Metformin Prevents Glucose-Induced Protein Kinase C-β2 Activation in Human Umbilical Vein Endothelial Cells Through an Antioxidant Mechanism

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Hyperglycemia determines the vascular complications of diabetes through different mechanisms: one of these is excessive activation of the isoform β2 of protein kinase C (PKC-β2). Metformin, a widely used antidiabetic agent, is associated with decreased cardiovascular mortality in obese type 2 diabetic patients. Therefore, we assessed the role of metformin in glucose-induced activation of PKC-β2 and determined the mechanism of its effect in human umbilical venous endothelial cells grown to either normo- (5 mmol/l) or hyperglycemia (10 mmol/l) and moderately and acutely exposed to 25 mmol/l glucose. We studied PKC-β2 activation by developing adenovirally expressed chimeras encoding fusion protein between green fluorescent protein (GFP) and conventional β2 isoform (PKC-β2–GFP). Glucose (25 mmol/l) induced the translocation of PKC-β2–GFP from the cytosol to the membrane in cells grown to hyperglycemia but not in those grown in normal glucose medium. Metformin (20 μmol/l) prevented hyperglycemia-induced PKC-β2–GFP translocation. We also assessed oxidative stress under the same conditions with a 4-((9-acridinecarbonyl)amino)-2,2,6,6-tetramethylpiperidin-oxyl, free radical (TEMPO-9-AC) fluorescent probe. We observed significantly increased radical oxygen species production in cells grown in hyperglycemia medium, and this effect was abolished by metformin. We show that in endothelial cells, metformin inhibits hyperglycemia-induced PKC-β2 translocation because of a direct antioxidant effect. Our data substantiate the findings of previous large intervention studies on the beneficial effect of this drug in type 2 diabetic patients. *Diabetes* 54:1123–1131, 2005

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Received for publication 12 November 2004 and accepted 15 December 2004.

AMPK, AMP-activated protein kinase; DPI, diphenyleneiodonium chloride; GFP, green fluorescent protein; HUVEC, human umbilical endothelial vein cell; PEG, polyethylene glycol; PKC, protein kinase C; PMA, phorbol myristic acid; ROS, reactive oxygen species; TEMPO-9-AC, 4-((9-acridinecarbonyl) amino)-2,2,6,6-tetramethylpiperidin-oxyl, free radical; UKPDS; U.K. Prospective Diabetes Study.

Substantial clinical and experimental evidence suggests that diabetes causes endothelial dysfunction (1): this condition includes decreased endothelium-dependent vasorelaxation, increased leukocyte-endothelial cell adhesion, and increased vascular permeability (2–4). One of the primary mechanisms that contribute to these alterations in diabetes appears to involve the activation of protein kinase C (PKC), a serine/threonine kinase that catalyzes the transfer of a phosphate group from ATP to various substrate proteins (5). To date, ~12 isoforms of PKC have been identified that differ in structure, substrate requirement, and tissue localization. Endothelial cells contain a specific PKC isoform, PKC-β2, whose activation leads to basement membrane thickening, extracellular matrix expansion, increased endothelial permeability, and cell turnover. Hyperglycemia can cause the activation of PKC-β2 through the de novo pathway of diacylglycerol synthesis (6). Hyperglycemia can induce oxidative stress through three main mechanisms: NAD(P)H oxidase, xanthine oxidase, and mitochondrial electron transport chain (7). Oxidative stress activates PKC in vascular tissues (7,8), possibly through the regulatory role of calcium-independent phospholipase A_2 (9). Thus, the activation of PKC in endothelial cells appears to be related to both metabolic derangement and glucose-induced oxidative stress.

Considerable data have accumulated over the past years indicating that elevated postprandial glucose concentration increases the risk of cardiovascular disease. Information from both in vitro and in vivo studies have provided plausible mechanisms by which increases in postprandial glucose can produce vascular damage: they include the activation of PKC (10,11). Recently, we demonstrated in vivo that this is also the case in humans (12).

