High Glucose Induces Antioxidant Enzymes in Human Endothelial Cells in Culture

Evidence Linking Hyperglycemia and Oxidative Stress

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It has been suggested that oxidative stress may play an important role in the pathogenesis of diabetic complications. Hyperglycemia may cause increased production of free radicals, and evidence supports a prominent role for these reactive molecules as mediators of endothelial cell dysfunction in diabetes. It has been demonstrated that active oxygen species induce antioxidant enzyme expression in some tissues, and this phenomenon is considered proof of an existing oxygen-dependent toxicity. In this study, human endothelial cells from umbilical vein, immortalized human endothelial cells, and immortalized human endothelial cells transfected to express high glutathione peroxidase levels were grown in normal and high-glucose conditions. High glucose delayed replication after 7 and 14 days of culture of human endothelial cells, both from umbilical vein and immortalized, while transfected cells were not affected. The activity and the mRNA expression of the antioxidant enzymes CuZn-superoxide-dismutase, Mn-superoxide-dismutase, catalase, and glutathione peroxidase were evaluated after 2, 7, and 14 days of culture. High glucose at days 7 and 14 induced an overexpression of CuZn-superoxide-dismutase, catalase, and glutathione peroxidase in both human endothelial cells from umbilical vein and immortalized human endothelial cells, while in transfected cells it did not. This study demonstrates that high glucose induces an increase in antioxidant enzyme levels in human endothelial cells, suggesting that elevated glucose levels may produce an oxidative stress in the cells. *Diabetes* 45:471–477, 1996

Under physiological conditions, glucose is prone to oxidation and consequently generates hydrogen peroxide and reactive intermediates such as hydroxyl-free radicals (1). It has been suggested that oxidative stress plays an important role in tissue damage associated with diabetes (2) and that peroxide formation is increased in elevated glucose solution (3). The sources of oxygen-derived free radicals in diabetes are not known, but it is possible that the sources may be from autoxidation of glucose (3), intermediate products of cycloxygenase catalysis (4), and/or intracellular production from mitochondria (5).

Because of their localization and functions, endothelial cells are good candidates for primary involvement in the pathogenesis of diabetic vascular complications. Chronic exposure to blood containing abnormally high levels of glucose, although the molecular mechanisms are unknown, may trigger initial damage within the intimal layer of the vessel walls and may therefore represent a prominent factor among the metabolic abnormalities potentially responsible for such complications (6).

High glucose has been reported to modify the behavior of in vitro cultured human endothelial cells. Lengthening of cell proliferation time and a slight increase in cell death have been reported (7). This phenomenon may be reversed by antioxidant substances (8).

Several studies have shown impairment of endothelium-dependent relaxations in diabetic animals and humans (9,10). The impairment of endothelial function seems to be hyperglycemia-dependent (11,12). Antioxidants may normalize the hyperglycemia-induced decreased endothelium-mediated relaxation in vivo (12,13) and in vitro (14).

These findings suggest that free radicals may play a role in the endothelial cell dysfunction caused by elevated glucose, although this hypothesis needs to be elucidated.

Eukariotes have developed several different antioxidant enzymes to detoxify reactive oxygen species. Current evidence indicates that the damaging effects of oxygen-derived free radicals are associated in different tissues with an elevation of antioxidant enzyme activity during exposure (15,16). The purpose of this study was to explore the possibility that endothelial cells may increase antioxidant enzyme generation in response to exposure to hyperglycemia and that elevated protection against free radicals may protect cells from hyperglycemia-induced delay in replication.

RESEARCH DESIGN AND METHODS

Cell cultures. Three different cell cultures were used in this study: *1*) Human endothelial cells from umbilical vein (HUVEC), from 10 umbilical cord veins, were established as previously described (17). Cells were checked for ability to produce factor VIII–related antigen by indirect immunofluorescence microscopy. The mouse monoclonal anti-human factor VIII–related antigen was from Dako (Carpintera, CA), and the goat anti-mouse IgG, fluorescein isothiocyanate conjugated, was from

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Received for publication 22 December 1994 and accepted in revised form 16 November 1995.

CuZnSOD, CuZn-superoxide-dismutase; ECV304, spontaneously transformed and immortalized human endothelial cells; ECV304-GPX, spontaneously transformed and immortalized human endothelial cells transfected to express high levels of glutathione peroxidase; GPX, glutathione peroxidase; GSH, reduced glutathione; HUVEC, human endothelial cells from umbilical vein; MnSOD, Mn-superoxide-dismutase; SSC, standard sodium citrate.

Kirkegaard and Perry Laboratories (Gaithersburg, MD). *2*) A spontaneously transformed and immortalized human endothelial cell (ECV304) line (a gift from Dr. Takahashi) was used. This cell line shows spontaneous phenotypic alterations, without evidence of virus implication, leading an indefinite life span (18). Although one of the endothelial cell markers, factor VIII–related antigen, is negative in this cell line, immunocytochemical staining for the lectin *Ulex europaens* I and anti-human endothelium as well as glomerular epithelium monoclonal antibody was positive (18). Angiotensin-converting enzyme activity and plasminogen activator inhibitor production were also demonstrated (18). This cell line has been used for the study of some physiologically active factors (18) and recently has been proposed as a valid tool for in vitro research (19). *3*) To culture spontaneously transformed and immortalized human endothelial cells transfected to express high levels of glutathione peroxidase (ECV304-GPX), a pD5 vector containing the gpt-resistance cassette was constructed. For this purpose, a 2.2-kb *Bam*HI fragment containing the SV40-*Escherichia coli* gpt-resistance cassette was removed from vector pMSG described by Lee et al. (20), a blunt-ended *Xba* I site of pD5. The pD5 vector (gift from K. Berkner, Zymogenetics) contains the adeno 5 major late promoter and the SV40 promoter and enhancer sequences.

