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ANTIOXIDANTS REVERSE THE ANTIPROLIFERATIVE EFFECT OF HIGH GLUCOSE AND ADVANCED GLYCOSYLATION END PRODUCTS IN CULTURED RAT MESANGIAL CELLS

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SUMMARY. High glucose and elevated levels of advanced glycosylation end products (AGEs) exert an antiproliferative effect on cultured mesangial cells. In view of the role of oxygen free radicals in the pathogenesis of diabetic nephropathy, we tested whether two endogenous antioxidants, taurine and vitamin E, ameliorate the effects of an elevated ambient glucose and/or AGEs on mesangial cell growth *in vitro*. Regardless of whether cell proliferation was assayed by the incorporation of [^{3}H] thymidine, direct cell counting or bromodeoxyuridine (BrdU) cell staining, both taurine and vitamin E reversed the inhibitory effect of high glucose and AGEs on mesangial cell growth. In conjunction with our previous studies indicating that taurine and vitamin E reduce collagen production in mesangial cells exposed to high glucose, these findings suggest that endogenous antioxidants attenuate diabetic glomerulosclerosis by interfering with the bioactivation of transforming growth factor-β.

The glomerular mesangial cell, a specialized, contractile, vascular pericyte, plays a central role in the pathogenesis of diabetic nephropathy (1). Abnormalities in mesangial cell growth and extracellular matrix production correlate with the progressive decline in glomerular filtration rate during the course of diabetic kidney disease (2). Oxidant injury, mediated directly by high glucose or by the accumulation of advanced glycosylation end products (AGEs), contributes to disturbances in mesangial cell function in diabetes (3,4). Thus, high glucose (5,6) and AGEs (7,8,9) have an antiproliferative effect on mesangial cells and promote enhanced production of extracellular matrix.

Taurine (2-aminoethane sulfonic acid) and vitamin E are abundant lipid soluble

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antioxidants in the kidney (10,11). Both agents prevent the high glucose-induced increase in lipid peroxidation and collagen production in cultured rat mesangial cells (12,13). In light of these studies, we conducted experiments to determine whether taurine and vitamin E reverse the inhibitory effect of high glucose and/or AGEs on mesangial cell growth.

METHODS

Cells: Rat mesangial cells were obtained from primary glomerular explants and used between passages 6-10 (14). On phase-contrast microscopy, the identity of mesangial cells was confirmed by their elongated, stellate or fusiform appearance; in addition, there was no inhibitory effect of puromycin aminonucleoside or D-valine on cell growth (14). Cells were plated at a density of 50,000 cells/well in 24 well plates and incubated at 37°C in a humidified atmosphere of 10% CO_2-90% air. In the bromodeoxyuridine (BrdU) cell staining studies, cells were plated on sterile round coverslips that were inserted in the bottom of the wells. Cells were maintained in Dulbecco's modified Eagle (DME) medium, supplemented with streptomycin, 100 μg/ml, penicillin, 100 U/ml and 10% fetal bovine serum. The effect of high glucose and BSA-AGE on renal tubular epithelial cell proliferation was examined using MDCK and LLC-PK_1 cells (passages 65-75) purchased from the American Type Culture Collection (Rockville, MD).

Production of AGEs: Glycosylation of bovine serum albumin (BSA) (50 mg/ml) was conducted at 37°C in 0.2 M sodium phosphate buffer (pH 7.4) with 0.5 M glucose and 0.5 mM EDTA. Samples were sterile filtered and maintained at pH 7.4 for 56 days. At the completion of the incubation period, the mixture was extensively dialyzed against PBS. AGEs-specific fluorescence determinations were performed at 450 nm during excitation at 390 nm using a spectrofluorophotometer (Shimadzu). A BSA-AGE standard was used as a reference and fluorescence intensity standards were used to calibrate and monitor performance of the spectrometer.

Experimental conditions: One day after the mesangial cells were plated, they were serum deprived for 24 h. At that time, the wells were randomly assigned to one of the following four experimental conditions: (1) Control: DME containing 5.6 mM glucose; (2) High glucose: DME supplemented with 27.8 mM glucose to a final concentration of 33.3 mM; (3) Low dose AGEs: DME + BSA-AGE, 25 μg/ml; and (4) High dose AGEs: DME + BSA-AGE, 250 μg/ml. In each condition, the media contained either no further additives, taurine 500 μM, vitamin E 100 μM or glycine 1 mM. In the high glucose studies, a hyperosmolality control consisted of DME + 27.8 mM mannitol. Cells were maintained for one week and the various test media were changed every 2-3 d.

