Free Radical Research, 2002 Vol. 36 (9), pp. 1017–1022

Characterization of Free Radical Defense System in High Glucose Cultured HeLa-tat Cells: Consequences for Glucose-induced Cytotoxicity

SOPHIE BOUVARD*[a], PATRICE FAURE[a,†], CORINNE ROUCARD[b], ALAIN FAVIER[a] and SERGE HALIMI[a]

[a]*Laboratoire de Biochimie, Faculté de Médecine, Université Joseph Fourier, 38000 Grenoble, France;* [b]*Groupe de Recherche sur les Lymphomes, Institut Albert Bonniot, Université Joseph Fourier, 38706 La Tronche, France*

Accepted by Professor B. Halliwell

(Received 16 May 2001; In revised form 7 November 2001)

HeLa cell line stably transfected with the tat gene from human immunodeficiency virus type 1 has a decreased antioxidant potential. In this work, we used this model to investigate the effect of a high glucose level (20 mM) on the glucose induced cytotoxicity and on the antioxidant system. In comparison to cell culture under control medium, HeLa-wild cell cultured under 20 mM glucose did not exhibit necrosis or apoptosis, contrary to HeLa-tat cell presenting a significant increase in necrotic or apoptotic state. Moreover after 48 h culture under high glucose level the HeLa-tat proliferation rate was not higher than the one of HeLa-wild cells. In HeLa-wild cell high glucose level resulted in an induction of glutathione reductase activity in opposition to HeLa-tat cells where no change was observed. High glucose level resulted in 20% increase in GSSG/GSH ratio in HeLa-wild cells and 38% increase in HeLa-tat cells. Moreover, high glucose level resulted in a dramatic cytosolic thiol decrease and an important lipid peroxidation in HeLa-tat cells. No significant change of these two parameters was observed in HeLa-wild cells. In both cell lines, high glucose resulted in an increase of total SOD activity, as a consequence of the increase in Cu,Zn-SOD activity. High glucose did not result in an increase of Mn-SOD activity in both cell lines. As a consequence of tat tranfection Mn-SOD activity was 50% lower in HeLa-tat cells in comparison to HeLa-wild cells. This work emphasizes the importance of the antioxidant system in the glucose induced cytotoxicity.

Keywords: Glucose; HeLa-wild; Free radicals; Antioxidant system

Abbreviations: Cu,Zn-SOD, Cu,Zn-dependent superoxide dismutase; DMSO, dimethylsulfoxide; DTNB, 5,4dithiobis(2-nitrobenzoic) acid; GPx, glutathione peroxidase; GRase, glutathion reductase; GSH, reduced glutathione; GSSG, oxidized glutathion; HIV, human immunodeficiency virus; ICP-MS, ???; NADPH, nicotinamide dinucleotide diphosphate; MDA, malondialdehyde; Mn-SOD, Mn-dependent superoxide dismutase; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PNPD, p-nitrophenyl disulfide; ROS, reactive oxygen species; -SH, thiol groups; Tat, transactivator protein of HIV-1; TFA, trifluroacetylacetone

INTRODUCTION

Free radicals in tissues are generated by both enzymatic and non-enzymatic pathways leading to the formation of reactive compounds by reduction or oxidation. Reactive oxygen species (ROS) in most cells can be involved in physiological functions,[1–4] but they may also cause cellular injury by hydroperoxidation, ischemia/reperfusion, inflammation, ionizing radiation exposure, diabetes and aging.[5,6] On the other hand, a number of enzymatic and nonenzymatic cellular antioxidative defenses protect cells from the attack of oxygen free radicals.[7,8] The most important and versatile protector is reduced glutathione (GSH).[9] GSH is present in most

*Sophie Bouvard was supported by Volvic Foundation.

