Apoptosis in Human Aortic Endothelial Cells Induced by Hyperglycemic Condition Involves Mitochondrial Depolarization and Is Prevented by *N*-Acetyl-L-Cysteine

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We investigated whether the dissipation of mitochondrial transmembrane potential ($\Delta\Psi_m$) was involved in apoptosis of cultured human aortic endothelial cells (HAECs) exposed to hyperglycemic conditions (30 mmol/L glucose). In parallel experiments, *N*-acetyl-L-cysteine (NAC) was added to the culture medium to verify whether this antioxidant may prevent apoptosis in these cells. The binding of annexin V and DNA fragmentation were measured, in addition to the production of reactive oxygen species (ROS), the number of cells with depolarized mitochondria, and the intracellular glutathione (GSH) content. As compared to the control (5 mmol/L glucose), high-glucose treatment increases both ROS generation and the number of cells binding annexin V. Moreover, a simultaneous decrease of intracellular GSH content was observed, which was accompanied by an increased number of cells showing both depolarized mitochondria and fragmented DNA. Incubation of HAECs with high glucose in the presence of 10 mmol/L NAC prevented the drop of intracellular GSH content, and decreased both ROS generation and the number of cells committed to apoptosis. These results suggest that high glucose triggers the same cascade of molecular events as do other apoptosis inducers in other cells. Among these events, the disruption of mitochondrial membrane barrier function might be decisive because it causes the release of soluble proteins from intermembrane space, which then induce nuclear apoptotic changes.

INJURY OF THE VASCULAR endothelium is a critical event in the pathogenesis of atherosclerosis. Importantly, endothelial cells (ECs) in lesion-prone regions are characterized by increased EC turnover rates, suggesting a mechanistic link between the 2 events.[1] The enhanced EC turnover is most likely secondary to an increase of apoptosis, since this process can be induced by several proatherosclerotic factors, such as inflammatory cytokines,[2] angiotensin II,[3] oxidized low-density lipoprotein,[4,5] and cholesterol oxides,[6] as well as oxidative stress.[7-10]

In addition, high glucose concentration, mimicking the diabetic condition, has been shown to trigger apoptosis in cultured ECs.[11-14] One possible mechanism leading to apoptosis is the generation of reactive oxygen species (ROS), which occurs in ECs cultured in high-glucose–containing media.[13-19] Recent studies emphasize the role of mitochondria in apoptosis signaling[20,21] and the reduction of mitochondrial $\Delta\Psi_m$ seems to constitute an early irreversible step of this process.[22-24] Taking into account this finding, we investigated the involvement of mitochondrial $\Delta\Psi_m$ disruption in human aortic endothelial cell (HAEC) apoptosis induced by incubation with high glucose. In parallel experiments, *N*-acetyl-L-cysteine (NAC) was added to the culture medium to test whether this antioxidant may prevent apoptosis in these cells.

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MATERIALS AND METHODS

Cell Culture

Cells were obtained from Clonetics (BioWhittaker; Cambridge Corp, East Rutherford, NJ) and were grown to confluence in endothelial basal medium supplemented with 10 ng/mL human recombinant epidermal growth factor, 1 μg/mL hydrocortisone, 12 μg/mL bovine brain extract, 2% fetal bovine serum (FBS), 50 ng/mL amphotericin B, and 50 ng/mL gentamicin, in a humidified atmosphere (5% CO_2 at 37°C). After splitting (trypsin/EDTA solution, 37°C, 5 minutes), the cells were grown at confluence and then incubated in medium 199 with 10% FBS, without growth factors (resting state), in the presence of 5 mmol/L (control), 30 mmol/L D-glucose alone, or in combination with 10 mmol/L NAC (2 hours preincubation). At different time points (6 to 72 hours), cells were collected and stained with the different fluorochromes. Cells up to passage 6 were used.

Measurement of ROS

The generation of ROS by HAECs was determined analyzing the oxidation of the nonfluorescent dichlorofluorescein diacetate (DCFH) to the fluorescent dichlorofluorescin (DCF),[25] and the fluorescence intensity was measured using a Victor spectrofluorimeter (excitation wavelength [ex], 488 nm; emission wavelength; [em], 525 nm; Wallac, Turku, Finland).

