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Bovine Retinal Pericytes Are Resistant to Glucose-Induced Oxidative Stress *In Vitro*

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ABSTRACT

Diabetic retinopathy is a sight-threatening complication of diabetes, and loss of pericytes represents early signs of its development. We tested the hypothesis that high glucose levels may induce signs of oxidative stress in cultured bovine retinal pericytes. Pericytes were exposed to either normal (5.5 m*M*) or high (22 m*M*) glucose levels for 1, 3, and 5 days. Signs of oxidative stress were measured by expression of copper/zinc superoxide dismutase, manganese superoxide dismutase, catalase, and glutathione peroxidase using real-time RT-PCR. To elucidate the role of oxidative stress, we also measured glutathione (GSH) concentration in the cells and investigated the impact of thiol-reactive metal ions and hydrogen peroxide (H_2O_2) on intracellular GSH. Despite the stimulation with high glucose, thiol-reactive metal ions, or H_2O_2, there was no clear increased expression of antioxidant enzymes or influence of GSH levels. Lipid peroxidation (malondialdehyde level) was increased in bovine aortic smooth muscle cells, but not in bovine retinal pericytes. The data indicate that pericytes do not develop oxidative stress in response to hyperglycemia. However, it is not definitively excluded that oxidative stress may occur after longer time periods of glucose stimulation. *Antioxid. Redox Signal.* 7, 1486–1493.

INTRODUCTION

Diabetic retinopathy is a sight-threatening long-term complication in both type 1 and type 2 diabetes (25). Proliferative retinopathy is present in 25% of patients with type 1 diabetes after 15 years of diabetes duration and in >50% after 20 years (25). Loss of retinal pericytes together with basement membrane thickening represents early signs of diabetic retinopathy, and pericyte loss has been considered to be the primary lesion in the development of retinopathy (13).

The pathogenesis of diabetic retinopathy is complex and not fully understood. However, the primary metabolic abnormality is hyperglycemia, and tight metabolic control has beneficial effects on both development and progression of diabetic microangiopathy in type 1 (42) as well as type 2 (43) diabetes. Although the pathogenesis of diabetic microangiopathy is probably multifactorial, it has been suggested that oxidative stress induced by hyperglycemia may play a role (6). Thus, hyperglycemia may increase the generation of free radicals through glucose autooxidation (21), glycoxidation (48), activation of protein kinase C (14), and increased flux in the polyol pathway leading to a state of pseudohypoxia (46). There are several endogenous enzyme systems that protect the tissues from oxidative stress, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx1) (34). SOD catalyzes the dismutation of superoxide to hydrogen peroxide (H_2O_2) and exists in the main subforms: cytosolic copper/zinc SOD (CuZnSOD), mitochondrial manganese

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SOD (MnSOD), and extracellular SOD. Catalase is present in peroxisomes and catalyzes the two-step conversion of H_2O_2 to water and oxygen. GPx1 is a selenoprotein located in the cytoplasm and the mitochondria (49). It reduces lipidic or nonlipidic hydroperoxides, as well as H_2O_2 while oxidizing two molecules of glutathione (GSH) (39). GSH, a major antioxidant, is a tripeptide synthesized from its constituent amino acids. It is well recognized that depletion of GSH is caused by several factors, such as oxidative stress by H_2O_2. We have also recently shown that GSH production is stimulated in the presence of agents that form complex/adducts with GSH, *e.g.*, thiol-reactive metals such as mercury ions (20). An active system for maintaining glutathione in its reduced form is normally present in the retina, and the retina has the ability to regulate the GSH concentration by a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent process (47). It is generally believed that exposure to oxidative stress is associated with an increased gene expression of these enzymes and increase in antioxidant enzyme activity.