A 1-kb cDNA fragment was removed by *Eco*RI digestion from pSPT19-glutathione peroxidase (GPX), which contains a bovine GPX cDNA insert. The pSPT19-GPX construct was a gift from G. Mullenbach (Chiron). The ends were filled in with Klenow enzyme, and *Bam*HI linkers were added to the blunt-ended GPX cDNA fragment before ligation into a unique *Bam*HI site of the pD5-gpt vector described above. The pD5-gpt-GPX vector was transfected into ECV304 according to Chen and Okayama (21). Resistant clones were selected for 9 days in medium containing 8% fetal calf serum, 250 μg/ml xanthine, 25 μg/ml mycophenolic acid, 2 μg/ml aminopterin, 10 μg/ml thymidine, and 15 μg/ml hypoxanthine.

All cells were grown in 199 medium (Sigma, St. Louis, MO) with 8% fetal calf serum (Gibco, Grand Island, NY), endothelial cell growth supplement (50 μg/ml) (Sigma), and heparin (5 μg/ml) (gift from Alfa-Wassermann, Bologna, Italy) in an atmosphere of 5% CO_2 at 36.5°C. Medium for ECV304-GPX included 50 ng/ml Na_2SeO_3 because GPX expression is Se-dependent. Cultures were fed twice a week with fresh medium. At confluence, they were passaged using a solution of 20 U/ml collagenase (Worthington, Freehold, NJ), 0.75 mg/ml trypsin (1:300, ICN Biomedicals, High Wycombe, Bucks, U.K.) and 2% heat-inactivated dialyzed chicken serum (Gibco) in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (22).

Study design. HUVEC, ECV304, and ECV304-GPX were seeded at a density of 2×10^4 on day 0. Cells were plated in 5 and 20 mmol/l glucose and grown for 14 days in these experimental conditions in the mediums previously described. Mediums were exchanged every 2 days. At days 2, 7, and 14, cells were resuspended with trypsin and counted. Each experimental point was the mean of six different dishes.

At the same time, HUVEC, ECV304, and ECV304-GPX were also seeded on day 0 into 10-cm petri dishes at a density of $\sim 1.3 \times 10^5$ cells and similarly grown in 5 and 20 mmol/l glucose for 14 days. At days 2, 7, and 14, cells were removed and assayed for both activity and mRNA expression of the following antioxidant enzymes: CuZn-superoxide-dismutase (CuZnSOD), Mn-superoxide-dismutase (MnSOD), catalase, and GPX. The results represent the means of six different experiments.

In all three types of cells, the levels of reduced glutathione (GSH) and the presence of normal glucose metabolism were also checked. Two different series of the three types of cell cultures were plated: one for GSH assay and one for glucose consumption and lactate and pyruvate production. In each series, cells were grown as described above in 5 and 20 mmol/l glucose for 14 days. At the end of incubations, the levels of GSH, glucose consumption, and pyruvate and lactate production were assayed. The results for each substance represent the means of six different experiments.

Assay procedures

Antioxidant enzyme activity. For the determination of catalase and GPX activities, the monolayers were rinsed twice with ice-cold phosphate-buffered saline and the cells were collected by scraping with a sterile rubber policeman. The cells were sedimented for 4 min at 1,600*g* and processed either for enzyme/protein or for mRNA analyses. For enzyme/protein lysates, cells were resuspended in 50 mmol/l potassium-phosphate buffer containing 0.5% Triton X-100 and sonicated (in an ice-water bath) for two 30-s bursts on a Branson sonicator B15 (position 2, continuous setting; Branson Ultrasonic, Danbury, CT) with a 30-s cooling interval. Total protein concentration was determined according to the procedure of Bradford (23). For catalase and GPX activities, sonicates were first spun 5 min at 800*g* (4°C). The supernatants were assayed according to the procedures of Clairborne (24) for catalase activity and Günzler and Flohé (25) for GPX activity.

For SOD measurements, cells were suspended in 100 mmol/l triethanolamine-diethanolamine buffer and homogenized with a teflon glass Dounce homogenizer. The homogenate was centrifuged at 105,000*g* for 1 h (4°C), and the supernatant was passed through a small Sephadex G25 (coarse) column to remove low–molecular weight substances that interfere with the enzyme assay according to the procedure of Paoletti et al. (26). An aliquot of the eluate was applied onto a 5.5% polyacrylamide gel to localize SOD activity according to the procedure of Beauchamp and Fridovich (27), with the exception that no tetramethylethylenediamine was used for staining. MnSOD activity was determined in mitochondrial fractions prepared by differential centrifugation. Mitochondria were disrupted by freezing-thawing in a high–ionic strength buffer (0.25 mmol sucrose, 0.12 mol KCl, 10 mmol Tris-HCl, pH 7.4). Mitochondrial membranes were removed by sedimentation at 105,000*g* for 1 h, and enzyme activity was measured in the supernatant.

