

Intermittent High Glucose Enhances Apoptosis Related to Oxidative Stress in Human Umbilical Vein Endothelial Cells

The Role of Protein Kinase C and NAD(P)H-Oxidase Activation

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The effects of intermittent and constant high glucose in the formation of nitrotyrosine and 8-hydroxydeoxyguanosine (markers of oxidative stress), as well as the possible linkage between oxidative stress and apoptosis in endothelial cells, have been evaluated. Stable high glucose increased nitrotyrosine, 8-hydroxydeoxyguanosine (8-OHdG), and apoptosis levels. However, these effects were more pronounced in intermittent high glucose. Protein kinase C (PKC) was elevated in both such conditions, particularly in intermittent glucose. The adding of the PKC inhibitors bisindolylmaleimide-I and LY379196, a specific inhibitor of PKC- β isoforms, normalized nitrotyrosine and reduced 8-OHdG concentration and cell apoptosis in both stable and intermittent high glucose. Similar results were obtained with the MnSOD mimetic Mn(III)tetrakis(4-benzoic acid)porphyrin chloride that normalized nitrotyrosine, 8-OHdG, and apoptosis and inhibited PKC activation. NAD(P)H oxidase was also measured. NAD(P)H oxidase components p47phox, p67phox, and p22phox was overexpressed during both stable and intermittent high glucose. PKC inhibition and MnSOD mimetic normalized this phenomenon. In conclusion, our study shows that the exposure of endothelial cells to both stable and intermittent high glucose stimulates reactive oxygen species overproduction also through PKC-dependent activation of NAD(P)H oxidase, leading to increased cellular apoptosis. Our data suggest that glucose fluctuations may also be involved in the development of vascular injury in diabetes. *Diabetes* 52:2795–2804, 2003

There is overwhelming evidence for an involvement of reactive oxygen species (ROS) in the pathogenesis of diabetes-associated vascular complications (1). Nishikawa et al. (2) recently showed that the causal link between elevated glucose and hyperglycemic damage is the increased production of superoxide by the mitochondrial electron transport chain. In addition to mitochondrial sources of ROS, superoxide anion can be derived from nitric oxide synthase (3) and NAD(P)H oxidases (4,5). Recently, it was shown that the latter enzymes, the membrane-associated NAD(P)H oxidases, are the primary physiological producers of superoxide in several animal models of vascular disease, including diabetes (6). It also has been suggested that high glucose levels may stimulate ROS production through protein kinase C (PKC)-dependent activation of NAD(P)H oxidase in smooth muscle cells and endothelial cells (7), whereas numerous studies have implicated increased PKC activity in the pathophysiology of diabetic vasculopathy (8).

A direct measurement of ROS production is not currently possible. However, it can be assessed indirectly by measurement of oxidative products because ROS, which are unstable molecules as a result of the presence of unpaired electrons in their molecular structure, undergo a series of interaction with biological macromolecules such as proteins, lipids, and DNA (9).

Two interesting markers of oxidative stress are available today. Nitrotyrosine is considered a marker for the presence of peroxynitrite, a powerful oxidant, derived from the reaction of superoxide and nitric oxide, because it is the product of the reaction of tyrosine residues in proteins with peroxynitrite (10). Recent studies demonstrated that in hyperglycemia, there is an augmented production of nitrotyrosine, both in vivo (11) and in vitro (12).

The oxidized nucleoside 8-hydroxydeoxyguanosine (8-OHdG) is known as a sensitive indicator of oxidative damage to DNA (13,14). Increased levels of 8-OHdG have been found in the kidney of diabetic rats and in tissue or body fluid in patients with diabetes (15–17).

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8-OHdG, 8-hydroxydeoxyguanosine; BOMO-I, bisindolylmaleimide-I; ELISA, enzyme-linked immunosorbent assay; HUVEC, human umbilical vein endothelial cell; MnTBAP, Mn(III)tetrakis(4-benzoic acid)porphyrin chloride; PKC, protein kinase C; ROS, reactive oxygen species.

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It was shown previously that hyperglycemia enhances free radical production, inducing oxidative damage, which in its turn activates the death pathways implicated in cell apoptosis and necrosis (18–20).

In a previous study, we demonstrated that intermittent high glucose is more dangerous than constant high glucose medium for human umbilical vein endothelial cells (HUVECs) in culture because in the former condition, there was a marked increase in cellular apoptosis (21). In the present study, we examined whether the increase in apoptosis levels observed in our experimental model is linked to free radical generation and oxidative stress damage, through nitrotyrosine and 8-OHdG measurement. Moreover, we evaluated the possible involvement of PKC and NAD(P)H oxidase activation in oxidative stress generation and apoptosis during both conditions of high glucose culture.

RESEARCH DESIGN AND METHODS

Unless otherwise specified, reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Isolation and culture of human endothelial cells. HUVECs were isolated and pooled from umbilical cords obtained from normal vaginal deliveries by the procedure described by Jaffe et al. (22). The cells were cultured in gelatin-coated 60-mm Petri dishes (Sarstedt) and grown in medium 199 (Gibco, Gaithersburg, MD) supplemented with 2 mmol/l glutamine (Gibco), 20% heat-inactivated fetal bovine serum (Gibco), 25 µg/ml endothelial cell growth supplement, 90 µg/ml heparin (Gibco), and 0.25 µg/ml Fungizone (Gibco). The Petri dishes were incubated at 37°C, in 5% CO₂/95% air gas mixture. Primary cultures were fluid changed 24 h after seeding and were subcultured on reaching confluence by the use of 0.01% trypsin-EDTA, inactivated by dilution. Cultured cells were identified as endothelial by their morphology and the presence of factor VIII-related antigen detected using indirect immunofluorescence as previously described (21). Only first- and second-passage HUVECs were used in the study to avoid age-dependent cellular modifications. HUVECs were seeded at equal density (1.3×10^5) in gelatin-coated 60-mm Petri dishes and allowed to attach overnight. Then they were exposed to the experimental conditions for 14 days. Therefore, three groups of cells were formed, each one receiving the following fresh media every 24 h: 1) continuous normal glucose medium (5 mmol/l), 2) continuous high glucose medium (20 mmol/l), and 3) normal and high glucose media alternating every 24 h. The potent PKC inhibitors bisindolylmaleimide-I (BIMI-I; 5 µmol/l) (23) and LY379196 (30 nmol/l), a specific PKC-β isoform inhibitor (24), a gift from Lilly Research Laboratories (Indianapolis, IN), and the MnSOD mimetic Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP; 100 µmol/l; Calbiochem, Darmstadt, Germany) were also added singularly to the three media previously described. Osmotic control was ensured by incubating cells with 20 mmol/l mannitol, continuously or in an alternating manner.