Previous studies have shown that diabetes increases oxidative stress in both humans and animals, playing a putative role in the development of diabetic complications (5, 6, 28, 30). Oxidative stress has also been shown to develop in the retina of diabetic and galactose-fed animals (16, 27, 28). The importance of oxidative stress is further supported by studies showing that dietary supplementation with antioxidants can inhibit abnormalities of retinal metabolism induced by diabetes (26–29). Taken together, these results indicate that oxidative stress is associated with and may play a role in the development of diabetic complications, such as retinopathy.

The aim of the present study was therefore to determine whether glucose exposure induces an activation of the defense system against oxidative stress, as evidence of increased oxidative stress. Cultured bovine pericytes were chosen because this cell type has been shown to be involved early in the development of diabetic retinopathy. Pericytes were cultured in normal (5.5 m*M*) and high (22 m*M*) glucose for 1, 3, and 5 days, and the mRNA expression of the antioxidative enzymes CuZnSOD, MnSOD, catalase, and GPx1 was measured using real-time polymerase chain reaction (PCR). Furthermore, in order to elucidate the role of oxidative stress, we also measured GSH concentration in the pericytes and investigated the impact of thiol-reactive metal ions and H_2O_2 on intracellular GSH in high and normal glucose environments. Malondialdehyde (MDA) was assayed in both smooth muscle cells and pericytes in order to demonstrate increased lipid peroxidation due to hyperglycemia-induced oxidative stress.

MATERIALS AND METHODS

Bovine retinal microvascular isolation and culture of pericytes

Bovine eyes were cleared from surrounding tissue, sterilized in Lugol solution (Sigma) for 40 min, and rinsed in phosphate-buffered saline (PBS) and PBS supplemented with penicillin–streptomycin. The eyes were cut 8 mm behind the iris; the retina was collected, transferred to Dulbecco's modified Eagle's medium (DMEM) (GIBCO), homogenized by crosscutting, and rinsed; and the different components were separated using nylon nets with different mesh sizes, initially 88 μm. Cell material remaining on the mesh was collected, and after collagenase type 1 (GIBCO) digestion (900 U/ml in DMEM for 30 min at 37°C), a second rinse was performed with a mesh size of 155 μm. Cell material filtering through was collected, and pericytes were filtered through a third mesh (55 μm), then seeded on petri dishes (20×57 cm^2), and incubated at 37°C with 5% CO_2. The medium was changed the following day and thereafter every 3–4 days. Passaging was performed at 70% confluence and was used for the experiments after second through fourth passages. No antibiotics were used. Pericytes were identified by morphology and reaction with monoclonal antibody 3G5 (35). Morphologically, pericytes appeared as large flattened cells with arborized plasma membranes. At confluence, pericyte monolayers were interspersed with multilayered nodules.

Smooth muscle cell explant cultures

Fresh bovine aortae (tied off at each end before removal) were obtained from the Meat Sciences Department of the University of Arizona. The intima and adventitia were removed by blunt dissection. The medial smooth muscle layer was minced into 1.0-mm cubes in a sterile 10-cm tissue culture petri dish to which 3 ml of growth medium (DMEM plus 10% fetal bovine serum) was added. The fragments were incubated for 1 h in a tissue culture incubator to allow the fragments to adhere, after which 7 ml of growth medium was then added. After 7 days the tissue fragments were removed, and the cells occupying the petri dish were washed with PBS, released with trypsin/EDTA, and passaged to fresh plates for expansion of the cell population. Smooth muscle cells were identified by morphology and reactivity with anti-smooth muscle actin antibody. Morphologically, the smooth muscle cells were seen to be bipolar cells that grew in multilayers at confluence with classic "hill and valley" appearance.

Stimulation

Pericytes were stimulated in holding medium with 22 m*M* glucose for 1, 3, or 5 days, and the control with 5.5 m*M* glucose. Both high and low glucose media were changed every second day. The low glucose medium was supplemented with 16.5 m*M* NaCl to correct for osmolarity. New medium with or without the test substances was added at the start of the experiments. The substances (H_2O_2 at 0.1, 1.0 and 5.0 μmol/L; and mercury chloride ($HgCl_2$) at 1 and 10 μmol/L) were prepared and added to the cell culture experiment immediately before the start of the experiment, either at 5.5 or 22 m*M* glucose. All concentrations were expressed as the final concentration in the medium.