Northern blot analysis. Total RNA was prepared according to the procedure of Chirgwin et al. (28). A total of 10 μg of total RNA was electrophoresed on a 1.4% agarose-formaldehyde gel and then transferred to gene screen membranes. The filters were prehybridized in 50 mmol Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate, 0.2% poly(vinylpyrrolidone), 0.2% ficoll, 5 mmol EDTA, 50% formamide, 0.2% bovine serum albumin, 1 × standard sodium citrate (SSC), and 150 μg/ml denatured salmon sperm DNA at 65°C for 6 h. Blots were hybridized with ^{32}P-labeled probes for human CuZnSOD (29), human catalase (29), human MnSOD (30), and bovine GPX (31) to a specific activity of 1×10^6 cpm/ml in hybridization fluid at 65°C overnight. The filters were washed at 65°C twice for 15 min with 2 × SSC-0.1% and twice for 15 min with 0.1 × SSC-0.1% sodium dodecyl sulfate and then subjected to autoradiography using an intensifying screen at −85°C. Densitometry was performed on an LKB laser scanning densitometer. Hybridization to glyceraldehyde-3-phosphate dehydrogenase cDNA was used as internal control to correct for loading inequalities.

Measurement of GSH, pyruvate, and lactate. For GSH determination, cells were washed with ice-cold phosphate-buffered saline, scraped with a rubber policeman, and sonicated on ice. The cell sonicate was treated with two volumes of ice-cold 3% sulfoxalicylic acid for 15 min on ice. After centrifugation at 10,000 rpm at 4°C, the supernatant was immediately frozen for measurement of GSH by the method described by Griffith (32).

To explore glucose consumption and pyruvate and lactate generation, cells were washed with ice-cold phosphate-buffered saline to remove

TABLE 1
Glutathione levels, lactate and pyruvate production, and glucose consumption in the different cell lines after 14 days of culture in normal and high-glucose conditions

	Glutathione (nmol/mg protein)	Lactate (nmol · μg DNA^{-1} · h^{-1})	Pyruvate (nmol · μg DNA^{-1} · h^{-1})	Glucose consumption (μmol · μg DNA^{-1} · h^{-1})
HUVEC (5 mmol/glucose)	45.2 ± 1.9	15.2 ± 2.1	1.2 ± 0.5	0.01 ± 0.003
HUVEC (20 mmol/glucose)	44.5 ± 1.3	14.9 ± 1.7	1.4 ± 0.3	0.04 ± 0.002*
ECV304 (5 mmol/glucose)	41.2 ± 1.9	14.8 ± 2.7	1.4 ± 0.5	0.01 ± 0.003
ECV304 (20 mmol/glucose)	42.9 ± 1.6	15.0 ± 2.2	1.3 ± 0.3	0.03 ± 0.004*
ECV304-GPX (5 mmol/glucose)	39.9 ± 2.6	14.7 ± 1.9	1.6 ± 0.7	0.01 ± 0.005
ECV304-GPX (20 mmol/glucose)	41.2 ± 1.3	15.1 ± 2.2	1.5 ± 0.5	0.04 ± 0.004*

Data are means ± SD. *$P < 0.01$ vs. control.

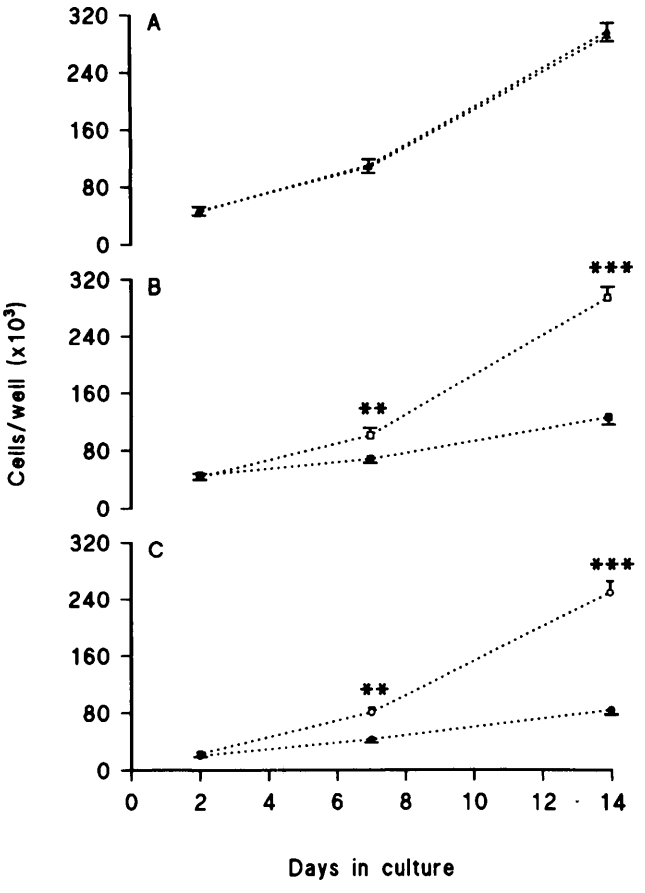

FIG. 1. Cell growth of the three human endothelial cell types cultured in 5 mmol/l (○, □) or 20 mmol/l (●, ■) glucose: ECV304-GPX (*A*); ECV304 (*B*); HUVEC (*C*). Results are expressed as means (n = 6); bars indicate SD. **P < 0.01; ***P < 0.001 vs. basal.

lactate and pyruvate in the culture medium and then incubated for 60 min at 37°C in serum-free medium containing either 5 or 20 mmol/l glucose, respectively. Pyruvate and lactate released in the medium were assayed according to Bergmeyer (33). The glucose level of the media was measured with a glucose analyzer (Beckman). Data were normalized for DNA content of each plate. DNA was measured according to Labarca and Paigen (34).