†Corresponding author. Laboratoire CardioStress et Pathologies Associées, Faculté de Médecine, Université Joseph Fourier, 38700 La Tronche, France. Tel.: +33-476637152. Fax: +33-476637152. E-mail: patrice.faure@ujf-grenoble.fr

ISSN 1071-5762 print/ISSN 1029-2470 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/107156021000006671

mammalian cells and plays an important role in many biological processes such as sulfur-containing amino acid metabolism, and participates in the cellular defense system against oxidative stress by reducing disulfide linked proteins and other cellular molecules, or by scavenging free radicals and reactive oxygen intermediates.[10]

There is strong evidence that glucose, under physiological conditions, is prone to oxidation and consequently generates hydrogen peroxide and reactive intermediates such as hydroxyl free radicals and α-hydroxyaldehydes.[11] It has been suggested that oxidative stress plays an important role in tissue damage associated with diabetes[12] and that peroxide formation is increased in parallel to elevated glucose oxidation.[13] In different studies, it has been suggested that variations in glucose concentrations are sufficient to induce cell death through a free radical-mediated mechanism,[14] delay in various phases of the cell cycle of human endothelial cells,[15] inhibition of endothelial cell replication[16] and to induce an oxidative stress in smooth muscle cells.[17]

The Tat protein from human immunodeficiency virus type 1 (HIV-1) is essential for efficient HIV-1 gene expression. Tat acts by binding to a transactivation response element, a RNA stem-loop structure located close to the 5′ end of HIV-1 transcripts.[18] The expression of some cellular genes and proteins is influenced either positively or negatively by HIV-1 Tat.[19,20] HIV-1 Tat is secreted by HIV-1-infected cells and can be taken up rapidly by other cells.[21] Thus, Tat can potentially influence cellular gene expression in infected and non-infected cells. Flores *et al.* have demonstrated that in HeLa cells transfected with Tat (HeLa-tat cells) the activity of the Mn dependent-superoxide dismutase (Mn-SOD) was decreased to 50% compared with the level measured in parental cells accompanied with a reduction of protein expression.[22] A likely consequence of decreased Mn-SOD activity is increased oxidative stress reflected by a decrease in the total amount of glutathione or a decreased ratio of GSH to oxidized glutathione (GSSG). Moreover, they have demonstrated that the total GSH content present in HeLa-tat cells decreased compared with control cells. Thus, HIV-1 Tat might provide the link between viral infection and the observed oxidative stress and consequent CD4 + T-cell depletion in AIDS.[22] In these conditions, HeLa-tat cells are a good model to investigate the implications of free radicals in cell damage.

In the present study, HeLa-tat and parental cells were used to explore the effect of high glucose concentrations, based on the fact that HeLa-tat present a lower antioxidant defense system, in order to examine the implications of low antioxidant protection in glucose-induced cytotoxicity.

EXPERIMENTAL PROCEDURES

Materials

Opti-MEM, fetal calf serum (FCS) were purchased from Gibco-BRL, (Grand Island, NY). The solution of 0.05% trypsine and 0.02% EDTA was from Gibco-BRL, (Grand Island, NY). MTT kit 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide), reduced nicotinamide dinucleotide diphosphate (NADPH) were from Boehringer Mannhein (Mannhein, Germany). Glucose, mannitol, p-nitrophenyl disulfide (PNPD), 5,4-dithiobis(2-nitrobenzoic) acid (DTNB) acridine orange, ethydium bromide, paraformaldehyde, Triton X-100, terbutyl hydroperoxide, thiobarbituric acid (TBA), 1,1,3,3,-tetraethoxypropane and dimethyl sulfoxide (DMSO) were from Sigma Chemical Company (St. Louis, MO). Nitric, hydrochloric acids, hydrogen peroxide (H_2O_2), pyridine, ethanolamine, 3-vinyl-pyridine butanol, metaphosphoric acid and hexane were purchased from Merck (Darmstadt, Germany) and ammonium acetate from Prolabo (Paris, France) and were high purity grade. Trifluoroacetylacetone (TFA) was purchased from Aldrich (Milwaukee, WI, USA).

Cell Cultures

The HeLa-tat and wild cell lines were obtained from NIH-AIDS Research and Reference Reagent Program (Besthesda, MD). Cells were cultured in Opti-MEM, in the presence of 4% FCS. Prior to experimental treatment, cells were grown to the same density (3×10^6 cells/ml). Cells were incubated during 24 and 48 h with different mediums.

Control medium: This comprised the standard culture medium described above containing 4% FCS and 5 mM glucose.

High-glucose medium: This was identical to control medium but supplemented with glucose to increase its concentration up to 20 mM.

