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Glucose-induced oxidative stress in mesangial cells

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Glucose-induced oxidative stress in mesangial cells.

Background. Hyperglycemia is a well-recognized pathogenic factor of long-term complications in diabetes mellitus. Hyperglycemia not only generates reactive oxygen species but also attenuates antioxidant mechanisms creating a state of oxidative stress.

Methods. Porcine mesangial cells were cultured in high glucose (HG) for ten days to investigate the effects on the antioxidant defenses of the cell.

Results. Mesangial cells cultured in HG conditions had significantly reduced levels of glutathione (GSH) compared with those grown in normal glucose (NG). The reduced GSH levels were accompanied by decreased gene expression of both subunits of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in de novo synthesis of GSH. Elevated levels of intracellular malondialdehyde (MDA) were found in cells exposed to HG conditions. HG also caused elevated mRNA levels of the antioxidant enzymes CuZn superoxide dismutase (SOD) and MnSOD. These changes were accompanied by increased mRNA levels of extracellular matrix proteins (ECM), fibronectin (FN) and collagen IV (CIV). Addition of antioxidants to high glucose caused a significant reversal of FN and CIV gene expression; α-lipoic acid also up-regulated γ-GCS gene expression and restored intracellular GSH and MDA levels.

Conclusions. The results demonstrate the existence of glucose-induced oxidative stress in mesangial cells as evidenced by elevated MDA and decreased GSH levels. The decreased levels of GSH are as a result of decreased mRNA expression of γ-GCS within the cell. Antioxidants caused a significant reversal of FN and CIV gene expression, suggesting an etiological link between oxidative stress and increased ECM protein synthesis.

Diabetic nephropathy is an important complication of both type 1 and 2 diabetes mellitus. A major histological characteristic of this disease is the enlargement of the glomerular mesangium due to the accumulation of extracellular matrix (ECM) proteins, synthesized by the mesangial cells. Mesangial cells are mononucleated cells embedded in extracellular matrix and are in contact through their processes with the basement membrane and endothelial lining of the glomerular capillaries [1]. Studies have indicated that hyperglycemia plays a central role in the development and progression of the glomerulosclerotic process in diabetes mellitus [2]. The structural changes of an enlarged mesangium in diabetes in vivo are reflected in an increase of ECM protein production by mesangial cells grown under high glucose conditions in vitro [3]. The mechanisms by which glucose stimulates an increase of ECM protein synthesis in diabetes may involve the polyol pathway [4], advanced glycosylation-end product formation [5], increased activity of protein kinase C [6] and increased production of transforming growth factor-β (TGF-β) [7].

Although considerable evidence exists linking glucose to the increased accumulation of ECM protein in diabetic nephropathy, relatively little is known concerning the oxidant/antioxidant status of glomerular mesangial cells in a high glucose medium nor whether glucose-induced oxidative stress plays an etiological role in increased ECM protein production by these cells. Glucose-induced oxidative stress is an attractive hypothesis for the pathogenesis of diabetic complications including nephropathy. Increases in gene expression of the antioxidant enzymes CuZn superoxide dismutase (SOD) and catalase [8] and increased levels of 8-hydroxydeoxyguanosine, a marker of oxidative tissue damage [9], have been reported in kidneys of diabetic rats. The protective effect that antioxidants have on certain aspects of nephropathy in diabetic animals has been reported. Thus, taurine and vitamin C effectively reduced glomerular hypertrophy, albuminuria, glomerular collagen and TGF-β accumulation in STZ rats [10–13]. In cultured mesangial cells, glucose-induced increases in malondialdehyde (MDA) and conjugated dienes were prevented by taurine and vitamin E [14]. These results suggest that oxidative stress may be associated with some of the changes of diabetic nephropathy.

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Glutathione (GSH), a major intracellular antioxidant, is depleted in erythrocytes [15], leukocytes [16] and platelets from human diabetic patients [17], porcine vascular smooth muscle cells [18], and in endothelial cells from diabetic rabbits [19]. The changes in GSH levels, which result in an oxidant/pro-oxidant imbalance, may predispose the cells to oxidant-induced damage and diabetic complications. GSH is synthesized from its constituent amino acids by the activity of two adenosine 5′-triphosphate (ATP)-dependent enzymes, γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase, with γ-GCS catalyzing the rate-limiting step. γ-GCS is a heterodimer composed of a heavy [γ-GCS(H)] and light [γ-GCS(L)] subunit. The heavy subunit exhibits catalytic activity [20] whereas the light subunit regulates the activity of the heavy subunit [21]. Several studies have demonstrated the reductions in gene expression of the heavy subunit and activity of γ-GCS in high glucose conditions [22–24]. However, regulation of γ-GCS activity at the transcriptional level with respect to both the light and heavy subunits has not been investigated in high glucose conditions.