Statistical analysis. All values in the text and figures are presented as means ± SD. All data were subjected to analysis of variance, followed by the Bonferroni correction for post hoc t test. Probabilities of 0.05 or less were considered to be statistically significant.

RESULTS

GSH content and pyruvate and lactate production were not significantly different between the various cell types and did not change in high glucose conditions (Table 1). Glucose consumption was significantly increased in high glucose medium (Table 1).

In high glucose medium, a marked delay in cell replication time occurred at day 7 and 14 for both HUVEC ($P < 0.001$) and ECV304 ($P < 0.001$) but not for ECV304-GPX (Fig. 1).

High glucose after 7 days was able to induce an increase of GPX ($P < 0.01$), CuZnSOD ($P < 0.01$), and catalase ($P < 0.01$) (Figs. 2 and 4–6) in HUVEC and in ECV304 in terms of both activity and mRNA expression. Similar data were shown after 14 days of exposure to hyperglycemia, even if the increase of GPX was less pronounced ($P < 0.05$) (Figs. 2 and 4–6). MnSOD did not change during the study (Figs. 3–6).

ECV304-GPX showed constitutively increased GPX activity and mRNA expression with respect to parent ECV304 (Figs. 5 and 6). In both high glucose–treated and untreated ECV304-GPX, CuZnSOD, MnSOD, and catalase activities and mRNA expression were constitutively lower compared with untransfected ECV304 (Figs. 2–4 and 6). High glucose did not stimulate antioxidant enzyme expression in these cells (Figs. 2–4 and 6).

DISCUSSION

Hyperglycemia is generally accepted to be the major cause of vascular complications in diabetic patients (35). A glucose-induced functional change in vascular endothelial cells could be a key event in the development of diabetic vascular complications (6). The involvement of oxidative stress has been proposed in the pathogenesis of diabetic vascular complications (36).

Eukariotes have developed several different antioxidant enzymes to detoxify reactive oxygen species. CuZnSOD is located primarily in the cytoplasm, whereas MnSOD, a structural distinct protein encoded by a different gene (37), is located primarily in the mitochondria. Both enzymes catalyze the reaction $O_2^- + O_2^- + 2H^+ = O_2 + H_2O_2$. H_2O_2 is converted to H_2O in the peroxisomes by the antioxidant enzyme catalase and in the cytoplasm by GPX (37).

The cells must maintain a balance between the normal

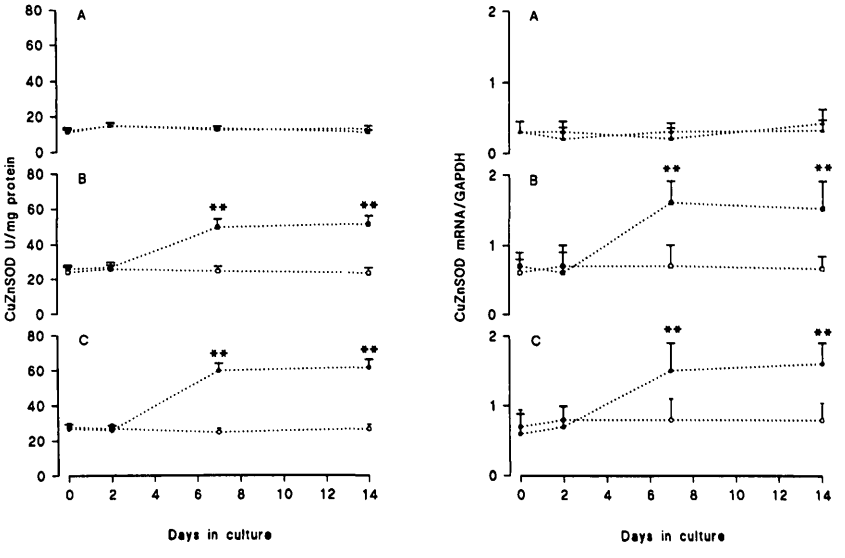

FIG. 2. Time course of CuZnSOD activity and mRNA expression of the three human endothelial cell types cultured in 5 mmol/l (○, □) or 20 mmol/l (●, ■) glucose: ECV304-GPX (*A*); ECV304 (*B*); HUVEC (*C*). Results are expressed as means (n = 6); bars indicate SD. **P < 0.01 vs. basal.