Mannitol osmotic control medium: This was similar to control medium (glucose 5 mM) except that it was supplemented with mannitol (15 mM) a nonpermeable solute which served as an osmotic control medium for the high-glucose medium.

Cytotoxicity Assay

The cytotoxic activity of glucose was determined by a colorimetric assay. Cell viability was determined by a modified MTT assay.[23] HeLa cells were seeded at a density of 3×10^3 cells per well in 96-well flat-bottomed microtiter plates and incubated at 37°C for 24 and 48 h in 0.2 ml culture medium Containing serial dilutions of glucose. After incubation, 10 μl of a MTT solution (5 mg/ml in PBS) was added. After

another 2 h incubation, supernatants were removed, followed by the addition in 100 μl DMSO to dissolve the formazan crystals. The absorbance readings for each well was performed at 550 nm in a plate reader. Survival was calculated as the percentage of the staining values of untreated cultures. Each concentration was tested in duplicate and the experiments were repeated three times.

Morphologic Study of Apoptosis

Apoptosis was evaluated by direct cell counting with morphologic features of apoptosis after acridine orange—ethydium bromide staining. HeLa-wild and HeLa-tat cells were stained with a solution of orange acridine (0.1 mg/ml) and ethydium bromide (0.1 mg/ml) and fixed in 1% paraformaldehyde in PBS, as previously described.[24] Cells were examined by fluorescence microscopy (Zeiss, Axiovert, Germany). Dead cells (orange), living cells (green, integrity cellular, normal aspect) and apoptotic cells (green, apoptotic bodies and nuclear fragmentation) were quantified.

Antioxidant Enzymes Activities

Cell supernatant was obtained after homogenization of tissue in a potter in hypotonic Tris–HCl buffer, pH 7.4. Glutathion peroxidase (GPX) activity was assessed as previously described[25] using terbutyl hydroperoxide. Results were expressed as micromoles of nicotinamide dinucleotide (NADP +) obtained per minute. Catalase activity was determined by the method of Beers[26] by following the decomposition of H_2O_2 at 240 nm. One unit of catalase is defined as 1 μmol H_2O_2 consumed per minute. Glutathione reductase (Grase) was determined by the method of Carlberg and Mannervik.[27] Results were expressed as micromoles of NADPH oxidized per minute

Cu,Zn-SOD activity was determined on the supernatant by monitoring the auto-oxidation of pyrogallol using the Marklund method.[28] The specific Cu,Zn-SOD inhibition by KCN (60 μl of KCN, 54 mM added to 300 μl of lysate) allows the Mn-SOD determination in the same conditions.[22] The result of three separate experiments are given. A normalization of enzyme soluble activities to protein content was performed.

Glutathione Determination

The cell homogenates were deproteinized by adding metaphosphoric acid 6%. After shaking, the mixture was centrifuged. If not performed immediately, the supernatant was stored at −80°C. Total cellular glutathione contents were evaluated as previously described.[29] The reduction rate of 5,4dithiobis (2-nitrobenzoic) acid (DTNB) into 5-thio-2-nitrobenzoate was spectrophotometrically evaluated at 412 nm. GSSG was determined under the same conditions after adjusting pH with ethanolamine and trapping GSH with 3-vinylpyridine. Results were the mean of three independent experiments.

Intracellular Thiols Measurement

HeLa cells were seeded at a density of 3×10^6 cells/ml culture medium in 25 cm^2 tissue culture flask and incubated at 37°C for 48 h. After the incubation, the supernatant was removed and cells were washed two times with phosphate buffered saline PBS (without Ca^{2+} and Mg^{2+}) and detached by incubation in PBS containing 0.05% Trypsin and 0.02% EDTA. The resulting cell suspension was centrifuged, and the pellet was resuspended with 350 μl of 2.5% DMSO in PBS. The p-nitrophenyl disufide (PNPD) is a liposoluble reagent used for thiol group measurements.[36] We developed a new method to measure thiol groups in intact cells. The compound was dissolved by heating at a concentration of 6 mg/ml in DMSO and centrifuged. The solution was diluted in DMSO (1:10). Cell suspension (350 μl) was incubated with 8 μl of PNPD solution for 20 min in the dark. Then, cells were lysed by freezing in N_2 liquid and defrosting successively. After centrifugation (10,000 rpm at 4°C), 350 μl of supernatant was removed in 96-well flat-bottomed microtiter plates for reading of the absorbance at 414 nm. In parallel, in the same conditions we performed a standard curve with *N*-acetyl cysteine solution (1 mM). Cell protein was determined by the technique of Lowry. The results were expressed in nmol −SH/mg of protein. Each concentration was tested in duplicate and the experiments were repeated three times.