Flow Cytometric Analyses

Flow cytometry used a Vantage flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with an argon laser (Innova 300, Coherent, Santa Clara, CA). Typically, forward and orthogonal scatter signals were used to gate out live cells, and 10,000 events were collected using log amplification.

To measure the number of cells binding annexin V, fresh HAECs (10^5 cells per test) were incubated in 250 μL of annexin V buffer containing 25 ng/mL fluorescein isothiocyanate (FITC)-conjugated annexin V for 10 minutes at room temperature in the dark.[26] Propidium iodide (PI) was used to exclude dead cells. The percentage of annexin V–positive cells (ex at 488 nm; em at 525 nm) was calculated within the viable population of cells (ie, PI-negative). Mitochondrial transmembrane potential was measured by using the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide (JC-1)[27] (ex at 488 nm, em at 525 and 590 nm). Intracellular glutathione (GSH) content was measured according to Hedley and

Chow,[28] using monobromobimane as fluorescent probe (multiline UV ex and em between 333 and 363 nm). To measure DNA fragmentation, the cells were incubated with the fluorescent dye PI (50 ng/mL in 0.1% sodium citrate containing 0.1% Triton X-100) for 30 minutes.[29] Cell debris was gated out based on light-scatter measurements before the single-parameter histograms were drawn (ex, 488 nm; em, 620 nm).

Statistical Analysis

Results are expressed as the mean ± SEM of at least 5 separate experiments performed in duplicate. Inter- and intra-assay variations did not exceed 5% and 18%, respectively. Statistical evaluation of the data was performed by use of unpaired Student's *t* test. A *P* value less than .05 was considered statistically significant.

RESULTS

To evaluate the effect of increased extracellular glucose concentration on intracellular ROS formation, the oxidation of DCFH to DCF was measured in HAECs cultured for 6 and 24 hours in the presence of 5 and 30 mmol/L glucose. Six-hour exposure to high glucose was not sufficient to increase ROS production, whereas 24 hours incubation in high glucose resulted in a statistically significant increase of DCF fluorescence (Table 1).

The increase of ROS generation was inhibited by preincubation with NAC, which also prevented the spontaneous ROS production. The changes observed in high-glucose–treated cells were not due to an osmotic effect, since mannitol (25 mmol/L + 5 mmol/L glucose) did not cause ROS production (data not shown). The number of HAECs committed to apoptosis has been determined measuring the loss of plasma membrane integrity by means of FITC-conjugated annexin V. Annexin V is a naturally occurring protein that binds to phosphatidylserine, a plasma membrane phospholipid that moves to the extracellular side of the plasma membrane early in apoptosis.[26] Using the expression of phosphatidylserine, the number of annexin-positive cells has been determined for different times (from 6 to 72 hours) after treatment with normal and high glucose concentrations (Fig 1). A time-dependent, almost linear increase of annexin-positive cells was observed subsequent to high-glucose incubation. Under the condition tested, the expression of phosphatidylserine was greatly reduced by NAC treatment.

Mitochondrial membrane potential was measured by means of JC-1, a lipophilic cationic fluorescent probe that incorporates into mitochondria. JC-1 either forms monomers (fluorescence in green, 525 nm) or, at high transmembrane potentials, aggregates (fluorescence in orange, 590 nm).[27]

Table 1. ROS Generation (DCF fluorescence)

	6 Hours	24 Hours
Control	3,774 ± 353	4,875 ± 356
Control + NAC	2,871 ± 190*	2,787 ± 108*
High glucose	4,190 ± 385	6,433 ± 606*
High glucose + NAC	3,617 ± 290	3,843 ± 347*†

NOTE. The concentrations of glucose were 5 mmol/L and 30 mmol/L, in control and high glucose cultures, respectively. The concentration of NAC was 10 mmol/L. Data are means ± SEM of 5 experiments.

*Statistically different from control or †from the corresponding samples incubated without NAC.