PKC activity. Detection of PKC activity was performed with PepTag assay (Promega, Madison, WI), which uses brightly colored, fluorescent peptide substrate that is highly specific for PKC. Phosphorylation by PKC of the specific substrate alters the peptide's net charge from +1 to -1. Samples were separated on a 0.8% agarose gel at 100 V for 15 min. Phosphorylated peptide migrated toward the anode (+); the nonphosphorylated one migrated toward the cathode (-). The gel was photographed on an ultraviolet transilluminator. Quantification of results was performed by densitometry.

PKC-βI and -βII expression. Assays were performed by Western blot analysis with specific antibodies directed against PKC-βI and -βII isoforms, which, among the various PKC isoforms, seem to be activated preferentially in the vasculature of diabetic animals (8). Protein expression was evaluated by Western blot analysis, using specific antibodies for each one, all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were washed three times in cold PBS and then lysed for 30 min at 4°C in buffer containing 1% Nonidet P-40, 50 mmol/l Tris-HCl (pH 7.5), 100 mmol/l NaCl, 5 mmol/l EDTA, and 1 mmol/l phenylmethylsulfonyl fluoride. After centrifugation at 13,000g for 10 min at 4°C, the supernatant was collected and the protein content of all sample was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). An identical amount of protein (20 µg) for each lysate was subjected to SDS-PAGE. The concentration of polyacrylamide gels was 8%. Proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories). The filters were

blocked for 2 h using 5% nonfat dried milk in TBS (50 mmol/l Tris, 0.15 mol/l NaCl [pH 7.5]) containing 0.1% Tween 20, washed with TBS, and incubated at room temperature for 2 h with the primary antibody at the suitable dilution: mouse monoclonal anti-cPKC-βI (1:100), rabbit polyclonal anti-cPKC-βII (1:200). Filters were later washed with TBS and incubated with 1:1,000 dilution of secondary anti-mouse or anti-rabbit IgG antibody, coupled with horseradish peroxidase. The enhanced chemiluminescence system (Amersham Pharmacia Biotech) was used for detection. Filters were subsequently exposed to Kodak Bio Max Light-1 films, and the intensity of Western blot signals was quantified by densitometry.

Nitrotyrosine measurement. After 7 and 14 days, culture cells were lysed as reported in the Western blot assays, and protein content was determined by the Bio-Rad protein assay kit. An identical amount of protein (50 µg) was applied to a Maxisorp ELISA plate (NUNC Brand Products) together with nitrated BSA standard samples, obtained as previously described (11), using 50 mmol/l Na₂CO₃-NaHCO₃ buffer at pH 9.6, and allowed to bind overnight at 4°C. Afterward, nonspecific binding sites were blocked with 1% BSA in PBS. The wells were incubated at 37°C for 1 h with a mouse monoclonal antibody anti-nitrotyrosine (5 µg/ml; Upstate Biotechnology, Lake Placid, NY) and then for 45 min at 37°C with a peroxidase-conjugated goat anti-mouse IgG secondary antibody diluted 1:1,000. After the plates were washed, the peroxidase reaction product was generated using a TMB peroxidase substrate. Plates were incubated for 10 min at room temperature, and the reaction was stopped with 50 µl per well of H₂SO₄ 0.5 mol/l and read at 450 nm in a microplate reader.

8-OHdG. 8-OHdG amount was determined in HUVEC DNA digests using Bioxytech 8-OHdG-EIA Kit, a competitive enzyme-linked immunosorbent assay (ELISA) purchased from OXIS Health Products (Portland, OR). HUVEC DNA was isolated using DNazol Reagent, purchased from Gibco (Life Technologies, Grand Island, NY), according to the manufacturer's instructions, and quantified using a spectrophotometer. Samples containing 400 µg of DNA were resuspended in 50 µl of reaction mixture, containing 100 mmol/l sodium acetate (pH 5.0) and 5 mmol/l MgCl₂, and digested with 1 µl of DNase I (Sigma-Aldrich), for 10 min at room temperature. DNA-digested samples were added to the microtiter plate precoated with 8-OHdG, and the assay was performed according to the manufacturer's instructions.

Apoptosis measurements

Bcl-2 expression. Bcl-2 levels in HUVEC cell lysates were detected by the use of a commercial kit, an ELISA purchased by Bender MedSystems Diagnostics (Wien, Austria), according to the manufacturer's instructions. The quantification was also performed with a Western blot assay, carried out using a specific mouse monoclonal IgG anti-Bcl-2 antibody (Santa Cruz), diluted 1:100, as described for PKC-β isoforms.

Caspase-3 activity and expression. The activity of caspase-3 was assayed by the use of Chemicon's CPP32/Caspase-3 Colorimetric Protease Assay Kit (Chemicon International, Temecula, CA). The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide after cleavage from the labeled substrate DEVD-*p*-nitroanilide. Caspase-3 expression was performed with a Western blot assay, as previously described for PKC-β isoforms, using a specific rabbit polyclonal IgG anti-caspase-3 antibody (Santa Cruz), diluted 1:200.

NAD(P)H oxidase. NAD(P)H oxidase expression was evaluated through the detection of its components p67phox, p47phox, and p22phox by the Western immunoblotting technique as previously described for PKC-β isoforms. Specific antibodies against the three NAD(P)H oxidase components were purchased from Santa Cruz Biotechnology, and they were used at the following dilutions: goat polyclonal anti-p67phox (1:100), goat polyclonal anti-p47phox (1:100), and goat polyclonal anti-p22phox (1:100). Secondary anti-goat IgG antibody (Santa Cruz) was used at 1:1,000 dilution.

Statistical analysis. All data are mean ± SD. Groups were compared using two-way ANOVA, and Bonferroni-Dunn's post hoc test was performed on raw data. Differences were considered significant at $P < 0.05$.

RESULTS

The results of six different experiments for each experimental condition were analyzed throughout a 14-day period.