In the present study the effects of glucose on the antioxidant defenses of the mesangial cell have been examined. In addition to studies of antioxidant enzyme gene expression, the effects of glucose on the chief antioxidant of the cell, GSH and the rate-limiting enzyme in GSH synthesis, γ-GCS, have been investigated. The relationships between glucose-induced oxidative stress and up-regulation of genes for ECM proteins have been investigated by the use of two structurally unrelated antioxidants: Trolox (a water soluble analog of α-tocopherol) and α-lipoic acid.

METHODS

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

Mesangial cell culture

Glomeruli were isolated under sterile conditions by selective sieving of finely chopped porcine renal cortex. Mesangial cells were cultured from isolated glomeruli in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) at 37°C containing 20% fetal calf serum (FCS), and antibiotics (60 μg/mL benzylpenicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin B). Cells were harvested by washing two times in phosphate-buffered saline (PBS) and briefly exposed to 0.125% trypsin (Difco, Detroit, MI, USA). Cells were confirmed as mesangial cells by their typical morphology and positive immunofluorescence staining against smooth muscle α-actin. For the different experiments cells were cultured for ten days under normal (5 mmol/L) and high (25 mmol/L) glucose conditions with the addition of Trolox (200 μmol/L), α-lipoic acid (50 μmol/L), H_2O_2 (100 μmol/L) and L-glucose (20 mmol/L) for various incubation periods. Growth medium was changed twice weekly and all cells were used at confluence between the 5th and 10th passages.

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 minutes at room temperature. A hemocytometer was used to count live and dead cells, and percent viability was calculated. Lactate dehydrogenase activities were measured in the supernatants using dry-film technology on a Vitros 750 analyzer (Johnson & Johnson Clinical Diagnostics, Amersham, UK).

Growth curve experiments

For all experiments cells were seeded into 25 cm^2 tissue culture flasks under experimental conditions. Following trypsinization, cell counts were performed on a Coulter counter (Coulter Electronics Ltd, Luton, UK).

Harvesting the mesangial cells

Monolayers were rinsed twice with ice-cold Hanks' balanced salt solution (HBSS; without calcium and magnesium and phenol red; Gibco) and the cells were collected using a disposable cell scraper (Sarstedt, Leicester, UK). The cells were centrifuged at 225 × *g* at 4°C for five minutes and the cell pellet was stored at −80°C before the stage of cell lysis and release of cell and organelle contents.

Cell lysis and homogenization

Pellets were resuspended in 0.1% Triton X-100 (BDH Chemicals, Poole, UK) in PBS (pH 7.4) and sonicated with two 15-second bursts on a sonicator (Lucas Dawe Ultrasonics, London, UK). The sample was centrifuged at 3000 × *g* for 30 minutes at 4°C, aliquots were removed and antioxidant enzyme activities were measured immediately. Samples for MDA were stored at −80°C prior to analysis. Samples for GSH were stabilized by the addition of 1 part 10% sulphosalicylic acid to 2 parts sample. After centrifugation at 13,000 × *g* for five minutes, the resulting supernatant was stored at −80°C.

Protein measurement

Total protein was measured using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce & Warriner Ltd, Chester, UK).

Malondialdehyde

Malondialdehyde is produced by the hydrolysis of lipid hydroperoxides, which react with thiobarbituric acid (TBA) to produce a complex that absorbs at 532 nm. To avoid interference of other substrates with TBA, the malondialdehyde-thiobarbituric acid adduct was measured

by high-performance liquid chromatography (HPLC) coupled with MDA fluorimetric detection [25].

Glutathione

Glutathione (GSH) was measured by the enzymatic recycling method in which GSH was oxidized by 5′5′-dithiobis-(2-nitrobenzoic acid) and reduced by nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of glutathione reductase.

The method of Griffith [26] was automated on the Cobas Fara centrifugal analyzer (Roche Products Ltd, Welwyn Garden City, UK).

Antioxidant enzyme activities

Total superoxide dismutase (SOD) activity was measured using the method of L'Abbe and Fischer [27], adapted for the Cobas Fara centrifugal analyzer. SOD activity is measured indirectly. Superoxide is generated enzymatically by xanthine plus xanthine oxidase, which reduces cytochrome c. SOD competes for known amounts of superoxide radicals and decreases the reduction of cytochrome c seen in the absence of the enzyme. Cu/ZnSOD activity was calculated by the subtraction of MnSOD activity from total SOD activity, as MnSOD activity is resistant to incubation at room temperature with potassium cyanide. The enzyme activity of glutathione peroxidase (GPX), which catalyses the oxidation of reduced GSH by hydrogen peroxide, was determined by the method of McMaster et al [28]. Catalase activity was measured using the method of Johansson and Borg [29] that was adapted for measurement on the Cobas Fara [30].

Preparation of cDNA

Oligo (dT)-primed first strand cDNA synthesis was carried out on total RNA (1 μg) with a reverse transcription system (Promega Corp, Madison, WI, USA) using superscript II Rnase H^- reverse transcriptase (RT; Gibco). The reaction was incubated at 42°C for 60 minutes, heated to 70°C for 10 minutes and then quick-chilled on ice.

