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Intercellular signalling within vascular cells under high D-glucose involves free radical-triggered tyrosine kinase activation

G. Schaeffer[1], **S. Levak-Frank**[1], **M. M. Spitaler**[1], **E. Fleischhacker**[1], **V. E. Esenabhalu**[1], **A. H. Wagner**[2], **M. Hecker**[2], **W. F. Graier**[1]

[1] Department of Medical Biochemistry and Medical Molecular Biology, University of Graz, Graz, Austria
[2] Department of Cardiovascular Physiology, University of Goettingen, Goettingen, Germany

Abstract

Aims/hypothesis. Diabetes mellitus is associated with endothelial dysfunction in human arteries due to the release of superoxide anions ($^{\cdot}O_2^-$) that was found to occur predominantly in smooth muscle cells (SMC). This study was designed to elucidate the impact of high glucose concentration mediated radical production in SMC on EC. Pre-treatment of vascular SMC with increased D-glucose enhanced release of $^{\cdot}O_2^-$.
Methods. Microscope-based analyses of intracellular free Ca^{2+} concentration (fura-2), immunohistochemistry (f-actin) and tyrosine kinase activity were performed. Furthermore, RT-PCR and Western blots were carried out.
Results. Interaction of EC with SMC pre-exposed to high glucose concentration yielded changes in endothelial Ca^{2+} signalling and polymerization of f-actin in a concentration-dependent and superoxide dismutase (SOD) sensitive manner. This interaction activated endothelial tyrosine kinase(s) but not NFκB and AP-1, while SOD prevented tyrosine kinase stimulation but facilitated NFκB and AP-1 activation. Erbstatin, herbimycin A and the src family specific kinase inhibitor PP-1 but not the protein kinase C inhibitor GF109203X prevented changes in endothelial Ca^{2+} signalling and cytoskeleton organization induced by pre-exposure of SMC to high glucose concentration. Adenovirus-mediated expression of kinase-inactive c-src blunted the effect of pre-exposure of SMC to high glucose concentration on EC.
Conclusions/interpretation. These data suggest that SMC-derived $^{\cdot}O_2^-$ alter endothelial cytoskeleton organization and Ca^{2+} signalling via activation of c-src. The activation of c-src by SMC-derived radicals is a new concept of the mechanisms underlying vascular dysfunction in diabetes. [Diabetologia (2003) 46:773–783]

Keywords AP-1, cytoskeleton, diabetes, hyperglycaemia, Ca^{2+}, NFκB, src, superoxide anion.

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Corresponding author: W. F. Graier, Department of Medical Biochemistry and Medical Molecular Biology, University of Graz, Harrachgasse 21/III, 8010 Graz, Austria
E-mail: wolfgang.graier@uni-graz.ac.at
Abbreviations: PP-1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)-pyrazolo[3,4-d]pyrimidine; GF 109203X, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide; AP-1, activator protein-1; DMEM, Dulbecco's Minimal Essential Medium; EC, endothelial cells; $[Ca^{2+}]$, intracellular free Ca^{2+} concentration; NFκB, nucear factor κB; SMC, smooth muscle cells; O_2^-, superoxide anions; SOD, superoxide dismutase.

In the genesis of diabetes-induced cardiovascular dysfunction a concerted malfunction of many cells like endothelial cells (EC), smooth muscle cells (SMC), macrophages and platelets is involved [1, 2, 3, 4, 5]. Therefore, the impact of hyperglycaemia on these cells and the mechanisms of the fatal interaction of blood cells with the vascular wall in diabetes have been extensively investigated. However, the cellular interplay between vascular cells under diabetic conditions has been rarely studied so far.

Recently, we have described that the increased release of superoxide anions ($^{\cdot}O_2^-$) from uterine arteries of diabetic patients was due to enhanced $^{\cdot}O_2^-$ production in SMC rather than in EC [6]. Although these

data might be related to this particular type of artery and are in contradiction with reports in murine models [7], it is unclear whether, and if so, how vascular SMC affect EC during diabetes. Such smooth muscle originated modulation of EC function adds to our recent paradigm in which vascular dysfunction in diabetes (and many other diseases) is thought to occur initially in EC that, in turn, affects the vascular SMC [1, 8].

Hyperglycaemia has been clearly shown to initiate activation of protein kinase C that is thought to be crucially involved in the endothelial dysfunction under hyperglycaemic conditions [9]. Besides protein kinase C, the modulation of a number of additional signal transduction pathways has been reported in hyperglycaemia [7]. Thus, it seems interesting to assess the endothelial target(s) of smooth muscle-derived factors in hyperglycaemia.

In this study we used a co-culture model of EC and SMC in order to elucidate how vascular SMC affect EC in the presence of high glucose concentrations. Notably, the signal molecule(s) of the cellular interplay between the two cell types of the vascular wall, the mechanism of EC manipulation and the consequences thereof were assessed using short time cultured endothelial and smooth muscle cells from porcine aortae.

Materials and methods

Materials. Foetal calf serum was from PAA Laboratories, Linz, Austria and cell culture chemicals were purchased at Life Technologies, Vienna, Austria. Six-well plates and inserts were purchased at BD Biosciences, Vienna, Austria. Fluorescent dyes were obtained from Molecular Probes, Leiden, Netherlands. DNase I was from Promega (Mannheim, Germany), reverse transcriptase from Invitrogen (Vienna, Austria) and DyNAzyme DNA II polymerase was obtained from Finnzymes (Vienna, Austria). Western blot and radioactive chemicals were from Amersham Biosciences, Vienna, Austria. Antibodies were obtained from Biomol, Hamburg, Germany. Erbstatin analog, herbimycin A, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo-[3,4-d]pyrimidine (PP-1) and 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF109203X) were purchased at Calbiochem-Novabiochem (Bad Soden, Germany).

Cell Culture. Endothelial cells (EC) were isolated from porcine aortae [10]. Fresh porcine aortae were incubated at 37°C with 200 U/ml collagenase (type II) in Dulbecco's Minimal Essential Medium (DMEM) plus 1% MEM essential amino acids, 1% MEM non-essential amino acids, 1% MEM vitamins and 1 mg/ml trypsin inhibitor type I. Isolated EC were cultured in Opti-MEM containing 3% FCS. Cell culture purity was confirmed by typical cobblestone morphology and the absence of SMC α-actin. Experiments were carried out with cells from 1st passage.

Porcine aortic smooth muscle cells (SMC) were isolated using the explant technique [6]. Endothelium and connective tissue were removed from 1 cm^2 pieces of the porcine aorta that was washed with cold phosphate buffered saline (in mmol/l: 137 NaCl, 2.7 KCl, 8 $Na_2HPO_4 \times 2\ H_2O$, 1.5 KH_2PO_4; pH 7.4; PBS). The remaining smooth muscle layer was cut into small pieces, covered with a sterile glass cover slip and cultured in Opti-MEM containing 3% foetal calf serum. For experiments, cells from the 1st passage were used. Cell culture purity was confirmed by positive α-actin staining.