Lipid Peroxidation Evaluation

For lipid peroxidation cells were lysed in deionized water by three freeze–defrost cycles. After 10 min of centrifugation 5000 rpm, (3200*g*) the membrane-containing pellet was resuspended in deionized water for TBARs and protein determination. Product of lipid peroxidation were evaluated after acid hydrolysis at 95°C to form malondialdhehyde (MDA).[30] MDA can react with thiobarbituric acid to yield and MDA–TBA adduct measured by fluorimetry after extraction with butanol. The wavelengths used were 532 nm (excitation) and 553 nm (emission). Values were reported to a calibration curve of 1,1,3,3-tetraethoxypropane. Results were expressed as μmol MDA/g of membrane-containing pellet protein.

TABLE I Effect of high glucose concentration on antioxidant enzyme activities and intracellular thiols in HeLa-wild (HW) and HeLa-tat (HT) cells. Metalloenzyme activities were measured in cell supernatant. Data are mean ± SD of three different experiments

	HW 5 mM G	HW 20 mM G	HT 5 mM G	HT 20 mM G
GPx (U/g protein)	41.5 ± 1.4*	19.1 ± 1.6*†	14.6 ± 1.4*	9.1 ± 0.6*†
Total SOD (U/mg prot)	6.1 ± 0.3	7.5 ± 0.4*	5.8 ± 0.2	7.1 ± 0.3*
Cu Zn SOD (U/mg prot)	3.9 ± 0.1	5.5 ± 0.2*	4.1 ± 0.2	4.7 ± 0.3*
Mn SOD (U/mg prot)	3.8 ± 0.2	3.7 ± 0.3	1.7 ± 0.09†	1.9 ± 0.08†
Catalase (U/mg protein)	8.1 ± 0.43	7.6 ± 0.43	7.9 ± 0.32	7.9 ± 0.30
Glutathione reductase (U/g protein)	15.23 ± 1.20*	27.4 ± 0.92*†	38.1 ± 2.40*	41.2 ± 1.70*†
GSSG/GSH × 10^3	0.74 ± 0.17*	0.90 ± 0.13*†	0.92 ± 0.15*	1.27 ± 0.23*†
Thiols (nmol–SH/mg protein)	14.1 ± 0.5	13.4 ± 0.3*	12.5 ± 0.4	6.9 ± 0.9*†

*$p < 0.05$ HeLa-tat cells compared with HeLa-wild cells for the same glucose level. †$p < 0.05$ compared with 5 mM glucose (control).

Statistical Analysis

All data in the text and figures are expressed as mean ± SD. In order to compare data from different groups, non-parametric Kruskal–Wallis tests with multiple comparison New Man Keuls tests and *t*-test were used (SigmaStat statistical software v 2.0, Jandel Scientifics). Statistical significance was set at $p \leq 0.05$.

RESULTS

For each parameter measured, to prove that the observed effect was due to intracellular process of glucose and not to hyperosmolarity, we incubated the cells with 5 mM glucose, and the corresponding concentration of mannitol to obtain 20 nM final. Mannitol had no effect on any measured parameter.

Proliferation Rate and Cytotoxicity

At time zero the number of cells for each group was set at 3000 per well. The proliferation rate of the HeLa-tat cells was higher for 5 mM glucose (4.7×10^3 and 1.2×10^4 after 24 and 48 h, respectively) in comparison to HeLa-wild (4×10^3 and 7.5×10^3 after 24 and 48 h, respectively, $p < 0.05$). Culture in 20 mM glucose resulted in a decrease in proliferation rate of HeLa-tat cells (4×10^3 and 6.9×10^3 after 24 and 48 h, respectively) which became similar to the one of the parental cells (4.1×10^3 and 7×10^3 after 24 and 48 h, respectively).

