvo J, Nelson SR, Kaplan N: Protein tyrosine phosphorylation in smooth muscle: a potential coupling mechanism between receptor activation and intracellular calcium. *Proc Soc Exp Biol Med* 214:285–301, 1997
45. Posch K, Simecek S, Wascher TC, Jürgens G, Baumgartner-Parzer S, Kostner GM, Graier WF: Glycated low-density lipoprotein attenuates shear stress–induced nitric oxide synthesis by inhibition of shear stress–activated L-arginine uptake in endothelial cells. *Diabetes*. 48:1331–1337, 1999