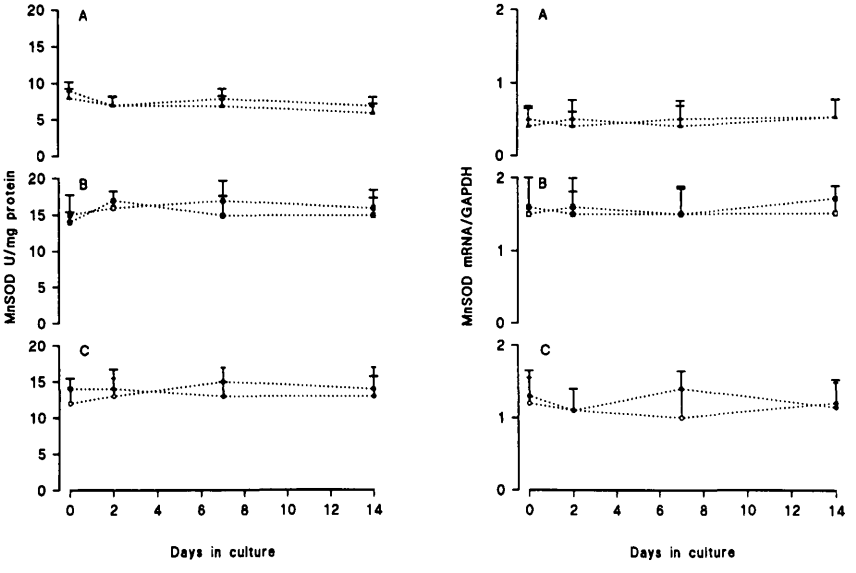

FIG. 3. Time course of MnSOD activity and mRNA expression of the three human endothelial cell types cultured in 5 mmol/l (○, □) or 20 mmol/l (●, ■) glucose: ECV304-GPX (*A*); ECV304 (*B*); HUVEC (*C*). Results are expressed as means (n = 6); bars indicate SD.

encounter of oxidants and antioxidant defense mechanisms (38). Removal of toxic oxygen metabolites is the putative function of antioxidant enzymes such as CuZnSOD, MnSOD, GPX, and catalase.

The primary cellular damage resulting from free radical formation falls into three major categories: reaction with sulfhydryl-containing proteins, nucleic acid base modifications and/or DNA-strand scission, and lipid peroxidation (39). It is demonstrated, however, that all these modifications induced by active oxygen species induce antioxidant enzyme expression in tissues (15,16,40,41). At the same time, this phenomenon is considered the proof of an existing oxygen-dependent toxicity (42,43).

It has already been demonstrated that oxidative stress induces antioxidative enzymes, including SOD, catalase, and GPX, in vascular endothelial cells (43). In this study, we have demonstrated that high glucose stimulates CuZnSOD, catalase, and GPX overexpression in human endothelial cells in culture. This finding suggests that high glucose might produce an oxidative stress in cells, which respond by increasing their antioxidant defenses.

The increase of intracellular antioxidant enzymes might be hypothesized to be a direct effect of increased intracellular glucose on gene expression, independent of oxidative stress. In this study, ECV304-GPX, unlike parent ECV304, does not delay replication time in high-glucose conditions. Moreover, ECV304-GPX cells, which are largely protected against oxidative stress, are not influenced by high glucose to modify their antioxidant enzyme pattern. This evidence shows that elevated intracellular defense against free radicals protects cells from high glucose–induced alteration, supporting the hypothesis that glucose may work through free radical production.

The experimental protocol commonly used to evaluate the protecting potential of the antioxidant enzymes or of an antioxidant substance is to expose cells to a free radical–generating generating system, then to add the different antioxidants and to test for their protective effect. However, the results of these experiments may be difficult to interpret because the enzymes are not in their natural location within the cells (41), while an antioxidant substance may induce intracellular antioxidative defenses by itself (44,45). It has been suggested, then, that increasing the intracellular amount of antioxidant enzymes may be a better choice (42). Because it has been shown that GPX is more potent, on a molar basis, than SOD and catalase to protect endothelial

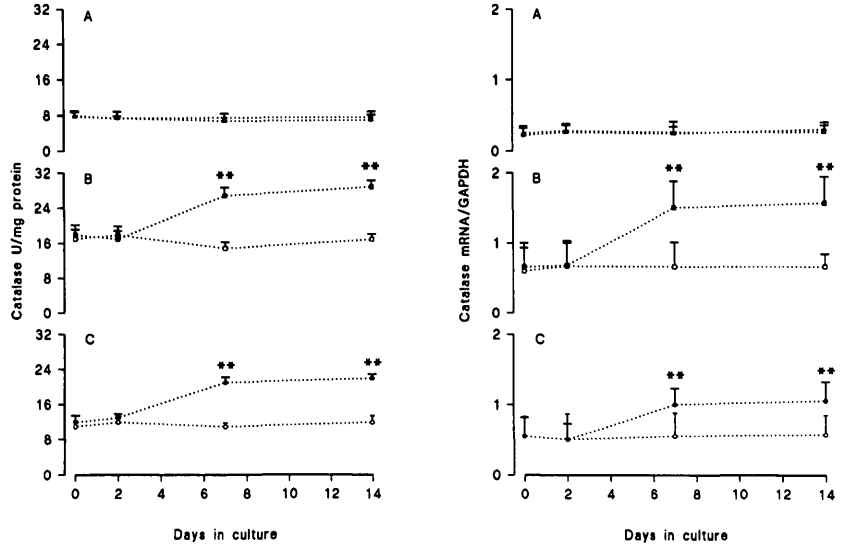

FIG. 4. Time course of catalase activity and mRNA expression of the three human endothelial cell types cultured in 5 mmol/l (○, □) or 20 mmol/l (●, ■) glucose: ECV304-GPX (*A*); ECV304 (*B*); HUVEC (*C*). Results are expressed as means (n = 6); bars indicate SD. $^{**}P < 0.01$ vs. basal.