Cell interaction experiments. We cultured SMC in six-well plates while EC were cultured on inserts in separate six-well plates. After reaching confluence, SMC were treated with DMEM containing the D-glucose concentration indicated. After 24 h, the SMC were washed twice with DMEM and the inserts with the EC were transferred into the smooth muscle containing six-well plates. Cell interaction was allowed in DMEM for the indicated time (Fig. 1).

Ca^{2+} measurement. Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using fura-2 as previously described [11]. Experiments were carried out in Hepes-buffered solution (HBS) with or without 2.5 mmol/l $CaCl_2$ (in mmol/l: 145 NaCl, 5 KCl, 1 $MgCl_2$, 10 Hepes-acid; pH 7.4) in suspended cells in order to allow basolateral interaction of EC with SMC and methodical limits to monitor the Ca^{2+} signalling in confluent EC grown on inserts. Due to uncertainties of the $[Ca^{2+}]_i$ calibration techniques, $[Ca^{2+}]_i$ is expressed as ratio (F_{340}/F_{380}) units (340/380 nm excitation at 510 nm emission).

Measurement of superoxide anions. O_2^- were photometrically measured by the reduction of ferricytochrome C as previously described [10, 12]. Confluent SMC were incubated at 37°C in Ca^{2+}-free PBS containing 10 μmol/l ferricytochrome C (horse heart type III) with or without SOD (476 U/ml; i.e. between 110 and 134 μg/ml protein) and the reduction of ferricytochrome C was followed at 550 nm for 30 min. The difference of absorption between samples with or without SOD corresponds to $^{\cdot}O_2^-$-mediated reduction of ferricytochrome C. Concentrations of $^{\cdot}O_2^-$ were calculated using the molar extinction coefficient of reduced ferricytochrome C (ε=21.000) [13].

Fluorescence microscopy. Fixation was done by incubating EC for 30 min at 4°C in PBS containing 10 μg/ml lysophosphatidylcholin, 3.5% formaldehyde, and 10 U/ml Bodipy 581/591 phalloidine. Fluorescence was monitored at 575 nm excitation and 630 nm emission with a 40× oil immersion objective (N.A. 1.3; Nikon, Vienna, Austria) of a fluorescence microscope (Eclipse TE300, Nikon) using a cooled CCD camera (Quantix, Roper Scientific, Acton, Mass., USA). Image analysis was done using Image Pro 3.0 (Media Cybernetics, Sliver Spring, Mass., USA) [14]. Out-of-focus fluorescence was eliminated by deconvolution using the iterative constrained interation algorithm (VayTek, Fairfield, Iowa, USA).

Western Blot analysis. Confluent EC were washed twice with chilled PBS and harvested by scraping. Cell lysates were prepared in Tris-buffer (in mmol/l: 50 Tris-HCl, 150 NaCl, 1 EGTA, 1 PMSF, 1 Na_3VO_4, 1 NaF, 0.3% sodium desoxycholate, 1 μg/ml aprotinin and 1 μg/ml leupeptin; pH 7.4), followed by three cycles of freezing/thawing. Equal amounts of protein were mixed with buffer containing 20% glycerol, 5% (w/v) SDS, 0.15% (w/v) bromphenol blue, 3% 2-mercaptoethanol and 63 mmol/l Tris-HCl (pH 6.8), boiled for 10 min at 95°C, separated on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose. The membrane was blocked for 3 h in 5% (w/v) non-fat instant milk in PBS containing 0.05% Tween-20 and probed at 4°C with the phosphotyrosine specific antibody 4G10 (1:1000 dilution) overnight. After washing with PBS containing 0.05% Tween 20, the membrane was incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:1000 dilution). Immunoreactivity was visualized using

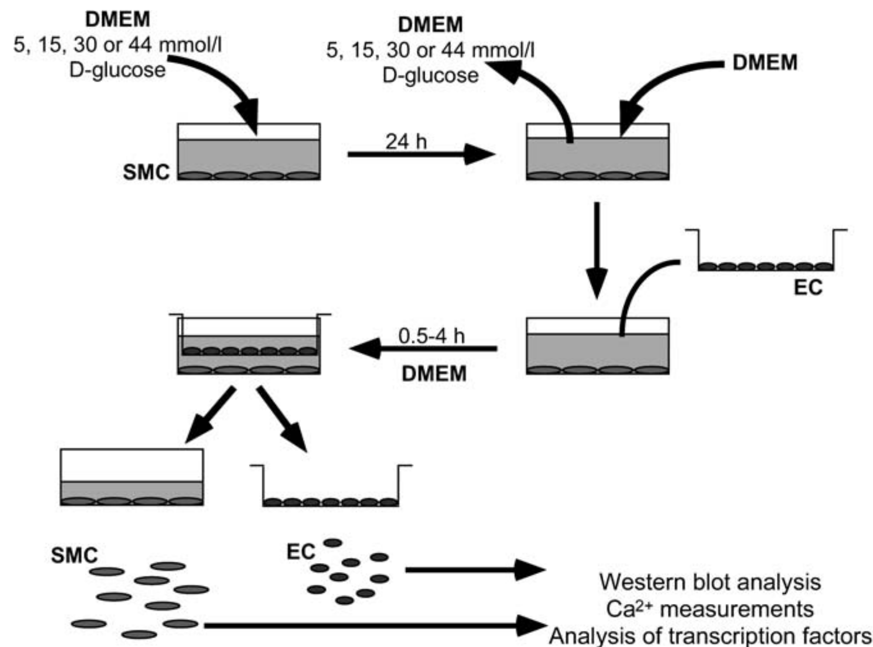

Fig. 1. Schematic presentation of the experimental protocol to study intercellular signalling between EC and SMC. Confluent SMC were incubated in DMEM containing D-glucose as indicated. After 24 h, cells were washed three times with DMEM and inserts with EC were placed on top of the well. After the indicated interaction period, cells were harvested separately for further analysis

enhanced chemiluminescence. For additional staining, the membrane was stripped and reprobed with the β-catenin specific antibody (1:1000 dilution).

Electromobility shift assay (EMSA) for NFκB. Cells were harvested and preparation of nuclear protein extracts, labelling and binding reaction were carried out [15]. The NFκB consensus oligonucleotide 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ was from Santa Cruz Biotechnology (Heidelberg, Germany). EMSAs were repeated four times and representative gels are shown.

