ELSEVIER

European Journal of Pharmacology 294 (1995) 221–229

ejp

Intracellular mechanism of high D-glucose-induced modulation of vascular cell proliferation

Wolfgang F. Graier [a,*], Isabella Grubenthal [a], Peter Dittrich [b], Thomas C. Wascher [c], Gerhard M. Kostner [a]

[a] *Department of Medical Biochemistry, University of Graz, Graz, Austria*
[b] *Department of Pharmacology and Toxicology, University of Graz, Graz, Austria*
[c] *Department of Internal Medicine, University of Graz, Graz, Austria*

Received 10 July 1995; revised 14 August 1995; accepted 18 August 1995

Abstract

Development of atherosclerosis in diabetes patients is thought to be associated with high D-glucose-induced changes in vascular cell proliferation. This study was designed to investigate the intracellular mechanisms of altered proliferation in porcine aortic endothelial and smooth muscle cells under high D-glucose conditions. Two different technical approaches were used for determination of cell proliferation, a cell counting procedure and bromodeoxyuridine incorporation. D-Glucose diminished endothelial cell proliferation (30.3%) and increased smooth muscle cell proliferation (143%) in a dose-dependent manner. Neither D-mannitol, sucrose nor L-glucose mimicked the effect of D-glucose. Inhibition of D-glucose uptake into vascular cells by cytochalasin B prevented the effect of high D-glucose on cell proliferation. The aldose-reductase inhibitors, sorbinil and zopolrestat, little affected high D-glucose-attenuated endothelial cell proliferation, while the enhanced proliferation of smooth muscle cells was prevented by aldose-reductase inhibitors. Elevation of cellular glutathione levels yielded protection of both cell types from high D-glucose-mediated changes in cell proliferation, suggesting that high D-glucose may act via generation of oxidative species. Finally, aminoguanidine was shown to constitute a very potent inhibitor of D-glucose-induced dysfunction in vascular cell proliferation. These data suggest that high D-glucose-induced changes in cell proliferation of endothelial and smooth muscle cells are related to specific D-glucose uptake rather than hyperosmolality. Aldose-reductase seems to be mainly involved in the effect of high D-glucose only on smooth muscle cell proliferation, while in endothelial cells there is (are) other factor(s) in addition to the sorbitol pathway involved in high D-glucose-induced changes in cell proliferation.

Keywords: Endothelial cell; Smooth muscle cell; Diabetes mellitus; Atherosclerosis; Aminoguanidine; Glutathione; Aldose-reductase

1. Introduction

In comparison to the general population, patients with diabetes mellitus are at a 4- to 6-fold increased risk of the development of atherosclerotic macro-vascular disease (Kannel, 1985; Keen and Jarret, 1979). Therefore, these patients have a substantially increased morbidity and mortality from coronary artery disease (Goldschmid et al., 1994) as well as an increased risk of lower limb amputation due to peripheral vascular disease (Most and Sinnock, 1983). Furthermore, the risk of restenosis after balloon angioplasty is increased in patients with diabetes mellitus (Pilger et al., 1991).

On one hand, micro-vascular disease (for review see Brownlee, 1985; Lorenzi et al., 1986), manifested as nephropathy, retinopathy and (at least partially) neuropathy, is established to be closely linked to the severity and duration of hyperglycemia and can be prevented or delayed by close glycemic control (DCCT Research Group, 1993). Macro-vascular disease of the diabetic patient, on the other hand, is supposed to be based on a complex network of several vascular risk factors (Paisey et al., 1984) including the insulin resistance syndrome (DeFronzo, 1992; Reaven, 1988).

Following the 'response to injury' hypothesis of Ross (1986) and Ross and Agius (1992) endothelial cell injury is thought to be one of the initial steps within

* Corresponding author. Department of Medical Biochemistry, University of Graz, Harrachgasse 21/III, A-8010 Graz, Austra. Tel.: +43-316-380-4202/4482; fax: +43-316-32 25 32.

SSDI 0014-2999(95)00534-X

the process of atherogenesis. Thus, an intact proliferative response of vascular endothelial cells is of outstanding interest for reparative processes at the lesion site. Later on in the development of atherosclerotic lesions, formation of neointima by migration of vascular smooth muscle cells from the media followed by proliferation of these cells is seen (Ross, 1986; Ross and Agius, 1992). Hyperglycemia has been shown to increase proliferation of cultured vascular smooth muscle cells (Lorenzi et al., 1985) as well as to reduce the proliferation of vascular endothelial cells (Natarajan et al., 1992). Therefore both processes suggest a direct contribution of hyperglycemia itself in the development of atherosclerosis in diabetes mellitus although the intracellular mechanisms involved in the effect of elevated glucose on vascular cell proliferation are widely unknown.

The aim of the present study was therefore to investigate which intracellular pathways might be involved in the effects of D-glucose on both vascular endothelial cell and vascular smooth muscle cell proliferation and whether a common mechanism, suggesting a possible common therapeutic approach, could be identified.

2. Materials and methods

2.1. Cell isolation and culture

Cells were isolated from fresh porcine aortae by enzymatic digestion.

For isolation of endothelial cells, porcine aortae were cut longitudinally and placed in a lucite chamber, washed twice with phosphate-buffered saline (PBS; in mM: 150 NaCl, 2.7 KCl, 8 Na_2HPO_4, 5 KH_2PO_4, pH 7.4) and incubated with minimum essential medium, Dulbecco modification (DMEM), containing 294 U/ml collagenase plus (in mg/ml): 2 bovine serum albumin, 1 trypsin inhibitor (soybean) and 0.4 DNAase I (Graier et al., 1993a, 1995). After 20 min at 37°C the cell suspension was centrifuged, resuspended in Opti/MEM containing 3% fetal calf serum and seeded in 10-cm plastic dishes for culture. After one week the cells were split 1:4 in 6-well clusters for experiments. All experiments were performed with endothelial cells in first passage. Cell culture was >99% pure, tested by the typical cobblestone morphology and the lack of immunofluorescence detection of included smooth muscle cells (α-actin).

Smooth muscle cells were also isolated from porcine aortae. After removal of the endothelium, a 1 × 1 cm piece was cut and washed twice with sterile PBS. Small parts of the media were prepared, placed in a Petri dish with Opti-MEM and covered with a cover slip. After about 1 week smooth muscle cells were harvested with 0.05% trypsin (type II, porcine pancreas) containing 0.02% Na_4-EDTA in PBS (pH 7.4) and split for further culture. Experiments were performed with smooth muscle cells from passages 2–4 in 6-well cluster plates. Purity was tested by typical morphology and immunofluorescence detection of included smooth muscle α-actin.

