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The effects of glucose-induced oxidative stress on growth and extracellular matrix gene expression of vascular smooth muscle cells

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Summary Vascular smooth muscle cell (VSMC) dysfunction plays a role in diabetic macrovasculopathy and this may include abnormalities in growth characteristics and the extracellular matrix. As the actual mechanisms by which glucose induces VSMC dysfunction remain unclear, the aim of this study was to assess the potential role of glucose-induced oxidative stress. Porcine aortic VSMCs were cultured for 10 days in either 5 mmol/l normal glucose or 25 mmol/l D-glucose (high glucose). There was evidence of oxidative stress as indicated by a 50% increase in intracellular malondialdehyde ($p < 0.05$), increased mRNA expression of CuZn superoxide dismutase and Mn superoxide dismutase (by 51% and 37% respectively, $p < 0.01$) and a 50% decrease in glutathione in 25 mmol/l D-glucose ($p < 0.001$). Growth was increased by 25.0% ($p < 0.01$). mRNA expression of extracellular matrix proteins (collagens I, III, IV and fibronectin) was not altered by high glucose in these experimental conditions. Repletion of glutathione with N-acetyl L-cysteine (1 mmol/l) in VSMC grown in high glucose was associated with reduction in malondialdehyde and restored growth to that of normal glucose. The water soluble analogue of vitamin E, Trolox (200 μmol/l), reduced malondialdehyde concentrations, but had no effect on glutathione depletion or the increased growth rate seen with high glucose. The addition of buthionine sulphoximine (10 μmol/l) to VSMC cultured in normal glucose reduced glutathione, increased malondialdehyde and increased growth to a similar extent as that found in high glucose alone. These results suggest that thiol status, rather than lipid peroxides, is a key factor in modulating VSMC growth and that mRNA expression of extracellular matrix proteins is not increased in VSMC under conditions of glucose-induced oxidative stress. [Diabetologia (1998) 41: 1210–1219]

Keywords Diabetic macrovasculopathy, oxidative stress, vascular smooth muscle cells, extracellular matrix, antioxidant enzymes, glutathione

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Abbreviations: VSMC, Vascular smooth muscle cells; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate buffered saline; SOD, superoxide dismutase; GPX, glutathione peroxidase; RT-PCR, reverse transcriptase mediated polymerase chain reaction; BSO, buthionine sulphoximine; TAE, Tris acetate EDTA; NAC, N-acetyl L-cysteine; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; YGCS, gamma glutamyl cysteine synthetase

Diabetes mellitus is a major risk factor for atherosclerosis. In population based studies diabetes is associated with an increased incidence of coronary heart disease [1], cerebrovascular disease and peripheral vascular disease [2]. The high prevalence of macrovascular disease is partly explained by the increased frequency of conventional risk factors and partly by risk factors specific for diabetes including hyperglycaemia, hyperinsulinaemia and dyslipidaemia. The view that diabetic vasculopathy is, at least in part, related to excess free radicals is supported by evidence such as increased lipid peroxidation [3] and reduced antioxidant reserve in patients with diabetes [4] and from studies showing a direct cytotoxic effect of free

radicals and their metabolic end-products (lipid hydroperoxides) on endothelial cells [5].

High ambient glucose has been shown to affect endothelial and other vascular cells [6], such as is shown by the induction of apoptosis in cultured endothelial cells [7] and endothelial dysfunction in vivo [8]. Dysfunction of vascular smooth muscle cells (VSMC) is also known to occur in diabetes. Their growth and proliferation in the arterial intima is one key feature in the atherosclerotic lesion [9] and hyperproliferation has also been shown in VSMC cultured in high glucose concentrations [10]. There is evidence to suggest that reactive oxygen intermediates can act as stimulants of proliferation for VSMC [10, 11], although there are a number of other recognised factors such as insulin [12], protein kinase C [13], and potent growth factors such as platelet-derived growth factor, fibroblast growth factor and heparin-binding epidermal growth factor-like growth factor [14]. The gene expression of the third potent growth factor can be increased by oxidative stress [14] and oxidant-mediated activation of protein kinase C has also been reported [15]. Studies also suggest that VSMC which have migrated to the intima in atherosclerotic lesions, have changed from a contractile to a synthetic phenotype which could have profound effects on the capacity of the cell to respond to various agonists [16]. Non-atherosclerotic changes could also be important in the development of diabetic macroangiopathy and VSMC may be intimately involved in this process. Increases in the extracellular matrix proteins, fibronectin and collagen IV have been reported in the tunica media in diabetic patients without atherosclerotic plaques [17]. Therefore glucose-induced dysfunction of VSMC could involve increased growth rates, alteration in phenotype and increased synthesis of extracellular matrix proteins. The role of glucose-induced oxidative stress in these processes has not been extensively investigated.

Although glucose-induced oxidative stress possibly is important in promoting VSMC growth, there is little doubt that oxidative stress can also induce cell death [18]; therefore both the degree of oxidant stress and cell type must have potential importance in the growth response. Endogenous antioxidant defences to protect against attack by reactive oxygen species include the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPX) and the non-enzymatic aqueous phase antioxidant glutathione, which is perhaps the most important and versatile protector [19]. The reduced glutathione-oxidised glutathione couple is the major redox buffer in the cell and is known to be involved (along with oxidative stress) in regulation and activation of transcription factors such as NF-κB (nuclear factor kappa B) and AP-1 (activator protein 1). Activation of these transcription factors leads to the rapid induction of genes encoding both growth and defence proteins [20, 21]. Our aim was to assess the evidence for the existence of glucose-induced oxidative stress in VSMC and its role in VSMC dysfunction as displayed by growth abnormalities and gene expression of extracellular matrix proteins.

Materials and methods

All materials were obtained from Sigma Chemical Co. Ltd (Poole, Dorset, UK) unless otherwise stated.