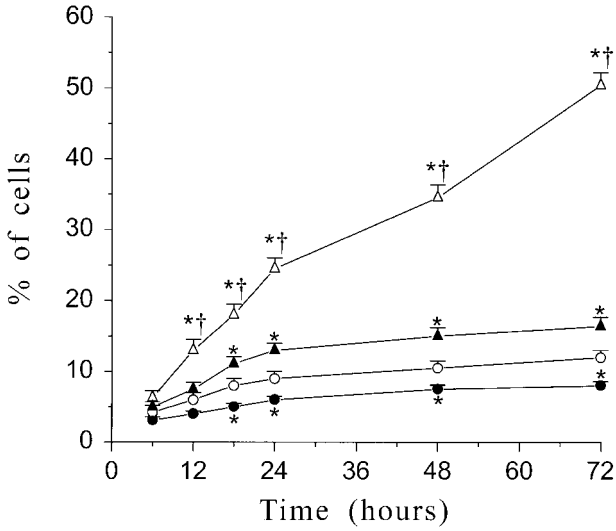

Fig 1. Time-dependent increase of the number of HAECs binding annexin V. Control (○); control + NAC (●); 30 mmol/L glucose (△); 30 mmol/L glucose + NAC (▲). *Statistically different from control or †from the corresponding samples incubated with NAC. Data are means ± SEM of 5 separate experiments.

Figure 2 depicts the results of 5 different experiments. Twenty-four–hour exposure to high sugar concentration resulted in a significant increase of the percentage of cells with depolarized mitochondria (27.3% in high glucose *v* 18.7% in control).

A further increase of the number of cells with damaged mitochondria occurred between 24 and 48 hours in high-sugar–treated cells and reached levels of 50%. Between 48 and 72 hours, the increase was less. Interestingly, NAC supplementation to the culture medium completely prevented the sugar- and time-dependent increase of the number of cells with depolarized mitochondria.

Intracellular GSH content was determined to ascertain its

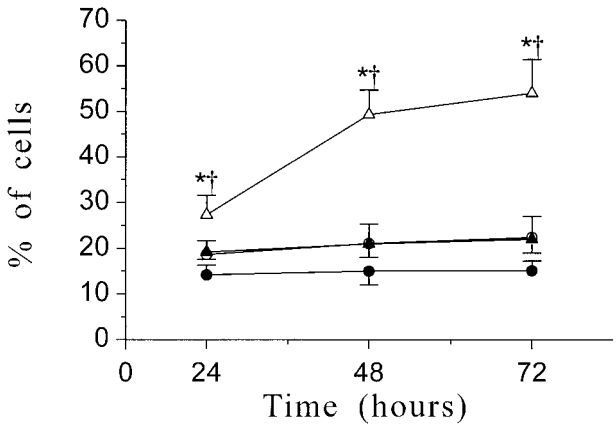

Fig 2. Time-dependent increase of the number of HAECs displaying depolarized mitochondria. Control (○); control + NAC (●); high glucose (△); high glucose + NAC (▲). *Statistically different from control or †from the corresponding samples incubated with NAC. Data are means ± SEM of 5 separate experiments.

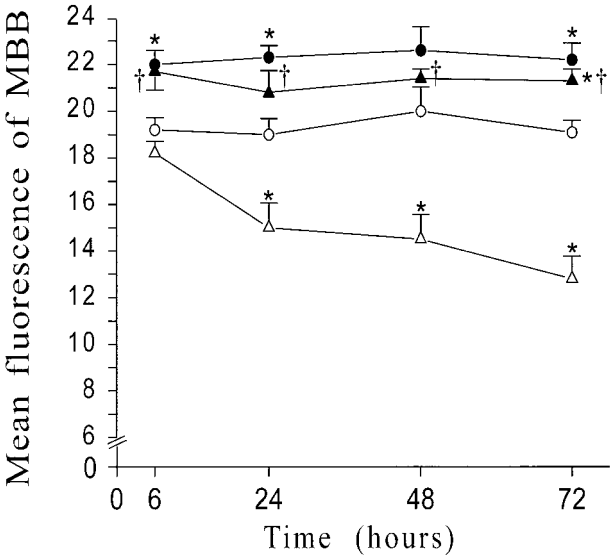

Fig 3. Intracellular GSH content as measured using the specific fluorescent probe, MBB. Control (○); control + NAC (●); high glucose (△); high glucose + NAC (▲). *Statistically different from control or †from the corresponding samples incubated with NAC. Data are means ± SEM of 5 separate experiments.

eventual contribution in apoptosis. In agreement with the occurrence of a peroxidative stress in cells, exposure to high glucose resulted in a decrease of GSH content (-26%) as compared to the control (Fig 3). This drop remained almost constant through the period considered, and was prevented by NAC preincubation.