2.2. Measurement of cell proliferation

Cell proliferation was determined by blind test cell counting using a Neubauer hemocytometer and a bromo-2′-deoxy-uridine enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Germany).

For counting cells were grown in a 6-well plate (diameter 36 mm), harvested with trypsin solution, centrifuged, resuspended in 1.00 ml DMEM and 20 μl cell suspension was transferred into the hemocytometer. Cells were counted with a 10× phase contrast objective (Nikon 10× Ph 1 DL, Japan). For each sample, ten areas were counted and the median was calculated as number of cells in a given sample. The numbers of cells/ml was calculated from the following equation: $N = n \times 10^4$ cells/ml (N, numbers of cells/ml; n, median of the cell numbers in the chamber areas).

Bromo-2′-deoxy-uridine ELISA measurements of cells were performed according to Magaud et al. (1988) and Houng et al. (1991). Briefly, cells were put in a 96-well plate in a concentration of 1000 cells/well. After an equilibration period of 24 h in Opti-MEM containing 3% fetal calf serum, bromo-2′-deoxy-uridine (final concentration: 10 μM) was added. After 12 h at 37°C, the solution was aspirated, the cells were washed twice with PBS and fixed (70% ethanol in 0.5 M HCl for 30 min at −20°C). After fixation, the cells remained attached to the plastic during the whole procedure described below. Cells were washed 3 times, incubated for 30 min at 37°C with nuclease and were washed 3 times. Monoclonal anti-bromo-2′-deoxy-uridine was added (Boehringer Mannheim, Germany) for 30 min at 37°C. After a further 3 times washing procedure, peroxidase-conjugated sheep anti-mouse IgG heavy and light chain (Boehringer Mannheim, Germany) was added and incubated for 30 min at room temperature. Peroxidase activity was detected in a microplate reader as the extinction at 405 nm with a reference wavelength at 490 nm. The ratio of the extinction at 450 nm and 490 nm just at the beginning of the peroxidase incubation was used as blank. The data are expressed as percent of the extinction of control cells (i.e. 5 mM D-glucose).

2.3. Materials

Cell culture chemicals were obtained from Gibco, Eggenstein, Germany. Fetal calf serum was from Se-

bak, Suben, Austria. All other chemicals were purchased from Sigma, Munich, Germany. Cell culture Petri dishes (diameter 10 cm) were from Corning, New York, USA and 6-well clusters were from Costar, Cambridge, MA, USA. The cell proliferation kit was obtained from Boehringer Mannheim, Germany.

2.4. Statistics

Experiments were performed with at least three different cell preparations in triplicate. Cell counting was performed as double-blind experiments. The data are expressed as means ± S.E. Statistical significance was calculated by paired Student's *t*-test in two groups (5 and 44 mM D-glucose). Analysis of variance (ANOVA) was used to compare more than two groups and significance was determined by Fisher's post-hoc B4s protected least-significant difference test. Statistical significance was defined as $P < 0.05$ and 'n.s.' defines values not statistically significantly different from the control.

3. Results

3.1. Endothelial cells

Within 72 h proliferation of endothelial cells was significant diminished in a concentration-dependent manner by elevation of the extracellular D-glucose concentration (Fig. 1A). In agreement with these experiments in which cell counting was used, elevation of extracellular D-glucose attenuated endothelial cell proliferation measured with the bromo-2'-deoxy-uridine incorporation technique (Fig. 1B).

Table 1
Effect of D-mannitol and sucrose on proliferation of endothelial cells

	Proliferation in % of control (i.e. 5 mM)					
	D-Mannitol		L-Glucose		Sucrose	
5 mM	98.2 ± 2.4	(12)	97.7 ± 7.2	(12)	109 ± 1.5	(8)
44 mM	105.3 ± 1.2	(12)	98.7 ± 4.1	(12)	105 ± 1.9	(8)

Cultured endothelial cell proliferation was determined by a cell counting technique, as described under Materials and methods, after 72 h in the presence of the sugar concentration indicated. Proliferation is expressed as percentage of the proliferation with 5 mM D-glucose. Numbers of experiments are listed in parentheses. All values are not significantly different from proliferation with 5 mM D-glucose.

Osmotic controls

In contrast to elevated D-glucose, addition of 5 and 44 mM D-mannitol failed to affect endothelial cell proliferation within 72 h, as measured by the cell counting technique (Table 1). While D-mannitol failed to alter proliferation measured by cell counting, D-mannitol affected the incorporation of bromo-2'-deoxy-uridine. This effect was not concentration-dependent, reflecting attenuation of bromo-2'-deoxy-uridine incorporation to 77.9 ± 4.1% ($n = 12$, $P < 0.05$ vs. 5 mM D-glucose) with 5 mM D-mannitol and 80.0 ± 4.6 with 44 mM D-mannitol ($n = 12$, $P < 0.05$ vs. 5 mM D-glucose; n.s. vs. 5 mM D-mannitol).

In addition to that of D-mannitol, the effects of different concentrations of L-glucose on endothelial proliferation were tested. Thereby, 5 as well as 44 mM

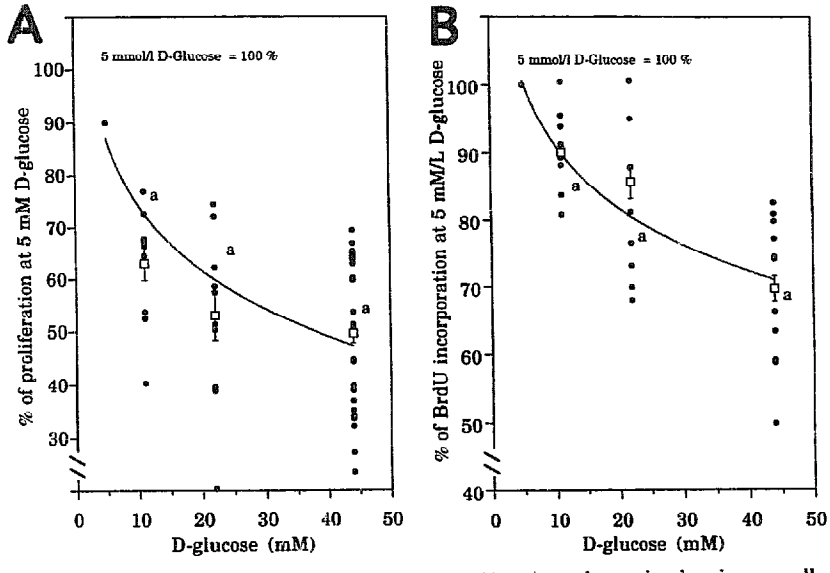

Fig. 1. Effect of various D-glucose concentrations on endothelial cell proliferation, determined using a cell counting technique (A) and bromo-2'-deoxy-uridine incorporation assay (B). Vascular endothelial cells were incubated for 72 h (A) or 36 h (B) in culture medium containing 5, 11, 22 or 44 mM D-glucose. Each point represents one single measurement and proliferation is expressed as percentage of the proliferation at 5 mM D-glucose. Open squares represent means ± S.E.M. Experiments ($n = 15$–66) were performed with cells from 11–33 different aortae. [a] $P < 0.05$ vs. proliferation in the presence of 5 mM D-glucose.