VSMC isolation and culture. We isolated VSMC from porcine aortae (donors were between 50–70 kg, of both sexes and less than 24 months old) obtained from the local abbatoir. The intima and adventitial layers were stripped and the medial segment cut into small pieces approximately 1 mm^2 and transferred onto the growing surface of 25 cm^2 tissue culture flasks (Corning, Bibby Sterilin Ltd, Stone, Staff, UK) pre-moistened with fetal calf serum (FCS, PAA Laboratories GmbH, Linz, Austria). Following this, Dulbecco's modified Eagle medium (DMEM, GibcoBRL, Life Technologies, Paisley, UK) supplemented with antibiotics (benzylpenicillin 60 μg/ml and streptomycin sulphate 100 μg/ml), amphotericin B (2.5 μg/ml, GibcoBRL) and 10 % (v/v) fetal calf serum (growth medium) was added to the flasks. The flasks were placed in an incubator (37 °C, 5 % CO_2) and left undisturbed for 7 days. After this the growth medium (DMEM/10 % fetal calf serum) was replaced twice weekly. Within 7–10 days VSMC grew out from the explants reaching confluence within 2–3 weeks and were harvested with 0.125 % trypsin (type II, porcine pancreas, Difco Laboratories Inc, Detroit, Mich., USA) and split for further culture into 75 cm^2 tissue culture flasks. Cells were confirmed as VSMC by their typical morphology and positive immunofluorescence staining against smooth muscle α-actin.

For the different experiments, cells were cultured for 10 days in the appropriate conditions. Growth medium was changed twice weekly. All experiments were done on cells up to and including subculture number 5 (preliminary experiments had shown no significant differences in morphology, growth rates or intracellular activities and concentrations of the analytes of interest up to this subculture).

Viability of cultures. Trypan blue solution (0.4 %) was added to aliquots of cell suspensions in a 1:1 ratio and allowed to stand for 10 min at room temperature. A haemocytometer was used to count live and dead cells, and per cent viabilities were calculated. Lactate dehydrogenase activities were also measured in the supernatants using dry-film technology on a Vitros 750 analyser (Johnson & Johnson Clinical Diagnostics, Amersham, Bucks, UK).

Growth curve experiments. For all experiments VSMC were seeded (1.5×10^5 cells) into 25 cm^2 tissue culture flasks (day 0) under the various experimental conditions. Following trypsinisation, cell counts were performed on a Coulter counter (Coulter Electronics Ltd, Luton, Beds, UK); three separate readings were taken and the mean calculated. Cell counts were done on days 2, 4, 6, 8 and 10 in all cases.

Assessment of mitotic and apoptotic rates. Cytospins (600 rpm × 10 min), approximately 10^5 cells per slide, were prepared following trypsinisation of VSMC cultured under the different conditions for 6, 8 and 10 days. VSMC were stained with standard Haematoxylin & Eosin (H&E) and Giemsa.

For apoptosis, cells were inspected (500 cells per slide) to identify the typical nuclear condensation changes of this condition. Mitosis was assessed by counting mitotic figures (expressed as % of total of 3000 cells). Flow cytometric analysis (Epics ELITE flow cytometer, Coulter Corporation, Fla., USA) of propidium iodide stained cells was also used to identify proliferating cells (% G2 + M + S). VSMC were trypsinised, fixed in 70 % ethanol and stained with propidium iodide (30 μg/ml in phosphate-buffered saline (PBS), pH 7.6, 30 min). At least 10 000 events were measured for each sample and red fluorescence (623 nm) was detected to determine DNA content per nucleus. DNA histograms were analysed using Wincycle Software (Phoenix Flow Systems, San Diego, Calif., USA) and the per cent G2 + M + S calculated.

Harvesting of VSMC for measurement of malondialdehyde (MDA), glutathione (GSH), enzyme activities and protein activities. Monolayers were rinsed twice with ice-cold Hanks Balanced Salt Solution (HBSS, without calcium, magnesium and phenol red, GibcoBRL) and the cells collected using a disposable cell scraper (Sarstedt Ltd, Leicester, UK). The cells were centrifuged at 225 *g* at 4 °C for 5 min and the cell pellet stored at –80 °C prior to the stage of cell lysis.

Cell lysis and homogenization. Cell pellets were removed from the freezer and resuspended in 0.1 % Triton X-100 (BDH Chemicals Ltd) in PBS (pH 7.4). Sonication was done with two 15 s bursts on a sonicator (Lucas Dawe Ultrasonics, London, UK), using a microtip (setting number 4, continuous cycle) with a 20 s cooling period separating the two bursts. An aliquot of the whole cell homogenate was removed for protein analysis. The rest of the homogenate was centrifuged at 3000 *g* for 30 min at 4 °C and aliquots of the resulting supernatant taken for the analyses outlined below. CuZnSOD, MnSOD and GPX activities were analysed immediately. CuZnSOD protein and malondialdehyde samples were stored at –80 °C prior to analysis. The samples for glutathione were stabilized by the addition of 1 part 10 % sulphosalicylic acid to 2 parts sample. After centrifugation at 13 000 *g* for 5 min, the resulting supernatant was stored at –80 °C.

Protein measurement. Total protein was measured using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce and Warriner Ltd, Chester, UK). Bovine serum albumin (BSA) was used as the standard.

Malondialdehyde. Malondialdehyde was measured by HPLC with fluorimetric detection [22].

Glutathione. This was measured by the method described by Griffith [23] which has been automated on the Cobas Fara centrifugal analyser (Roche Products Ltd, Welwyn Garden City, Herts, UK).

CuZnSOD protein concentration. The concentration of CuZnSOD protein was measured using an ELISA [24].

CuZnSOD, MnSOD & GPX activities. Total superoxide dismutase (SOD) activity was measured using the method of L'Abbe and Fischer [25] adapted for the Cobas Fara centrifugal analyser. MnSOD activity is resistant to incubation at room temperature with 2 mmol/l potassium cyanide (BDH Chemicals Ltd) for 45 min. CuZnSOD activity was subsequently calculated by the subtraction of MnSOD activity from total SOD activity. The enzyme activity of GPX was determined by the method of McMaster et al. [26].