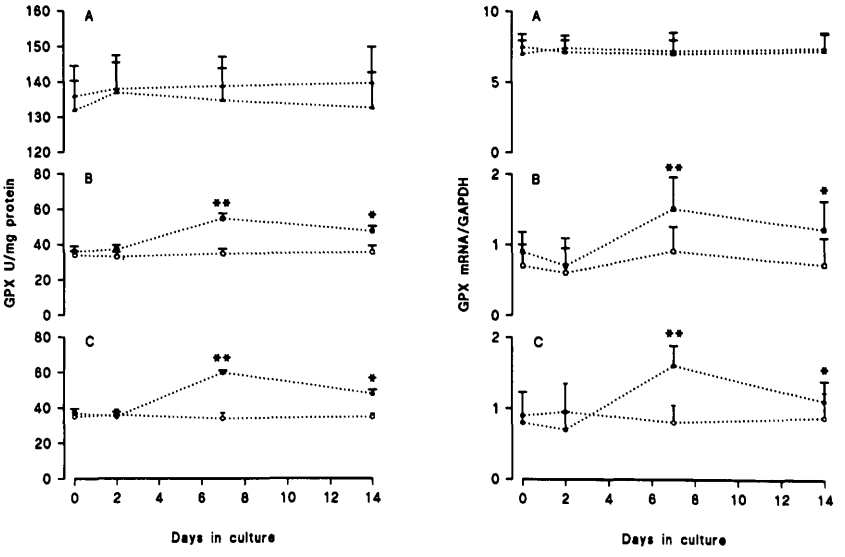

FIG. 5. Time course of GPX activity and mRNA expression of the three human endothelial cell types cultured in 5 mmol/l (○, □) or 20 mmol/l (●, ■) glucose: ECV304-GPX (*A*); ECV304 (*B*); HUVEC (*C*). Results are expressed as means (n = 6); bars indicate SD. *P < 0.05; **P < 0.01 vs. basal.

cells from oxidative stress (46), we decided to use transfected cells expressing high intracellular levels of GPX. These cells exhibit, in both normal and high-glucose conditions, a glucose metabolism equivalent to that of parent ECV304 and HUVEC, as revealed by lactate and pyruvate production and by glucose consumption. These results concerning endothelial lactate production and glucose consumption are consistent with those of Asahina et al. (47) and Danne et al. (48).

ECV304-GPX, possessing very high GPX levels, downregulates the expression of other antioxidant enzymes. This phenomenon is consistent with the evidence that cells must precisely regulate the balance between antioxidant enzymes, dysregulation being dangerous for their normal life (38). However, this new situation can oppose the high glucose–stimulated CuZnSOD and catalase increase and the high glucose–induced delay in cell replication in these cells, while the simultaneous increase of three antioxidant enzymes in nontransfected cells does not.

In our opinion, the evidence that endothelial cells cultured in high glucose delay the replication time, while the antioxidant defenses increase, is not contradictory. The increase of intracellular antioxidant defenses in elevated glucose conditions only indicates that high glucose may produce an oxidative stress in the cells. However, optimal protection against free radicals is achieved only when an appropriate balance between the activities of these enzymes is obtained (41).

Interpretation of the deleterious effects of free radicals has to be analyzed not only as a function of the amount of free radicals produced but also relative to the efficiency and to the activities of these enzymatic and chemical antioxidant systems (41). The threshold of protection can indeed vary dramatically as a function of both the activity and the balance of these enzymes (41).

In other terms, we suggest that in our study, the increase of antioxidant enzymes demonstrates that high glucose produces an oxidative stress in endothelial cells but that, depending on the balance between the various enzymes that can be achieved by the cells, this phenomenon is not necessarily good for the cells (41).

It has been suggested that GSH content is a rate-limiting step for eliminating an oxidative stress in HUVEC (49). According to Kashiwagi et al. (49), our results confirm that GSH content does not change in high-glucose conditions. This result agrees with the evidence that GSH does not play a role in regulating antioxidant enzyme expression in HUVEC (50). Moreover, our data are consistent with the evidence that in T47D human breast cells at the same level of GPX expression induced in our transfected cells, GSH does not change significantly in comparison to that in parental cells (51). It is interesting to note that T47D human breast

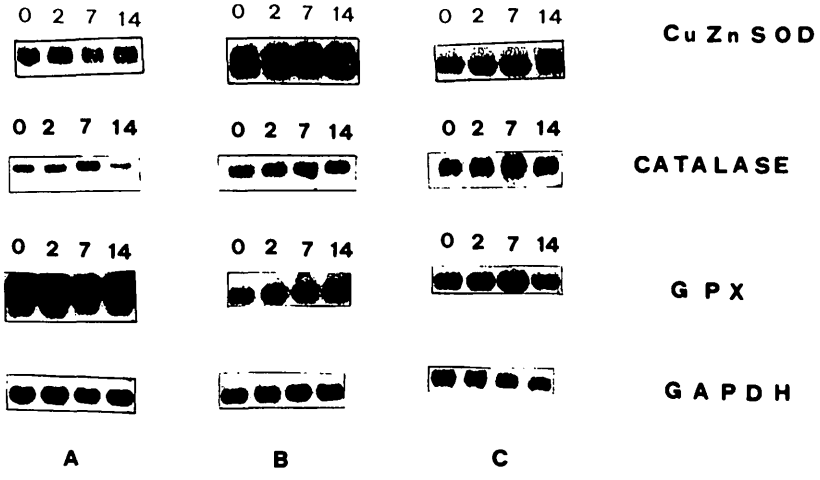

FIG. 6. Representative Northern blot analysis using cDNA probes for human CuZnSOD, human MnSOD, human catalase, and bovine GPX in ECV304-GPX (*A*), ECV304 (*B*), and HUVEC (*C*) exposed to 20 mmol/l glucose. Each line of each expperiment indicates 0, 2, 7, and 14 days of exposure, respectively.

cells with increased GPX activity without GSH increase are resistant to the oxidative stress (51).