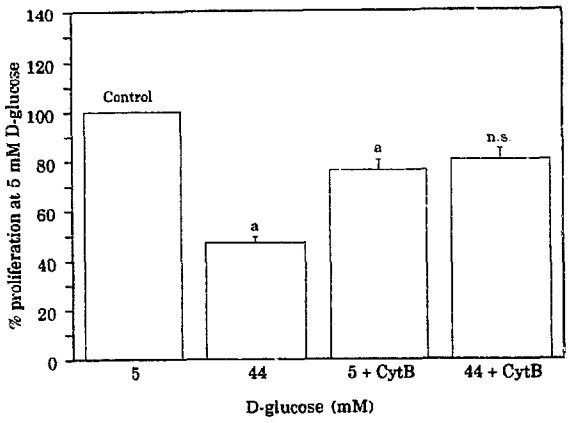

Fig. 2. Vascular endothelial cell proliferation within 72 h in culture medium containing 5 or 44 mM D-glucose in the presence and absence of 1 μM cytochalasin B. Proliferation was measured using a cell counting technique and is expressed as percentage of the proliferation with 5 mM D-glucose. [a] $P < 0.001$ vs. 5 mM D-glucose, n.s. not significant ($n = 18$ from 12 different aortae).

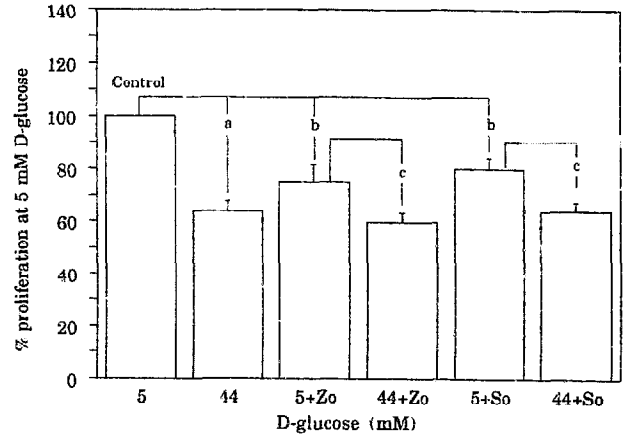

Fig. 3. Proliferation of vascular endothelial cells in 5 or 44 mM D-glucose-containing medium in the presence and absence of an aldose reductase inhibitor, sorbinil (100 μM) or zopolrestat (100 μM). Cell proliferation was measured using a cell counting technique and is expressed as percentage of the proliferation with 5 mM D-glucose in the absence of an aldose-reductase inhibitor. [a] $P < 0.001$ and [b] $P < 0.05$ vs. proliferation with 5 mM, [c] $P < 0.05$ vs. 5 mM D-glucose in the presence of the aldose-reductase inhibitor indicated ($n = 20$ from 10 different aortae).

L-glucose had no effect on endothelial proliferation measured using cell counting (Table 1). In agreement with our findings with D-mannitol, L-glucose diminished bromo-2'-deoxy-uridine incorporation in a concentration-independent manner (data not shown). Similarly to D-mannitol and L-glucose, sucrose failed to affect endothelial proliferation at a concentration of 5 mM (Table 1).

Pharmacological modulation

Inhibition of D-glucose uptake by cytochalasin B prevented the inhibitory effect of a pathological, high D-glucose concentration on endothelial proliferation. Cell proliferation measured by a cell counting technique was markedly diminished in the presence of 44 mM D-glucose. Co-incubation with 1 μM cytochalasin B itself affected cell proliferation in the presence of 5 mM D-glucose. However, increasing D-glucose to 44 mM did not further diminish cell proliferation in the presence of 1 μM cytochalasin B (Fig. 2).

Inhibition of aldose-reductase by sorbinil or zopolrestat weakly affected the high D-glucose-mediated effect of endothelial cell proliferation. Increasing D-glucose to 44 mM diminished cell proliferation ($\Delta = -37.2\%$). Both aldose-reductase inhibitors themselves reduced proliferation of endothelial cell in the presence 5 mM D-glucose (Fig. 3). Co-incubated with 44 mM D-glucose, 100 μM sorbinil or 100 μM zopolrestat reduced the anti-proliferation effect of 44 mM D-glucose ($\Delta = -16.2\%$ and $\Delta = -15.5\%$, respectively; Fig. 3).

Aminoguanidine, an inhibitor of the formation of advanced glycated end-products (Brownlee, 1994; Tilton et al., 1993; Vlassara et al., 1992) and inducible and constitutive nitric oxide synthases (Misko et al., 1993), prevented the high D-glucose-mediated attenuation of endothelial cell proliferation (Fig. 4). While aminoguanidine (1 μM) itself weakly diminished cell proliferation, it completely prevented the decrease in cell proliferation caused by elevating extracellular D-glucose from 5 mM to 44 M (Fig. 4). Similar results were obtained using bromo-2'-deoxy-uridine incorporation (data not shown). In contrast to aminoguanidine, N^{ω}-nitro-L-arginine failed to prevent the anti-proliferating effect of 44 mM D-glucose on endothelial cells. Reduction of cell proliferation by 44 M D-glucose in the absence of N^{ω}-nitro-L-arginine ($70.4 \pm 2.0\%$, $n = 8$, $P < 0.05$ vs. 5 mM D-glucose) was almost identi-