PCR amplification and quantification of PCR products

Equal amounts (3 μL) of the reverse transcription reaction were subjected to polymerase chain reaction (PCR) amplification at a final concentration of 1 × PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 9), 0.1% Triton X-100), 25 μmol/L dNTP, 100 pmol each 5′ and 3′ primers, 10 μCi [α^{32}P] dCTP, 5 U Taq polymerase and $MgCl_2$ to a final concentration of 1.5 mmol/L in a total volume of 100 μL. The amplification profile involved denaturation at 94°C for 45 seconds, primer annealing at 60°C for one minute and extension at 72°C for one minute. During PCR, aliquots were withdrawn at regular intervals (15, 20, 25, 30, 35, 40 or 45 cycles) of amplification. An aliquot was electrophoresed in 1.4% agarose and radioactivity was determined on a Bio-Rad Molecular Imager system. The amount of radioactivity from PCR products was plotted against the number of PCR cycles to determine the exponential phase of the reaction. Target mRNA was then co-amplified with the internal control (GAPDH), which acted as a control for sample-to-sample variation in RT and PCR reaction and monitored the extent of degradation and recovery of RNA. Following co-amplification, 20 μL of PCR product was electrophoresed in agarose gel and analyzed on a Molecular Imager. Band intensities were quantified following normalization with respect to internal control. Primer sequences are detailed in Table 1.

Northern blot analysis

Total RNA was isolated from mesangial cells by the acid-guanidinium thiocyanate-phenol-chloroform method as described by Chomczynski and Sacchi [31]. Thirty micrograms of RNA were electrophoresed on a 1% agarose-formaldehyde gel. Denatured RNA was transferred to a charged nylon filter (Hybond N^+, Amersham, UK). Hybridization was performed in a Techne hybridization oven (Techne Ltd, Cambridge, UK) using cDNA probes to fibronectin, collagen IV, [Human Genome mapping project (HGMP, UK)] and collagen I (mouse α1 chain; kindly supplied by Prof. Eero Vuorio, Department of Medical Biochemistry and Molecular Biochemistry, University of Turku, Turku, Finland). A porcine cDNA probe to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was prepared by RT-PCR and used as a "housekeeping" gene. Prehybridization was at 60°C for 30 minutes following the addition of Quickhyb hybridization solution (Stratagene, Cambridge, UK). cDNA probes (75 to 100 ng) were labeled using the Prime-It II Random Primer Labeling Kit (Stratagene) and 50 μCi ^{32}P dCTP (Amersham). Hybridization was carried out for two hours at 65°C, following which the membrane was washed (42°C) twice in 2 × standard sodium citrate (SSC) containing 0.1% sodium dodecyl sulfate (SDS) for 15 minutes, then once with 1 × SSC (0.1% SDS) and once with 0.2 × SSC, (0.1% SDS). The membranes were analyzed on a Bio-Rad molecular Imager system GS-525 (Bio-Rad, Herts, UK). Band intensities were quantified using volume analysis, correcting for loading inequalities with GAPDH.

RESULTS

Cell viability

There were no significant differences in cell viability between cells cultured in 5 or 25 mmol/L D-glucose as assessed by trypan blue exclusion; there were also no increases in lactate dehydrogenase activity in the supernatants under any conditions (data not shown).

Table 1. Primer sequences of antioxidant enzymes, GAPDH and γ-GCS subunits used in quantitative PCR reactions

	Sense	Antisense
MnSOD		
Acc No X64057	GGAATTCCAGCTGCACCACAGCGAGC	GGAATTCGATCCCCAGCAGCGGAACC
Cu/ZnSOD		
Acc No AJ010339	GGAATTCGGAGATAATACACAAGGCTG	GGAATTCTTCGTGTACCACCATTGTGC
GPX		
Acc No AJ5010340	CGAATTCGCCAAGAATGAGGAGATCC	CGAATTCGAAAGCGACGGCTGTACC
γ-GCS heavy		
Acc No M90656	GGAATTCTTGGAGGCCATGTGGACC	GGAATTCAACTCCCTCATCCATCTGG
γ-GCS light		
Acc No L35546	GGAATTCAACCAAGTTAATCTTGCCTCC	GGAATTCTGCCTCAATGACACCATTTAC
GAPDH		
Acc No U48832	GGAATTCAGGGACTCATGACCACGG	GGAATTCTTGGAGGCCATGTGGACC

Abbreviations are in the text.

Cell counts

The culture of cells in 25 mmol/L D-glucose resulted in a 24% reduction in cell number compared to those cultured in 5 mmol/L D-glucose (data not shown).