It has been proposed that hyperglycemia may induce O_2^- overproduction in cells through an imbalance in NADH/NAD^+ ratio (52). If this is true, then intracellular antioxidant enzymes could play a crucial role in detoxifying intracellular O_2, and it is reasonable that antioxidant enzymes would be increased. This study, then, supports this hypothesis.

In conclusion, this study demonstrates that high glucose induces an increase in antioxidant enzyme levels in human endothelial cells through increased gene expression. This finding suggests that high glucose may act by generating an oxidative stress in the cells. However, this effect is not sufficient to protect the cells against the high glucose–induced delay in cell replication. A protection is obtained only when very high levels of the antioxidant enzyme GPX are present in the cells. The role of antioxidant enzymes induced by elevated glucose in the cells, their complex interaction, and their role on hyperglycemia-induced endothelial cell alterations need further elucidations.

ACKNOWLEDGMENTS

This work was supported by National Council of Research (Grants 92.01005.CT04 and 93.04317.CT04), Lions Club Udine Castello, and Bayer Pharmacology S.p.A.

REFERENCES

1. Wolff SP, Dean RT: Glucose autoxidation and protein modification: the potential role of oxidative glycosylation in diabetes. *Biochem J* 245:243–250, 1987
2. Baynes JW: Role of oxidative stress in development of complications in diabetes. *Diabetes* 40:405–412, 1991
3. Hunt JV, Smith CCT, Wolff SP: Autoxidative glycosylation and possible involvement of peroxides and free radicals in LDL modification by glucose. *Diabetes* 39:1420–1424, 1990
4. Mason RP, Kalyanaraman B, Tainer BE, Eling TE: A carbon-centered free radical intermediate in the prostaglandin synthase oxidation of arachidonic acid. *J Biol Chem* 255:5019–5022, 1980
5. Boveris A: Mitochondrial production of superoxide and hydrogen peroxide. *Adv Exp Med Biol* 78:67–82, 1977
6. Lorenzi M, Cagliero E: Pathobiology of endothelial and other vascular cells in diabetes mellitus: call for data. *Diabetes* 40:653–659, 1991
7. Lorenzi M, Cagliero E, Toledo S: Glucose toxicity for human endothelial cells in culture: delayed replication, disturbed cell cycle, and accelerated death. *Diabetes* 34:621–627, 1985
8. Curcio F, Ceriello A: Decreased cultured endothelial cell proliferation in high glucose medium is reversed by antioxidants: new insights on the pathophysiological mechanism of diabetic vascular complications. *In Vitro Cell & Dev Biol* 28A:787–790, 1992
9. Durante W, Sen AK, Sunahara FA: Impairment of endothelium-dependent relaxation in aortae from spontaneously diabetic rats. *Br J Pharmacol* 94:463–468, 1988
10. Saenz de Tejada I, Goldestein I, Azadzoi K, Choen RA: Impaired neurogenic and endothelium-dependent relaxation of human penile smooth muscle: the pathophysiological basis for impotence in diabetes mellitus. *N Engl J Med* 320:1025–1030, 1989
11. Tesfamariam B, Brown ML, Deykin D, Cohen RA: Elevated glucose impairs endothelium-dependent relaxations by activating protein kinase C. *J Clin Invest* 87:1643–1648, 1991
12. Marfella R, Verrazo G, Acampora R, La Marca C, Giunta R, Lucarelli C, Paolisso G, Ceriello A, Giugliano D: Glutathione reverses systemic hemodynamic changes induced by acute hyperglycemia in healthy subjects. *Am J Physiol* 268:E1167–E1173, 1995
13. Ceriello A, Quatraro A, Giugliano D: Diabetes mellitus and hypertension: the possible role of hyperglycaemia through oxidative stress. *Diabetologia* 36:265–266, 1993
14. Tesfamariam B, Cohen RA: Free radicals mediate endothelial dysfunction caused by elevated glucose. *Am J Physiol* 264:H859–H864, 1992
15. Ichikawa I, Kiyama S, Yoshiok T: Renal antioxidant enzymes: their regulation and function. *Kidney Int* 45:1–9, 1994
16. Clerch LB, Massaro D: Tolerance of rats to hyperoxia: lung antioxidant enzyme gene expression. *J Clin Invest* 91:499–508, 1993
17. Lorenzi M, Cagliero E, Markey B, Henricksen T, Witzum JL, Sampietro T: Interaction of human endothelial cells with elevated glucose concentrations and native and glycosylated low density lipoproteins. *Diabetologia* 26:218–222, 1984
18. Takahashi K, Sawasaki Y, Hata JI, Mukai K, Goto T: Spontaneous transformation and immortalization of human endothelial cells. *In Vitro Cell & Dev Biol* 25:265–274, 1990
19. Takahashi K, Sawasaki Y: Rare spontaneously transformed human endothelial cell line provides useful research tool. *In Vitro Cell & Dev Biol* 28:380–382, 1992
20. Lee F, Mulligan R, Berg P, Ringold G: Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumor virus chimaeric plasmids. *Nature* 294:228–232, 1981
21. Chen C, Okayama H: High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* 7:2745–2752, 1987
22. Coon HG: Clonal stability and phenotypic expression of chick cartilage cells in vitro. *Proc Natl Acad Sci USA* 55:66–73, 1966