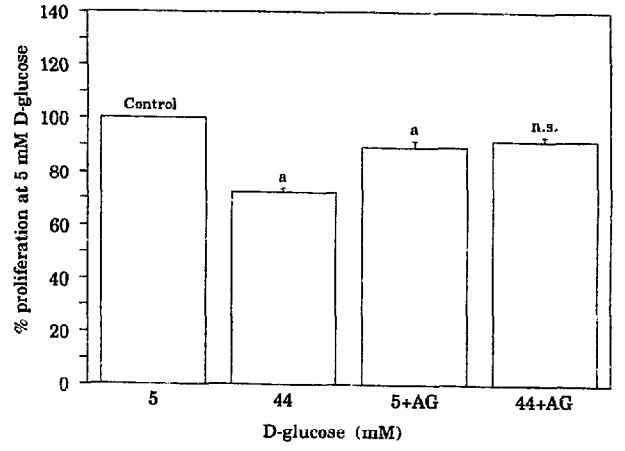

Fig. 4. Inhibitory effect of aminoguanidine (AG; 1 μM) on endothelial cell proliferation in culture medium containing 5 or 44 mM D-glucose for 72 h. Endothelial cell proliferation was determined by cell counting. Columns represent the percentage of the proliferation obtained in the presence of 5 mM D-glucose without aminoguanidine. [a] $P < 0.001$ and [b] $P < 0.05$ vs. proliferation with 5 mM D-glucose in the absence of aminoguanidine ($n = 12$ from six aortae).

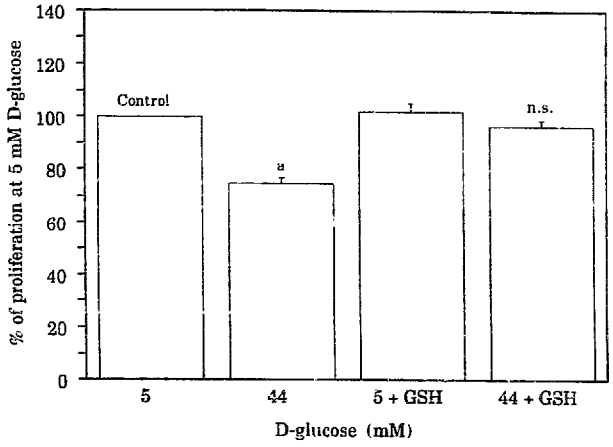

Fig. 5. Effect of glutathione (GSH) on endothelial cell proliferation in media containing 5 or 44 mM D-glucose. Cells were treated for 72 h in the media indicated and proliferation was measured by cell counting. Proliferation measured by cell counting is expressed as percentage of that obtained in 5 mM D-glucose-containing media without GSH. [a] $P < 0.001$ vs. proliferation with 5 mM D-glucose and n.s. not significant ($n = 13$ from seven aortae).

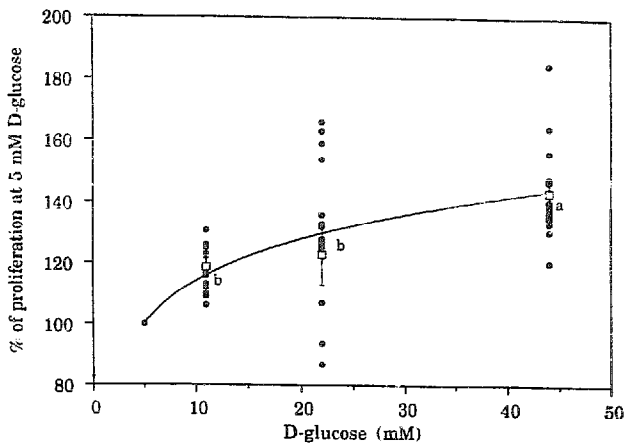

Fig. 7. Summary of the effect of various D-glucose concentrations on vascular smooth muscle proliferation within 72 h. Cell proliferation was measured by cell counting after 72 h in 5, 11, 22, and 44 mM D-glucose and results are expressed as percentage of proliferation in 5 mM D-glucose. Open squares represent mean ± S.E.M. [b] $P < 0.001$ and [a] $P < 0.05$ vs. proliferation with 5 mM D-glucose-containing culture media ($n = 16$–36 from 3–18 aortae).

cal to that obtained with 44 M D-glucose in the presence of 100 μM N^{ω}-nitro-L-arginine (68.5 ± 1.0%, $n = 8$, $P < 0.05$ vs. 5 mM D-glucose ± N^{ω}-nitro-L-arginine, n.s. vs. 44 mM without N^{ω}-nitro-L-arginine). N^{ω}-Nitro-L-arginine itself had no effect on endothelial proliferation (99.6 ± 1.0, $n = 8$, n.s. vs. in the absence of N^{ω}-nitro-L-arginine).

Co-incubation with glutathione (GSH) also prevented high D-glucose-mediated decreases in endothelial cell proliferation (Fig. 5).

Incubation of endothelial cells for 48 h with 30 μU/ml xanthine oxidase in the presence of 1 mmol/l hypoxanthine decreased endothelial cell proliferation (Fig. 6). This effect of the xanthine oxidase/hypoxanthine reaction on endothelial cell proliferation could be prevented by co-incubation with aminoguanidine or GSH (100 μmol each; Fig. 6).

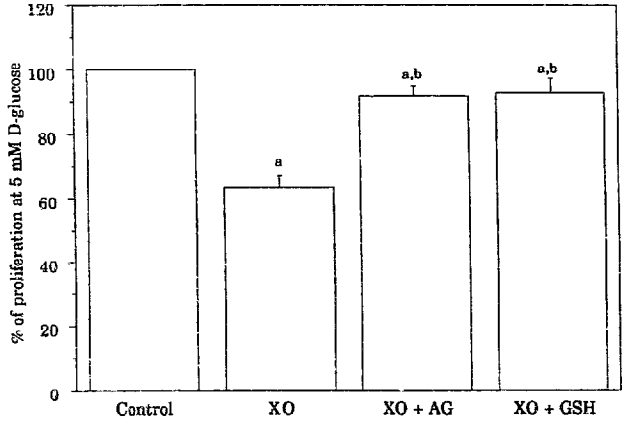

Fig. 6. Effect of the xanthine oxidase/hypoxanthine reaction on endothelial cell proliferation. Cells were treated for 48 h with 30 μU/ml xanthine oxidase in the presence of 1 mM hypoxanthine (initial concentration). Proliferation is expressed as the percentage of that measured in the absence of xanthine oxidase/hypoxanthine. [a] $P < 0.001$ vs. proliferation in the absence of xanthine oxidase/hypoxanthine and [b] $P < 0.001$ vs. proliferation in the presence of xanthine oxidase/hypoxanthine ($n = 12$ from four aortae).

3.2. Smooth muscle cells

Elevation of the extracellular D-glucose concentration from 5 to 11, 22 and 44 mM resulted in an increased proliferation of smooth muscle cells (Fig. 7).