The occurrence of DNA fragmentation, as a late feature of apoptosis, was determined by flow cytometric measurement as the percentage of nuclei with hypoploid DNA content. This procedure has an excellent correlation with colorimetric and electrophoretic methods for the detection of DNA fragments.[29,30] The drop of $\Delta\Psi_m$ in high-glucose–treated cells was accompanied by DNA fragmentation (Fig 4). Consistent with the other parameters considered in this study, a time-dependent reduction of nuclei with diploid DNA content and a concomitant increase in the hypoploid DNA peak has been observed in cells exposed to high glucose (Fig 4). NAC treatment prevented both spontaneous and sugar-induced DNA fragmentation.

DISCUSSION

A number of studies have described apoptosis in ECs induced by different stimuli,[2-10] including high glucose concentration.[11-14] In most of these experiments, human umbilical vein endothelial cells (HUVECs) or bovine aortic endothelial cells (BAECs) were used, but the involvement of mitochondrial permeability transition in the process of HAECs apoptosis has never been investigated.

In agreement with the results on ECs of different origin,[13-18] short-term incubation of HAECs with high glucose induces ROS generation. This is accompanied by consumption of intracellular GSH, drop of $\Delta\Psi_m$, and DNA fragmentation. The production of ROS seems to play a central role in the induction of EC apoptosis by sugars; the mechanisms causing this increase are still a matter of debate. Proposed mechanisms relate to metal-catalyzed glucose oxidation in endothelial cells[31] or to glycation reactions.[32] However, recently, Nishikawa et al,[17] using BAECs, provided evidence that hyperglycemia-induced intracellular ROS are produced by the proton electrochemical gradient generated by the mitochondrial electron transport chain. In addition, the same investigators showed that each of the 3 main pathways important in the pathogenesis of diabetic complications—activation of protein kinase C,[33] excessive production of advanced glycation end-products,[34] and increased flux through the polyol pathway[35]—are all dependent on elevated ROS production by the electron transport chain.

The reduction of intracellular GSH concentration might be due to the concomitant increase of ROS production and the decrease of NADPH, the critical substrate required to maintain levels of GSH. Indeed, Zhang et al[14] reported that incubation of BAECs in high-glucose–containing media resulted in a decrease of glucose-6-phosphate dehydrogenase (G6PD) activity. It is well known that this enzyme plays an essential role in the regulation of oxidative stress by primarily regulating NADPH, the main cellular reductant.[36] This decrease was accompanied by a decrease of intracellular GSH content and by apoptosis. Of note, G6PD inhibitors lead to an increase in ROS and a decrease in both NADPH and GSH levels, as well as in an increase of cells committed to apoptosis.[14] Other investigators[37] failed to demonstrate a decrease of GSH concentration subsequent to incubation of HUVECs in medium containing 20 mmol/L for 14 days. This discrepancy may be explained taking into account the difference in the concentration of glucose used, as well as in the time of analysis of GSH. Indeed, it is possible that the peroxidative stress induced by 20 mmol/L glucose might be compensated by the increased activity of antioxidant enzymes[37] or that the analysis of GSH content was performed in a selected population of cells, ie, those not committed to apoptosis. In fact, it is well known that cells undergoing apoptosis detach from the adhering surface. If 20 mmol/L glucose induces apoptosis in some cells, these could be eliminated when the medium was changed every 2 days.

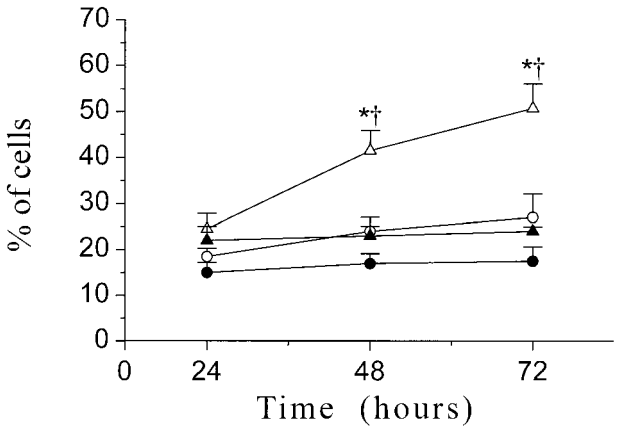

Fig 4. Time-dependent increase of the percentage ofHAECs showing fragmented DNA. Control (○); control + NAC (●); high glucose (△); high glucose + NAC (▲). *Statistically different from control or †from the corresponding samples incubated with NAC. Data are means ± SEM of 5 separate experiments.