The spontaneous apoptotic levels in both HeLa-wild and HeLa-tat were <5%. We observed that 27% of HeLa-tat cell cultured with 20 mM glucose led to morphologic features of apoptosis, chromatin condensation and alteration. At this glucose level no morphologic features of apoptosis in HeLa-wild cells was observed.

Antioxidant Enzyme Activities and Cell Damage

In control glucose medium, GPx activity was strongly decreased in HeLa-tat cells in comparison to HeLa-wild (Table I). High glucose level induced a significant decrease in GPx activity in HeLa-wild cells (53%) and in HeLa-tat cells (37%). No significant difference in catalase activity was observed.

In comparison to HeLa-wild cells, HeLa-tat cells exhibited a significant increase in GRase activity. High glucose level resulted in a 80% increase in this enzyme activity in HeLa-wild, but no significant

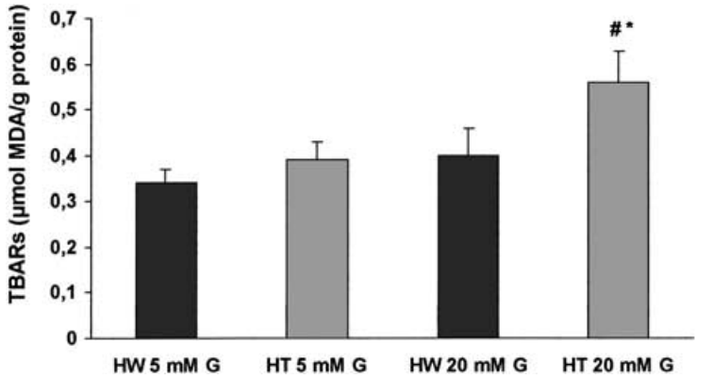

FIGURE 1 Effects of glucose on lipid peroxidation in HeLa-wild and HeLa-tat cells. Cells were lysed in deionized water by freeze–defrost cycles. After 10 min of centrifugation 5000 rpm the membrane-containing pellet was resuspended in deionized water for TBARs and protein determination. After acid hydrolysis MDA-Thiobarbituric acid adducts were measured by fluorometry. Each value represents the mean ± SD of results from three experiments. Black: HeLa-wild, dashed: HeLa-tat *$p < 0.05$ compared with 5 mM glucose (control), #$p < 0.05$ HeLa-tat cells compared with HeLa-wild cells for the same glucose level.

increase in HeLa-tat cells. GSSG/GSH was increased in HeLa-tat. High glucose level resulted in a 20% increase of this ratio in HeLa-wild cells and a 38% increase in HeLa-tat cells. No change in cytosolic thiol content with 20 mM glucose was observed in HeLa-wild cells, contrary to HeLa-tat cells where thiol groups were reduced to 43% (Table I).

The formation of TBARs was not significantly different between HeLa-wild and HeLa-tat. High glucose level induced only in HeLa-tat a significantly increase in the formation of the marker of lipid peroxidation (Fig. 1)

Total SOD and Cu,Zn-SOD activities were not significantly different between HeLa-wild and HeLa-tat cells. We confirm that HeLa-tat cells exhibit a 50% decrease in Mn-SOD activity in comparison to parental cells (Table I). When cultured in 20 mM glucose total and Cu,Zn-SOD activities increase in both cell lines, and no change was observed in Mn-SOD activity.

DISCUSSION

Our experiments show that cells exhibiting a low antioxidant defense system are prone to an important glucose-induced cytotoxicity. Oxidative stress is one of the important mediators of cytotoxicity in diabetes.[31] High glucose generates reactive oxygen species (ROS) as a result of glucose auto-oxidation, metabolism, and formation of advanced glycosylation end products. The concept of ROS-induced tissue injury has recently been revised with the appreciation of new roles of ROS in metabolic diseases such as diabetes. Direct evidence for the generation of ROS has previously been reported in other cells by Graier *et al*.[32] These authors have shown that ROS are released in dependence on the concentration of glucose and that even small variations of glucose have a distinct influence on the release of ROS.

In control medium Hela-tat 50% loss in Mn-SOD activity confirms the deleterious effect of tat protein on antioxidants. Therefore this model is convenient in studying the consequences of low antioxidant defense of glucose-induced toxicity.