RT-PCR for AP-1. Total endothelial RNA was isolated by using RNeasy kit (Qiagen, Vienna, Austria). 3 μg thereof were treated with RQ1 RNase-free DNase I for 15 min at 37°C and subsequently used as a template for first-strand cDNA-synthesis in a 30 μl reaction containing 0.5 μmol/l dNTPs (Roth, Graz, Austria), 15 U RNAguard, 3.3 μM random hexameric primers (Amersham Biosciences, Vienna, Austria), 10 μmol/l dithiothreitol and 200 U Moloney murine leukaemia virus reverse transcriptase (Invitrogen) in first-strand buffer for 1 h at 37°C. Following heat inactivation at 75°C for 10 min, 2.5 μl of the cDNA was used as a template for PCR with specific primers for the human c-jun subunit of the transcription factor AP-1 (5′-ACG ACC TTC TAT GAC GAT GC-3′ and 5′-GTG TTC TGG CTG TGC AGT TC-3′) yielding a 360 bp product. 50 μl PCR reaction mixture contained 0.2 μmol/l dNTPs, 10 μmol/l of each primer and 1 U DyNAzyme II DNA polymerase (Finnzymes) in PCR buffer.

Measurement of tyrosine kinase activity. Endothelial tyrosine kinase activity was measured by using a customized photometric protein tyrosine kinase kit from Calbiochem-Novabiochem [12, 16]. After interaction period cell lysates were obtained by sonification in chilled lysis buffer containing in mmol/l: 20 Tris, 50 NaCl, 1 EDTA, 1 EGTA, 0.2 PMSF, 0.2 mercaptoethanol plus 1 μg/ml pepstatin and 0.5 μg/ml leupeptin (pH 7.4). Phosphorylation was measured by horseradish peroxidase-labelled phosphotyrosine specific antibody and photometrically monitored at 450 nm.

Tyrosine kinase activity was also monitored in single EC by monitoring fluorescence resonance energy transfer (FRET) using a probe for tyrosine phosphorylation of the CrkII adaptor protein (Picchu-936X) [17]. In addition, the inactive mutant Picchu-938X was used. Cells were transfected with 2 μg cDNA/ml of the respective PicchuX and FRET was monitored at 440 nm excitation and 480/535 nm emission using a beam splitter (MultSpec, Visitron, Puchheim, Germany) mounted onto the CCD camera.

Statistical analysis. Data represent the means±SEM. Analysis of variance was done and statistical significance was evaluated using Scheffe's post hoc F test. A p value of less than 0.05 was considered statistically significant.

Results

Endothelial Ca^{2+} signalling. After interaction of EC with SMC pre-exposed to elevated D-glucose for 4 h (Fig. 1), bradykinin-initiated intracellular Ca^{2+} release was increased by 59% compared with EC that interacted with SMC pre-exposed to 5 mmol/l D-glucose (Fig. 2A; n=33, p<0.05). Furthermore, capacitative Ca^{2+} entry due to depletion of intracellular Ca^{2+} stores was increased by 170% in EC that interacted with high D-glucose pre-exposed SMC (Fig. 2A; n=33, p<0.05). Changes in endothelial Ca^{2+} signalling critically depended on the concentration of D-glucose in which SMC were held for 24 h (Fig. 1) prior to the interaction period with EC (Fig. 2B). In contrast to pathological D-glucose concentrations, preincubation of SMC with 44 mmol/l D-mannitol for 24 h did not

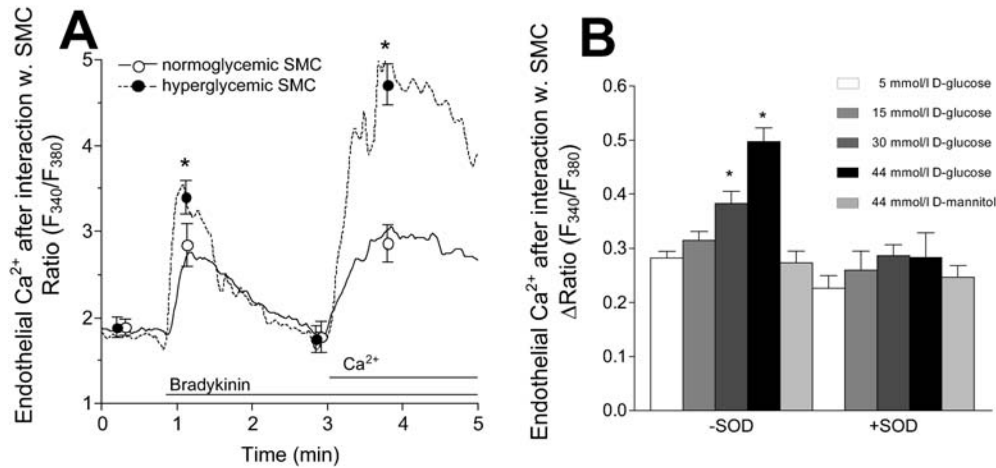

Fig. 2A, B. Effect of intercellular signalling between EC and SMC on endothelial Ca^{2+} signalling. After the interaction procedure shown in Fig. 1, EC were loaded with 2 μmol/l fura-2/AM. $[Ca^{2+}]_i$ is expressed in ratio units F_{340}/F_{380}. (**A**) Representative tracings of the observed changes in endothelial Ca^{2+} signalling in response to 100 nmol/l bradykinin in nominal Ca^{2+} free solution followed by the addition of 2.5 mmol/l extracellular Ca^{2+} (i.e. capacitative Ca^{2+} entry). (**B**) Concentration response relationship between the D-glucose concentration the SMC got exposed to prior interaction (4 h in the absence or presence of 200 U/ml SOD) with the EC and the changes in endothelial Ca^{2+} signalling in response to 100 nmol/l bradykinin in the presence of 2.5 mmol/l extracellular Ca^{2+}. *$p<0.05$ vs endothelial Ca^{2+} signalling after an interaction with normoglycaemic (i.e. 5 mmol/l D-glucose) SMC (n=6–33)

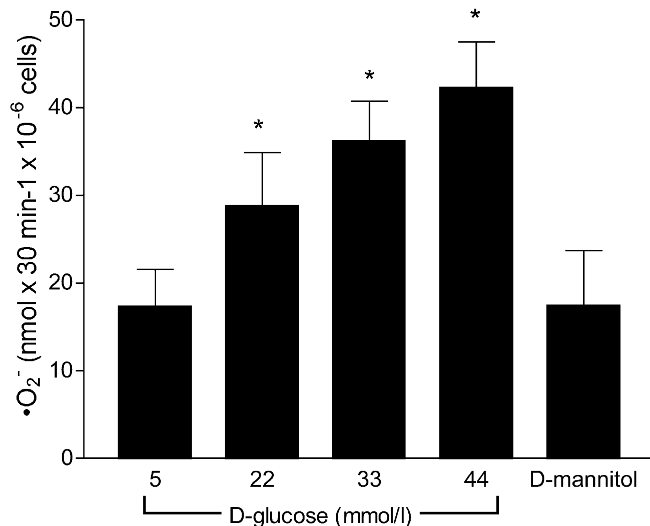

Fig. 3. Effect of elevated D-glucose on $\cdot O_2^-$ release from cultured SMC. Confluent SMC were incubated for 24 h in DMEM containing D-glucose or D-mannitol (44 mmol/l) as indicated. The release of $\cdot O_2^-$ was measured by monitoring the SOD-sensitive reduction of ferricytochrome C. *$p<0.05$ vs normal D-glucose conditions (i.e. 5 mmol/l D-glucose, n=6–9)

result in altered endothelial Ca^{2+} signalling after cell interaction (Fig. 2B). Changes in endothelial Ca^{2+} signalling by interaction with high D-glucose pre-exposed SMC represented a time-dependent phenomenon that started after 30 min and peaked after 4 h of interaction (data not shown). Moreover, interaction of EC with high D-glucose pre-exposed SMC 10 h after high D-glucose was removed from the SMC, did not affect endothelial Ca^{2+} signalling (data not shown).