Osmotic controls

As shown in Table 2, neither of the sugars affected cell proliferation at a concentration of 5 mM. Furthermore, high D-mannitol and sucrose concentrations (44 mM) failed to mimic the proliferating effect of 44 mM D-glucose on smooth muscle cells.

Pharmacological modulation

The effect of cytochalasin B, an inhibitor of D-glucose uptake into smooth muscle cells, was investigated.

Table 2
Effect of D-mannitol and sucrose on proliferation of vascular smooth muscle cells

	Proliferation as % of control (i.e. 5 mM)					
	D-Glucose		D-Mannitol		Sucrose	
5 mM	100	(18)	101.6 ± 2.7	(28)	99.7 ± 2.0	(8)
44 mM	139.7 ± 1.9 [a]	(18)	96.2 ± 6.5 [b]	(28)	99.1 ± 2.6 [b]	(8)

Cultured smooth muscle cell proliferation was determined by a cell counting technique as described under Materials and methods, after 72 h in the presence of the sugar concentration indicated. Proliferation is expressed as percentage of the proliferation with 5 mM D-glucose. Numbers of experiments are listed in parentheses. [a] $P < 0.05$ vs. proliferation at 5 mM D-glucose. [b] $P < 0.05$ vs. proliferation with 44 mM D-glucose.

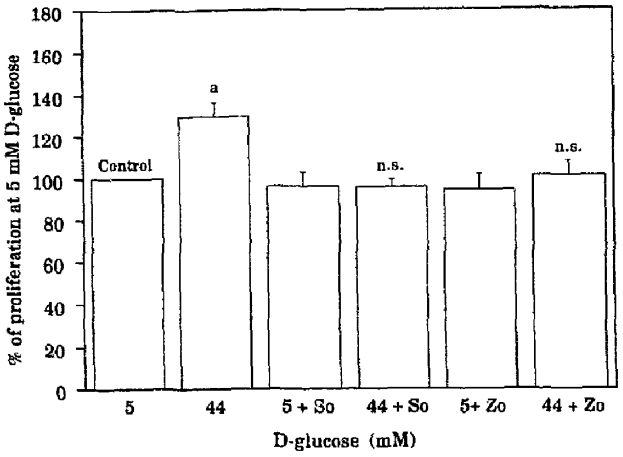

Fig. 8. The aldose-reductase inhibitors, sorbinil and zopolrestat, prevented the effect of high D-glucose on vascular smooth muscle proliferation. Smooth muscle cells were cultured for 72 h in culture media containing 5 or 44 mM D-glucose in the absence or presence of the aldose-reductase inhibitor indicated (100 μM each). Cell proliferation was evaluated by cell counting technique and results are given as percentage of proliferation in 5 mM D-glucose-containing media in the absence of an aldose-reductase inhibitor. [a] $P < 0.001$ and n.s., not significant vs. proliferation with 5 mM D-glucose ($n = 8$ from four aortae).

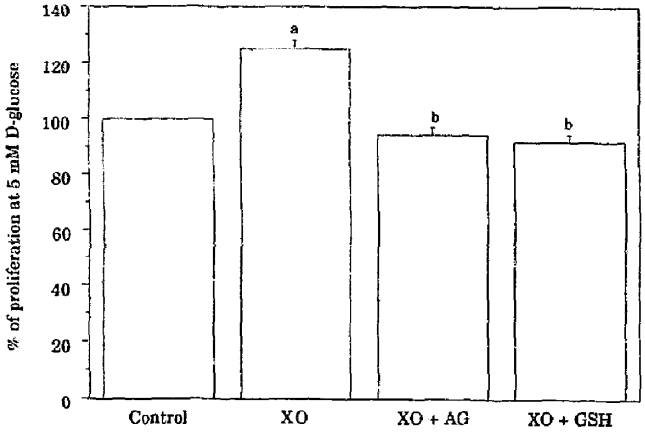

Fig. 9. Effect of xanthine oxidase/hypoxanthine reaction on smooth muscle cell proliferation. Cells were treated for 48 h with 30 μU/ml xanthine oxidase in the presence of 1 mM hypoxanthine (initial concentration). Proliferation was measured by a cell counting technique. Results are expressed as the percentage of the proliferation obtained in the absence of xanthine oxidase/hypoxanthine. [a] $P < 0.001$ vs. proliferation in the absence of xanthine oxidase/hypoxanthine and [b] $P < 0.001$ vs. proliferation in the presence of xanthine oxidase/hypoxanthine ($n = 6$ from two aortae).

Cytochalasin B (1 μM) per se decreased smooth muscle cell proliferation in 5 mM D-glucose to 67.3 ± 2.8 ($n = 5$, $P < 0.05$ vs. in the absence of cytochalasin B) similar to its effect on endothelial cells (see Fig. 3). However, in the presence of 1 μM cytochalasin B the high D-glucose-mediated increase in cell proliferation was completely prevented (65.3 ± 1.9, $n = 5$, n.s. vs. 5 mM in the presence of cytochalasin B).

The increase in smooth muscle cell proliferation caused by 44 mM D-glucose was found to be due to aldose-reductase activity. Therefore, the effects of the aldose-reductase inhibitors, sorbinil or zopolrestat, on the high D-glucose-mediated enhanced smooth muscle proliferation were investigated. As shown in Fig. 8, both compounds completely prevented the stimulatory effect of high D-glucose on cell proliferation, while they did not affect proliferation in 5 mM D-glucose.

Similar to its effect on high D-glucose-modulated endothelial cell proliferation, aminoguanidine prevented high D-glucose-mediated changes in smooth muscle cell proliferation (Table 3). Co-incubation with glutathione (GSH) also affected high D-glucose-mediated enhanced proliferation of vascular smooth muscle cells. As demonstrated in Table 2, the effect of 44 mM D-glucose on smooth muscle cell proliferation was abolished in the presence of 100 μM GSH.

Xanthine oxidase in the presence of 1 mmol/l hypoxanthine yielded increase in smooth muscle cell proliferation (Fig. 9). The effect of the xanthine oxidase/hypoxanthine reaction was prevented by co-incubation with aminoguanidine or GSH (100 μmol/l each; Fig. 9).