A number of enzymatic and non-enzymatic cellular antioxidative defenses protect cells from the attack of ROS. It has already been demonstrated that oxidative stress induces antioxidative enzymes, including SOD, catalase, and glutathione peroxidase.

A previous report[33] suggested that the glutathione redox cycle is the key step to scavenging H_2O_2 in cytosol as well as in the mitochondria, which may contribute to the integrity of the cell.[34] Several studies have shown that the redox cycle is regulated by the intracellular content of thiol rich proteins, and by GPx and glutathione reductase activities as well as NADPH levels.[35,36] Therefore any decrease in those enzyme activities and substrate levels may profoundly impair free radical-scavenging activity, resulting in exacerbated cell damage following exposure to free radicals generated by glucose. The glutathione redox cycle is a key step in the scavenging mechanism of glucose-dependent free radical.[14] The levels of intracellular thiol groups, mainly GSH but also metallothionein and other protein thiol groups contribute to the regulation of redox homeostasis in cells. Therefore, the thiol rich compounds are antioxidant, which may regulate the intracellular metabolism defending biological structures and functions from the noxious attack by ROS. Our findings concerning cell thiol content and reduced to oxidized glutathione ratio, suggest that at high concentrations, glucose induces a decrease in cysteine-rich molecules. Interestingly, the total thiol groups content in HeLa-tat are lower and the TBARs formation is important in HeLa-tat cells cultured in 20 mM glucose. Consequently the higher lipid peroxidation of Tat cells cultured under high glucose could be a consequence of the lower Se-GPx activity, coupled with an induced activity of Cu,Zn-SOD. Hence an increased SOD activity coupled with a lower GPx activity results in an increased peroxide formation which are not detoxified by peroxidase. Ceriello *et al*.[37] showed that high glucose induced an overexpression of antioxidant enzymes in immortalized human endothelial cells, while in transfected cells with GPX it did not. This underlines the importance of GPX in the prevention of glucose-induced free radical production. In opposition to other results[38] in our conditions no induction in Mn-SOD in both cell lines is observed. As a consequence HeLa-tat are more sensitive to the cytotoxic effect of glucose than the parental cell line as no significant difference in glucose-induced cytotoxicity is observed in parental cells cultured in 20 mM glucose. This could be due to the short term incubation of the cells with glucose. It is also interesting to observe that HeLa-tat cells cultured in 20 mM glucose lose their higher proliferation rate described previously.[39] This result has to be put in parallel with the necrosis and apoptosis process observed for the high glucose level. Increase oxidative stress may push the cells in different pathways: necrosis or apoptosis. Whether one or the other is chosen depends on the redox state of the cell. Although signal transduction pathways linked to high glucose have not been fully elucidated, the current data provide evidence that ROS generated by glucose metabolism may act as an integral signaling molecules in addition to low antioxidant defense conditions, leading to apoptosis. In conclusion exposure of HeLa-tat cells to high glucose levels worsens antioxidant depletion. The biological consequences of such changes of the cellular redox state

relate not only to an altered capacity to eliminate ROS, but also to an increased susceptibility of the cells to glucose-induced cytotoxicity. These results emphasize the implications of free radical defense system in glucose induced cytotoxicity.

References

[1] Martin, W., Loschen, G., Gunzler, W. and Floche, L. (1985) "Superoxide dismutase inhibits LTB4-induced leukotaxis", *Agents Actions* **16**, 48–49.

[2] Freed, B.M., Patterson, D.A., Rapoport, R., Lawrence, D.A. and Lampert, N. (1988) "Inhibition of human T-cell responses by hydrogen peroxide", *Pharmacol. Ther.* **39**, 267–268.

[3] Del Principe, D., Menichelli, A., De Matteis, W., Di Corpo, M.L., Di Giulio, S. and Finazzi-Agro, A. (1985) "Hydrogen peroxide has a role in the aggregation of human platelets", *FEBS Lett.* **185**, 142–146.

[4] Whorton, A.R., Montgomery, M.E. and Kent, R.S. (1985) "Effect of hydrogen peroxide on prostaglandin production and cellular integrity in cultured porcine aortic endothelial cells", *J. Clin. Invest.* **76**, 295–302.