PKC activity and expression. After 7 days, the activity of PKC showed a marked increase in HUVECs that were exposed to the 20 mmol/l glucose condition and even more in cells that were incubated with fluctuating glucose concentrations ($P < 0.01$ by ANOVA). After 14 days of incubation, PKC activity augmented even further, showing greater values in the oscillating glucose condition as

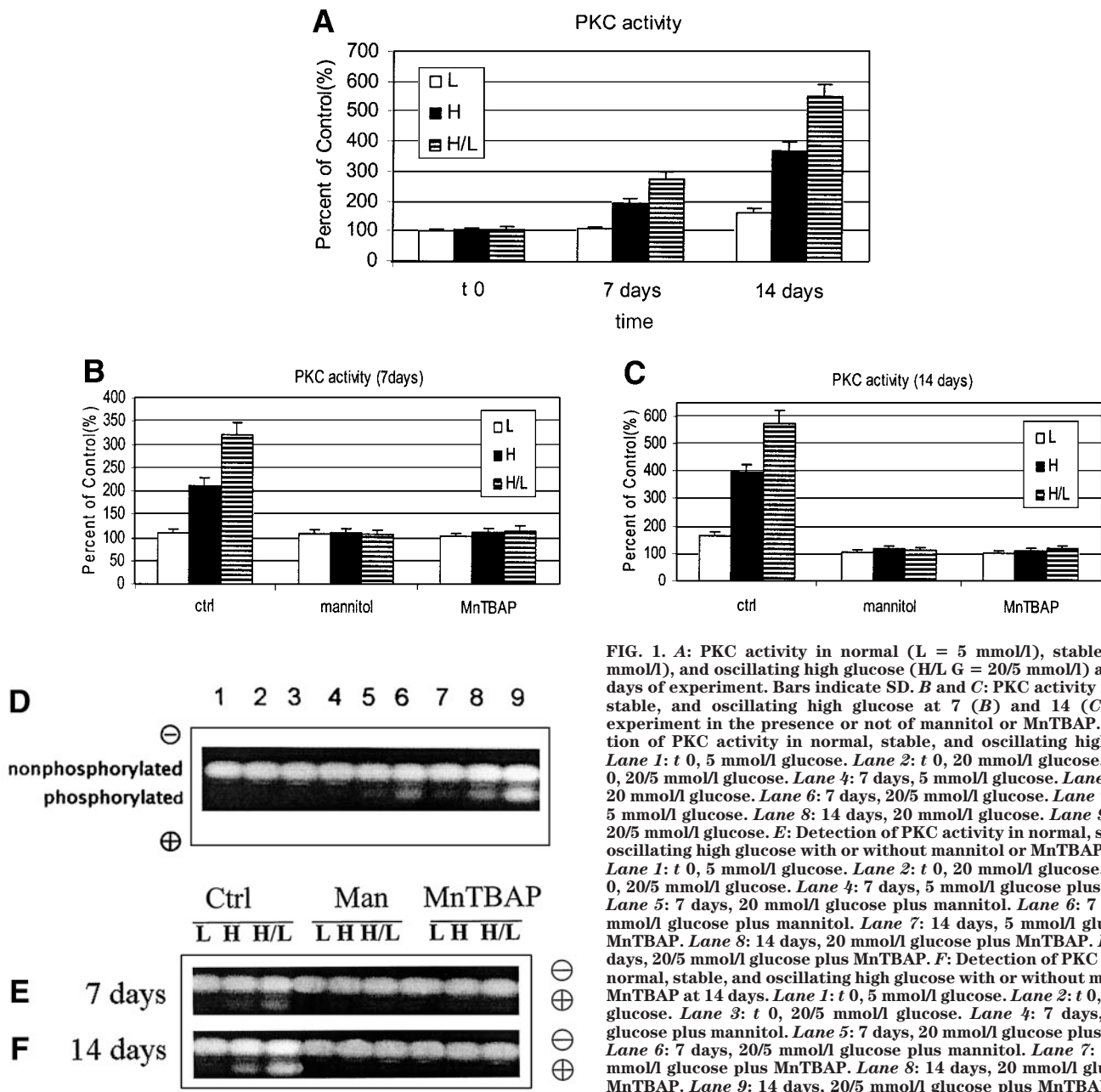


FIG. 1. **A:** PKC activity in normal (L = 5 mmol/l), stable (H = 20 mmol/l), and oscillating high glucose (H/L G = 20/5 mmol/l) at 7 and 14 days of experiment. Bars indicate SD. **B** and **C:** PKC activity in normal, stable, and oscillating high glucose at 7 (**B**) and 14 (**C**) days of experiment in the presence or not of mannitol or MnTBAP. **D:** Detection of PKC activity in normal, stable, and oscillating high glucose. Lane 1: t 0, 5 mmol/l glucose. Lane 2: t 0, 20 mmol/l glucose. Lane 3: t 0, 20/5 mmol/l glucose. Lane 4: 7 days, 5 mmol/l glucose. Lane 5: 7 days, 20 mmol/l glucose. Lane 6: 7 days, 20/5 mmol/l glucose. Lane 7: 14 days, 5 mmol/l glucose. Lane 8: 14 days, 20 mmol/l glucose. Lane 9: 14 days, 20/5 mmol/l glucose. **E:** Detection of PKC activity in normal, stable, and oscillating high glucose with or without mannitol or MnTBAP at 7 days. Lane 1: t 0, 5 mmol/l glucose. Lane 2: t 0, 20 mmol/l glucose. Lane 3: t 0, 20/5 mmol/l glucose. Lane 4: 7 days, 5 mmol/l glucose plus mannitol. Lane 5: 7 days, 20 mmol/l glucose plus mannitol. Lane 6: 7 days, 20/5 mmol/l glucose plus mannitol. Lane 7: 14 days, 5 mmol/l glucose plus MnTBAP. Lane 8: 14 days, 20 mmol/l glucose plus MnTBAP. Lane 9: 14 days, 20/5 mmol/l glucose plus MnTBAP. **F:** Detection of PKC activity in normal, stable, and oscillating high glucose with or without mannitol or MnTBAP at 14 days. Lane 1: t 0, 5 mmol/l glucose. Lane 2: t 0, 20 mmol/l glucose. Lane 3: t 0, 20/5 mmol/l glucose. Lane 4: 7 days, 5 mmol/l glucose plus mannitol. Lane 5: 7 days, 20 mmol/l glucose plus mannitol. Lane 6: 7 days, 20/5 mmol/l glucose plus mannitol. Lane 7: 14 days, 5 mmol/l glucose plus MnTBAP. Lane 8: 14 days, 20 mmol/l glucose plus MnTBAP. Lane 9: 14 days, 20/5 mmol/l glucose plus MnTBAP.

compared with stable high glucose ($P < 0.01$ by ANOVA; Fig. 1D). PKC activity was not affected by osmolarity, whereas MnTBAP in the media normalized PKC activation induced by both stable and intermittent high glucose (Fig. 1E and F). Quantification of PKC activity, performed by densitometry, is presented in Fig. 1A–C.