[^{3}H]-Thymidine incorporation assay: After a 7 day incubation in the test media, 0.25 μCi [^{3}H]-thymidine was added to each well for 6 h. Mesangial cells were washed twice with PBS and then 200 μl ice cold 5% trichloroacetic acid was added to each well and incubated at 4°C for 1 h. The supernatant was discarded and 200 μl of 0.25 N NaOH was added to each well and incubated overnight at 4°C. An aliquot of each well was removed for protein determination and the rest was placed in a scintillation vial for counting. The results are expressed as counts/well.

Cell counting: After 7 days, 500 μl of a trypsin solution was added to each well for 15-20 min. The wells were scraped to insure complete removal of all of the cells, samples were brought up to 1 ml with PBS and gently agitated to obtain a homogeneous suspension. An aliquot was placed in a hemocytometer for direct cell counting. The results, cell number/ml, are expressed as a percentage of the total cell count in DME, 5.6 mM glucose with no additives.

5'-Bromo-2'-deoxyuridine (BrdU) cell staining: After 7 days of growth on sterile coverslips in 24 well plates, BrdU, 10 μg/ml, was added to each well for 16-18 h. Mesangial cells were

rinsed twice with 10 mM sodium phosphate buffer, pH 7.4, and fixed with 3.7% formaldehyde for 10 min. The wells were washed twice with buffer and then a 1.5% bovine serum albumin (BSA) solution for 20 min. Cells were incubated with anti-BrdU antibody (Becton-Dickinson), 40 μl/10 ml, for 60 min, washed with buffer for 10 min, and then incubated with 50 μl/10 ml biotinylated antibody in 1.5% BSA for 60 min. After washing with buffer, the Vectastain (Vector Laboratories) was added for 60 min, followed by the addition of a peroxidase substrate, 0.02% H_2O_2 in 1 mg/ml diaminobenzidine tetrahydrochloride for 5 min. Coverslips were washed with tap water for 5 min, then dipped serially in 95% alcohol, Eosin Y, 95% alcohol, 100% alcohol, xylene and 100% alcohol. Coverslips were mounted on slides; the number of stained cells was tabulated in a minimum of 100 cells per specimen and expressed as a percentage.

Protein assay: The protein contents of the cell homogenates were determined using a Coomassie blue reagent (BioRad, Richmond, CA).

Statistical methods: Results are presented as mean $\pm$ SEM. The means between the experimental groups were compared using analysis of variance; post hoc inter-group comparisons were made using the Bonferroni correction.

RESULTS

Effect of high glucose: Exposure of mesangial cells to 33.3 mM glucose led to a 35% decrease in cell proliferation based upon reduced incorporation of [^{3}H]-thymidine (Figure 1). The inhibitory action of an elevated ambient glucose on mesangial cell proliferation was not a consequence of hypertonicity of the medium because mannitol did not reproduce the effect of high glucose, control DME, 6404$\pm$426 *versus* DME + 27.8 mM mannitol, 7227$\pm$651 counts/well.

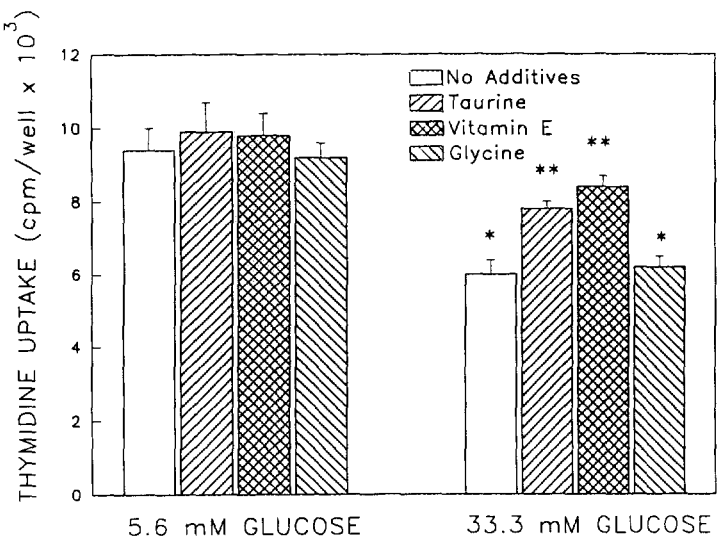

Figure 1. Thymidine incorporation in cultured rat mesangial cells exposed to normal (5.6 mM) or high (33.3 mM) glucose. * $P<0.05$ *versus* DME + 5.6 mM glucose, all conditions. ** $P<0.05$ *versus* DME + 33.3 mM glucose + 500 μM taurine & DME + 33.3 mM glucose + 100 μM vitamin E.