If 200 U/ml SOD was present during the 4-h interaction between EC and high D-glucose pre-exposed SMC endothelial Ca^{2+} signalling was normalized (Fig. 2B) while 500 U/ml catalase during interaction could not prevent alterations in endothelial Ca^{2+} signalling by high D-glucose pre-exposed SMC. If EC were exposed for 4 h to DMEM that was preconditioned for 6 h by SMC, which were pre-treated for 24 h under hyperglycaemic conditions, only a small increase in endothelial Ca^{2+} signalling was detectable (data not shown).

Release of $\cdot O_2^-$ from smooth muscle cells in response to elevated D-glucose. Preincubation of SMC in medium with elevated D-glucose concentration increased the release of $\cdot O_2^-$ in a concentration-dependent manner (Fig. 3). In contrast, incubation of SMC in DMEM containing 44 mmol/l D-mannitol did not affect their $\cdot O_2^-$ release (Fig. 3).

Changes in endothelial cell cytoskeleton arrangement by SMC-derived radicals. In EC that were exposed for 4 h to high D-glucose pre-exposed SMC strong f-actin polymerization was observed (Fig. 4). This effect was prevented when SOD (200 U/ml) was present during the 4-h interaction. In line with these findings, exposure of EC for 1 h to DMEM containing the $\cdot O_2^-$ generating mixture of 1 mmol/l hypoxanthine and 300 μU/ml xanthine oxidase yielded SOD-sensitive (200 U/ml) but catalase-insensitive (500 U/ml) stress fibre formation (data not shown).

Effect of intercellular signalling on endothelial transcription factor activation. To verify additional effects

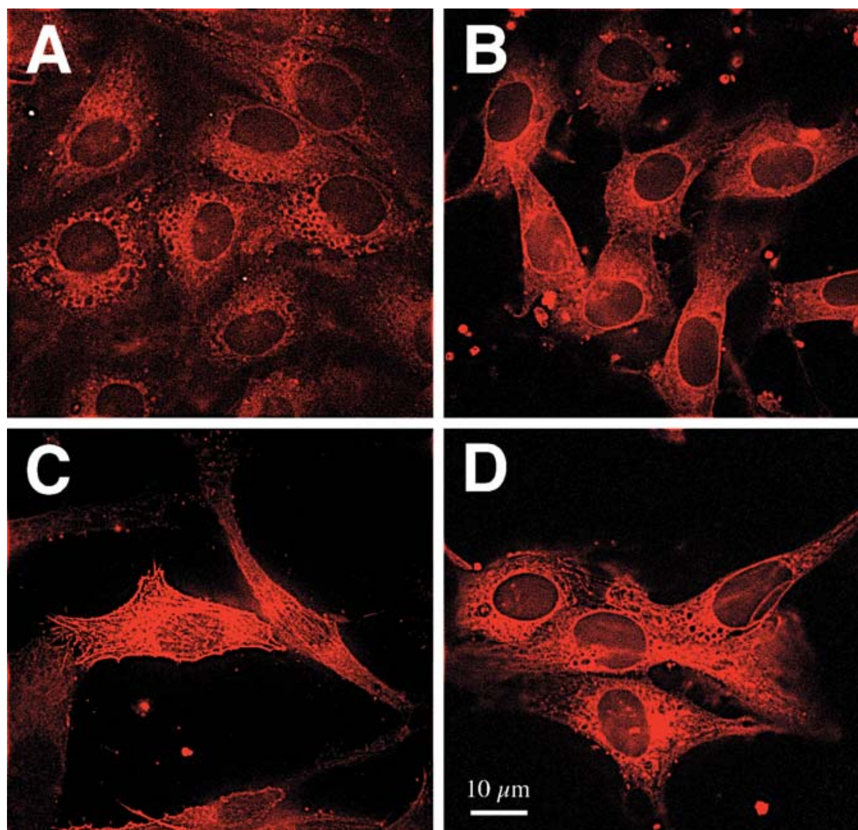

Fig. 4A–D. The interaction of endothelial cells with hyperglycemia-preexposed SMC alters endothelial cytoskeleton organization in an SOD-sensitive manner. EC were put for 4 h in DMEM on top of SMC that were pretreated for 24 h in DMEM containing 5 (**A** and **B**) or 44 mmol/l (**C** and **D**) D-glucose in the absence (**A** and **C**) or presence (**B** and **D**) of 200 U/ml SOD. EC were fixed and f-actin was stained using Bodipy 581/591 phalloidine. Fluorescence was monitored at 584 nm excitation and 630 emission using a deconvolution fluorescence microscope as described previously [6] (*n*=15)

of interaction of EC with high D-glucose pre-exposed SMC, endothelial NFκB activation was studied using EMSA. Interaction of EC for 4 h with untreated (lane 1) as well as high D-glucose pre-exposed SMC (lane 2) slightly activated endothelial NFκB as compared to EC that did not interact with SMC (lane 8). Nevertheless, no differences in NFκB activation were found between EC that could interact with untreated or high D-glucose pre-exposed SMC. In contrast, in the presence of SOD (200 U/ml) NFκB activation occurred in cells that were incubated with high D-glucose pre-exposed SMC (lane 4) but not in cells that interfered with untreated SMC (lane 3) (Fig. 5).

The $\cdot O_2^-$-generating mixture of 1 mmol/l hypoxanthine and 300 μU/ml xanthine oxidase only slightly stimulated NFκB (lane 5) while in the presence of SOD (200 U/ml) this effect was more pronounced (lane 6). Hydrogen peroxide (H_2O_2; 10 μmol/l) also caused strong NFκB stimulation (lane 7) that was prevented by catalase (500 U/ml; data not shown).