The Western blot analysis of electrophoresed proteins present in lysed cells confirmed that after 7- and 14-day culture, the two PKC isoforms β I and β II were increased in the conditions of high constant and intermittent glucose ($P < 0.01$ by ANOVA). In cells that were cultured in 5 mmol/l glucose, PKC- β I and - β II were significantly lower than in cells that were cultured in constant 20 mmol/l glucose ($P < 0.01$ by ANOVA) and that MnTBAP normalized this phenomenon (Fig. 2A and B). Consistently, mannitol did not influence PKC- β I and - β II expression (Fig. 2B). Representative Western blot and intensity of signals, analyzed by densitometry, are shown in Fig. 2.

Nitrotyrosine measurements. After 7 days of experimentation, nitrotyrosine content increased in the stable 20 mmol/l glucose condition, in comparison with the 5 mmol/l condition, but not as much as in the fluctuating glucose condition ($P < 0.01$ by ANOVA). The adding of the PKC total inhibitor or the selective PKC- β inhibitor prevented the increase of nitrotyrosine concentration in both high glucose and intermittent high glucose (Fig. 3A). After 14 days, when no inhibitory substance was added, the nitrotyrosine content increased even further in both the 20 mmol/l and the intermittent glucose compared with the 5 mmol/l condition (Fig. 3B). The adding of the two PKC inhibitory substances equally inhibited the increase of nitrotyrosine in the 20 mmol/l and intermittent glucose studies as compared with the same case in which no inhibitor was added (Fig. 3B). MnTBAP also normalized nitrotyrosine formation, whereas mannitol did not show any effect (Fig. 3B).

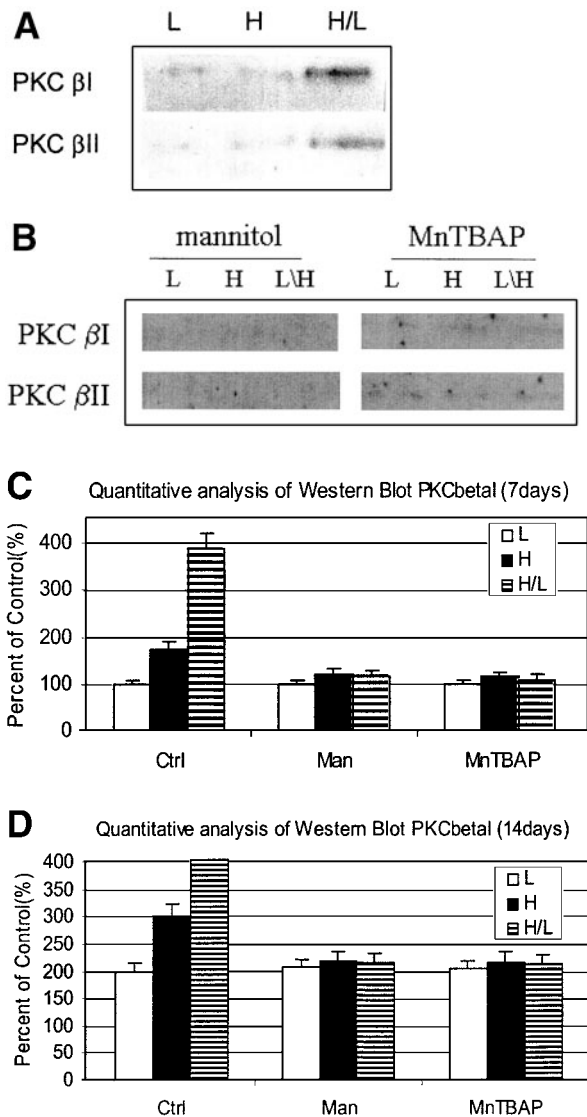


FIG. 2. Protein expression of PKC-βI and -βII. **A:** Representative Western blot after 14 days of culture (normal, L = 5 mmol/l; stable, H = 20 mmol/l; and oscillating high glucose, H/L G = 20/5 mmol/l). **B:** Representative Western blot after 14 days of culture with mannitol or MnTBAP (normal, L = 5 mmol/l; stable, H = 20 mmol/l; and oscillating high glucose, H/L G = 20/5 mmol/l). **C and D:** Quantification of signal intensity by densitometry of PKC-βI after 7 (**C**) and 14 (**D**) days of experiment (normal, L = 5 mmol/l; stable, H = 20 mmol/l; and oscillating high glucose, H/L G = 20/5 mmol/l). Bars indicate SD. **E and F:** Quantification of signal intensity by densitometry of PKC-βII after 7 (**E**) and 14 (**F**) days of experiment (normal, L = 5 mmol/l; stable, H = 20 mmol/l; and oscillating high glucose, H/L G = 20/5 mmol/l). Bars indicate SD.

8-OHdG. Seven days after the beginning of the experiment, the level of 8-OHdG rose in cells that were exposed to stable high glucose and substantially more in cells that were exposed to the intermittent high glucose ($P < 0.01$; Fig. 3C). When cells were treated with the two PKC inhibitors, there was an equal reduction in 8-OHdG production in both the 20 mmol/l and intermittent glucose states (Fig. 3C). After 14 days of culture, the amount of 8-OHdG increased in constant 20 mmol/l glucose and in the fluctuating condition, but in the latter, the content more than doubled ($P < 0.01$; Fig. 3D). The presence of PKC inhibitors blocked the 8-OHdG production in both conditions (Fig. 3D). MnTBAP normalized 8-OHdG formation, whereas mannitol did not show any effect at both 7 and 14 days (Fig. 3D).

Apoptosis measurements

Bcl-2 expression. Bcl-2 ELISA and Western blot analysis, after 7 days of testing, showed that Bcl-2 protein significantly decreased in the fluctuating situation compared with either normal or stable high glucose ($P < 0.01$; Fig. 4). The addition of the two PKC inhibitory drugs produced a significant reduction in the decrease of Bcl-2 expression in both stable high and intermittent glucose ($P < 0.01$; Fig. 4).