Evidence of glucose-induced oxidative stress

Intracellular MDA concentrations were significantly higher in cells cultured in 25 mmol/L D-glucose compared with those cultured in 5 mmol/L D-glucose (0.51 ± 0.02 vs 0.78 ± 0.056 nmol/mg protein, 5 vs. 25 mmol/L D-glucose, $P < 0.001$, $N = 7$; Fig. 1A). This increase was not seen in cells cultured in the presence of 5 mmol/L D-glucose + 20 mmol/L L-glucose (results not shown). GSH concentrations were significantly decreased in cells cultured in 25 mmol/L D-glucose by 50% (1.0 ± 0.17 vs 0.48 ± 0.07 nmol/mg protein, 5 vs. 25 mmol/L D-glucose, $P < 0.05$, $N = 5$; Fig. 1B). The decrease in GSH was not seen in cells cultured in 5 mmol/L D-glucose + 20 mmol/L L-glucose (results not shown). This reduction in intracellular GSH was accompanied by a glucose-induced decrease in gene expression of both γ-GCS(H) (67.0 ± 10.9% of 5 mmol/L glucose, $P < 0.05$, $N = 4$) and γ-GCS(L) (54.1 ± 7.6% of 5 mmol/L glucose, $P < 0.05$, $N = 4$), as determined by semiquantitative PCR (Table 2).

The antioxidant enzyme activities are demonstrated in Table 3 and show no significant differences in activities between mesangial cells cultured in 5 and 25 mmol/L D-glucose. The results of mRNA expression for each of the antioxidant enzymes determined by RT-PCR are displayed in Figure 2. When the volume of mRNA expression of 5 mmol/L D-glucose is taken as 100% for each enzyme, the results demonstrate significant increases in the gene expression of CuZnSOD (1.3-fold) and MnSOD (1.3-fold) with no change in GPX gene expression.

Gene expression of extracellular matrix proteins

The steady-state mRNA levels for each of the extracellular matrix proteins and the housekeeping gene GAPDH were determined by Northern analysis and results are displayed in Table 4. Cells cultured in 25 mmol/L D-glucose had increased gene expression for fibronectin (430 ± 33%) and collagen IV (244 ± 21%), but not for collagen I (96 ± 5%).

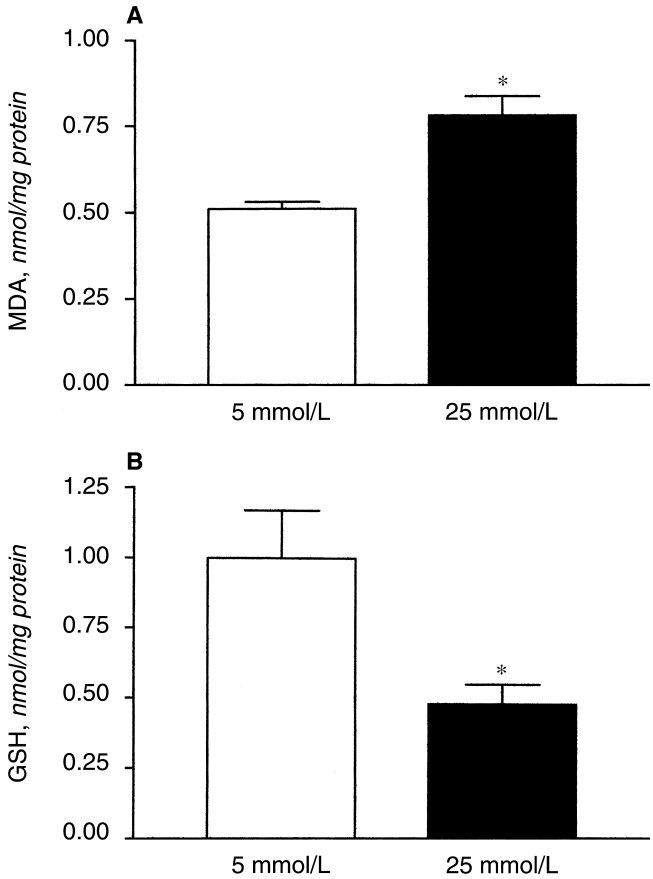

Fig. 1. (*A*). Intracellular malondialdehyde (MDA) in mesangial cells cultured in 5 mmol/L and 25 mmol/L D-glucose for 10 days. Results are expressed as mean ± SEM, *$P < 0.001$ vs. 5 mmol/L D-glucose, $N = 7$; *B*). Intracellular glutathione (GSH) in mesangial cells cultured in 5 mmol/L and 25 mmol/L D-glucose for 10 days. Results are expressed as means ± SEM, *$P < 0.05$ vs. 5 mmol/L D-glucose, $N = 5$.