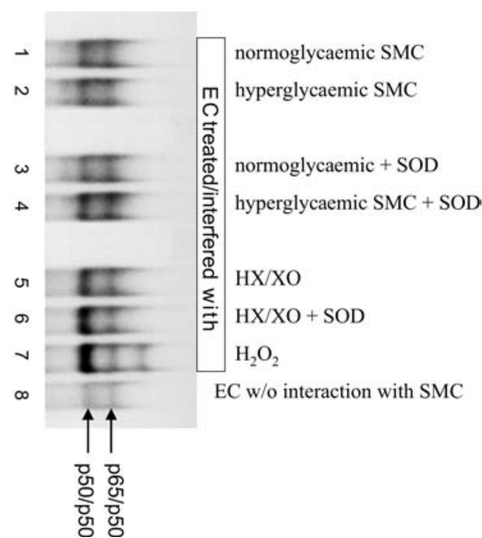

Fig. 5. The interaction of EC with high D-glucose pre-exposed SMC does not indicate activation of endothelial NFκB. Representative analysis on the dimerization of the p65 and the p50 subunits for NFκB stimulation by EMSA in EC. EC were incubated for 4 h in DMEM with SMC that were pretreated for 24 h in DMEM containing 5 (*lane 1*) or 44 mmol/l (*lane 2*) D-glucose or in DMEM containing 200 U/ml SOD and 5 (*lane 3*) or 44 mmol/l D-glucose (*lane 4*). Alternatively, EC were treated for 1 h with 1 mmol/l hypoxanthine and 300 μU/ml xanthine oxidase in the absence (*lane 5*) or in the presence of SOD (*lane 6*). *Lane 7* shows NFκB activation in EC treated for 1 h with 10 μmol/l hydrogen peroxide (H_2O_2). *Lane 8* represents NFκB in endothelial cells after 4 h in DMEM (5 mmol/l D-glucose) without any further treatment

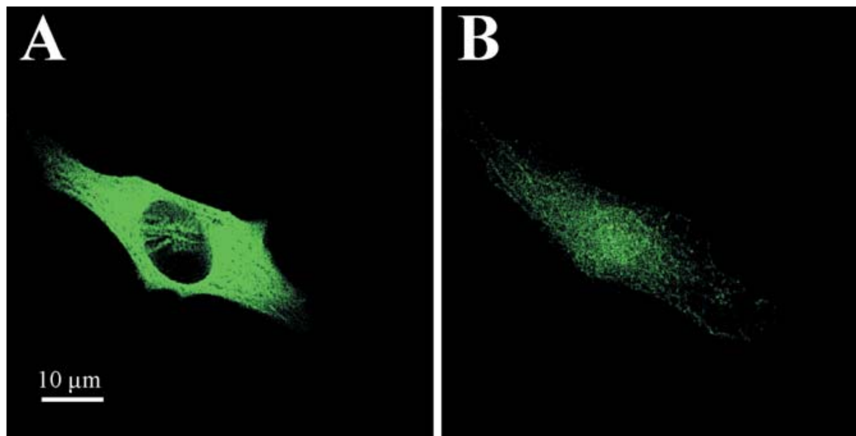

Fig. 6A, B. The nuclear translocation of p65-GFP during interaction with high D-glucose–pre-exposed SMC requires the presence of SOD. EC were transfected with the p65-GFP construct and after two days cells were put on top of SMC that were preincubated for 24 h in DMEM containing 44 mmol/l D-glucose. No nuclear translocation was found within 2 h of interaction in DMEM (5 mmol/l D-glucose) (**A**), while 45 min after addition of 200 U/ml SOD a clear nuclear translocation of p65-PFP was observed (*n*=5) (**B**)

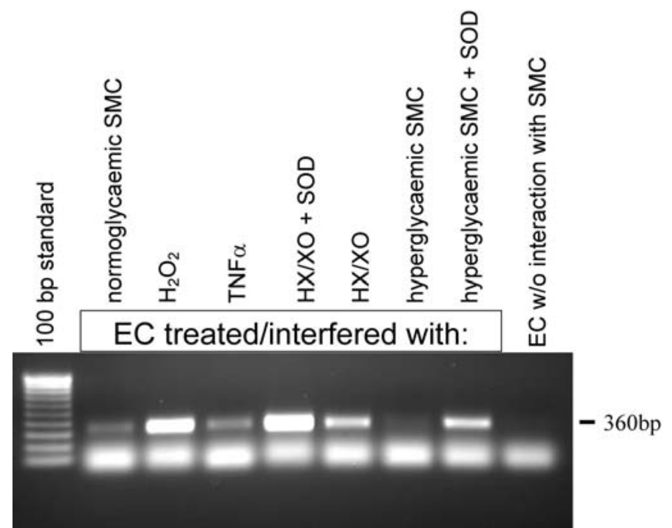

Fig. 7. Interaction of EC with high D-glucose–pre-exposed SMC does not enhance expression of AP-1. Representative RT-PCR analysis of AP-1 expression in EC without any treatment (EC w/o interaction with SMC), 10 μmol/l hydrogen peroxide (H_2O_2), 10 ng/ml TNFα, hypoxanthine/xanthine oxidase (1 mmol/l, 300 μU/ml; HX/XO) in the presence or absence of 200 U/ml SOD (4 h each). In addition, EC were incubated for 4 h in DMEM (5 mmol/l D-glucose) with SMC that were pretreated for 24 h in DMEM containing 5 (normoglycaemic SMC), 44 mmol/l D-glucose (hyperglycaemic SMC) or 44 mmol/l D-glucose plus 200 U/ml SOD (hyperglycaemic SMC+SOD)

In line with these findings, no nuclear translocation of NFκB was observed in EC that interacted with high D-glucose pre-exposed SMC (Fig. 6A). However, if 200 U/ml SOD were present a NFκB translocation to the nucleus occurred (Fig. 6B).

Similar to our findings on NFκB, endothelial AP-1 expression was not augmented by incubation with either untreated or high D-glucose pre-exposed SMC (Fig. 7), while in the presence of SOD (200 U/ml) an upregulation of AP-1 occurred. The expression of AP-1 initiated by the $^{\cdot}O_2^-$-generating mixture of hypoxanthine (1 mmol/l) and xanthine oxidase (300 μU/ml) was increased in the presence of SOD (200 U/ml). TNFα (10 ng/ml) and H_2O_2 (10 μmol/l) also yielded strong AP-1 activation (Fig. 7).

Effect of intercellular signalling on endothelial tyrosine kinase activity. We have shown that exposure of EC to the $^{\cdot}O_2^-$-generating mixture of hypoxanthine/xanthine oxidase results in activation of tyrosine kinase(s). To test whether $^{\cdot}O_2^-$ derived from high D-glucose pre-exposed SMC are responsible for tyrosine phosphorylation in EC, tyrosine kinase activity was monitored in EC homogenates after interaction with untreated and high D-glucose pre-exposed SMC. In EC that interacted for 4 h with high D-glucose pre-exposed SMC tyrosine kinase activity increased depending on the amount of D-glucose to which the SMC had been exposed to (Fig. 8A). Co-incubation with SOD (200 U/ml) during interaction with high D-glucose pre-exposed SMC prevented the increase in tyrosine kinase activity in EC. Furthermore, tyrosine phosphorylation of the cytoskeleton anchor protein β-catenin was increased after interaction with high D-glucose pre-exposed SMC in a SOD-sensitive manner (Fig. 8B). In line with these findings, incubation of EC with exogenously generated $^{\cdot}O_2^-$ (hypoxanthine/xanthine oxidase; 1 mmol/l and 300 μU/ml) for 4 h also resulted in tyrosine phosphorylation of β-catenin (data not shown).