After 14 days of experiment, Bcl-2 expression, determined with the two methods described, showed a further decrease in stable and intermittent high glucose (Fig. 4). The adding of BIM-1 and of LY379196 was still effective in avoiding Bcl-2 decrease (Fig. 4). MnTBAP was also able to preserve Bcl-2, whereas mannitol did not show any effect (Fig. 4).

Caspase-3 activity and expression. Seven days after testing, caspase-3 activity showed a marked increase in stable high glucose and in normal glucose did not ($P < 0.01$). The activity was even higher in the intermittent glucose ($P < 0.01$ vs. stable high glucose, $P < 0.001$ vs. normal glucose; Fig. 5). When PKC inhibitors were added in all three glucose conditions, the caspase-3 increase that was previously observed showed a decrease both in stable high glucose and in intermittent high glucose ($P < 0.01$; Fig. 5). After 14 days, the activity of caspase-3 further increased in the intermittent glucose condition; adding the two PKC inhibitors was still effective in reducing its raising up ($P < 0.01$; Fig. 5). Adding MnTBAP normalized caspase-3, whereas mannitol did not have any effect (Fig. 5).

Caspase-3 expression, performed with the Western blot

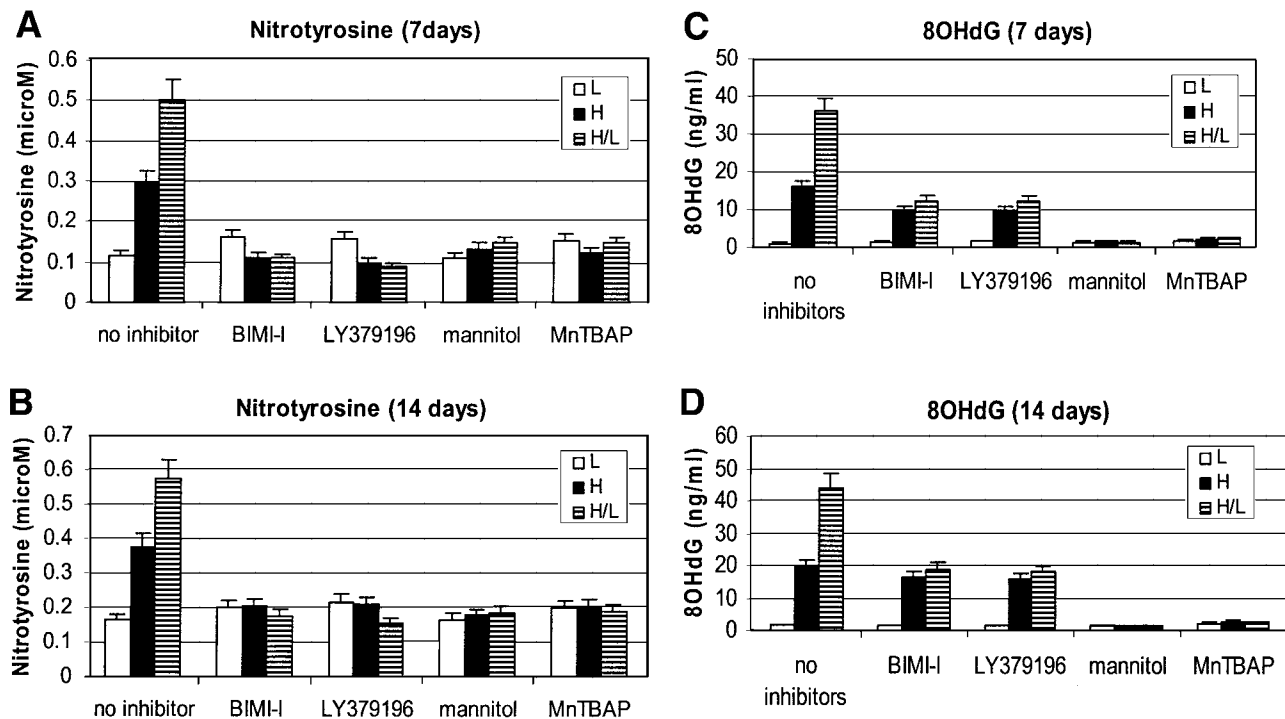


FIG. 3. Oxidative stress markers measurement. *A* and *B*: Nitrotyrosine content in HUVEC cell lysates after 7 (*A*) and 14 (*B*) days of experiment, when cells were exposed to 5 mmol/l glucose (L), 20 mmol/l glucose (H), or 20/5 mmol/l glucose (H/L), without PKC inhibitors or with the addition of inhibitory drug BIM-I or LY379196, mannitol, or MnTBAP. Bars indicate SD. *C* and *D*: 8-OHdG content in HUVEC DNA at 7 (*C*) and 14 (*D*) days of experiment, when cells were exposed to 5 mmol/l glucose (L), 20 mmol/l glucose (H), or 20/5 mmol/l glucose (H/L), without PKC inhibitors or with the addition of inhibitory drug BIM-I or LY379196, mannitol, or MnTBAP. Bars indicate SD.

assay, after 7 days of testing raised in both stable and intermittent high glucose, but in the last condition, the increase was substantial (Fig. 5). The two PKC inhibitory drugs showed a decrease in caspase-3 expression both in cells cultured in stable high glucose and in those grown in the intermittent glucose medium (Fig. 5). Caspase-3 protein after 14 days increased its expression in stable and even more in intermittent high glucose; the adding of PKC inhibitors confirmed the reduction of caspase-3 expression increase (Fig. 5). MnTBAP inhibited the caspase-3 increase; that in mannitol remained unchanged (Fig. 5).

NAD(P)H oxidase. Where no PKC inhibitor was added, the NAD(P)H-oxidase components increased in cells in stable high glucose and even more in those cultured in intermittent high glucose at both 7 and 14 days ($P < 0.01$; Fig. 6). The adding of BIM-I or LY379196 produced a comparable reduction in p47phox, p67phox, and p22phox expression in both stable and intermittent high glucose (Fig. 6). A similar effect was obtained by MnTBAP, whereas mannitol did not showed any effect (Fig. 6). The intensity of the Western blot signals quantified by densitometry confirmed these results (Fig. 6).

DISCUSSION

Several lines of evidence suggest that endothelial dysfunction and damage represent early steps in the development of vascular complications in diabetes (25). Hyperglycemia is the central initiating factor for all types of diabetic microvascular disease (26,27), and it also may be involved in the pathogenesis of macrovascular complications (28). Oxidative stress has recently been proposed as the unifying factor for the damaging effect of hyperglycemia (2).