Table 2. mRNA expression of γ-GCS(H) and γ-GCS(L) subunits in mesangial cells: Effects of 10 days culture in D-glucose (25 mmol/L) with the addition of Trolox (200 μmol/L) and α-lipoic acid (50 μmol/L)

	5 mmol/L glucose	25 mmol/L glucose	25 mmol/L glucose + 200 μmol/L Trolox	25 mmol/L glucose + 50 μmol/L lipoic acid
			%	
γ-GCS(H)	100	67.0 ± 10.9[a]	100.1 ± 8.9[b]	58 ± 3.2[a]
γ-GCS(L)	100	54.1 ± 7.6[a]	122.4 ± 11.4[b]	101.9 ± 11.1[b]

mRNA was measured by semiquantitative PCR and results are expressed as means ± SEM.
[a] $P < 0.05$ vs. 5 mmol/L D-glucose, $N = 4$
[b] $P < 0.05$ vs. 25 mmol/L D-glucose, $N = 4$

Table 3. Intracellular enzyme activities in mesangial cells cultured for 10 days in either 5 or 25 mmol/L D-glucose

Enzyme activity	5 mmol/L D-glucose	25 mmol/L D-glucose
CuZnSOD *U/mg protein*	51.5 ± 3.5	61.0 ± 1.5
MnSOD *U/mg protein*	48.6 ± 3.1	43.3 ± 1.01
GPX *mU/mg protein*	3.9 ± 0.12	4.6 ± 0.22
Catalase *U/mg protein*	29.0 ± 2.9	25.7 ± 1.2

Results are expressed as mean ± SEM, $N = 4$.

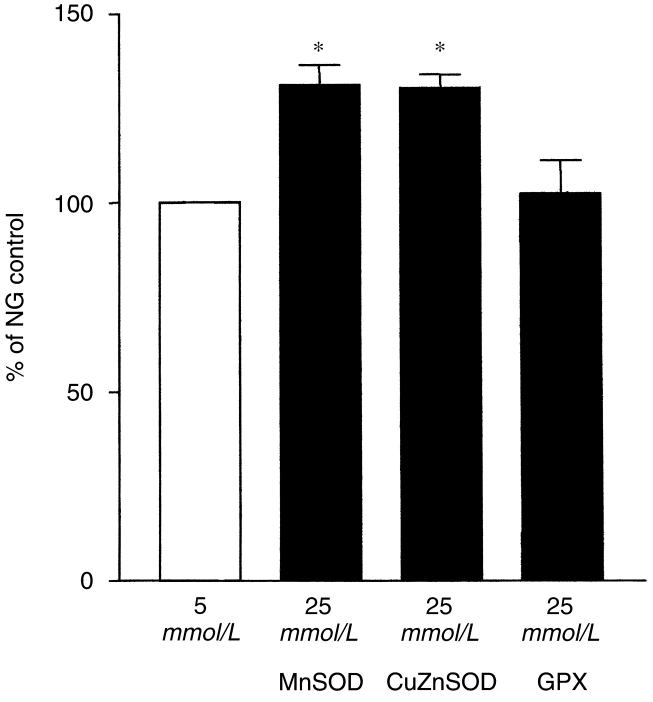

Fig. 2. Semiquantitative polymerase chain reaction (PCR) measurement of mRNA for antioxidant enzymes, Mn superoxide dismutase (SOD), Cu/ZnSOD and glutathione peroxidase (GPX), in mesangial cells cultured in 5 mmol/L and 25 mmol/L D-glucose for 10 days. Results are expressed as means ± SEM, *$P < 0.05$ vs. 5 mmol/L D-glucose, $N = 4$.

Addition of oxidants and antioxidants

Addition of the oxidant, hydrogen peroxide (H_2O_2, 100 μmol/L) for twelve hours to mesangial cells cultured in normal glucose resulted in a significant decrease of intracellular GSH levels and increased intracellular MDA levels when compared to that of 5 mmol/L D-glucose alone (Table 5).

Table 4. Northern blot analysis of mRNA expression of extracellular matrix proteins in mesangial cells cultured for 10 days in either 5 or 25 mmol/L D-glucose

Extracellular matrix protein	5 mmol/L D-glucose	25 mmol/L D-glucose
	%	
Fibronectin	100	430 ± 33[a]
Collagen IV	100	244 ± 21[a]
Collagen I	100	96 ± 5
GAPDH	100	116 ± 7.6

Results are expressed as mean ± SEM, $N = 4$.
[a] $P < 0.05$ vs. 5 mmol/L D-glucose

Table 5. Intracellular GSH and MDA levels in mesangial cells cultured in 5 and 25 mmol/L D-glucose for 10 days with the addition of hydrogen peroxide (H_2O_2) (100 μmol/L, 12 hours)

	5 mmol/L glucose	25 mmol/L glucose	5 mmol/L glucose + 100 μmol/L H_2O_2
		%	
GSH	100	51 ± 6[a]	33 ± 8[a]
MDA	100	160 ± 7[a]	187 ± 2[a]

Results are expressed as percentages (%) of 5 mmol/L D-glucose (mean ± SEM, $N = 3$).
[a] $P < 0.05$ vs. 5 mmol/L D-glucose

The addition of antioxidants to cells cultured in 5 mmol/L D-glucose showed no significant changes in intracellular MDA and GSH levels when compared to cells culture in 5 mmol/L D-glucose alone (Table 6).