This result was further confirmed when tyrosine kinase activation in response to exogenous $^{\cdot}O_2^-$ (hypoxanthine/xanthine oxidase; 1 mmol/l and 500 μU/ml) was investigated in single EC using the fluorescence probe for tyrosine kinase activity Picchu-936X. Tyrosine phosphorylation increased predominantly on the edge of the cell within 30 min of $^{\cdot}O_2^-$ exposure, while

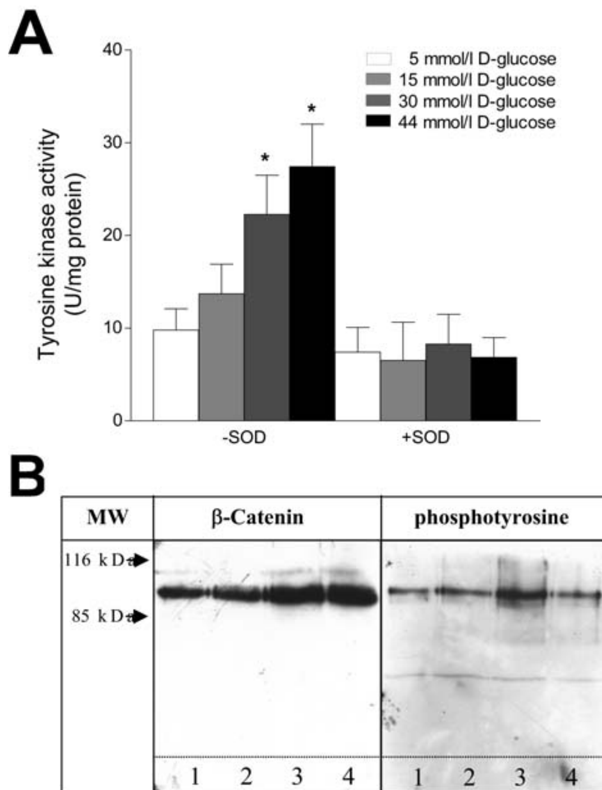

Fig. 8A, B. Interaction of EC with high D-glucose–pre-exposed SMC results in tyrosine kinase activation and tyrosine phosphorylation of β-catenin in a SOD-sensitive manner. (**A**) EC were put for 4 h in DMEM in the absence (–SOD) or presence of 200 U/ml SOD (+SOD) on top of SMC that were pretreated for 24 h in DMEM containing the D-glucose concentration indicated. Tyrosine kinase activity was monitored (*n*=6). *$p<0.05$ vs kinase activity in cells after interaction with normoglycaemic SMC. (**B**) Western blot analysis (*n*=3) of EC that were incubated in DMEM in the absence (*lane 1 and 3*) and presence of 200 U/ml SOD (*lane 2 and 4*) with SMC pretreated for 24 h in DMEM containing 5 (*lane 1 and 2*) or 44 mmol/l D-glucose (*lane 3 and 4*). The same blot was labelled with anti-phosphotyrosine (*right*), stripped and reprobed with anti-β-catenin (*left*)

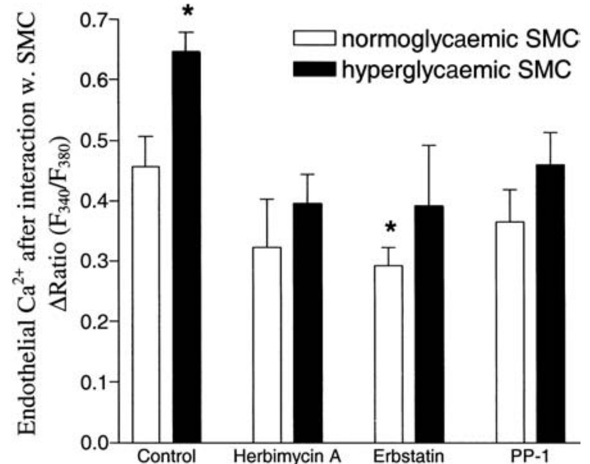

Fig. 9. Inhibition of tyrosine kinase(s) prevents alteration of endothelial Ca^{2+} signalling upon interaction with high D-glucose–pre-exposed SMC. EC were incubated with SMC that were pretreated for 24 h in DMEM containing 5 (normoglycaemic SMC) or 44 mmol/l D-glucose (hyperglycaemic SMC) for 4 h in the absence (*Control*) or presence of either herbimycin A (2 μmol/l), erbstatin analog (Erbstatin, 10 μmol/l) or PP-1 (10 μmol/l). Columns indicate the mean±SEM of the increase in cytosolic Ca^{2+} concentration in response to 100 nmol/l bradykinin in the presence of 2.5 mmol/l extracellular Ca^{2+}. *$p<0.05$ vs endothelial Ca^{2+} signalling in cells after interaction with normoglycaemic SMC (*n*=12)

no effect of hypoxanthine/xanthine oxidase on the inactive mutant Picchu-938X was found (data not shown).

Contribution of tyrosine kinase(s) to endothelial cell adaptation upon interaction with high D-glucose pre-exposed smooth muscle cells. To test which tyrosine kinase is involved in the changes of endothelial Ca^{2+} signalling upon interaction with high D-glucose pre-exposed SMC, the non-specific tyrosine kinase inhibitors erbstatin analog (10 μmol/l), herbimycin A (2 μmol/l) and the src-family specific inhibitor PP-1 (10 μmol/l) were used. All inhibitors prevented changes in Ca^{2+} signalling in EC that interacted with high D-glucose pre-exposed SMC (Fig. 9). Furthermore, all tyrosine kinase inhibitors but not the protein kinase C inhibitor GF109203X (5 μmol/l) prevented stress fibre formation in EC exposed to high D-glucose-pretreated SMC (data not shown).

To further elucidate the involvement of c-src kinase in the observed changes in EC upon interaction with high D-glucose pre-exposed SMC, a kinase-inactive c-src (KI-src) was expressed in EC by infection with adenovirus encoding KI-src. As a control, EC were infected with adenovirus encoding LacZ. Expression of KI-src per se did not affect Ca^{2+} signalling but prevented the stimulatory effect of interaction with high D-glucose pre-exposed SMC (Fig. 10). In contrast, in EC that were infected with the control virus (i.e. LacZ) a similar enhancement of the Ca^{2+} signalling by high D-glucose pre-exposed SMC as in non-infected cells was observed (Fig. 10).