4. Discussion

In agreement with Lorenzi et al. (1985) and Santilli (1992), we found that high D-glucose concentrations (> 30 mM) attenuated endothelial cell proliferation by

Table 3
Effect of various concentrations of aminoguanidine (AG) and glutathione (GSH) on high D-glucose-mediated increased proliferation of vascular smooth muscle cells

	Proliferation in % of control (i.e. 5 mM)									
	None		AG						GSH	
			1 μM		10 μM		100 μM		100 μM	
5 mM	100	(36)	100.7 ± 1.4	(8)	–	–	95.3 ± 4.9	(4)	97.5 ± 0.7	(8)
44 mM	143.7 ± 3.4 [a]	(36)	106.8 ± 8.7 [b]	(8)	110.0 ± 8.1 [b]	(8)	92.6 ± 7.77	(8)	99.2 ± 2.8 [b]	(8)

Cultured smooth muscle cell proliferation was determined by a cell counting technique, as described under Materials and methods, after 72 h in normal (5 mM) and high (44 mM) D-glucose-containing media containing concentrations of aminoguanidine or glutathione as indicated. Proliferation is expressed as percentage of the proliferation with 5 mM D-glucose. Numbers of experiments are listed in parentheses. [a] $P < 0.05$ vs. proliferation with 5 mM D-glucose. [b] $P < 0.05$ vs. proliferation with 44 mM D-glucose.

approximately 40%. Lorenzi et al. (1986) reported that cell proliferation diminished by high D-glucose might the result of increasing DNA damage. For these experiments endothelial cells were incubated with 30 mM D-glucose for 9–14 days. Whether similar effects of high D-glucose contributed to the effect of high D-glucose in our experiments (24–72 h high D-glucose treatment) remains to be investigated.

We cannot yet explain the marked effect of D-mannitol and L-glucose in our bromo-2'-deoxy-uridine experiments. No difference between 5 and 44 mM D-mannitol and L-glucose was found. Since this phenomenon was not observed in cell counting experiments, we speculate that the effect of these sugars in the bromo-2'-deoxy-uridine technique is an interaction with bromo-2'-deoxy-uridine labeling per se rather than an inhibitory effect on cell proliferation.

In contrast to the decrease of endothelial cell proliferation with high D-glucose, vascular smooth muscle proliferation was enhanced by approximately 50% by incubation with high D-glucose. Similar data were obtained by Natarajan et al. (1992). The uptake of D-glucose in both types of cells studied constitutes an insulin-independent process due to GluT 1 transporter (Lienhard et al., 1992; Mueckler, 1990). The reason for the controversial effect of elevated D-glucose on proliferation of endothelial cells and smooth muscle cells is unclear. This paradox might be related to the differences in the GluT 1 protein expression during high D-glucose treatment. While the concentration of GluT 1 protein in smooth muscle has been shown to be markedly decreased in the presence of high D-glucose, this phenomenon could not be observed in endothelial cells (Kaiser et al., 1993). Moreover, endothelial D-glucose uptake depends strictly on extracellular D-glucose concentration in an almost linear manner (Graier et al., 1993b). Thus, one might speculate that, in smooth muscle cells, there is an auto-regulatory mechanism protecting against an overload by D-glucose. In contrast, D-glucose uptake in endothelial cells may result in D-glucose toxicity at higher D-glucose concentrations.

In a previous study we reported that the GluT 1 inhibitor, cytochalasin B, prevented high D-glucose-mediated changes in endothelial Ca^{2+} signaling (Graier et al., 1993b).

In this study, cytochalasin B caused a significant decrease in cell proliferation of endothelial and smooth muscle cells. However, cytochalasin B prevents high D-glucose-mediated changes in cell proliferation of endothelial cells and smooth muscle cells. This could suggest that this effect is due to D-glucose uptake rather to hyperosmolality. This hypothesis is confirmed by the fact that D-mannitol, L-glucose and sucrose failed to mimic the effect of high D-glucose on smooth muscle cell and endothelial cell proliferation. The metabolism of D-glucose also depends on the amount of D-glucose within the cells. Ruderman et al. demonstrated that, under high D-glucose conditions, up to one-third of the D-glucose is metabolized by the sorbitol pathway (Ruderman et al., 1992). Tesfamariam et al. provided evidence that decreased endothelium-dependent relaxation in response to acetylcholine is prevented by inhibition of aldose reductase, a key enzyme in the sorbitol pathway (Tesfamariam et al., 1992, 1993). In contrast, aldose reductase inhibitors have been shown to be less effective to prevent high D-glucose-mediated changes in endothelial cell proliferation (Lorenzi et al., 1987; Vlassara et al., 1992). Similar results were also shown in pericytes (Chibber et al., 1994). In agreement with these reports, aldose reductase inhibition by sorbinil or zopolrestat in our experiments diminished but did not abolish the effect on high D-glucose-modulated proliferation of endothelial cells. Thus, our data suggest that, in vascular endothelial cells, factors other than and in addition to the sorbitol pathway contribute to the decrease in proliferation induced by high D-glucose. For example, formation of prostanoids induced by high D-glucose treatment is not altered by aldose-reductase inhibition (Tesfamariam and Cohen, 1992) and elevation of D-glucose has been shown to enhance 15-HETE release and diacylglycerol formation in endothelial cells (Brown et al., 1988). In view of the expected mitogenic properties of these compounds, one might speculate that besides an involvement of aldose-reductase, formation of 15-HETE, some prostaglandin(s) or activation of protein kinase C might be involved in the D-glucose-mediated decrease of endothelial cell proliferation.

In contrast to endothelial cell proliferation, high D-glucose-mediated increases in smooth muscle cell proliferation were abolished by aldose-reductase inhibitors. Turner and Biermann (1978) reported that sorbitol per se increased proliferation of fibroblasts. Our results suggest that in vascular smooth muscle cells, the sorbitol pathway plays a crucial role in the high D-glucose-mediated increase in cell proliferation.

In vascular smooth muscle, high D-glucose might lead to a 'pseudo hypoxia' due to sorbitol-dehydrogenase-linked changes in the NADH/NAD ratio, as proposed by Williamson et al. (1993). The reported changes in the NADH/NAD ratio are thought to result in the formation of superoxide anion which may contribute to the high D-glucose-mediated changes in cell function. It has been demonstrated by several groups that elevation of D-glucose results in the generation of reactive species, such as free radicals (Langenstroer and Pieper, 1992; Tesfamariam and Cohen, 1992) or hydrogen peroxide (Langenstroer and Pieper, 1992). Furthermore, peroxide formation by auto-oxidation of D-glucose in vitro was described (Hunt et al., 1990). The hypothesis of the involvement of oxidative species

in the observed changes in vascular cell proliferation is supported by our findings that the xanthine oxidase/hypoxanthine reaction mimicked the effects of high D-glucose on cell proliferation. Furthermore, the observed changes in cell proliferation caused by high D-glucose treatment or xanthine oxidase/hypoxanthine are prevented by increasing the intracellular glutathione (GSH) level with exogenous GSH (from 1.89 $\pm$ 0.04 (n = 30) to 10.72 $\pm$ 0.63 pmol/10^6 cells after 24 h in 10 mM GSH-containing medium (n = 9) in endothelial cells), which has been shown to protect cells against injury induced by some oxidative species (Tsan et al., 1989). Although the aldose-reductase-derived formation of oxidative species seems to totally account for changes in cell proliferation in smooth muscle cells only, while an additional factor(s) is involved in endothelial cells, our results suggest that, in vascular endothelial and smooth muscle cells, oxidative species derived from high D-glucose metabolism play a crucial role in the reported changes in cell proliferation in the presence of high D-glucose.