Addition of the antioxidant, Trolox (vitamin E analog, 200 μmol/L), to mesangial cells cultured in high glucose conditions resulted in a small increase of intracellular GSH levels, but no significant change in MDA levels (Table 6). The addition of Trolox to high glucose was associated with significant increases in the gene expression of both subunits of γ-GCS, so that they were restored to or above that found with 5 mmol/L glucose alone; γ-GCS(H) 100.1 ± 8.9% of 5 mmol/L glucose (cf 25 mmol/L glucose, $P < 0.05$, $N = 4$) and γ-GCS(L) 122.0 ± 11.4% of 5 mmol/L glucose (cf 25 mmol/L glucose, $P < 0.01$, $N = 4$; Table 2). When added to 25 mmol/L

Table 6. Intracellular GSH and MDA levels in mesangial cells cultured in 5 and 25 mmol/L D-glucose with the addition of Trolox (200 μmol/L) or α-lipoic acid (50 μmol/L) for 10 days

	5 mmol/L glucose	25 mmol/L glucose	5 mmol/L glucose + Trolox	5 mmol/L glucose + lipoic acid	25 mmol/L glucose + Trolox	25 mmol/L glucose + lipoic acid
				%		
GSH	100	51 ± 6[a]	97 ± 3	107 ± 3	82 ± 9[b]	121 ± 12[b]
MDA	100	160 ± 7[a]	107 ± 2	104 ± 1	147 ± 3[a]	105 ± 3[b]

Results are expressed as percentages (%) of 5 mmol/L D-glucose (mean ± SEM, $N = 3$).
[a] $P < 0.05$ vs. 5 mmol/L D-glucose
[b] $P < 0.05$ vs. 25 mmol/L D-glucose

Table 7. Effect of Trolox (200 μmol/L) on mRNA expression of extracellular matrix proteins in mesangial cells cultured for 10 days in either 5 or 25 mmol/L D-glucose as measured by Northern blot analysis

Extracellular matrix protein	5 mmol/L D-glucose	25 mmol/L D-glucose	25 mmol/L D-glucose + 200 μmol/L Trolox
		%	
Fibronectin	100	352 ± 24[a]	110 ± 23
Collagen IV	100	313 ± 43[a]	110 ± 11

Results are expressed as mean ± SEM, $N = 4$.
[a] $P < 0.05$ vs. 5 mmol/L D-glucose

Table 8. Effect of α-lipoic acid (50 μmol/L) on mRNA expression of extracellular matrix proteins in mesangial cells cultured for 10 days in either 5 or 25 mmol/L D-glucose as measured by Northern blot analysis

Extracellular matrix protein	5 mmol/L D-glucose	25 mmol/L D-glucose	25 mmol/L D-glucose + 50 μmol/L lipoic acid
		%	
Fibronectin	100	258 ± 44[a]	145 ± 4
Collagen IV	100	228 ± 12[a]	150 ± 6

Results are expressed as mean ± SEM, $N = 3$.
[a] $P < 0.05$ vs. 5 mmol/L D-glucose

glucose, Trolox also caused a significant reversal of the increased gene expression of extracellular matrix proteins found in the presence of 25 mmol/L glucose alone: thus, fibronectin, 110 ± 23% of 5 mmol/L glucose and collagen IV, 110 ± 11% of 5 mmol/L glucose (Table 7).

The addition of the antioxidant α-lipoic acid to 25 mmol/L glucose significantly increased intracellular GSH levels compared to that found with 25 mmol/L glucose alone (Table 6). The antioxidant also significantly reduced intracellular MDA levels compared to that found with 25 mmol/L D-glucose alone (Table 6). α-Lipoic acid also increased the mRNA expression of γ-GCS(L) in the presence of 25 mmol/L glucose to 101.9 ± 11.1% of 5 mmol/L glucose (cf 25 mmol/L glucose alone, $P < 0.05$, $N = 4$; Table 2). However, in contrast to Trolox, the addition of α-lipoic acid to 25 mmol/L glucose had no effect on γ-GCS(H) mRNA expression, 67.4 ± 18.8% of 5 mmol/L glucose, $N = 4$; Table 2). The addition of this antioxidant to 25 mmol/L glucose did, however, reverse the increased gene expression of fibronectin (to 145 ± 4% of 5 mmol/L glucose) and collagen IV (to 150 ± 6% of 5 mmol/L glucose) caused by 25 mmol/L glucose alone (Table 8).

DISCUSSION

Although glucose-induced oxidative stress is an attractive hypothesis for the pathogenesis of diabetic complications including nephropathy, very little work has been published concerning the ability of cultured mesangial cells to exhibit oxidative stress. Our current study clearly demonstrates that the culture of mesangial cells in high glucose environments causes oxidative stress evidenced by increased levels of MDA when mesangial cells were exposed to high glucose. This is in agreement with previous studies [32].