In agreement with our findings on Ca^{2+} signalling, formation of stress fibres in EC by interaction with high D-glucose pre-exposed SMC was prevented in cells transfected with KI-src but not in the respective control cells (Fig. 11).

Discussion

We have shown that SMC, which were exposed to elevated D-glucose concentration affect EC by the release of a diffusible factor that could be scavenged by SOD, thus pointing to $^{\cdot}O_2^-$ as paracrine molecules. While no activation of NFκB and AP-1 was found in

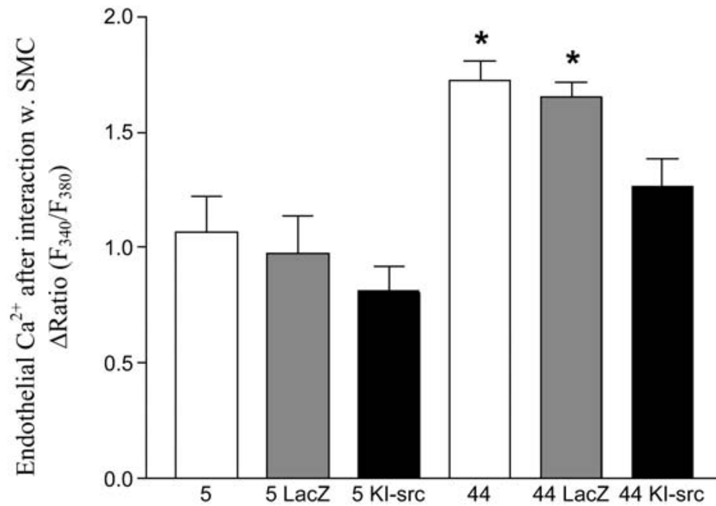

Fig. 10. Adenovirus-mediated transfection of EC with a kinase-inactive c-src but not LacZ prevents changes in endothelial Ca^{2+} signalling by high D-glucose–pre-exposed SMC. EC were infected with 1000 m.o.i. of adenovirus encoding kinase-inactive src (*KI-src*) or LacZ. After 5 days the cells were incubated for 4 h in DMEM with SMC that were pretreated for 24 h in DMEM containing 5 or 44 mmol/l D-glucose. *$p<0.05$ vs EC incubated with normoglycaemic SMC (n=6)

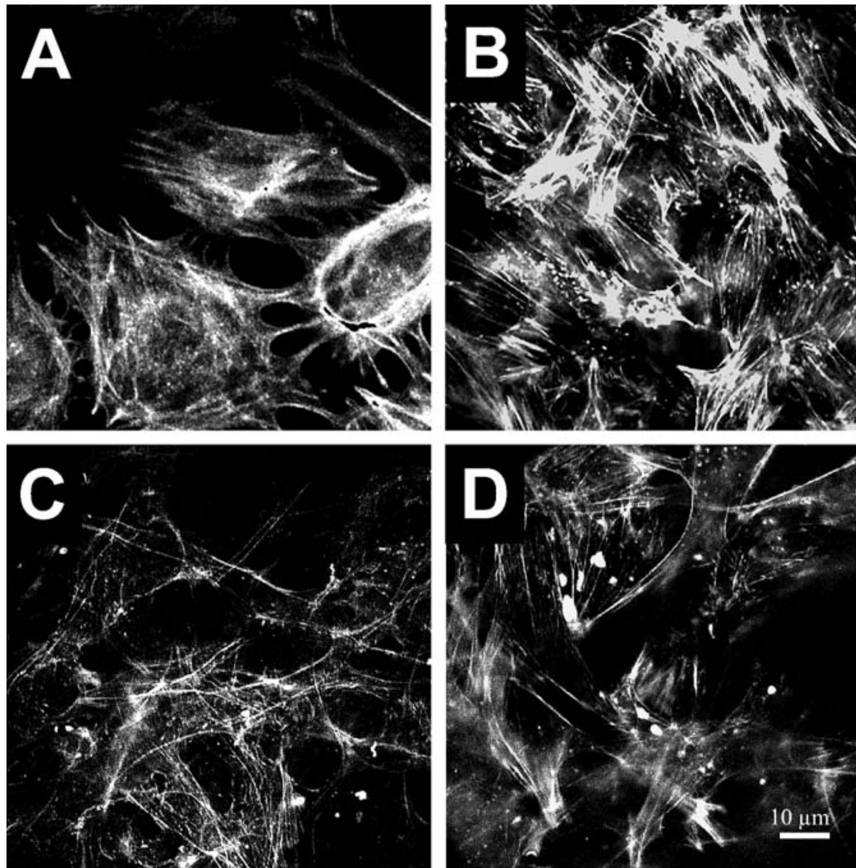

Fig. 11A–D. Adenovirus-mediated transfection of EC with a kinase-inactive c-src prevents changes in endothelial cytoskeleton organization by high D-glucose–pre-exposed SMC. EC were infected with 1000 m.o.i. of adenovirus encoding kinase-inactive c-src (**C, D**). After 5 days the cells were incubated for 4 h in DMEM with SMC that were pretreated for 24 h in DMEM containing 5 (**A, C**) or 44 mmol/l D-glucose (**B, D**)

the absence of SOD, a stimulation of endothelial src kinase occurred, that in turn, resulted in reorganization of the cytoskeleton and alterations in Ca^{2+} signalling. These data suggest, that elevated D-glucose not only affects EC directly, but initiates the release of diffusible radicals from smooth muscle cells that, in turn, alter endothelial function via activation of the tyrosine kinase src.

The intercellular signalling between SMC that were pre-treated with high D-glucose for 24 h and EC was initially shown by studying endothelial Ca^{2+} signalling. Notably, endothelial Ca^{2+} signalling has been shown to be a suitable marker for cell dysfunction initiated by a variety of stimuli such as *E. coli* lipopolysaccharides (LPS; [19]), peroxides [20, 21, 22], oxidized low density lipoprotein [23], streptozotocin-induced diabetes [24, 25] and hyperglycaemia [10, 11, 26]. The latter was causally linked to generation of $^{\cdot}O_2^-$ in EC [10, 27]. In line with these reports, the interaction of EC with high D-glucose pre-exposed SMC augmented the increase in cytosolic Ca^{2+} in response to bradykinin. As the magnitude of this effect correlated with the concentration of D-glucose the SMC had been exposed to, we suggest that D-glucose treatment of the SMC triggers the release of an intercellular signalling molecule in a concentration-dependent manner. The release of this paracrine molecule continues even after removal of the D-glucose excess and, in turn, alters endothelial Ca^{2+} signalling. However, high D-glucose pre-exposed *SMC* that were kept for further 10 h under normoglycaemic conditions did not influence endothelial Ca^{2+} homeostasis. These data suggest that D-glucose-triggered release of signalling molecules from the SMC is reversible. Furthermore, pretreatment of the SMC with high concentrations of D-mannitol had no effect on endothelial Ca^{2+} signalling excluding hyperosmolarity as the cause for SMC activation. Thus, we wanted to find out which signalling molecule is released from the SMC that affects EC function.