Extraction of RNA from VSMC. Total cellular RNA was extracted from cultured cells by the acid-guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi [27].

Northern blot analysis. Aliquots (30 μg) of total RNA were separated by electrophoresis on a 1 % agarose-formaldehyde gel. Transfer of the denatured RNA was to a charged nylon filter (Hybond N^+, Amersham Internat plc, Little Chalford, Bucks, UK). Hybridization was done in a Tecne Hybridizer HB-1D hybridization oven (Techne Ltd, Duxford, Cambridge, UK) using specific porcine cDNA probes to CuZnSOD, MnSOD and GPX mRNA prepared by reverse-transcriptase mediated polymerase chain reaction (RT-PCR) as described in the following section. A porcine cDNA probe to glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was also prepared by RT-PCR and used as a "house-keeping" gene. Prehybridization was at 65 °C for 30 min following the addition of Quickhyb hybridization solution (0.2 ml per cm^2, Stratagene Ltd, Cambridge, UK.). cDNA probes (75–100 ng) were labelled using the Prime-It II Random Primer Labelling kit (Stratagene) and 50 μCi ^{32}P dCTP (Amersham, Redivue, 370 MBq/ml). Hybridization was carried out for 2 h at 65 °C following which the membrane was washed (at 42 °C) twice in 2 × standard sodium citrate (SSC), 0.1 % sodium dodecyl sulphate (SDS) for 15 min, once with 1 × SSC, 0.1 % SDS for 15 min and finally with 0.2 × SSC, 0.1 % SDS for 15 min.

The membranes were analysed on a Bio-Rad Molecular Imager system GS-525 (Bio-Rad Laboratory Ltd, Hemel Hempstead, Herts, UK). Band intensities were quantified using the attached software by volume analysis with local background subtraction. GAPDH mRNA expression was used to correct for loading inequalities.

Reverse Transcriptase-mediated Polymerase Chain Reaction (RT-PCR). The following PCR primers were used (EcoRI internal restriction sites underlined):

I. MnSOD (Acc No. X64057); CBS1: GGAATTCCAGCTGCACCACAGCGAGC (100 pmol/reaction); CBA1: GGAATTCGATCCCCAGCAGCGGAACC (100 pmol/reaction)

II. CuZnSOD (Acc No. P04178); CBS2: GGAATTCT(T/C)CA(T/C)GTNCA(T/C)CA(A/G)TT(T/C)GG (640 pmol/reaction); CBA2: GGAATTCC(A/G)TCNGG(T/C)TT(T/C)TC(A/G)TGNAC (1280 pmol/reaction)

III. GPX I [28–30] ; CBS3: GGGAATTCCCNTG(T/C)AA(T/C)CA(A/G)TT(T/C)GG (320 pmol/reaction); CBA3: GGGAATTCG(T/C)(T/C)T(C/G)(A/G/T)AT(A/G)TCNG-G(T/C)TC (1290 pmol/reaction)

IV. GAPDH (Acc Nos. U48832, X94251); CBS4: GGGAATTCACNATGGA(A/G)AA(A/G)GCNGG (320 pmol/reaction); CBA4: GGAATTCTTGGAGGCCATGTGGACC (100 pmol/reaction)

Oligo(dT) primed first strand cDNA synthesis was carried out on total RNA (1 μg) with a reverse transcription system (Promega Corp, Madison, Wis., USA) but using superscript II Rnase H^- reverse transcriptase (GibcoBRL). PCR reagents were obtained from Promega. An aliquot (3 μl) of the first strand cDNA reaction was added directly to the PCR mix containing 0.25 mmol/l of each deoxynucleotide triphosphate (dNTPs)(dATP, dCTP, dGTP, dTTP), 5.0 U Taq DNA polymerase, sense and antisense primers in 100 μl of 1 × Taq DNA polymerase buffer [50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 9.0), 0.1 % Triton X-100]. $MgCl_2$ to a final concentration of 1.5 mmol/l was also added. A Perkin Elmer DNA Thermal Cycler (Perkin Elmer, Beaconsfield, Bucks, UK) was used with the following cycling times and temperatures; 94 °C for 2 min,

Table 1. Intracellular malondialdehyde and glutathione in VSCM cultured for 10 days under varying conditions

Culture conditions	MDA (nmol/mg protein)	GSH (nmol/mg protein)
5 mmol/l D-glucose	1.20 ± 0.12	1.36 ± 0.16
25 mmol/l D-glucose	1.81 ± 0.25[a]	0.67 ± 0.09[b]
5 mmol/l D-glucose + 20 mmol/l mannitol	1.34 ± 0.16	1.68 ± 0.22
5 mmol/l D-glucose + 20 mmol/l L-glucose	1.15 ± 0.15	1.23 ± 0.16

MDA, malondialdehyde, GSH, glutathione, $n = 12$ independent experiments (in triplicate), [a] $p < 0.05$, [b] $p < 0.001$, 5 mmol/l vs 25 mmol/l D-glucose

followed by 5 cycles of 50 °C for 1 min, 72 °C for 40 s, 94 °C for 45 s, and 30 cycles of 60 °C for 1 min, 72 °C for 40 s and 94 °C for 45 s, and then 72 °C for 5 min. Reaction products (10 μl) were separated by 1.2 % agarose gel electrophoresis in 1 × Tris acetate EDTA (TAE) buffer and visualised by staining with ethidium bromide (0.5 μg/ml).

Subcloning. Purification of PCR products was done using the Wizard PCR purification kit (Promega) and subcloned into pGEM-T (Promega). Verification of transformation was done by PCR screening of individual colonies (clones).

DNA sequencing and sequence analysis. The sequence of cDNA inserts was verified. Plasmid DNA was denatured and sequenced in both directions using sequenase V2.0 (USB, Cleveland, Ohio, USA).

mRNA expression of extracellular matrix proteins. VSMC were grown for a period of 10 days in the presence of either 5 mmol/l or 25 mmol/l D-glucose. Total RNA was extracted as described above. cDNA to fibronectin and collagen IV were obtained from Human Genome Mapping Project (HGMP, Cambridge, UK). Plasmids pMCol1a-1 (collagen I, mouse) and pRGR5 (collagen III, rat) were obtained as gifts from Professor Eero Vuorio (Department of Medical Biochemistry and Molecular Biology, University of Turku, Turku, Finland) [31, 32]. Northern blotting and hybridization was as described previously with the exception that the hybridization temperature was reduced to 60 °C.