Glutathione is one of the most important of the intracellular antioxidants. It is synthesized in a two-step reaction from glutamate, cysteine (a thiol containing amino acid) and glycine by the activity of γ-GCS and glutathione synthetase. Reduced GSH is the predominant antioxidant species within the cell, and during conditions of oxidative stress GSH is oxidized to GSSG via a recycling mechanism, allowing the scavenging of reactive oxygen species. In the presence of high glucose there was an approximate 50% reduction of GSH. Interestingly mesangial cells contain significantly lower amounts of GSH in comparison to aortic vascular smooth muscle cells of the same species [33]. The increase in MDA levels may be due to the poor antioxidant capacity of mesangial cells as a consequence of low GSH levels, which were lowered further in the presence of high concentrations of glucose. While the supplementation of cells cultured in high glucose with α-lipoic acid caused a complete reversal of MDA levels, this effect was not observed with Trolox. Therefore, the early decreases in GSH levels may be the primary event leading to oxidative stress-induced tissue damage in the cell.

The reduction of GSH may be brought about through a variety of mechanisms including increased breakdown of GSH by γ-glutamyltranspeptidase, increased export of

GSSG from the cell, or a reduction in de novo synthesis through reduced activity of γ-GCS. Previous reports have shown a decrease in thiol transport, a reduction in GSH content and decreased activity of γ-GCS in erythrocytes from subjects with non–insulin-dependent diabetes mellitus [24]. Decreased GSH content and γ-GCS activity has been reported from rat embryos exposed to high glucose conditions [34]. Decreased expression of γ-GCS(H) has been observed in mouse endothelial cells exposed to high glucose [23]. For the first time, to our knowledge, we have shown an approximately 30% decrease in gene expression of both subunits of γ-GCS when mesangial cells are cultured in conditions of high glucose.

Trolox, a vitamin E analog, results in a significant increase in the gene expression of both the heavy and light subunits of γ-GCS. However, this increase in γ-GCS gene expression did not result in a significant elevation in GSH levels. Yosida et al found that in K562 cells grown in 27 mm glucose for seven days there was an increase in γ-GCS protein, but a gradual decrease in γ-GCS activity throughout the experimental period, suggesting that γ-GCS is inactivated in diabetic conditions [24].

In contrast to treatment with Trolox, α-lipoic acid supplementation significantly increases GSH levels. As well as functioning as an antioxidant, α-lipoic acid can increase de novo GSH synthesis by improving cystine utilization. Intracellularly α-lipoic acid is reduced to dihydrolipoate, which is released from the cell where it reduces extracellular cystine to cysteine. Cysteine is taken up by the cell via the Na^+-dependent neutral amino acid transport system, ASC, thereby bypassing the cystine transport system x_c^-, and results in GSH synthesis [35]. Cystine transport is mediated by a Na^+-independent anionic amino acid transport system x_c^-, which exchanges cystine for intracellular glutamate [36] and is inhibited by glutamate. Intracellularly cystine is reduced to cysteine, which is utilized for GSH de novo synthesis. Inhibition of cystine influx has been shown to decrease intracellular GSH and conversely induction of cystine transport increases GSH content [37] therefore system x_c^- is important in maintaining GSH levels in various cell types [38–41]. In human vascular smooth muscle cells, the uptake rate of cystine decreased with increasing glucose concentrations [42], and supplying cysteine in the form of α-lipoic acid resulted in restoration of GSH accompanied by an increase in the gene expression of the light subunit [43]. These results suggest that the decrease in intracellular GSH in high glucose conditions results from a combination of decreased expression of γ-GCS, the rate-limiting enzyme in de novo GSH synthesis, reduced availability of cyst(e)ine, the rate-limiting substrate and, as shown by others [24], a reduction in the specific activity of the enzyme in the presence of high glucose. However, it is possible that the restoration of GSH levels may be due to the additional effects of α-lipoic acid on gene expression of the γ-GCS subunits.

The detailed mechanism of gene regulation of γ-GCS is not completely understood [44], but several studies have demonstrated the presence of activator protein-1 (AP-1), AP-2, antioxidant response elements (ARE)/electrophile response elements (EpRE), and sequence specific transcription factor (SP-1) binding sites in the 5′ region upstream of the transcription start site of the two subunits of γ-GCS [45, 46]. Agents such as xenobiotics induce transcription of γ-GCS via ARE/EpRE, which bind AP-1–like and NF-E2–like transcription factors [47]. AP-1 is known to be activated by high glucose conditions [48], but no studies to date have examined the activity of NF-E2 transcription factors in cells exposed to high glucose. It is well established that γ-GCS gene expression can be induced by oxidative stress [49–51]; however, we have demonstrated that exposure to high glucose conditions decreases mRNA expression of both subunits in mesangial cells and human vascular smooth muscle cells [43]. Lu et al found that in retinal Muller cells, GSH depletion for 18 hours induced the gene expression of both subunits, but after seven days in 28 mmol/L glucose the gene expression was reduced [52]. Therefore, glucose or indeed reactive oxygen species themselves may have a direct effect on the transcriptional activity of γ-GCS subunits.