RNA isolation

RNA was isolated using Tri reagent (Sigma) according to the protocol of the manufacturer. Twelve dishes of pericytes were exposed to each glucose condition. To acquire enough RNA, two dishes were combined in a total of 1.5 ml of reagent yielding six RNA samples for each glucose level. In

short, culture dishes were rinsed twice with ice-cold PBS, then the Tri reagent was added directly to the dishes, and the cells were lysed using a rubber policeman. The pellet was finally dissolved in 22.5 µl of diethyl pyrocarbonate-treated water. The amount and purity were determined by spectrophotometry and agarose gel electrophoresis. Each RNA sample was then subjected to the PCR procedure six times.

Real-time RT-PCR

cDNA was synthesized from 2 µg of total RNA primed with 250 ng of random hexamer using Superscript II (Invitrogen) according to the protocol of the manufacturer. TaqMan primers and probes against CuZnSOD, MnSOD, catalase, GPx1, and cyclophilin B (CypB) were designed using the PrimerExpress software (Applied Biosystems), spanning over an intron–exon boundary and blast (nblast) for specificity. The following primers and probes from MWG Biotech were used (all sequences are 5′ to 3′): (a) *CuZnSOD:* forward, CGG TGG GCC AAA AGA TGA; reverse, ACA CCG TTT TTG TCA GCT GTC A; probe, FAM-TTG CCC AGG TCT CCA ACA TGC CTC T-TAMRA. (b) *MnSOD:* forward, CAA CGT CGC CGA GGA GAA; reverse, ATG CCC TTT TCA TCT TTC TCT GC; probe, FAM-ACC GGG AGG CGC TGG AG-TAMRA. (c) *Catalase:* forward, CAC ACC TGA AGG ATC CGG A; reverse, TGG TGC AGA GAG ACT CAG GGC; probe, FAM-TGG TCT GGG ACT TCT GGA GCC T-TAMRA. (d) *Gpx1:* forward, CCC CTG CAA CCA GTT TGG; reverse, CGG ACG TAC TTC AGG CAA TTC; probe, FAM-CAG GAA AAC GCC AAG AAC GAG GAG ATC. (e) *CypB:* forward, GAG ACG GCA CTG GAG GTA AGA G; reverse, GCC CAT AGT GTT TAA GCT TGA AGT TC; probe, FAM-ATC TAC GGT GAA CGC TTC CCC GAT G-TAMRA.

All mRNA sequences were bovine except for catalase, for which only the amino acid sequence is published. A BLAST (tblastn) search showed the highest homology with the catalase mRNA for dog (*Canis familiaris*), which therefore was used. Samples ($n = 6$ for each group) were run in duplicate on a 7900HT system (Applied Biosystems) with TaqMan Universal PCR Master Mix, 900 n*M* primer, and 250 n*M* probe. If the standard deviation of the duplicate Ct values differed by > 0.16, the samples were rerun. The template input amount was normalized for each sample using CypB as an endogenous control. The relative amount of each transcript was calculated using the comparative Ct method with the corresponding low glucose sample as calibrator (according to the protocol from Applied Biosystems). CypB was constant in all samples and was therefore regarded as a valid endogenous control (data not shown).