Other materials. D-glucose, D-mannitol, L-glucose, N-acetyl L-cysteine and Trolox were obtained from Sigma Chemical Co. (Poole, Dorset, UK). L-Buthionine-S,R,-sulphoximine (99 %, BSO) was obtained from Janssen Chimica (Acros, Newton, Cheshire, UK).

Statistical analysis. The normality of data was assessed using the Kolmogorov-Smirnov Normality test; comparison between groups was assessed by analysis of variance (ANOVA) and paired *t* tests. Northern blots were analysed by paired *t* tests. All results are expressed as mean ± SEM.

Results

Cell viability. In all experiments more than 92 % of VSMC were viable as assessed by trypan blue dye exclusion and lactate dehydrogenase activities in the supernatant were not different from those cultured in 5 mmol/l D-glucose (results not shown).

Evidence of glucose-induced oxidative stress. In VSMC cultured in 25 mmol/l D-glucose for 10 days intracellular malondialdehyde was increased (by 50 %) ($p < 0.05$), and glutathione was decreased (by 50 %) ($p < 0.001$), but this was not found in either equimolar L-glucose (glycation control) or mannitol (osmotic control) (Table 1). Glutathione disulphide was less than 3.0 % of total glutathione in 5 mmol/l and less than 3.6 % in 25 mmol/l D-glucose (Table 1) and there was no difference between 5 mmol/l and 25 mmol/l D-glucose ($n = 4$ experiments, results not shown).

In the presence of 25 mmol/l D-glucose ($n = 7$) mRNA expression of CuZnSOD was increased 1.51-fold and that of MnSOD 1.37-fold. There was no alteration in GPX mRNA expression (Fig. 1). In a smaller number of experiments ($n = 3$) mRNA expression of CuZnSOD and MnSOD were not altered in the presence of either equimolar L-glucose or mannitol (Table 2).

The concentration of CuZnSOD protein was increased in 25 mmol/l compared with 5 mmol/l D-glucose (8.38 ± 0.49 vs 4.01 ± 0.34 ng/mg protein, $p < 0.001$, $n = 12$). This was not found with either L-glucose or mannitol (4.65 ± 0.25 and 3.78 ± 0.29 ng/mg protein respectively). Enzymatic activities of CuZnSOD, MnSOD and GPX were similar for cells grown in 5 mmol/l and 25 mmol/l D-glucose (CuZnSOD: 62.2 ± 4.1 vs 64.0 ± 4.5 U/mg protein; MnSOD: 3.4 ± 0.4 vs 2.9 ± 0.3 U/mg protein; GPX: 25.0 ± 2.5 vs 16.8 ± 3.2 mU/mg protein, 5 mmol/l vs 25 mmol/l D-glucose, $n = 12$).

Table 2. mRNA of antioxidant enzymes in VSMC cultured for 10 days in 5 mmol/l D-glucose, 25 mmol/l D-glucose, 5 mmol/l D-glucose plus 20 mmol/l L-glucose and 5 mmol/l D-glucose plus 20 mmol/l mannitol

Antioxidant enzyme	5 mmol/l D-glucose	25 mmol/l D-glucose	5 mmol/l D-glucose + 20 mmol/l L-glucose	5 mmol/l D-glucose + 20 mmol/l mannitol
CuZn SOD	1.0	1.51 ± 0.09[a]	1.09 ± 0.10	1.14 ± 0.09
Mn SOD	1.0	1.37 ± 0.10[a]	0.93 ± 0.11	1.11 ± 0.07
GPX	1.0	1.09 ± 0.07		

Volume analysis corrected for glyceraldehyde-3 phosphate dehydrogenase; 5 mmol/l = 1.0, $n = 7$ independent experiments for 5 mmol/l vs 25 mmol/l D-glucose, $n = 3$ for 5 mmol/l D-glucose, 5 mmol/l D-glucose plus L-glucose and 5 mmol/l D-glucose plus mannitol. [a] $p < 0.01$ cf 5 mmol/l D-glucose

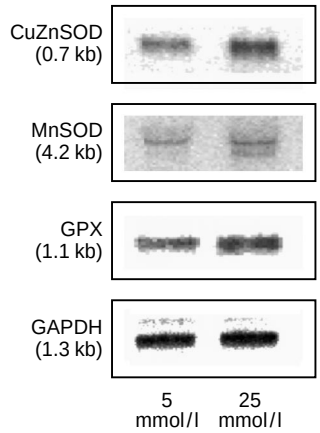

Fig. 1. mRNA expression of CuZnSOD (0.7 kb), MnSOD (4.2 kb) and glutathione peroxidase (GPX, 1.1 kb) in VSMC cultured for 10 days in either 5 mmol/l or 25 mmol/l D-glucose

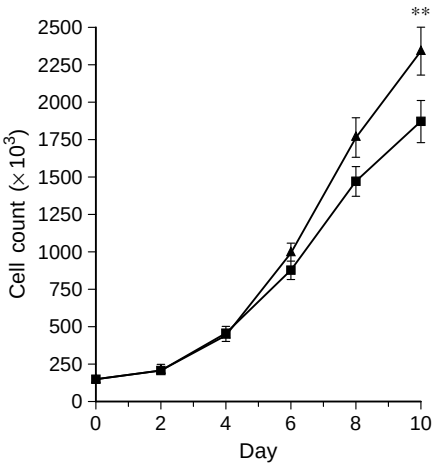

Fig. 2. Growth curves of VSMC cultured in 5 mmol/l and 25 mmol/l D-glucose for 10 days. $^{**}p < 0.01$, 25 mmol/l vs 5 mmol/l. ($n = 4$) —■— 5 mmol/l D-glucose, —▲— 25 mmol/l D-glucose

There was an increased number of cells in 25 mmol/l D-glucose from 6 days onwards. VSMC were increased by 25.0 ± 1.1 % compared with 5 mmol/l D-glucose at day 10 ($p < 0.01$) (Fig. 2). This increase was not repeated in equimolar L-glucose or mannitol (results not shown). There were no differences in mRNA expression of the extracellular matrix proteins in VSMC cultured in 25 mmol/l D-glucose (Table 3, Fig. 3).