Although in normal subjects plasma glucose concentration is strictly controlled within a narrow range, in patients with diabetes, the plasma glucose concentration often changes markedly within a single day. It is now recognized that both hyperglycemia at 2 h during an oral glucose challenge and glucose fluctuations per se are strong predictors of both cardiovascular disease (29) and microangiopathic complications (30), and it has been suggested that these "hyperglycemic spikes" may play a direct and significant role in the pathogenesis of vascular diabetic complications (31). Moreover, there is growing evidence that an acute increase of glycemia is accompanied by an oxidative stress generation (32) that may contribute to the generation of an endothelial dysfunction (17,18). Thus, clinical evidence suggests that in vivo glucose fluctuations may be dangerous for endothelial cells and that this effect should be mediated by an oxidative stress.

In this study, we proposed an experimental model in which primary cultures of human endothelial cells are exposed to intermittent high glucose, a condition that partly mimics what really happens in vivo in patients with diabetes. In agreement with previous studies (7,12,15–17), we found that stable high glucose produced an increase in apoptosis and in oxidative stress generation. Moreover, according to our previous report (21), we confirm that intermittent glucose seems to worsen the pro-apoptotic effects of high glucose. Because cells that are cultured in the intermittent high glucose condition present larger amounts of both nitrotyrosine and 8-OHdG, compared with constant high and normal glucose, our data suggest that in such conditions, there is an enhancement of oxidative stress generation that, convincingly, may favor

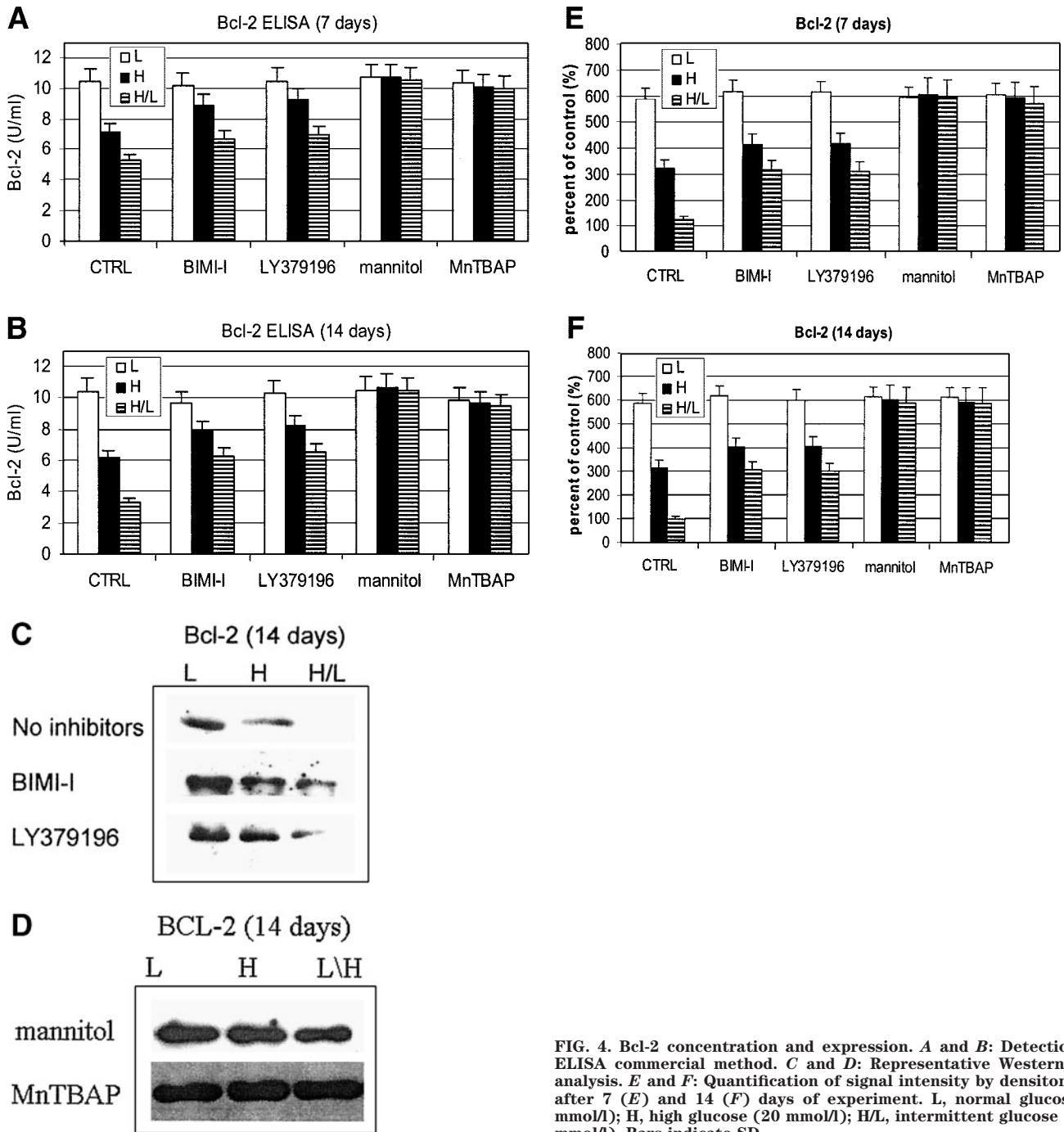


FIG. 4. Bcl-2 concentration and expression. *A* and *B*: Detection by ELISA commercial method. *C* and *D*: Representative Western blot analysis. *E* and *F*: Quantification of signal intensity by densitometry after 7 (*E*) and 14 (*F*) days of experiment. L, normal glucose (5 mmol/l); H, high glucose (20 mmol/l); H/L, intermittent glucose (20/5 mmol/l). Bars indicate SD.

enhanced apoptosis. This hypothesis is further confirmed by the evidence that by adding MnTBAP (a compound able to inhibit oxidative stress generation) normalizes nitrotyrosine and 8-OHdG and preserves endothelial cells from apoptosis induced by both stable and intermittent high glucose.