GSH concentration

After 1 day, cells were washed twice in ice-cold PBS and homogenized in 500 µl of phosphate buffer and 30 µl of 0.1 *M* dithiothreitol using a glass homogenizer. The homogenate was incubated at 37°C for 15 min, and then 100 µl of 15% sulfosalicylic acid was added. Samples were kept at 4°C for 30 min and then centrifuged (12,000 *g* for 5 min), and the supernatant was kept frozen at −70°C until analysis. For analysis, we used a high-performance liquid chromatography method, using isocratic reversed-phase ion-pair liquid chromatography at pH 2.4 and postcolumn derivatization with 4,4′-dithiopyridine and colorimetric detection at 324 nm. The cellular content of GSH is expressed as the amount (nanomoles) per milligram of cell protein. Protein content was analyzed according to Lowry *et al.* (32).

MDA concentration

Ten petri dishes of smooth muscle cells and 10 petri dishes of pericytes were maintained in 5.5 m*M* glucose or 22 m*M* glucose containing media for 5 days. Cells were washed twice with PBS and scraped into 1 ml of 20 m*M* phosphate buffer containing 5 m*M* butylated hydroxytoluene. The harvested cells were homogenized by sonication on ice with two 5-s bursts at 20 W separated by a 1-min cooling period. Aliquots of the homogenate were used for protein assay and measurement of MDA using the BIOXYTECH LPO-586 colorimetric assay kit for lipid peroxidation (OxisResearch, Portland, OR, U.S.A.) according to the manufacturer's instructions. All assays were performed in triplicate, and MDA levels were calculated as nanomoles per milligram of protein. Specific MDA levels were normalized to the mean level of the 5.5 m*M* glucose control and expressed as a percentage of the control level.

Statistics

Mann–Whitney's two-tailed test was used for evaluating differences between groups.

RESULTS

mRNA of antioxidative enzymes

The relative expression of the pericyte mRNA transcript for CuZnSOD, MnSOD, catalase, and GPx1 in normal (5.5 m*M*) glucose medium or high (22 m*M*) glucose medium after 1, 3, or 5 days of stimulation is shown in Figs. 1–4.

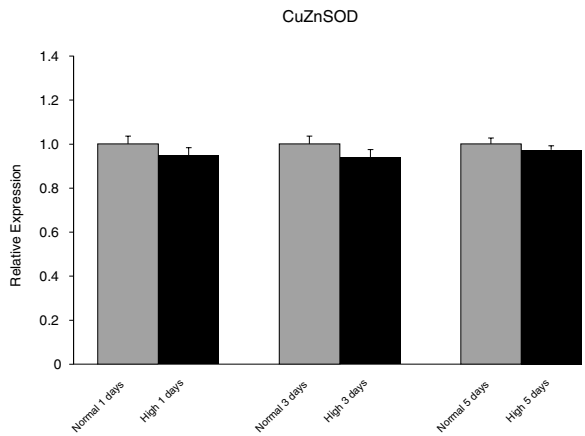

FIG. 1. Relative expression of the pericyte CuZnSOD transcript in normal (5.5 m*M*) versus high (22 m*M*) glucose media after 1, 3, or 5 days of stimulation. Error bars represent the relative range of the standard error of the mean of samples in each group ($n = 6$ in each group).

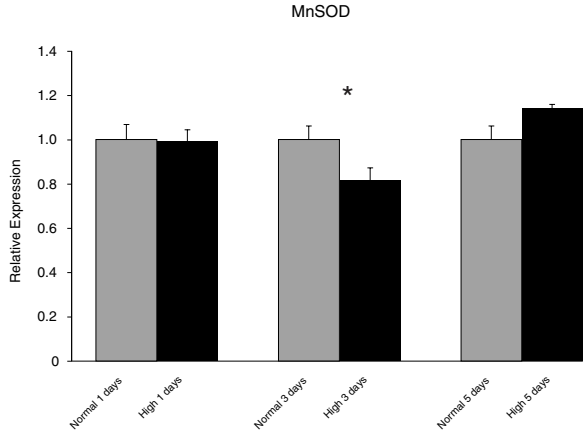

FIG. 2. Relative expression of the pericyte MnSOD transcript in normal (5.5 m*M*) versus high (22 m*M*) glucose media after 1, 3, or 5 days of stimulation. Error bars represent the relative range of the standard error of the mean of samples in each group ($n = 6$ in each group). *$p = 0.037$.