Effects of antioxidants. The following antioxidants were added to VSMC cultured in 25 mmol/l D-glucose; N-acetyl L-cysteine (NAC, 0.5 mmol/l and 1.0 mmol/l) and Trolox (200 μmol/l) over the 10 day period of culture. Concentrations of NAC greater than 1 mmol/l were associated with reduced cellular viability. The addition of NAC (0.5 mmol/l and 1.0 mmol/l) and Trolox (200 μmol/l) reduced intracellular malondialdehyde concentrations to levels which were not significantly different from those of VSMC cultured in 5 mmol/l D-glucose. Only NAC (and in particular the 1 mmol/l concentration) had an effect in increasing the glutathione concentration towards that of VSMC cultured in 5 mmol/l D-glucose (Fig. 4).

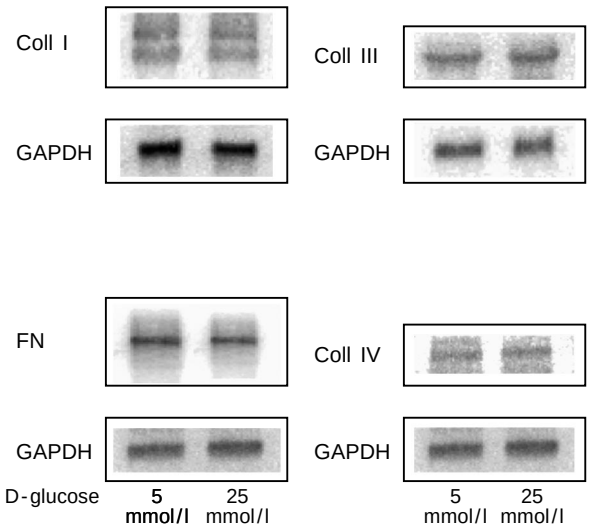

Fig. 3. mRNA expression of the extracellular matrix proteins, collagens I (5.4 kb, 6.4 kb), III (6 kb) and IV (6.5 kb) and fibronectin (7.9 kb) in VSMC cultured for 10 days in either 5 mmol/l or 25 mmol/l D-glucose

Independent experiments ($n = 3$) suggest that although Trolox reduced the increased mRNA expression to normal, 0.5 mmol/l and 1 mmol/l NAC had no effect in doing so (Table 4, Fig. 5).

The addition of both 0.5 mmol/l and 1.0 mmol/l NAC to VSMC cultured in 25 mmol/l D-glucose for 10 days normalised their growth (25 mmol/l D-glucose; growth increased by 24.5 ± 2.6 % compared with 5 mmol/l D-glucose at day 10, $p < 0.05$: 25 mmol/l D-glucose plus 0.5 mmol/l NAC; increased by 2.9 % ± 5.8 %: 25 mmol/l D-glucose plus 1 mmol/l NAC; decreased by 5.5 ± 7.5 %, $n = 3$, growth curves not shown). Trolox (200 μmol/l) failed to reduce the increased growth of VSMC cultured in 25 mmol/l D-glucose (growth curve not shown). VSMC numbers were increased by 24.5 ± 2.6 % in 25 mmol/l D-glucose and by 28.9 ± 7.5 % in 25 mmol/l D-glucose plus Trolox at day 10 ($n = 3$, $p < 0.05$ for each condition).

Effects of buthionine sulphoximine (BSO). The concentration of BSO added to VSMC cultured in 5 mmol/l D-glucose was titrated to produce a similar reduction in GSH as was seen in 25 mmol/l D-glucose (range 0.5–10 μmol/l). The addition of 10 μmol/l BSO to VSMC cultured in 5 mmol/l D-glucose led to a significant reduction in GSH (0.65 ± 0.12 vs 1.87 ± 0.22 nmol/mg protein, 5 mmol/l D-glucose plus 10 μmol/l BSO vs 5 mmol/l D-glucose, $n = 5$, $p < 0.01$) and an

Table 3. mRNA expression of fibronectin and collagens I, III and IV in VSMC cultured for 10 days in either 5 mmol/l or 25 mmol/l D-glucose

	5 mmol/l D-glucose	25 mmol/l D-glucose
Fibronectin	1.0	1.07 ± 0.07
Collagen I	1.0	1.02 ± 0.12
Collagen III	1.0	0.95 ± 0.06
Collagen IV	1.0	1.07 ± 0.13

Volume analysis corrected for glyceraldehyde-3 phosphate dehydrogenase. 5 mmol/l = 1.0, (n = 5 independent experiments for fibronectin and collagen IV, n = 3 for collagen I and III)

Table 4. mRNA expression of CuZnSOD and MnSOD in VSMC cultured for 10 days under differing conditions

Condition	CuZnSOD	MnSOD
5 mmol/l D-glucose	1.0	1.0
25 mmol/l D-glucose	1.50 ± 0.11	1.33 ± 0.10
25 mmol/l + 1 mmol/l NAC	1.39 ± 0.13	1.52 ± 0.17
25 mmol/l + 200 μmol/l Trolox	1.16 ± 0.18	1.08 ± 0.15

n = 3 independent experiments, 5 mmol/l = 1.0

increase in malondialdehyde (1.69 ± 0.13 vs 1.05 ± 0.08 nmol/mg protein, $n = 5$, $p < 0.05$) (Fig. 6)

In two experiments, mRNA expression of CuZnSOD and MnSOD was increased in VSMC cultured in 5 mmol/l D-glucose plus 10 μmol/l BSO compared with 5 mmol/l D-glucose (CuZnSOD; by 1.83-fold and 1.20-fold, MnSOD; by 1.52-fold and 1.19-fold).