Similar to GSH, aminoguanidine prevented high D-glucose-mediated changes in proliferation of vascular endothelial and smooth muscle cells in our experiments. This is in agreement with the results of Chibber et al. (1994) and Hammes et al. (1991) who reported that aminoguanidine prevents high D-glucose-mediated decreases in pericytes and retinal endothelial cell proliferation, respectively. However, the concentrations necessary in this study were about 100 times higher than in the present experimental protocols. This might be related to the mechanism of the inhibitory action of aminoguanidine, resulting in prevention of high D-glucose-mediated changes in vascular cell proliferation. Since L-NNA, a well known inhibitor of constitutive as well as inducible forms of nitric oxide (NO) synthase (Misko et al., 1993), did not mimic the effect of aminoguanidine in our studies, the inhibition of NO synthase(s) as a mechanism of aminoguanidine action can clearly be excluded. Aminoguanidine has also been shown to be an inhibitor of aldose-reductase (Kumari et al., 1989). We cannot exclude the possibility that aminoguanidine acts at least in part by inhibition of aldose-reductase, especially in smooth muscle cells (see the discussion above), but there seems to be an additional component of aminoguanidine action to cause its potency. Inhibition of the formation of advanced glycosylation end-products (Brownlee et al., 1986) by aminoguanidine is proposed to constitute a main mechanism for aminoguanidine prevention of diabetes-mediated vascular dysfunction (Ruderman et al., 1992). Due to the short-term incubation with D-glucose in this study, involvement of slowly formed advanced glycosylation end-products seems to be negligible in our experiments. However, aminoguanidine has been shown to interfere also with the early steps in the formation of glycation products (i.e. Schiff base and Amadori product), which might be related to its free radical scavenging properties (Brownlee, 1994; Tilton et al., 1993). The hypothesis that aminoguanidine might prevent high D-glucose-mediated changes of vascular cells by free-radical scavenging is in agreement with our results obtained with GSH. Convincingly, aminoguanidine also prevented changes in endothelial and smooth muscle cell proliferation induced by incubation with xanthine oxidase/hypoxanthine.

Thus, we speculate that high D-glucose treatment attenuates endothelial cell proliferation by generation of free radicals (Tesfamariam and Cohen, 1992) and/or oxidative species (Langenstroer and Pieper, 1992) partially derived from the sorbitol pathway. In smooth muscle cells, however, high D-glucose seems to increase cell proliferation mainly by aldose-reductase-derived mechanisms, possibly also via free radicals or oxidative species. Therefore, we suggest that D-glucose-induced formation of reactive species might constitute a common pathological phenomenon in vascular endothelial and smooth muscle cells in the development of diabetic macro-angiopathy. These findings might be of particular interest in the search for new therapeutic strategies in the treatment and prevention of atherosclerosis in diabetes.

Acknowledgements

The authors wish to thank Dr. Peter J. Oates, Pfizer, Inc., Groton, CT, USA for the gift of zopolrestat. This work was supported by grants from the Austrian National Bank (Jubiläumsfondsprojekt 4715 and 5406) and the Austrian Research Foundation (P10029-Med.) as an associate member of a project of the European Union (EureCa: No. BMH1-CT94-1375).

References

Brown, M.L., J.A. Jakubowski, L. Leventis and D. Deykin, 1988, Elevated glucose alters eicosanoid release from porcine aortic endothelial cells, J. Clin. Invest. 82, 2136

Brownlee, M., 1985, Microvascular disease and related abnormalities: their relation to control of diabetes, in: Joslin's Diabetes Mellitus, 12th edn., eds. A. Marble, L.P. Krall, R.F. Bradley, A.R. Christlieb and J.S. Soeldner (Lea and Febiger, Philadelphia, PA) p. 185.

Brownlee, M., 1994, Glycation and diabetic complications, Diabetes 43, 836.

Brownlee, M., H. Vlassara, A. Kooney, P. Ulrich and A. Cerami, 1986, Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking, Science 232, 1629.

Chibber, R., P.A. Molinatti, J.S.K. Wong, D. Mirlees and E.M. Kohner, 1994, The effect of aminoguanidine and tolrestat on glucose toxicity in bovine retinal capillary pericytes, Diabetes 43, 758.

Diabetic Control and Complication Research Group (DCCT Research Group), 1993, The effect of intensive treatment of dia-

betes on the development and progression of long-term complications in insulin-dependent diabetes mellitus, New Engl. J. Med. 329, 977.

DeFronzo, R., 1992, Insulin resistance, hyperinsulinemia and coronary heart disease: a complex metabolic web, J. Cardiovasc. Pharmacol. 20 (Suppl. 11), S1.

Goldschmid, M.G., E. Barret-Connor, S.L. Edelstein, D.L. Wingard, B.A. Cohn and W.H. Herman, 1994, Dyslipidemia and ischemic heart disease mortality among men and women with diabetes, Circulation 89, 991.

Graier, W.F., W.R. Kukovetz and K. Groschner, 1993a, cAMP enhances agonist-induced Ca^{2+} entry into endothelial cells by activation of potassium channels and membrane hyperpolarization, Biochem. J. 291, 263.

Graier, W.F., T.C. Wascher, L. Lackner, H. Toplak, G.J. Krejs and W.R. Kukovetz, 1993b, Exposure to elevated D-glucose concentrations modulates vascular endothelial cell vasodilatatory response, Diabetes 42, 1497.

Graier, W.F., S. Simecek and M. Sturek, 1995, Cytochrome P450 mono-oxygenase-regulated signalling of Ca^{2+} entry in human and bovine endothelial cells, J. Physiol. (London) 482, 259.

Hammes, H.P., S. Martin, K. Federlin, K. Geison and M. Brownlee, 1991, Proc. Natl. Acad. Sci. USA 88, 11555.