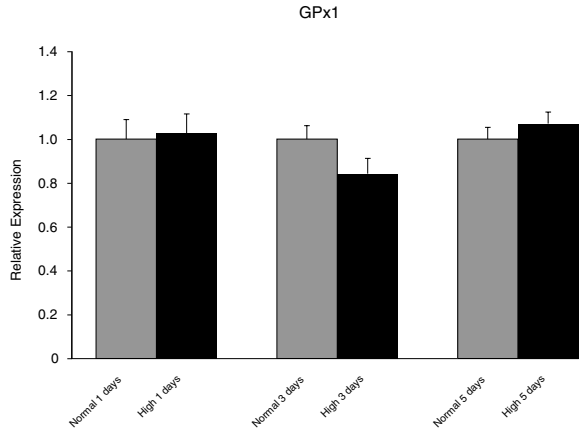

FIG. 4. Relative expression of the pericyte GPx1 transcript in normal (5.5 m*M*) versus high (22 m*M*) glucose media after 1, 3, or 5 days of stimulation. Error bars represent the relative range of the standard error of the mean in each group ($n = 6$ in each group).

The relative expression of the pericyte CuZnSOD transcript was similar at all time points in normal and high glucose media (Fig. 1), whereas the MnSOD transcript was similar in normal and high glucose media after 1 and 5 days of stimulation, but showed a slight decrease (20%; $p = 0.037$) after high glucose stimulation for 3 days (Fig. 2).

The pattern of relative expression of the pericyte catalase transcript at day 1 was essentially unchanged at 3 and 5 days with only a slightly higher value (10%) in high glucose medium, which was statistically significantly increased after 1 ($p = 0.037$), 3 ($p = 0.037$), and 5 ($p = 0.025$) days (Fig. 3).

The relative expression of GPx1 was similar after 1 and 5 days of glucose stimulation between normal and high glucose values. After 3 days of glucose stimulation, the relative expression of the pericyte GPx1 transcript was slightly lower ($p = 0.078$) in the high glucose stimulation group (Fig. 4).

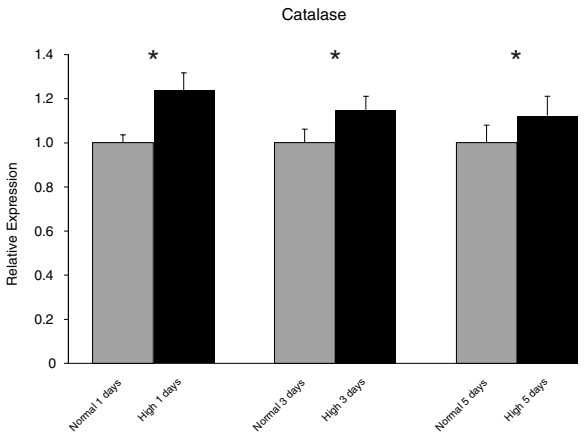

FIG. 3. Relative expression of the pericyte catalase transcript in normal (5.5 m*M*) versus high (22 m*M*) glucose media after 1, 3, or 5 days of stimulation. Error bars represent the relative range of the standard error of the mean of samples in each group ($n = 6$ in each group). 1 day: *$p = 0.037$; 3 days: *$p = 0.037$; 5 days: *$p = 0.025$.

GSH concentration

There was a nonsignificant tendency toward lower GSH concentration in pericytes stimulated with high glucose. Stimulation with thiol-reactive mercury ions at 10 μmol/L increased ($p = 0.082$) intracellular GSH concentration, but only in the cells incubated in the normal (5.5 m*M*) glucose environment. The addition of H_2O_2 (0.1, 1.0, and 5.0 μmol/L) did not change the cellular GSH concentration, at either normal or high (22 m*M*) glucose (Fig. 5). Cells incubated with higher concentration of H_2O_2 (10 umol/L) showed a decrease in cell protein and intracellular GSH levels, indicating cell death (data not shown).