TABLE I

RMC PROLIFERATION: DIRECT CELL COUNTING*

	GLUCOSE		BSA-AGE	
	5.6 mM	33.3 mM	25 μg/ml	250 μg/ml
DME, No additives	100(8)	71±2(8)[a]	60±7(8)[a]	31±4(8)[a]
+ 500μM Taurine	98±2(8)	91±5(8)[b]	117±16(8)[c]	68±8(8)[d]
+ 100 μM Vitamin E	103±8(6)	94±5(6)[b]	73±9(8)[a]	61±7(8)[d]
+ 1 mM glycine	102±6(4)	74±5(4)[a]	71±5(7)[a]	36±5(8)[a]

* Data are expressed as a percentage of the total cell count in DME, 5.6 mM glucose with no additives. Results: Mean ± SEM. Values in parentheses indicate sample number.

[a] $P<0.01$ *versus* DME, 5.6 mM glucose with no additives.

[b] $P<0.05$ *versus* DME, 33.3 mM glucose with no additives.

[c] $P<0.01$ *versus* DME, 25 μg/ml BSA-AGE with no additives.

[d] $P<0.01$ *versus* DME, 250 μg/ml BSA-AGE with no additives.

The adverse effect of high glucose on mesangial cell growth was confirmed by a 29% reduction in the cell count (Table I) as well as a 60% decrease in BrdU staining, compared to cells grown in standard DME containing 5.6 mM glucose (Table II).

Addition of 500 μM taurine or 100 μM vitamin E to high glucose media reversed the antiproliferative effect of an elevated ambient glucose level and restored the incorporation of [^{3}H]-thymidine to normal (Figure 1). Vitamin E was slightly more effective than taurine at promoting mesangial cell growth in high glucose media in the thymidine uptake assay. Cell counts (Table I) and the percentage of BrdU stained cells were normalized to a comparable degree by the two antioxidants. Neither taurine nor vitamin E had an effect on mesangial cell proliferation in control DME containing a normal ambient glucose level. Glycine (1 mM) alone did not alter mesangial cell growth under control conditions and it did not prevent the antiproliferative effect of high glucose.

TABLE II

RMC PROLIFERATION: BrdU CELL STAINING*

	GLUCOSE		BSA-AGE	
	5.6 mM	33.3 mM	25 μg/ml	250 μg/ml
DME, No additives	11.7±1.5	4.7±0.3[a]	3.5±0.3[a]	2.3±0.5[a]
+ 500μM Taurine	10.5±0.5	10.2±0.9[b]	8.8±0.2[c]	3.4±0.3[d]
+ 100 μM Vitamin E	10.1±0.6	10.4±0.9[b]	9.2±0.4[c]	7.4±0.5[d]
+ 1 mM glycine	10.1±0.7	5.9±0.7[a]	3.8±0.4[a]	2.3±0.6[a]

* Data represent the number of stained cells as a percentage of the total number of cells. Results: Mean ± SEM (n=5-6, for all conditions).

[a] $P<0.01$ *versus* DME, 5.6 mM glucose with no additives.

[b] $P<0.05$ *versus* DME, 33.3 mM glucose with no additives.

[c] $P<0.01$ *versus* DME, 25 μg/ml BSA-AGE with no additives.

[d] $P<0.01$ *versus* DME, 250 μg/ml BSA-AGE with no additives.

TABLE III

RENAL TUBULAR EPITHELIAL CELL PROLIFERATION: THYMIDINE UPTAKE*

	GLUCOSE		BSA-AGE	
	5.6 mM	33.3 mM	25 μg/ml	250 μg/ml
MDCK (n=12)	1258±198	1520±153	1052±61	1017±70
LLC-PK$_1$ (n=10)	6825±945	5540±603	5112±324	5472±766

* Data are expressed as counts/well. Results: Mean ±SEM.

High glucose did not significantly alter thymidine incorporation by MDCK or LLC-PK$_1$ cells (Table III).

Effect of AGEs: Addition of BSA-AGE to external media caused a dose-dependent inhibition of [^{3}H]-thymidine incorporation in cultured mesangial cells (Figure 2). Both the low concentration, 25 μg/ml, and the high concentration of BSA-AGE, 250 μg/ml, significantly reduced mesangial cell proliferation compared to control DME. The antiproliferative effect of BSA-AGE was confirmed by a 40-70% decrease in direct cell counts (Table I) and a 70-80% reduction in BrdU staining (Table II).