23. Bradford M: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
24. Clairborne A: Catalase activity. In *Handbook of Methods for Oxygen Radical Research.* Greenwald R, Ed. Boca Raton, FL, CRC Press, 1985, p. 283–284
25. Günzler W, Flohé L: Glutathione peroxidase. In *Handbook of Methods for Oxygen Radical Research.* Greenwald R, Ed. Boca Raton, FL, CRC Press, 1985, p. 285–290
26. Paoletti F, Aldinucci D, Mocali A, Caparrini A: A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissue extracts. *Anal Biochem* 154:536–541, 1986
27. Beauchamp B, Fridovich I: Superoxide dismutase improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 44:276–287, 1971
28. Chirgwin J, Przbyla A, MacDonald R, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294–5299, 1979
29. Amstad P, Peskin A, Shah G, Mirault ME, Moret R, Zbinden I, Cerutti P: The balance between Cu,Zn-superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to oxidative stress. *Biochemistry* 30:9305–9313, 1991
30. Ho YS, Crapo JD: Isolation and characterization of complementary DNAs encoding human manganese-containing superoxide dismutase. *FEBS Lett* 229:256–260, 1988
31. Mullenbach GT, Tabrizi A, Irvine BD, Bell GI, Tainer JA, Hallewell RA: cDNAs of three glutathione peroxidases: selenocystine incorporation. In *Oxy-radicals in Molecular Biology and Pathology.* Cerutti P, Fridovich I, McCord JM, Eds. New York, Alan R. Liss, 1988, p. 313–326
32. Griffith OW: Determination of glutathione and gluthatione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 106:207–212, 1980
33. Bergmeyer HV (Ed.): *Methods of Enzymatic Analysis.* 2nd English ed. New York, Academic, 1974
34. Labarca C, Paigen K: A simple, rapid and sensitive DNA assay procedure. *Anal Biochem* 102:344–352, 1980
35. The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 329:977–986, 1993
36. Tesfamariam B: Free radicals in diabetic endothelial cell dysfunction. *Free Radical Biol & Med* 16:383–391, 1994
37. Halliwell B, Gutteridge JMC: *Free Radicals in Biology and Medicine.* Oxford, Oxford University Press, 1989
38. Cerutti P: Prooxidant states and tumor promotion. *Science* 227:375–381, 1985
39. Freeman BA, Crapo JD: Biology of disease: free radicals and tissue injury. *Lab Invest* 47:412–426, 1982
40. Shull S, Heintz NH, Periasamy M, Manohar M, Jansse YMW, Marsch JP, Mossman BT: Differential regulation of antioxidant enzymes in response to oxidants. *J Biol Chem* 266:24398–24403, 1991
41. Michiels C, Raes M, Toussaint O, Remacle J: Importance of Se-glutathione peroxidase, catalase and Cu/Zn-SOD for cell survival against oxidative stress. *Free Radical Biol & Med* 17:235–248, 1994
42. Beckman JS, Freeman BA: Antioxidant enzymes as mechanic probes of oxygen-dependent toxicity. In *Physiology of Oxygen Radicals.* Taylor AE, Matalon S, Ward P, Eds. Bethesda, MD, American Physiological Society, 1986, p. 39–53
43. Lu D, Maulik N, Moraru II, Kreutzer DL, Das DK: Molecular adaptation of vascular endothelial cells to oxidative stress. *Am J Physiol* 264:C715–C722, 1993
44. Conti M, Couturier M, Lemonnier A, Lemonnier F: Effects of alpha-tocopherol on antioxidant enzyme activity in human fibroblast cultures. *Int J Vitam Nutr Res* 63:71–76, 1993
45. Wan XS, St Clair DK: Thiol-modulating agents increase manganese superoxide dismutase activity in human lung fibroblasts. *Arch Biochem Biophys* 304:89–93, 1993
46. Michiels C, Toussaint O, Remacle J: Comparative study of oxygen toxicity on human fibroblasts and endothelial cells. *J Cell Physiol* 144:295–302, 1990
47. Asahina T, Kashiwagi A, Nishio Y, Ikebuchi M, Harada N, Tanaka Y, Takagi Y, Saeki Y, Kikkawa R, Shigeta Y: Impaired activation of glucose oxidation and NADPH supply in human endothelial cells exposed to

H2O2 in high-glucose medium. *Diabetes* 44:520–526, 1995
48. Danne T, Spiro MJ, Spiro RG: Effect of high glucose on type IV collagen reproduction by cultured glomerular epithelial, endothelial, and mesangial cells. *Diabetes* 42:170–177, 1993
49. Kashiwagi A, Asahina T, Ikebuchi M, Tanaka Y, Takagi Y, Nishio Y, Kikkawa R, Shigeta Y: Abnormal glutathione metabolism and increased cytotoxicity caused by H2O2 in human umbilical vein endothelial cells cultured in high glucose medium. *Diabetologia* 37:264–269, 1994
50. Jornot L, Junod AF: Variable glutathione levels and expression of antioxidant enzymes in human endothelial cells. *Am J Physiol* 8:L482–L489, 1993
51. Mirault ME, Tremblay A, Beadoin N, Tremblay M: Overexpression of seleno-glutathione peroxidase by gene transfer enhances the resistance of T47D human breast cells to clastogenic oxidants. *J Biol Chem* 266:20752–20760, 1991
52. Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamura T, Nyengaard JR, Van Den Enden, Kilo C, Tilton RG: Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes* 42:801–813, 1993