MDA concentration

In bovine aortic smooth muscle cells, we observed an increase of ~22% in MDA levels in cells maintained in 22 m*M* > glucose compared with cells maintained in 5.5 m*M* glucose for 5 days (Fig. 6). The observed difference was not statistically significant (Table 1). In pericytes, however, there was no difference seen in MDA levels, and in the cells maintained in 22 m*M* glucose for 5 days the trend was to a lower MDA

TABLE 1. MDA CONCENTRATION IN BOVINE AORTIC SMOOTH MUSCLE CELLS AND BOVINE RETINAL PERICYTES EXPOSED TO HYPERGLYCEMIC AND EUGLYCEMIC CONDITIONS FOR 5 DAYS *IN VITRO*

	MDA concentration (nmoles/mg protein ± S.E.M.)	
Glucose concentration	*Smooth muscle cells*	*Pericytes*
5.5 m*M* Glucose	5.9 ± 1.7	19.4 ± 3.2
22 m*M* Glucose	7.3 ± 1.6	13.1 ± 4.4

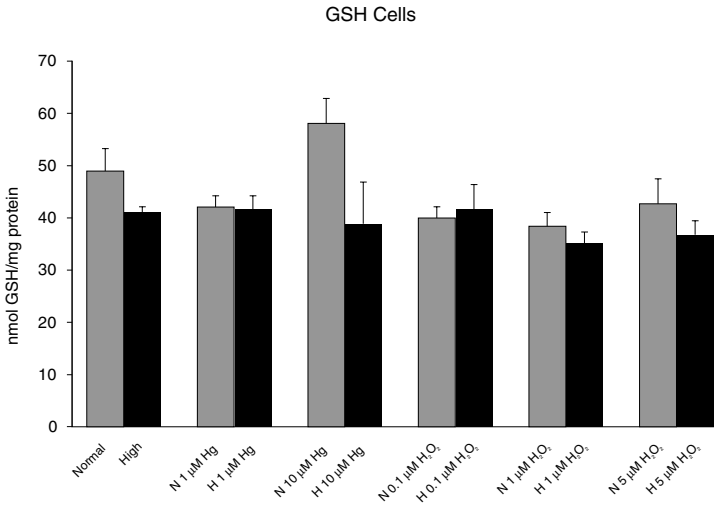

FIG. 5. Concentration of GSH (nmol) per mg of total protein in pericytes with normal (5.5 m*M*) versus high (22 m*M*) glucose stimulation with 1 or 10 µ*M* Hg or 0.1, 1.0, or 5.0 µ*M* H_2O_2 (*n* = 6 in each group). Values are given as means ± SEM.

level than in the 5.5 m*M* control. No statistically significant difference was seen in the pericyte cultures.

DISCUSSION

It has been assumed that high glucose levels might induce oxidative stress within cells (6). The principal result of the present study, however, is a lack of a substantially increased expression of antioxidative enzymes in pericytes exposed to high glucose for 1, 3, and 5 days. Although some small changes were seen, their pathophysiological relevance is questionable. This is supported by the observation of an increase in MDA levels in smooth muscle cells, but not in pericytes under the same conditions. Our results are thus in agreement with the only other published study (37), which also showed induction of oxidative stress by hyperglycemia in smooth muscle cells, but not in pericytes. There was only a tendency toward a reduction in GSH levels within cells incubated in high glucose, and exposure to H_2O_2 did not have any influence. The difference in GSH results from those obtained by Sharp *et al.* (37) cannot be definitely explained as we do not have the raw data from that study. However, it seems likely that the quantitative difference in our results may be ascribable, at least in part, to methods of protein assay. Sharpe *et al.* used the bicinchoninic acid method and we used the Lowry method. No colorimetric assay method gives absolute protein values as the standard curve is generated with a single protein, and therefore the protein assay yields a relative protein level. This can vary according to which protein is used as a standard and which color chemistry used.