The addition of 10 μmol/l BSO to VSMC cultured in 5 mmol/l D-glucose was associated with an increase in VSMC growth to a similar extent as demonstrated in 25 mmol/l D-glucose (Fig. 7).

Mitotic and apoptotic rates. There was no evidence of apoptosis, as assessed by morphological criteria, in any of the above conditions (5 mmol/l D-glucose, 25 mmol/l D-glucose, 25 mmol/l D-glucose plus NAC, 25 mmol/l D-glucose plus Trolox and 5 mmol/l D-glucose plus BSO, n = 3 for each condition) at 6, 8 and 10 days. The absence of a sub G1 peak also suggested the absence of apoptosis. Mitosis as measured by mitotic figures and % G2 + M + S at days 6, 8 and 10 showed a tendency to increase in 25 mmol/l D-glucose, 25 mmol/l D-glucose plus 200 μmol/l Trolox and 5 mmol/l D-glucose plus BSO and reduce in 25 mmol/l D-glucose plus 1 mmol/l NAC (Table 5).

Discussion

This work has shown that glucose causes oxidative stress in VSMC in culture. There has been evidence of oxidant damage in the form of increased malondialdehyde and conjugated dienes in human endothelial cells cultured in 20 mmol/l D-glucose for 10 days [33], but the existence of glucose-induced

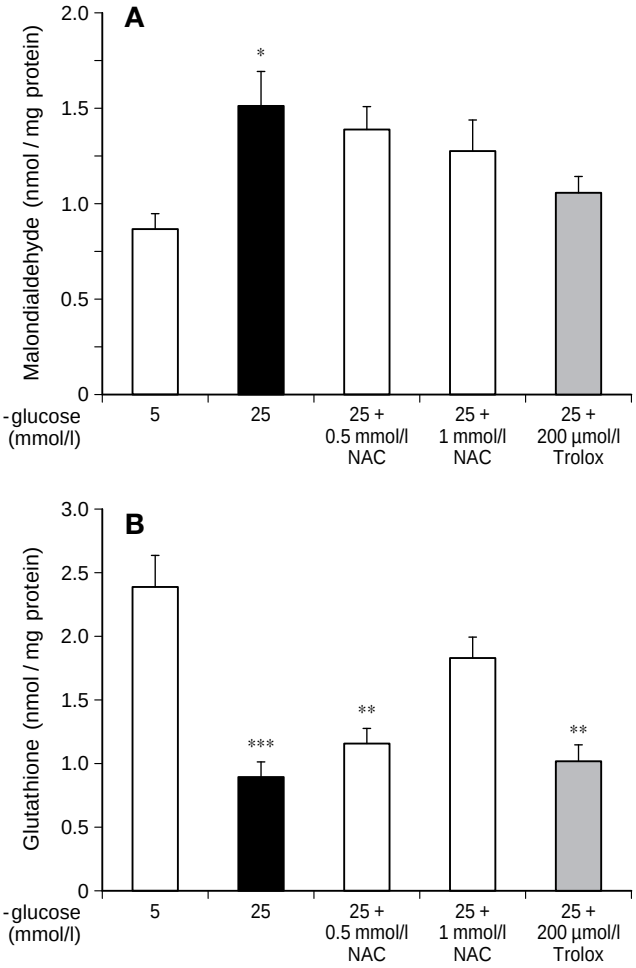

Fig. 4. A Intracellular malondialdehyde in VSMC cultured in 5 mmol/l D-glucose, 25 mmol/l D-glucose, 25 mmol/l D-glucose plus 0.5 mmol/l, 1.0 mmol/l N-acetyl cysteine (NAC) and 25 mmol/l D-glucose plus 200 μmol/l Trolox for 10 days. *$p < 0.05$ cf 5 mmol/l D-glucose. n = 5 (in duplicate). **B** Intracellular glutathione in VSMC cultured in 5 mmol/l D-glucose, 25 mmol/l D-glucose, 25 mmol/l D-glucose plus 0.5 mmol/l, 1.0 mmol/l N-acetylcysteine (NAC) and 25 mmol/l D-glucose plus 200 μmol/l Trolox for 10 days. **$p < 0.01$, ***$p < 0.001$ cf 5 mmol/l D-glucose. n = 5 (in duplicate)

oxidant damage in VSMC has not been reported previously.

Various sources of free radicals can contribute to oxidative stress in diabetes, including monosaccharide autoxidation, non-enzymatic glycation of proteins, increased polyol pathway activity and reduced antioxidant reserve. Although malondialdehyde is believed to be a sensitive indicator of free radical activity, oxidative stress does occur in the absence of lipid peroxidation. The upregulation of mRNA expression of antioxidant enzymes is another indicator of intracellular oxidant stress. The increase in MnSOD mRNA expression, in particular, is in keeping with its well recognised induction in response to oxidative stress [34] and increased MnSOD mRNA expression has been noted in endothelial cells cul-

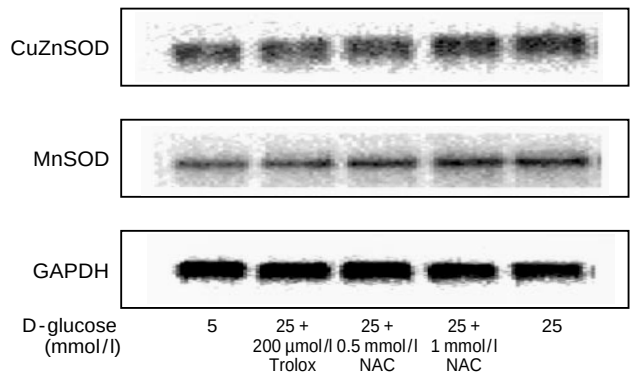

Fig. 5. mRNA expression of CuZnSOD and MnSOD in VSMC cultured for 10 days in 5 mmol/l D-glucose, 25 mmol/l D-glucose and 25 mmol/l D-glucose plus 0.5 mmol/l, 1 mmol/l N-acetyl cysteine (NAC)/200 μmol/l Trolox. GAPDH was used for background correction