[5] Bulkley, G.B. (1983) "The role of oxygen free radicals in human disease processes", *Surgery* **94**, 407–411.

[6] Oberley, L.W. (1988) "Free radicals and diabetes", *Free Radic. Biol. Med.* **5**, 113–124.

[7] Machlin, L.J. and Benedich, A. (1987) "Free radical tissue damage: protective role of antioxidant nutrients", *FASEB J.* **1**, 441–445.

[8] Buettner, G.R. (1993) "The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate", *Arch. Biochem. Biophys.* **300**, 533–543.

[9] Bump, E.A., Yu, N.Y., Taylor, Y.C., Brown, J.M., Travis, E.L. and Boyd, M.R. (1983) "Radiosensitization and Chemiosensitization by Diethylmaleate", In: Nygaard, O.F. and Simic, M.G., eds, Radioprotectors and Anticarcinogens (Academic Press, New York), pp 297–324.

[10] Meister, A. (1985) "Glutathione synthetase from rat kidney", *Methods Enzymol.* **113**, 571–585.

[11] Wolff, S.P. and Dean, R.T. (1987) "Glucose autoxidation and protein modification. The potential role of autooxidative glycosylation in diabetes", *Biochem. J.* **245**, 243–250.

[12] Baynes, J.W. (1991) "Role of oxidative stress in development of complications in diabetes", *Diabetes* **40**, 405–412.

[13] Hunt, J.V., Smith, C.C. and Wolff, S.P. (1990) "Autoxidative glycosylation and possible involvement of peroxides and free radicals in LDL modification by glucose", *Diabetes* **39**, 1420–1424.

[14] Donnini, D., Zambito, A.M., Perrella, G., Ambesi-Impiombato, F.S. and Curcio, F. (1996) "Glucose may induce death through a free radical-mediated mechanism", *Biochem. Biophys. Res. Commun.* **219**, 412–417.

[15] Lorenzi, M., Nordberg, J.A. and Toledo, S. (1987) "High glucose prolongs cell-cycle traversal of cultured human endothelial cells", *Diabetes* **36**, 1261–1267.

[16] Stout, R.W. (1982) "Glucose inhibits replication of cultured human endothelial cells", *Diabetologia* **22**, 51–55.

[17] Sharpe, P.C., Liu, W.H., Yue, K.M., McMaster, D., Catherwood, M.A., McGinty, A.M. and Trimble, E.R. (1998) "Glucose-induced oxidative stress in vascular contractile cells: comparison of aortic smooth muscle cells and retinal pericytes", *Diabetes* **47**, 801–809.

[18] Berkhout, B., Silverman, R.H. and Jeang, K.T. (1989) "Tat trans-activates the human immunodeficiency virus through a nascent RNA target", *Cell* **59**, 273–282.

[19] Dingwall, C., Ernberg, I., Gait, M.J., Green, S.M., Heaphy, S., Karn, J., Lowe, A., Singh, M., Skinner, M.A. and Valerio, R. (1989) "Human immunodeficiency virus 1 tat protein binds trans-activation-responsive region (TAR) RNA *in vitro*", *Proc. Natl Acad. Sci. USA* **86**, 6925–6929.

[20] Buonaguro, L., Barillari, G., Chang, H.K., Bohan, C.A., Kao, V., Morgan, R., Gallo, R.C. and Ensoli, B. (1992) "Effects of the human immunodeficiency virus type 1 Tat protein on the expression of inflammatory cytokines", *J. Virol.* **66**, 7159–7167.

[21] Scala, G., Ruocco, M.R., Ambrosino, C., Mallardo, M., Giordano, V., Baldassarre, F., Dragonetti, E., Quinto, I. and Venuta, V. (1994) "The expression of the interleukin 6 gene induced by the human immunodeficiency virus 1 TAT protein", *J. Exp. Med.* **179**, 961–971.

[22] Flores, S.C., Marecki, J.C., Harper, K.P., Bose, S.K., Nelson, S.K. and McCord, J.M. (1993) "Tat protein of human immunodeficiency virus type 1 represses expression of manganese superoxide dismutase in HeLa cells", *Proc. Natl Acad. Sci. USA* **90**, 7632–7636.