Previous studies showed that high glucose produces both an increase in intracellular oxidative stress and an upregulation of PKC activity (2,7). In our experiments, the exposure of HUVECs to high glucose concentrations induced an increase in β I and β II isoform expression and in the biological activity of PKC, which is greater in the fluctuating than in the stable high glucose condition. According to Brownlee et al. (2), the activation of PKC

seems to be dependent on free radical generation, because inhibiting oxidative stress significantly reduces PKC activation. However, the PKC inhibitors BIMI-I and the selective PKC β inhibitor LY379196 were equally, even only in part, effective in diminishing the production of nitrotyrosine and 8-OHdG, and reducing the NAD(P)H activation and apoptosis in stable high glucose and in intermittent high glucose conditions. Even though the precise mechanisms underlying this phenomenon may require further clarification, it should be suggested that free radicals produced by high glucose activate PKC and that through NAD(P)H-related overgeneration of oxidative stress may amplify apoptosis of endothelial cells.

Our results also support the hypothesis that NAD(P)H-

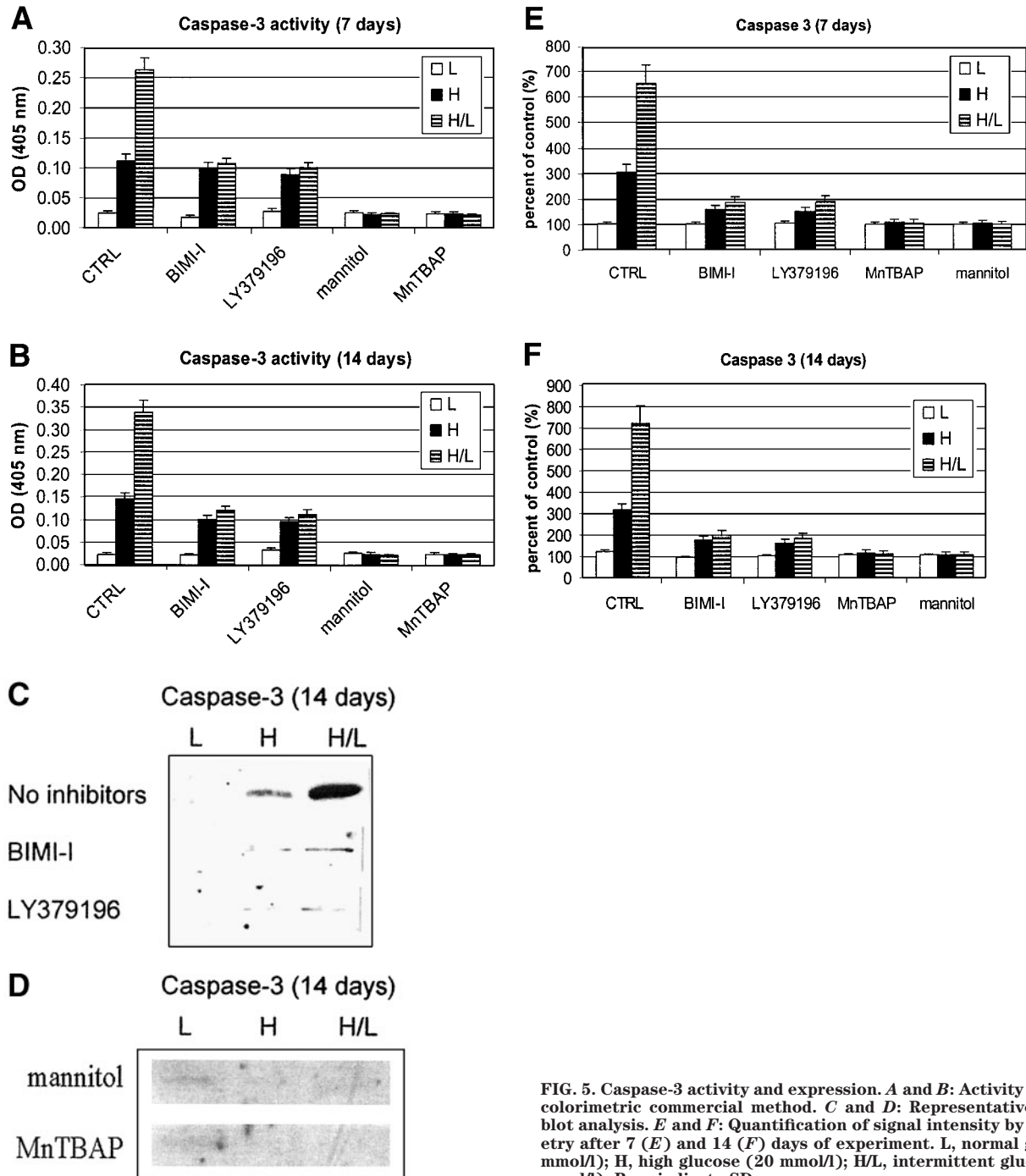


FIG. 5. Caspase-3 activity and expression. *A* and *B*: Activity assay by a colorimetric commercial method. *C* and *D*: Representative Western blot analysis. *E* and *F*: Quantification of signal intensity by densitometry after 7 (*E*) and 14 (*F*) days of experiment. L, normal glucose (5 mmol/l); H, high glucose (20 mmol/l); H/L, intermittent glucose (20/5 mmol/l). Bars indicate SD.

oxidase-dependent free radical production in high glucose is PKC dependent. PKC inhibitors, in fact, were equally effective as MnTBAP in reducing the overexpression of NAD(P)H-oxidase components. Our data, however, show that PKC inhibition normalizes nitrotyrosine, whereas 8-OHdG was only partially reduced.

Nitrotyrosine is considered a marker for the presence of peroxynitrite, a powerful oxidant, derived from the reaction of superoxide and nitric oxide, because it is the product of the reaction of tyrosine residues in proteins with peroxynitrite (10). The oxidized nucleoside 8-OHdG is known as a sensitive indicator of oxidative damage to DNA (13,14). This chemical modification of DNA is produced by the action of reactive oxygen/nitrogen/chlorine

species. PKC blocking may reduce superoxide anion production (7) and consequently peroxynitrite generation, but nothing can be said about other reactive species, able to damage DNA, that may be produced during hyperglycemia. Besides this, our results suggest that the PKC pathway may not be the only one that leads to Bcl-2 and caspase-3 activation, because PKC inhibitors are not able to normalize Bcl-2 and caspase-3 levels completely. Even in this case, Bcl-2 and caspase-3 could be triggered by different radical species than peroxynitrite, which may recruit different pathways. This hypothesis is supported by our data showing that scavenging free radicals with an antioxidant compound, such as MnTBAP, reduces both nitrotyrosine and 8-OHdG and normalizes Bcl-2 and