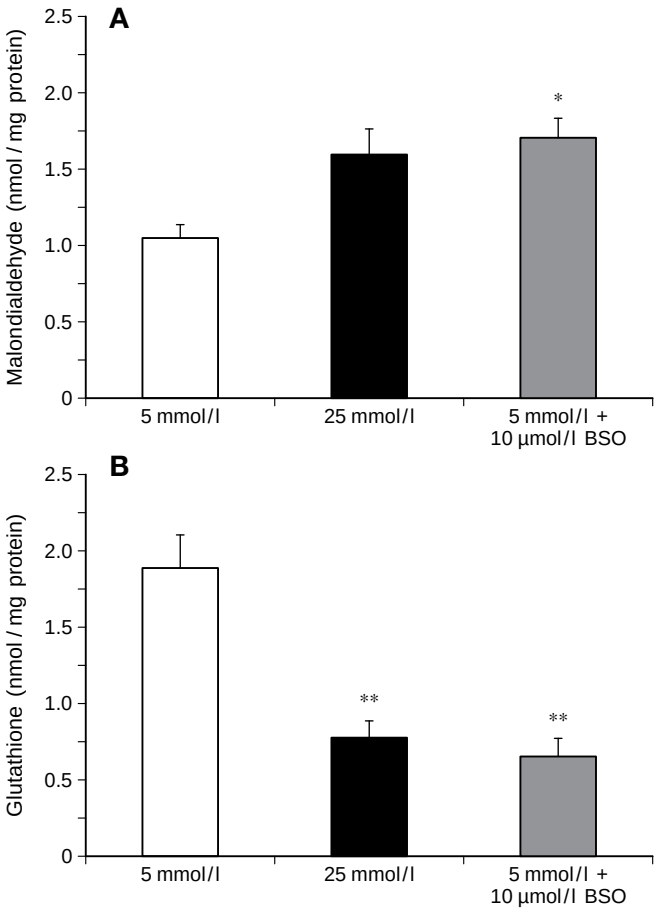

Fig. 6. **A** Intracellular malondialdehyde in VSMC cultured in 5 mmol/l D-glucose, 25 mmol/l D-glucose and 5 mmol/l D-glucose plus 10 μmol/l buthionine sulphoximine (BSO) for 10 days. *$p < 0.05$ cf 5 mmol/l D-glucose. $n = 5$. **B** Intracellular glutathione in VSMC cultured in 5 mmol/l D-glucose, 25 mmol/l D-glucose and 5 mmol/l D-glucose plus 10 μmol/l buthionine sulphoximine (BSO) for 10 days. **$p < 0.01$ cf 5 mmol/l D-glucose. $n = 5$

tured in high glucose concentrations [35]. Increases in CuZnSOD mRNA concentrations have also been reported in endothelial cells under similar conditions [35] and in diabetic rat kidneys [36]. These results show, for the first time, the ability of the VSMC to respond to glucose-induced oxidative stress by increasing the mRNA levels of the SOD enzymes (both CuZnSOD and MnSOD). The increase in CuZnSOD protein concentration is consistent with the increase in mRNA expression and the failure to detect an increase in actual enzyme activity can be explained by the fact that CuZnSOD activity is inhibited by glycation of the enzyme [37]; it can also be inactivated by free radicals. The failure to show an increase in MnSOD enzymatic activity despite the increase in mRNA expression was unexpected. Since MnSOD resides within the mitochondria, glycation would seem to be an unlikely event, however, inactivation by increased superoxide radicals is possible.

Glutathione is a key aqueous phase antioxidant in cells and it appears to be important in the VSMC. Its depletion has been reported in plasma, erythrocytes, leucocytes and platelets from patients with diabetes mellitus. Decreases have also been reported in endothelial cells from diabetic rabbits [38] but not in human endothelial cells cultured in 20 mmol/l D-glucose for 14 days [39]. There have been no previous reports on glutathione concentrations in VSMC. A possible explanation for the decrease in glutathione could be that the depletion is the primary event which subsequently predisposes the cell to oxidant-induced damage. Several studies have found reductions in both the gene expression and activity of the key rate-limiting enzyme in glutathione synthesis, gamma glutamyl cysteine synthetase (γ-GCS) with high glucose concentrations [40, 41]. It has been suggested that the glucose-derived modification of the γ-GCS

Table 5. Mitotic indices of VSMC at days 6, 8 and 10 cultured in different conditions

Condition	Mitotic figures %			% G2 + M + S		
	Day 6	Day 8	Day 10	Day 6	Day 8	Day 10
5 mmol/l D-glucose	2.34 ± 0.29	1.47 ± 0.48	1.93 ± 0.62	30.3 ± 6.7	18.8 ± 2.1	17.2 ± 2.2
25 mmol/l D-glucose	2.64 ± 0.21	1.71 ± 0.40	1.89 ± 0.61	29.8 ± 6.0	22.3 ± 3.9	21.2 ± 2.6
25 mmol/l + 200 μmol/l Trolox	2.82 ± 0.19	1.76 ± 0.55	2.12 ± 0.80	27.7 ± 6.4	18.2 ± 1.6	25.3 ± 4.5
25 mmol/l + 1 mmol/l NAC	1.59 ± 0.43	1.18 ± 0.31	1.54 ± 0.46	28.6 ± 7.5	15.6 ± 2.0[a]	16.0 ± 1.1
5 mmol/l + 1 μmol/l BSO	2.46 ± 0.22	1.73 ± 0.57	1.85 ± 0.43	30.1 ± 6.1	23.9 ± 5.4	22.0 ± 0.9

$n = 4$ independent experiments for mitotic figures and % G2 + M + S, [a] $p < 0.05$ cf 5 mmol/l D-glucose