Based on our recent observation of an increased $^{\cdot}O_2^-$ production in SMC under diabetic [6] and hyperglycaemic conditions [28], the role of $^{\cdot}O_2^-$ as a prime candidate was studied by adding SOD during cell interaction. The result that SOD treatment abrogated the stimulatory effect of high D-glucose pre-exposed SMC on endothelial Ca^{2+} signalling strongly supports this hypothesis. This was further corroborated by the observation that SMC released $^{\cdot}O_2^-$ if treated with increased D-glucose (but not mannitol) in a concentration-dependent manner. Moreover, D-glucose-dependent $^{\cdot}O_2^-$ release from porcine aortic SMC is in line with our previous report that the release of $^{\cdot}O_2^-$ is enhanced in uterine arteries of diabetic patients [6]. The source of SMC $^{\cdot}O_2^-$ and the mechanisms of its release under elevated D-glucose conditions are still a matter of debate, but NAD(P)H oxidase [29, 30, 31, 32, 33] and mitochondria [34, 35] have been discussed frequently to contribute to increased $^{\cdot}O_2^-$ production in diabetes [7]. Overall, recent literature and our present data suggest that under hyperglycaemic conditions $^{\cdot}O_2^-$ release from SMC is augmented and affects EC function.

This notion is further supported by our data on stress fibre formation in EC exposed to high D-glucose-pre-treated SMC, a phenomenon that could be prevented by SOD. Notably, the cytoskeleton and in particular f-actin bundles have been shown to contribute to Ca^{2+} signalling [36, 37, 38, 39]. Thus, it is tempting to speculate that the observed effect on Ca^{2+} signalling is causally linked to alteration of the cytoskeleton. A reorganization of the cytoskeleton in vascular cells by free radicals has been reported presumably due to activation of the Rho GTPase family member Rac [40, 41, 42, 43, 7] that, in turn, triggers activation of the transcription factor NFκB [44]. In addition to NFκB, AP-1 was found to be activated by peroxides in human microvascular EC [45]. In line with these findings, we observed activation of NFκB and upregulation of AP-1 in EC upon interaction with the high D-glucose pre-exposed SMC only in the presence of SOD which converts the SMC-derived $^{\cdot}O_2^-$ to H_2O_2. Moreover, hypoxanthine/xanthine oxidase, a predominantly $^{\cdot}O_2^-$ generating system [46], had only minor effects on the activity of these two transcription factors. These data point to diversity in the effects of H_2O_2 and $^{\cdot}O_2^-$ on regulation of the inflammatory transcription factors NFκB and AP-1 in EC.

In contrast, the interaction of EC with high D-glucose pre-exposed SMC resulted in activation of tyrosine kinase(s) that was dependent on the concentration of D-glucose used. Since co-incubation with SOD prevented endothelial tyrosine kinase(s) activation, one may assume that this $^{\cdot}O_2^-$-dependent tyrosine kinase(s) activation is crucial for modulation of endothelial function by high D-glucose pre-exposed SMC. This hypothesis is further confirmed by our finding of a SOD-sensitive tyrosine phosphorylation of the cytoskeleton anchor protein β-catenin in EC by high D-glucose pre-treated SMC. Moreover, activation of endothelial tyrosine kinase(s) by extracellullar $^{\cdot}O_2^-$ was further shown in single cells using Picchu-936X, a recently introduced molecular sensor for tyrosine kinase activity [17].

Besides the wide range tyrosine kinase inhibitors erbstatin analog and herbimycin A [47], the rather selective src family inhibitor PP-1 [48] prevented the above mentioned changes in EC function upon interaction with high D-glucose pre-exposed SMC thus, pointing to an involvement of src family member(s) therein. This assumption was further supported by the findings that transfection of EC with dominant negative c-src kinase diminished the observed changes in Ca^{2+} signalling and stress fibre formation. These data suggest that $^{\cdot}O_2^-$ derived from high D-glucose pre-exposed SMC activate endothelial src kinase that, in turn, alters cytoskeleton and Ca^{2+} signalling.

Our findings that the protein kinase C inhibitor GF109203X [49, 50] failed to prevent endothelial tyrosine kinase activation by high D-glucose pre-exposed SMC suggest that an activation of protein kinase C is not involved in the activation of src by $^{\cdot}O_2^-$. In consideration of the stimulation of protein kinase C-β during hyperglycaemia [9], the tyrosine

kinase activation reported herein represents a new pathway in diabetes. While the exact mechanism of src kinase activation is unclear, it seems obvious that extracellular $\cdot O_2^-$ but not H_2O_2 mediate this effect. Alternatively, increased tyrosine phosphorylation could be the result of inhibition of src selective tyrosine phosphatases as it was recently shown by H_2O_2 [51]. However, basal src kinase activity seemed to be rather low in the EC used, thus favouring the hypothesis that $\cdot O_2^-$ derived from high D-glucose pre-exposed SMC activate endothelial src kinase by unknown mechanisms.

Since all reported phenomena (i.e. changes in Ca^{2+} signalling, tyrosine kinase activation and stress fibre formation) were sensitive to the presence of SOD within the interaction period and could further be mimicked by a direct treatment of endothelial cells with the $\cdot O_2^-$ generating mixture xanthine oxidase/hypoxanthine, we speculate that smooth muscle-derived $\cdot O_2^-$ serve as intercellular messenger between the two types of vascular cells. However, our findings do not exclude additional signalling molecules like hsp 90 that was recently found to constitute a smooth muscle-derived messenger molecule under oxidative stress conditions [52].

Our data that SOD was actually prerequisite in order to initiate NFκB/AP-1 activation but prevented changes in Ca^{2+} signalling, src activation and stress fibre formation in EC by high D-glucose pre-exposed SMC point to two distinct pathways as potential targets under hyperglycaemic conditions. It is tempting to speculate that depending on the activity/expression of SOD in the vascular bed one or the other (or both) of the two signalling pathways gets activated under hyperglycaemic conditions. In view of the reported alterations of SOD expression during prolonged hyperglycaemic conditions [7, 53] it seems possible that the src pathway represents an initial target during acute hyperglycaemia while prolonged hyperglycaemia favours activation of the NFκB/AP-1 pathway. The actual consequences of such switch need to be further explored.

In conclusion, our data suggest that $\cdot O_2^-$ derived from hyperglycaemia pre-exposed SMC activate EC src kinase independently of protein kinase C. The enhanced tyrosine kinase activity initiates f-actin polymerization, phosphorylation of β-catenin and alters endothelial Ca^{2+} signalling. Our work unmasks considerable differences between the effects of $\cdot O_2^-$ and H_2O_2 on transcription factor activation and point to a surprising versatility in radical-mediated signal transduction.

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