Consistent with a pivotal role of peroxidative stress in inducing EC apoptosis are the results obtained treating the cells with NAC, which may exerts its effect by increasing the cellular GSH content. The mechanism though which GSH exerts its protection is under intense scrutiny during the last few years. Although ECs contain catalase and superoxide dismutase, the major mechanism they employ in detoxifying ROS is the GSH redox cycle,[38-40] and this feature may already be sufficient to explain the beneficial effect of NAC treatment. However, if the disruption of mitochondrial membrane barrier function marks a phase of irreversible commitment to apoptosis,[22-24] then GSH may also play a direct role in protecting mitochondria. Indeed, mitochondrial depolarization during the apoptotic process seems to be due to the opening of high-conductive, cyclosporine A (CsA)-sensitive channels,[41] and all the hypothesized mechanisms involve the peroxidation of specific thiols at mitochondrial membrane level.[42] One of these mechanisms is the binding of mitochondrial cyclophillin to the adenine nucleotide translocase (ANT) in the inner mitochondrial membrane,[43] and this binding is enhanced by thiol modification of the ANT caused by oxidative stress.[44] After the pore opening, cytochrome c, and possibly other apoptosis-inducing factors, redistributes from the mitochondrion to the cytosol[45,46] and induces nuclear apoptosis with the help of additional molecules.[21] In addition to the protective effect against HAECs apoptosis, several studies showed protective effects of NAC and other thiols in high-glucose–induced endothelial dysfunction. The impairments of endothelium-dependent relaxation, both in vitro[47,48] and in experimental animals,[49,50] as well as the increase of serum adhesion molecule-1 concentration in non–insulin-dependent diabetic patients,[51] were corrected by NAC or other thiols.

In summary, the present data show that incubation of HAECs with elevated levels of glucose induces a peroxidative stress that results in a decrease of intracellular GSH content, mitochondrial depolarization, and apoptosis. Preincubation with NAC, which prevents the drop of intracellular GSH, almost completely prevents mitochondrial de-energization and apoptosis. This suggests that high glucose triggers the same cascade of molecular events as do other apoptosis inducers in other cells, in which mitochondrial de-energization plays a key role.[20-24] Taking into account that apoptotic vascular ECs become procoagulant[52] and contribute to establish a proinflammatory milieu,[53] the present data also suggest that blocking mitochondrial depolarization with antioxidants or specific inhibitors of pore opening[5,54] might offer an additional strategy for the potential prevention of diabetic complications.

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REFERENCES

1. Caplan BA, Schwartz CJ: Increased endothelial cell turn-over in areas of in vivo Evans Blue uptake in the pig aorta. Atherosclerosis 17:401-407, 1973

2. Robaye B, Mosselmans R, Fiers W, et al: TNF induces apoptosis (programmed cell death) in normal endothelial cells. Am J Pathol 138:447-451, 1991

3. Dimmeler S, Rippmann V, Weiland U, et al: Angiotensin II induces apoptosis of human endothelial cells: Protective effect of nitric oxide. Circ Res 81:970-976, 1997

4. Dimmeler S, Haendeler J, Galle J, et al: Oxidized low density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases: A mechanistic clue to the response to injury hypothesis. Circulation 95:1760-1763, 1997

5. Walter DH, Haendeler J, Galle J, et al: Cyclosporin A inhibits apoptosis of human endothelial cells by preventing release of cytochrome c from mitochondria. Circulation 98:1153-1157, 1998

6. Lizard G, Deckert V, Dubrez L, et al: Induction of apoptosis in endothelial cells treated with cholesterol oxides. Am J Pathol 148: 1625-1638, 1996