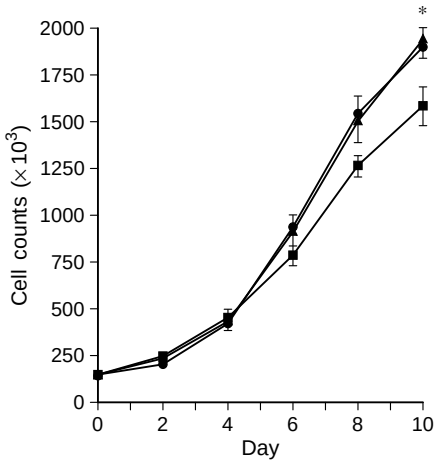

Fig. 7. Growth curves of VSMC cultured in 5 mmol/l D-glucose, 25 mmol/l D-glucose and 5 mmol/l D-glucose plus 10 μmol/l buthionine sulphoximine (BSO) for 10 days. *$p < 0.05$, 25 mmol/l and 5 mmol/l plus 10 μmol/l BSO vs 5 mmol/l. $n = 3$ —■— 5 mmol/l D-glucose, – –▲– – 25 mmol/l D-glucose, ···◆··· 5 mmol/l D-glucose + 10 μmol/l BSO

enzyme, possibly by glycation, predisposed the erythrocyte to oxidative stress [41]. This mechanism could also be the one responsible for decreased glutathione in VSMC which would predispose them to oxidant-mediated damage. It is also possible that reactive oxygen species rather than glucose itself are responsible for inactivation of γ-GCS; therefore, glucose-induced oxidative stress could be the cause, rather than the effect of γ-GCS inactivation.

The increased replication rate of VSMC in high ambient glucose is consistent with the results of others [10, 42]. In a study with hepatic stellate cells, malondialdehyde added to the substratum of the cells led to an increase in growth [43]; this provides evidence that products of free radical damage can stimulate cellular growth, at least when added exogenously. The effects of the antioxidants NAC and Trolox and of the γ-GCS inhibitor BSO [44], made it possible to define the importance of thiol status on growth characteristics of VSMC. The first antioxidant, NAC, is a thiol-containing compound which enters cells readily and serves as a precursor for glutathione by replenishing the intracellular pool of cysteine, so that the availability of this amino acid is no longer a limiting factor in glutathione synthesis [45]; NAC also possesses inherent free-radical scavenging effects itself [46]. The second antioxidant Trolox, a water soluble vitamin E analogue, was used rather than α-tocopherol because, in contast to α-tocopherol, it does not inhibit PKC [47]; it exerts major chain-breaking antioxidant effects and is readily transported across the cell membrane. In the 5 mmol/l glucose, BSO lowered glutathione and raised malondialdehyde to levels found with high glucose; it had the same effect on cell growth as 25 mmol/l glucose and this suggests that intracellular oxidative stress could be an important activator of VSMC growth. It was not clear from the BSO experiments whether lipid peroxides or thiol status (or both) is the most important in regulating growth. Addition of NAC, which led to repletion of glutathione and reduction in malondialdehyde, restored growth curves to normal even though the upregulation of mRNA for CuZnSOD and MnSOD was unaltered. Glutathione concentrations remained low in the presence of Trolox but treatment with Trolox was associated not only with reduction of malondialdehyde but also with normalisation of mRNA for CuZnSOD and MnSOD; it had no effect on the growth curves of VSMC. It would seem, therefore, that the effect of glucose on growth in VSMC is mediated through alteration of thiol status rather than lipid peroxides, although effects of the second cannot be excluded entirely as small intracellular increases are possibly not detected by measurement of malondialdehyde. By contrast to the effect of extracellular malondialdehyde on growth of stellate cells [43], cellular concentrations did not seem to have a direct effect on growth of VSMC.

Reductions in intracellular glutathione concentrations would be expected to have a profound effect on total thiol status and on cellular redox potential. It is known that redox status alters the activity of the transcription factors NF-κB and AP-1 as well as the tumour suppressor gene p53 in a complex manner [48]. NF-κB influences the activity of c-myc which is involved in control of the cell cycle [49]; c-fos and c-jun (AP-1) have been implicated in signal transduction processes leading to proliferation [50], and the tumour suppressor gene, p53, is involved in the apoptotic process [51]. Therefore activation of one or other of these transcription factors by altered redox status could potentially be involved in growth of VSMC. The evidence from this work suggests that lipid peroxides do not have a major role in growth regulation when VSMC are exposed to high concentrations of glucose.

Our results also show that aortic VSMC cultured in 25 mmol/l D-glucose do not display increased gene expression of the extracellular matrix proteins, fibronectin and collagens I, III and IV at 10 days. This suggests that either increased mRNA expression of extracellular matrix proteins does not occur in VSMC in hyperglycaemic conditions or that changes become apparent at other time points. There are numerous reports of increased extracellular matrix proteins (both mRNA expression and protein concentrations) at variable time points and glucose concentrations in differing cell types. For example, in human endothelial cells, mRNA expression of both fibronectin and collagen IV were increased (by approximately 150%) after 17 days exposure to 30 mmol/l glucose [52], whereas others have shown similar changes in endothelial cells after only 5 days in 30 mmol/l glucose [53]. In human mesangial cells cultured in

30 mmol/l D-glucose, increased mRNA expression of fibronectin, laminin and collagen IV were not apparent at 7 days but were increased at 14 days [54]. There is some suggestion that formation of AGEs (advanced glycation end-products) is an important pathogenic mechanism in upregulation of gene expression of extracellular matrix proteins [55], and under these circumstances the 10 day period of culture could be insufficient. Although increased amounts of these proteins have been identified in the aortae of diabetic subjects [17], their actual cell of origin (endothelial or VSMC), was not clearly identified. Indeed, endothelial cells could be the main source for extracellular matrix expansion. The absence of endothelial influences on VSMC in the tissue culture conditions could also explain the lack of response.

In conclusion, this work has shown the existence of glucose-induced oxidative stress in VSMC and this could be an important mechanism in induction of VSMC dysfunction. The results suggest that thiol status, rather than lipid peroxides, is a key factor in modulating VSMC growth in the presence of high glucose concentrations.

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