It is interesting to note that only the light subunit changed with lipoic acid treatment. However it is now known that isoform dysregulation can occur between the two subunits [44]. The subunit genes are located on different chromosomes and mRNA levels and ratios of both subunits vary widely between different human tissues [53]. An investigation of the effects of α-lipoic acid (100 μmol/L, 6 to 14 hours) on GSH levels and γ-GCS (H) mRNA expression in human Jurkat cells found that GSH levels were increased but γ-GCS(H) mRNA expression was unaffected by the addition of α-lipoic acid [54]. While the heavy subunit possesses the catalytic activity and is subject to negative feedback inhibition by GSH, the kinetic properties are significantly influenced by light subunit binding [21]. The magnitude of the modulatory effect of γ-GCS(L) is species- and cell-dependent, but in all studies to date, the availability of the light subunit enhances the catalytic activity of γ-GCS(H) [21, 44, 55]. γ-GCS(L) enhances the binding of γ-GCS (H) to its substrates, so the elevation in GSH as a result of α-lipoic acid treatment may be the result of increased association of the two subunits, thereby enabling greater interaction with the increased cysteine made available through the conversion from cystine. Trolox, possibly through the reduction of ROS, increases the gene expression of γ-GCS but fails to significantly elevate intracellular GSH because of the reduced availability of cyst(e)ine, the rate-limiting substrate in de novo GSH synthesis.

Oxidative stress also was evidenced by a small but significant increase in the gene expression for CuZnSOD and MnSOD in mesangial cells cultured in high glucose environments. Changes in gene expression were analyzed using semiquantitative RT-PCR, which permits the detection of small changes in gene expression as co-amplification of a target mRNA and an internal control occurs at the same time [56]. The increase in MnSOD gene expression is in keeping with its well-recognized induction in response to oxidative stress [57]. Increased gene expression of Mn and CuZnSOD have been previously reported in various other cells [18, 58], but our results show, to our knowledge for the first time, the ability of mesangial cells to respond to glucose-induced oxidative stress by increasing the mRNA levels of the SOD enzymes. It is interesting to note that changes in gene expression fail to be translated into changes in enzymatic activity. It has been reported that CuZnSOD activity is inhibited due to glycation of the enzyme [59], but it also can be inactivated by free radicals. We have previously shown that glucose-induced increases in gene expression for CuZnSOD in vascular smooth muscle cells is associated with an increase in CuZnSOD protein synthesis without increases in enzyme activity [18].

We observed a substantial increase in the gene expression of fibronectin (FN) and collagen IV when mesangial cells were cultured in high ambient glucose. This is in agreement with previously published reports [60, 61]. Although changes in gene expression for collagen I were not detected in porcine mesangial cells, a previous report [62] has observed an increase in collagen I ($\alpha 2$ chain) gene expression in a differentiated mouse mesangial line when cultured in high glucose. The authors noted that the stimulation by high glucose of $\alpha 2$ transcripts were evident as early as 24 hours and persisted at 72 hours. This discrepancy may be explained by a species difference or due to the different incubation times in high glucose. To investigate whether glucose-induced oxidative stress is associated with increased ECM protein synthesis, mesangial cells were cultured in 25 mmol/L glucose with the addition of two structurally unrelated antioxidants. Trolox is a water-soluble vitamin E analog that, unlike α-tocopherol, does not inhibit protein kinase C (PKC) [63]. Both Trolox and α-lipoic acid prevented up-regulation of FN and collagen IV gene expression by glucose, suggesting that this up-regulation may be associated with oxidative stress. In the case of Trolox, this was achieved without significant restoration of GSH levels. It has been reported that the glucose-induced increase in protein collagen levels can be reversed by the addition of taurine and vitamin E [14, 32]. These authors suggest that the taurine/vitamin E mode of action is in the inhibition of advanced glycation end product (AGE) accumulation. It has previously shown that AGE can stimulate the production of FN in mesangial cells [64]. However, it is known that the formation of AGE triggers a series of autoxidation reactions that stimulate the formation of free radicals [65, 66]. The fact that two structurally unrelated antioxidants can inhibit glucose-induced FN and collagen IV gene expression suggests that their mechanism of action involves the quenching of free radicals. To our knowledge this is the first report showing that Trolox and α-lipoic acid are capable of reversing the increased FN gene expression.

In conclusion, we have demonstrated the existence of glucose-induced oxidative stress in mesangial cells as evidenced by elevated MDA and decreased GSH levels. The decreased levels of GSH are accompanied by a reduction in the mRNA expression of both subunits of γ-GCS and can be ameliorated by the addition of the antioxidant α-lipoic acid, which improves cysteine utilization within the cell. To combat the oxidative insult mesangial cells increase gene expression of the antioxidant enzymes CuZnSOD and MnSOD, however, under these conditions this is not translated into increased enzyme activity. The glucose-induced increase in FN gene expression can be partially reversed by the addition of two structurally unrelated antioxidants, Trolox and α-lipoic acid. Thus, oxidative stress associated with hyperglycemia plays an important part in the development and progression of diabetic nephropathy. Understanding the mechanisms involved in oxidative stress associated with hyperglycemia should help delineate further the pathogenesis of diabetic nephropathy.

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