7. Del Bono DP, Yang WD: Exposure to low concentrations of hydrogen peroxide causes delayed endothelial cell death and inhibits proliferation of surviving cells. Atherosclerosis 114:235-245, 1995

8. Hermann C, Zeiher AM, Dimmeler S: Shear stress inhibits H_2O_2-induced apoptosis of human endothelial cells by modulation of the glutathione redox cycle and nitric oxide synthase. Arterioscler Thromb Vasc Biol 17:3588-3892, 1997

9. Lelli JL, Becks LL, Dabrowska MI, et al: ATP converts necrosis to apoptosis in oxidant-injured endothelial cells. Free Radic Biol Med 25:694-702, 1998

10. Suhara T, Fukuo K, Sugimoto T: Hydrogen peroxide induces up-regulation of FAS in human endothelial cells. J Immunol 160:4042-4047, 1998

11. Baumgartner-Parzer SM, Wagner L, Pettermann M: High glucose-triggered apoptosis in cultured endothelial cells. Diabetes 44: 1323-1327, 1995

12. Du XL, Sui GZ, Stockklauser-Farber K, et al: Induction of apoptosis by high proinsulin and glucose in cultured human humbelical vein endothelial cells is mediated by reactive oxygen species. Diabetologia 41:249-256, 1998

13. Feng MH, Shing HL, Chiau SL: Nitric oxide prevents apoptosis of human endothelial cells from high glucose exposure during early stages. J Cell Biochem 75:258-263, 1999

14. Zhang Z, Apse K, Pang J, et al: High glucose inhibits glucose-6-phosphate dehydrogenase via cAMP in aortic endothelial cells. J Biol Chem 275:400042-400047, 2000

15. Cosentino F, Hishikawa K, Katusic ZS, et al: High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. Circulation 96:25-28, 1997

16. Giardino I, Edelstein D, Brownlee M: Nonenzymatic glycosylation in vitro and in bovine endothelial cells alters basic fibroblast growth factor activity. A model for intracellular glycosylation in diabetes. J Clin Invest 94:110-117, 1994

17. Nishikawa T, Edelstein D, Du XL, et al: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 404:787-790, 2000

18. Mazière C, Auclair M, Rose-Robert F, et al: Glucose-enriched medium enhances cell-mediated low density lipoprotein peroxidation. FEBS Lett 363:277-279, 1995

19. Graier WF, Simecek S, Kukovetz W, et al: High glucose-induced changes in endothelial Ca^{2+}/EDRF signaling are due to generation of superoxide anions. Diabetes 45:1386-1395, 1996

20. Kroemer G, Zamzami N, Susin SA: Mitochondrial control of apoptosis. Immunol Today 18:44-51, 1997

21. Li P, Nijhawan D, Budihrdjo I, et al: Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479-489, 1997

22. Zamzami N, Marchetti P, Castedo M, et al: Reduction in mito-

chondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J Exp Med 181:1661-1672, 1995

23. Zamzami N, Marchetti P, Castedo M, et al: Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med 182:367-377, 1995

24. Petit PX, Lecoeur H, Zorn E, et al: Alterations in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. J Cell Biol 130:157-167, 1995

25. Possel H, Noak H, Augustin W: 2,7-Dihydrodichlorofluorescein diacetate as a fluorescent marker for peroxynitrite formation. FEBS Lett 416:175-178, 1997

26. Vermes I, Haanen C, Steffen-Nakken H, et al: A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. J Immunol Methods 184:39-51, 1995

27. Cossarizza A, Kalashnikova G, Grassilli E, et al: Mitochondrial modifications during apoptosis: A study at the single cell level. Exp Cell Res 214:323-330, 1994

28. Hedley DW, Chow S: Evaluation of methods for measuring cellular glutathione content using flow cytometry. Cytometry 15:349-358, 1994

29. Nicoletti I, Migliorati G, Pagliacci MC, et al: A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining by flow cytometry. J Immunol Methods 139:271-279, 1991

30. Telford WG, King LE, Fraker PJ: Comparative evaluation of several DNA binding dyes in the detection of apoptosis-associated chromatin degradation by flow cytometry. Cytometry 13:137-143, 1992

31. Wolf SP, Dean RT: Glucose autoxidation and protein modification: The potential role of oxidative glycosylation in diabetes. Biochem J 245:243-250, 1987