Supplementation of the incubation media with 500 μM taurine or 100 μM vitamin E restored mesangial cell growth towards normal at both concentrations of BSA-AGE,

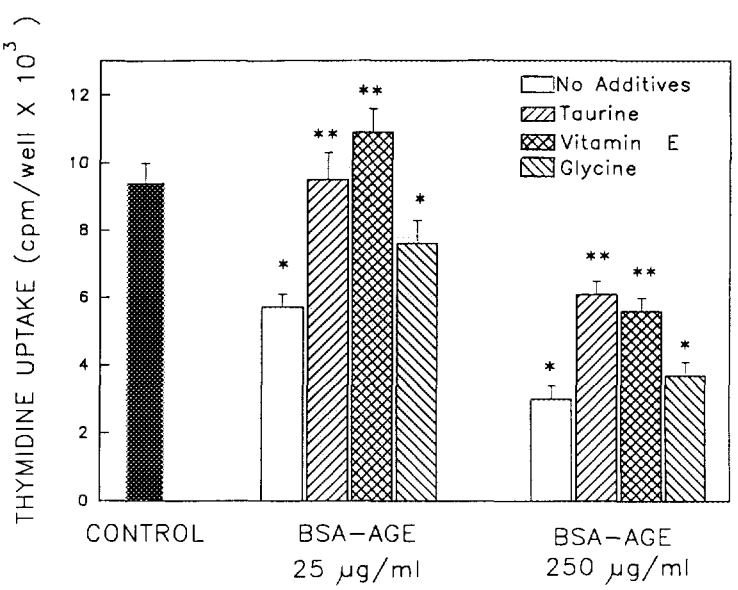

Figure 2. Thymidine incorporation in cultured rat mesangial cells exposed to BSA-AGE, 25-250μg/ml. * P<0.01 *versus* control. ** P<0.05 versus BSA-AGE + no additives. BSA-AGE + glycine.

regardless of whether proliferation was assayed by [^{3}H]-thymidine incorporation (Figure 2), cell counting (Table I) or BrdU staining (Table II). The two endogenous antioxidants were equally effective in restoring mesangial cell growth following exposure to low or high-dose BSA-AGE. Compared to taurine and vitamin E, glycine was ineffective at reversing the antiproliferative effect of BSA-AGE on mesangial cells.

BSA-AGE, 25-250 μg/ml, did not significantly reduce thymidine uptake by MDCK or LLC-PK$_1$ cells (Table III).

DISCUSSION

The major finding of these studies is that the antiproliferative effect of high glucose and AGEs in cultured mesangial cells can be prevented by the endogenous antioxidants, taurine and vitamin E. Our results are in accord with previous work in which superoxide dismutase, catalase and glutathione restored the prolonged replication time in cultured human endothelial cells exposed to high glucose (15). There have been no studies of the effect of antioxidants on AGEs-induced alterations in cell growth.

We cannot determine from these experiments whether high glucose and AGEs modulate mesangial cell growth by the same or different mechanisms. Mesangial cells express insulin-independent facilitative glucose transporters and may be highly susceptible to glucose-mediated oxidant damage (16). Mesangial cells also possess specific receptors for AGEs on their membrane surface (7).

Taurine and vitamin E are antioxidants that act by halting the progression of oxidant-generating reactions rather than by scavenging a particular free radical species (17,18). The response to taurine was amino acid specific, indicated by the failure of glycine to restore mesangial cell growth following exposure to high glucose or AGEs. Neither taurine nor vitamin E significantly reduce BSA-AGE formation *in vitro* (unpublished observation). Therefore, it is likely that taurine and vitamin E exert their beneficial effect within the mesangial cell. These antioxidants may modify the antiproliferative response to high glucose or AGEs by altering gene transcription of factors that regulate cell growth (19).

High glucose and AGEs cause oxygen free radical damage to mesangial cells, resulting in inhibition of growth and enhanced extracellular matrix production. The inverse relationship between mesangial cell proliferation and matrix synthesis following diabetic injury is paradigmatic of the cellular response to various experimental conditions

and the addition of exogenous stimuli (20,21). We speculate that high glucose and AGEs increase the bioactivation of transforming growth factor-6 (TGF-6) by mesangial cells and that the endogenous antioxidants, taurine and vitamin E, protect mesangial cells against diabetic injury by inhibiting the gene expression and/or synthesis of this cytokine (22).

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