In the present study, we chose to use NaCl as an osmotic control. Alditols such as mannitol or sorbitol are often used as a control for hyperglycemic conditions as they are not taken up or metabolized by cells and are considered to be physiologically inert, with the exception of osmotic effects on water transport. However, many reports in the literature indicate that hyperosmolar levels of sorbitol and mannitol have other effects on tissue cultured cells. For example, sorbitol and mannitol have been shown to have mild toxic effects on rat cardiomyocytes (50), and hyperosmolar concentrations of sorbitol activate the epidermal growth factor receptor (EGFR) in human keratinocytes (10). Experiments using specific inhibitors of EGFR phosphorylation showed that the increased amount of activated receptors was the result of a decreased rate of dephosphorylation. Furthermore, sorbitol treatment resulted in a strong activation of stress kinase p38. Additionally, it has been shown that in retinal pigment epithelial cells, hyperosmolar mannitol caused an increase in mRNA for lysyl oxidase (36). In a study of lipid peroxidation in human peritoneal mesothelial cells, hyperglycemia caused lipid peroxidation, but mannitol did not; however, both glucose and mannitol stimulated fibronectin synthesis (8). A

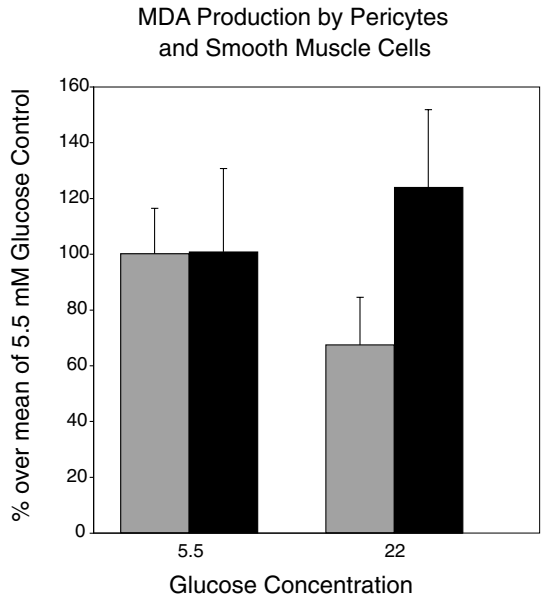

FIG. 6. Relative MDA levels in bovine aortic smooth muscle cells and pericytes exposed to 5.5 and 22 m*M* glucose for 5 days. Gray bars, pericytes; black bars, smooth muscle cells. Error bars show standard error of the mean.

study of the osmotic effects of mannitol and NaCl showed that they had equivalent effects on aortic endothelial cells (33), which at the high levels used in that study (300 milliosmolar and above) caused incremental increases in apoptosis. As NaCl and mannitol produce equivalent effects, we opted to use NaCl as an osmotic control because it is cell-permeant, as is glucose.