[23] Sladowski, D., Steer, S.J., Clothier, R.H. and Balls, M.J. (1993) "An improved MTT assay", *Immunol. Methods* **157**, 203–207.

[24] Gitler, C. and Londner, M. (1995) "Use of p-nitrophenyl disulfide to measure reductive capacity of intact cells", *Methods Enzymol.* **150**, 279–286.

[25] Emonet, N., Leccia, M.T., Favier, A., Beani, J.C. and Richard, M.J. (1997) "Thiols and selenium: protective effect on human skin fibroblasts exposed to UVA radiation", *J. Photochem. Photobiol.* **40**, 84–90.

[26] Wheeler, C.R., Salzman, J.A., Elsayed, N.M., Omaye, S.T. and Korte, D.W. (1990) "Automated assays for superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activity", *Ann. Biochem.* **184**, 193–199.

[27] Carlberg, I. and Mannervik, B. (1985) "Assay of glutathione reductase activity", *Methods Enzymol.* **11**(3), 484–490.

[28] Marklund, S. and Marklund, G. (1974) "Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase", *Eur. J. Biochem.* **47**, 469–474.

[29] Akerboom, T.P. and Sies, H. (1981) "Assay of glutathione, glutathione disulfide and glutathione mixed disulfides in biological sample", *Methods Enzymol.* **77**, 373–382.

[30] Richard, M.J., Portal, B., Meo, J., Coudray, C., Hadjian, A. and Favier, A. (1992) "Malondialdehyde kit evaluated for determined plasma and lipoproteins fractions that react with thiobarbituric acid", *Clin. Chem.* **38**, 704–709.

[31] Ha, H. and Lee, H.B. (2000) "Reactive oxygen species as glucose signaling molecules in mesangial cells cultured under high glucose", *Kidney Int.* **77**, S19–S25.

[32] Graier, W.F., Simecek, S., Kukovetz, W.R. and Kostner, D.M. (1996) "High D-glucose-induced changes in endothelial Ca^{2+}/EDRF signaling are due to generation of superoxide anions", *Diabetes* **45**, 1386–1396.

[33] Chance, B., Sies, H. and Boveris, A. (1979) "Hydroperoxide metabolism in mammalian organs", *Physiol. Rev.* **59**, 527–605.

[34] Schrafstatter, I.U., Hinshaw, D.B., Hyslop, P.A., Spragg, R.G. and Cochrane, C.G. (1985) "Glutathione cycle activity and pyridine nucleotide levels in oxidant-induced injury of cells", *J. Clin. Invest.* **76**, 1131–1139.

[35] Kashiwagi, A., Asahina, T., Ikebuchi, M., Tanaka, Y., Takagi, Y., Nishio, Y., Kikkawa, R. and Shigeta, Y. (1994) "Abnormal glutathione metabolism and increased cytotoxicity caused by H_2O_2 in human umbilical vein endothelial cells cultured in high glucose medium", *Diabetologia* **37**, 264–269.

[36] Gibbs, P.N., Gore, M.G. and Jordan, P.M. (1985) "Investigation of the effect of metal ions on the reactivity of thiol groups in human 5-aminolaevulinate dehydratase", *Biochem. J.* **225**, 573–580.

[37] Ceriello, A., Dello Russo, P., Amstad, P. and Cerutti, P. (1996) "High glucose induces antioxidant enzymes in human endothelial cell in culture. Evidence linking hyperglycemia and oxidative stress", *Diabetes* **45**, 471–477.

[38] Sharpe, P.C., Liu, W.H., Yue, K.K., McMaster, D., Catherwood, M.A., McGinty, A.M. and Trimble, E.R. (1998) "Glucose induced oxidative stress in vascular contractile cells: comparison of aortic smooth cells and retinal pericytes", *Diabetes* **47**, 801–809.

[39] Seve, M., Favier, A., Osman, M., Hernandez, D., Vaitaitis, G., Flores, N.C., McCord, J.M. and Flores, S.C. (1999) "The human immunodeficiency virus-1 tat protein increases cell proliferation, alters sensitivity to zinc chelator-induced apoptosis, and changes Sp1 DNA binding in HeLa cells", *Arch. Biochem. Biophys.* **361**, 165–172.