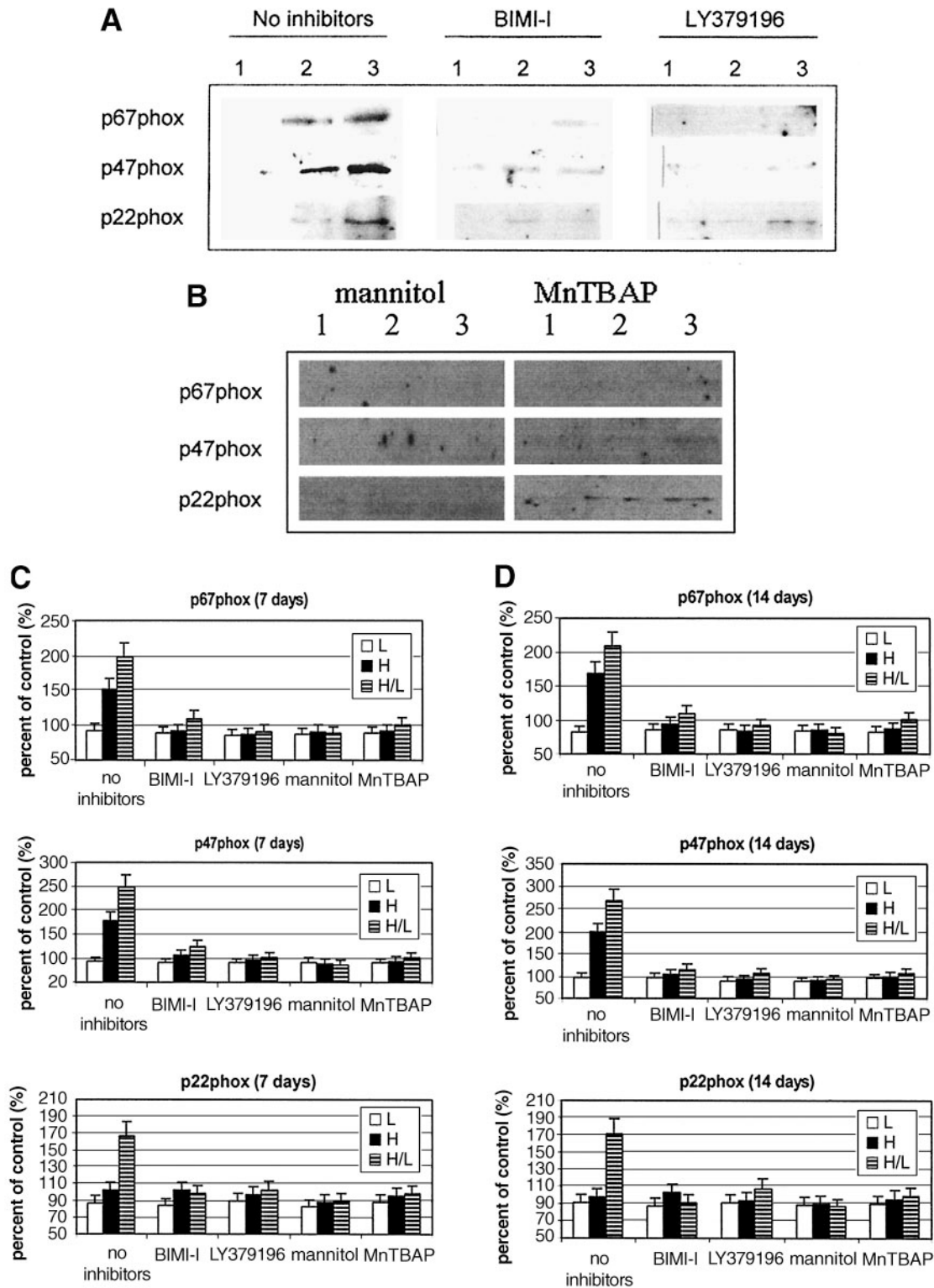


FIG. 6. A: Western blot analysis in HUVECs for p67phox, p47phox, and p22phox expression after 14 days, without PKC inhibitors or with the addition of inhibitory drug BIMI-I or LY379196 30 nmol/l. *Lane 1*, normal glucose (5 mmol/l); *lane 2*, high glucose (20 mmol/l); *lane 3*, intermittent glucose (20/5 mmol/l). **B:** Western blot analysis in HUVECs for p67phox, p47phox, and p22phox expression after 14 days, with mannitol or MnTBAP. L, normal glucose (5 mmol/l); H, high glucose (20 mmol/l); H/L, intermittent glucose (20/5 mmol/l). **C and D:** Quantification of Western blot signal intensity by densitometry, when cells were exposed to 5 mmol/l glucose (L), 20 mmol/l glucose (H), or 20/5 mmol/l glucose (H/L). Bars indicate SD.

caspase-3. Our data also suggest the main role played by the PKC-β isoform in this contest: the selective PKC-β inhibitor LY379196 produced the same effects as total PKC

inhibitor BIMI-I in diminishing nitrotyrosine and 8-OHdG overproduction, reducing apoptosis, and controlling the expression of NAD(P)H-oxidase components.

Cumulating *in vitro* evidence suggests that glucose variations may be more dangerous for the cells than stable high glucose. Mesangial cells that are cultured in periodic high glucose concentration increase matrix production more than cells that are cultured in high stable glucose (33). Similarly, fluctuations of glucose display a more dangerous effect than stable high glucose on tubulointerstitial cells, in terms of collagen synthesis and cell growth (34). Our data show that this phenomenon is true also for HUVECs and, for the first time, suggest that oxidative stress, also through PKC-dependent activation of NAD(P)H oxidase, may be involved.

Our data verify that the pathways involved in the damaging effect of intermittent high glucose on HUVECs are, at least in part, the same as those working in stable high glucose concentrations. However, they are consistently enhanced in such a condition. At present, the molecular mechanisms specifically triggered on cultured HUVECs by periodically changing glucose concentrations are not known. A possible explanation is that during chronic exposure to high glucose, some metabolic variations induced by this constant situation might change or feed back regulatory cell controls, partially counteracting the glucose toxic effect. Intermittent exposure to high glucose might reduce such adaptation, causing more pronounced toxicity. Further studies are needed to elucidate this point better.

In conclusion, our study shows for the first time that the apoptosis of HUVECs exposed to intermittent high glucose may be related to a ROS overproduction, through PKC-dependent activation of NAD(P)H oxidase. In our opinion, these data support the evidence that *in vivo* glucose fluctuation may be involved in the development of oxidative stress and vascular injury.

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