Loss of pericytes is an early morphological sign of diabetic retinopathy development. It is clear, however, that the pathogenesis of this diabetic complication is complex. The present results do not support the hypothesis that oxidative stress is of major importance for pericyte loss. This information is of importance in the search for therapeutic targets for prevention of sight-threatening stages of retinopathy (39). Our findings are also, to some extent, supported by the study of Kowluru *et al.* (31). In that study, diabetic rats were fed a dietary supplement of vitamins C and E for 18 months. It inhibited the development of acellular capillaries in the retinas, whereas the number of pericyte ghosts was not statistically influenced. However, when the rats were fed a multiantioxidant mixture consisting of vitamin C, Trolox, vitamin E, *N*-acetylcysteine, β-carotene, and selenium, the formation of both pericyte ghosts and acellular capillaries was inhibited. In a study by Hammes *et al.* (18), male streptozotocin (STZ) diabetic Lewis rats treated with the antioxidant and lipid-lowering compound nicanartine for 6 months showed an amelioration in the reduction of pericyte numbers, but no effect was observed on the formation of acellular capillaries, indicating that antioxidant therapy was of limited benefit in that rodent model of STZ-induced diabetes. This is in accordance with our previous results where we found no effect on the formation of acellular capillaries in diabetic rats fed the antioxidant probucol for 6 months (3). This finding is further supported by a study of diabetic rats given the water-soluble antioxidant Trolox for 5 months, which only partially normalized the diabetes-induced changes in the ratio of retinal capillary endothelial cells to pericytes (4). The somewhat contradictory results between different studies may depend to some extent on the level of glycemia, because it has been shown that endothelial cell proliferation requires higher glucose levels than pericyte loss (19). Thus, the results are to some extent conflicting, and may favor the hypothesis that oxidative stress is not a major cause of diabetic retinopathy.

The lack of increased expression of scavenging enzymes in pericytes exposed to high glucose in our study might be explained by previous findings that endogenous and scavenging protectors such as vitamin E and GSH are depleted in diabetes (1, 2) and antioxidant enzymes such as SOD and catalase are less active (11, 22, 44, 45). In the present study, there was a slight decrease in GSH concentration within cells incubated in high glucose, and thiol-reactive ions only increased GSH concentration in cells incubated in normal glucose levels. An alternative explanation might be that oxidative damage is tissue-specific. This is supported by the finding that the antioxidant probucol had no effect on albuminuria in STZ-diabetic rats over 32 weeks (39), whereas a significant effect of probucol on nerve conduction velocity and nerve blood flow was observed after only 1 and 2 months of diabetes, even in the presence of the prooxidant primaquine (9).

The present and previous results are thus not conclusive and favor mechanisms other than oxidative stress to contribute to the development of cellular damage and retinopathy. Chronic aspirin consumption was reported many years ago to be associated with protection from diabetic retinopathy, suggesting the possibility that the prostaglandin system might be involved in the development. However, results from clinical studies have been conflicting, showing a modest inhibitory effect in one (12) but no effect in another study (15). In a recent study of dogs with diabetes for 5 years, aspirin inhibited the development of retinal hemorrhages and acellular capillaries, but had no effect on the development of pericyte ghosts (24). A common early lesion of diabetic retinopathy in humans and many animal models of diabetes is capillary basement membrane thickening, which could contribute to impaired endothelial-pericyte communication, capillary contractility, or appropriate cell interaction (7, 23). In a recent 4-year study, the nonsteroidal antiinflammatory drug Sulindac was given to beagle dogs with STZ/alloxan-induced diabetes. Basement membrane thickening was prevented, an effect that was independent of inhibition of polyol pathway activity, advanced glycation, or oxidative stress, but the precise mode of action of this drug has to be further investigated (17). These results indicate that inflammation might be involved in the development of some of the pathologies of diabetic retinopathy.

In conclusion, the present data indicate that, under the experimental conditions used, pericytes do not develop oxidative stress in response to hyperglycemia, and that oxidative stress may not be a major cause of pericyte loss in the early development of diabetic retinopathy. However, although MDA levels do not change, this does not definitively exclude the presence of oxidative stress and that longer time periods of glucose exposure may be necessary.

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ABBREVIATIONS

CuZnSOD, copper/zinc superoxide dismutase; CypB, cyclophilin B; DMEM, Dulbecco's modified Eagle's medium; EGFR, epidermal growth factor receptor; GSH, glutathione; GPx1, glutathione peroxidase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; MnSOD, manganese superoxide dismutase; PBS, phosphate-buffered saline; RT-PCR, reversed transcription–polymerase chain reaction; SOD, superoxide dismutase; STZ, streptozotocin.

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