Houng, P.L.T., A.H.J. Kolk, T.A. Eggelte, C.P.H.J. Verstijnen, H. Gilis and J.T. Hendriks, 1991, Measurements of antigen specific lymphocyte proliferation using 5-bromo-deoxyuridine incorporation, J. Immunol. Methods 130, 243.

Hunt, J.V., C.C.T. Smith and S.P. Wolff, 1990, Autooxidative glycosylation and possible involvement of peroxidase and free radikals in LDL modification of glucose, Diabetes 39, 1420.

Kaiser, N., S. Sasson, E.P. Feener, N. Boukobza-Vardi, S. Higashi, D.E. Moller, S. Davidheiser, R.J. Przybylski and G.L. King, 1993, Differential regulation of glucose transport and transporters by glucose in vascular endothelial and smooth muscle cells, Diabetes 42, 80.

Kannel, W.B., 1985, Lipids, diabetes and coronary heart disease: insights from the Framingham study, Am. Heart J. 110, 1100.

Keen, H. and H.J. Jarret, 1979, The WHO multinational study of vascular disease in diabetes, Diabetes Care 2, 187.

Kumari, K., U.S. Bansal and M.K. Sahib, 1989, Inhibition of diabetic associated complications by nucleophilic compounds, Diabetes 40, 1079.

Langenstroer, P. and G. Pieper, 1992, Regulation of spontaneous EDRF release in diabetic rat aorta by oxygen free radicals, Am. J. Physiol. 263, 257.

Lienhard, G.E., J.W. Slot, D.E. James and M.M. M, 1992, How cells absorb glucose, Sci. Am. 1, 34.

Lorenzi, M., E. Cagliero and S. Toledo, 1985, Glucose toxicity for human endothelial cells in culture: delayed replication, disturbed cell cycle, and accelerated death, Diabetes 34, 621.

Lorenzi, M., D.F. Montisano, S. Toledo and A. Barrieux, 1986, High glucose induces DNA damage in cultured human endothelial cells, J. Clin. Invest. 77, 322.

Lorenzi, M., S. Toledo, G.R. Boss, M.J. Lane and D.F. Montisano, 1987, The polyol pathway and glucose 6-phosphate in human endothelial cells cultured in high glucose concentrations, Diabetologia 30, 222.

Magaud, J.-P., I. Sargent and D.Y. Mason, 1988, Detection of human white cell proliferative responses by immunoenzymatic measurement of bromodeoxyuridine uptake, J. Immunol. Methods 103, 95.

Misko, T.P., W.M. Moore, T.P. Kasten, G.A. Nickols, J.A. Corbett, R.G. Tilton, M.L. McDanie, J.R. Williamson and M.G. Currie, 1993, Selective inhibition of the inducible nitric oxide synthase by aminoguanidine, Eur. J. Pharmacol. 233, 119.

Most, R.S. and P. Sinnock, 1983, The epidemiology of lower-extremity amputations in diabetic individuals, Diabetes Care 6, 87.

Mueckler, M., 1990, Family of glucose-transporter genes: implications for glucose homeostasis, Diabetes 39, 6.

Natarajan, B., N. Gonzales, L. Xu and L. Nadler, 1992, Vascular smooth muscle cells exhibit increased growth in response to elevated glucose, Biochem. Biophys. Res. Commun. 187, 552.

Paisey, R.B., G. Arredono, A. Villalobos, O. Lozano, L. Guevara and S. Kelly, 1984, Association of differing dietary, metabolic and clinical risk factors with macrovascular complications of diabetes: a prevalence study of 503 Mexican type 2 diabetic subjects, Diabetes Care 7, 421.

Pilger, E., J. Lammer, H. Bertuch, G. Stark, M. Decrinis, K.P. Pfeiffer and G.J. Krejs, 1991, Nd:YAG laser with sapphire tip combined with balloon angioplasty in peripheral arterial occlusions, Circulation 83, 141.

Reaven, G.M., 1988, Role of insulin resistance in human disease, Diabetes 37, 1595.

Ross, R., 1986, The pathogenesis of atherosclerosis – an uptake, New Engl. J. Med. 314, 488.

Ross, R. and L. Agius, 1992, The process of atherogenesis – cellular and molecular interaction – from experimental animal-models to humans, Diabetologia 35, S34.

Ruderman, N., J. Willamson and M. Brownlee, 1992, Glucose and diabetic vascular disease, FASEB J. 6, 2905.

Santilli, S.M., 1992, The effect of diabetes on the proliferation of aortic endothelial cells, Ann. Vasc. Surg. 6, 503.

Tesfamariam, B. and R.A. Cohen, 1992, Free radicals mediate endothelial cell dysfunction caused by elevated glucose, Am. J. Physiol. 263, H321.

Tesfamariam, B., M.L. Brown and R.A. Cohen, 1992, Aldose reductase and myo-inositol in endothelial cell dysfunction caused by elevated glucose, J. Pharmacol. Exp. Ther. 263, 153.

Tesfamariam, B., J.J. Palacino, R.M. Weisbrod and R.A. Cohen, 1993, Aldose reductase inhibition restores endothelial cell function in diabetic rabbit aorta, J. Cardiovasc. Pharmacol. 21, 205.

Tilton, R.G., K. Chang, K.S. Hasan, S.R. Smith, J.M. Petrash, T.P. Misko, W.M. Moore, M.G. Currie, J.A. Corbett, M.L. McDaniel and J.R. Williamson, 1993, Prevention of diabetic vascular dysfunction by guanidines – inhibition of nitric oxide synthase versus advanced glycation end-product formation, Diabetes 42, 221.

Tsan, M.-F., J.E. White and C.L. Rosano, 1989, Modulation of endothelial GSH concentrations: effect of exogenous GSH and GSH monoethylester, J. Appl. Physiol. 66, 1029.

Turner, J.L. and E.L. Biermann, 1978, Effect of glucose and sorbitol on proliferation of cultured human fibroblasts and aterial smooth muscle cells, Diabetes 27, 583.

Vlassara, H., H. Fuh, Z. Makita, S. Krungkrai, A. Cerami and R. Bucala, 1992, Exogenous advanced glycosylation end products induce complex vascular dysfunction in normal animals: a model for diabetic and aging complications, Proc. Natl. Acad. Sci. USA 89, 12043.

Williamson, J.R., K. Chang, M. Frangos, K.S. Hasan, Y. Ido, T. Kawamuea, J.R. Nyengaard, M. vandenEnden, C. Kilo and R.G. Tilton, 1993, Hyperglycemic pseudohypoxia and diabetic complications, Diabetes 42, 801.