32. Sakurai T, Tsuchiya S: Superoxide production from nonenzymatically glycation protein. FEBS Lett 236:406-410, 1988

33. Koia D, King GL: Protein kinase C activation and development of diabetic complications. Diabetes 47:859-866, 1998

34. Brownlee M: Advanced protein glycosylation in diabetes and aging. Ann Rev Med 46:223-234, 1995

35. Lee AY, Chung SK, Chung SS: Demonstration that polyol accumulation is responsible for diabetic cataract by use of transgenic mice expressing the aldose reductase gene in lens. Proc Natl Acad Sci USA 92:2780-2784, 1995

36. Tian WN, Braunstein LD, Pang J, et al: Importance of glucose-6-phospate dehydrogenase activity for cell growth. J Biol Chem 273: 10609-10617, 1998

37. Ceriello A, Sello Russo P, Amstad P, et al: High glucose induces antioxidant enzymes in human endothelial cells in culture. Evidence linking hyperglycemia and oxidative stress. Diabetes 45:471-477, 1996

38. Suttorp N, Toepfer W, Roka L: Antioxidant defense mechanism of endothelial cells:glutathione redox cycle versus catalase. Am J Physiol 251:C671-C680, 1986

39. Harla JM, Levine JD, Callahan KS, et al: Glutathione redox cycle protects cultured endothelial cells against lysis by extracellular generated hydrogen peroxide. J Clin Invest 73:706-713, 1984

40. Toborek M, Barger SW, Mattson MP, et al: Role of glutathione redox cycle in TNF-α mediated endothelial cell dysfunction. Atherosclerosis 117:179-188, 1995

41. Kroemer G, Petit PX, Zamzami N, et al: The biochemistry of programmed cell death. FASEB J 9:1277-1287, 1995

42. Beatrice MC, Siers DL, Pfeiffer DR: The role of glutathione in the retention of Ca^{++} by liver mitochondria. J Biol Chem 259:1279-1287, 1984

43. Costantini P, Chernyak BV, Petronilli V, et al: Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. J Biol Chem 271:6746-6751, 1996

44. Halestrap AP, Kerr PM, Javadov S, et al: Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. Biochem Biophys Acta 1366:79-94, 1998

45. Kluck RM, Bossy-Wetzel E, Green DR, et al: The release of cytochrome c from mitochondria: A primary site for Bcl-2 regulation of apoptosis. Science 275:1132-1136, 1997

46. Susin SA, Lorenzo HK, Zamzami N, Marzo I, et al: Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441-446, 1999

47. Pieper GM, Dondlinger L: Glucose elevations alter bradykinin-stimulated intracellular calcium accumulation in cultured endothelial cells. Cardiovasc Res 34:169-178, 1997

48. Pieper GM, Dondlinger LA: Antioxidant pyrrolidine dithiocarbamate prevents defective bradykinin-stimulated calcium accumulation and nitric oxide activity following exposure of endothelial cells to elevated glucose concentration. Diabetologia 41:806-812, 1998

49. Pieper GM, Siebeneich W: Oral administration of the antioxidant, N-acetylcysteine, abrogates diabetes-induced endothelial dysfunction. J Cardiovasc Pharmacol 32:101-105, 1998

50. Mayhan WG, Patel KP: Treatment with dimethylthiourea prevents impaired dilatation of the basilar artery during diabetes mellitus. Am J Physiol 274:H1895-1901, 1998

51. De Mattia G, Bravi MC, Laurenti O, et al: Reduction of oxidative stress by oral N-acetylcysteine treatment decreases plasma soluble vascular adhesion molecule-1 concentrations in non-obese, non-dyslipemic, normotensive, patients with non-insulin-dependent diabetes. Diabetologia 41:1392-1396, 1998

52. Bombeli T, Karsan A, Tait JF, et al: Apoptotic vascular endothelial cells become procoagulant. Blood 89:2429-2442, 1997

53. Herbert MJ, Gulians SR, Mackenzie HS, et al: Apoptosis of endothelial cells is associated with paracrine induction of adhesion molecules. Am J Pathol 152:523-532, 1988

54. Bernardi P, Broekemeier KM, Pfeiffer DR: Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane. J Bioenerg Biomembr 26: 509-517, 1994