Strict metabolic control is a paramount measure to prevent the onset and slow down the progression of long-term diabetic vascular complications in diabetic patients. Specifically, in obese type 2 diabetic patients, the U.K. Prospective Diabetes Study (UKPDS) has shown that intensive glucose control with metformin decreases the risk of diabetes-related end points (13); therefore, there might be a specific protective vascular effect of this drug beyond its antihyperglycemic action. Metformin not only lowers blood glucose concentration but also inhibits adipose tissue lipolysis, reduces circulating free fatty acids, reduces the rate of formation of advanced glycation end

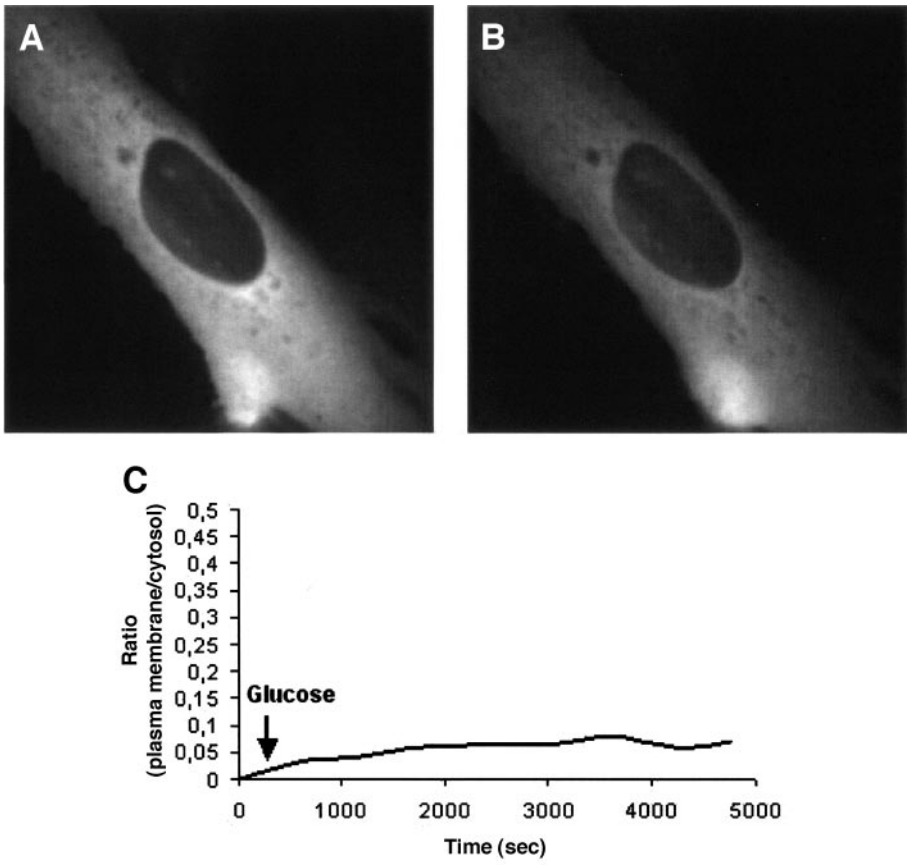

FIG. 1. Effect of acute hyperglycemia (25 mmol/l) on translocation of PKC-β2–GFP in HUVECs grown at 5 mmol/l glucose for 48 h. Images were recorded under basal conditions at 5 mmol/l glucose (*A*) and 2 h after exposure to 25 mmol/l glucose (*B*). *C*: The time course of plasma membrane translocation of PKC-β2–GFP, expressed as the increase in fluorescence ratio with respect to 0 (calculated as the ratio of plasma membrane and cytosol average intracellular fluorescence, obtained from multiple regions inside the cytosol and on the cell membrane), measured for 2 h. The graph is representative of three similar experiments.

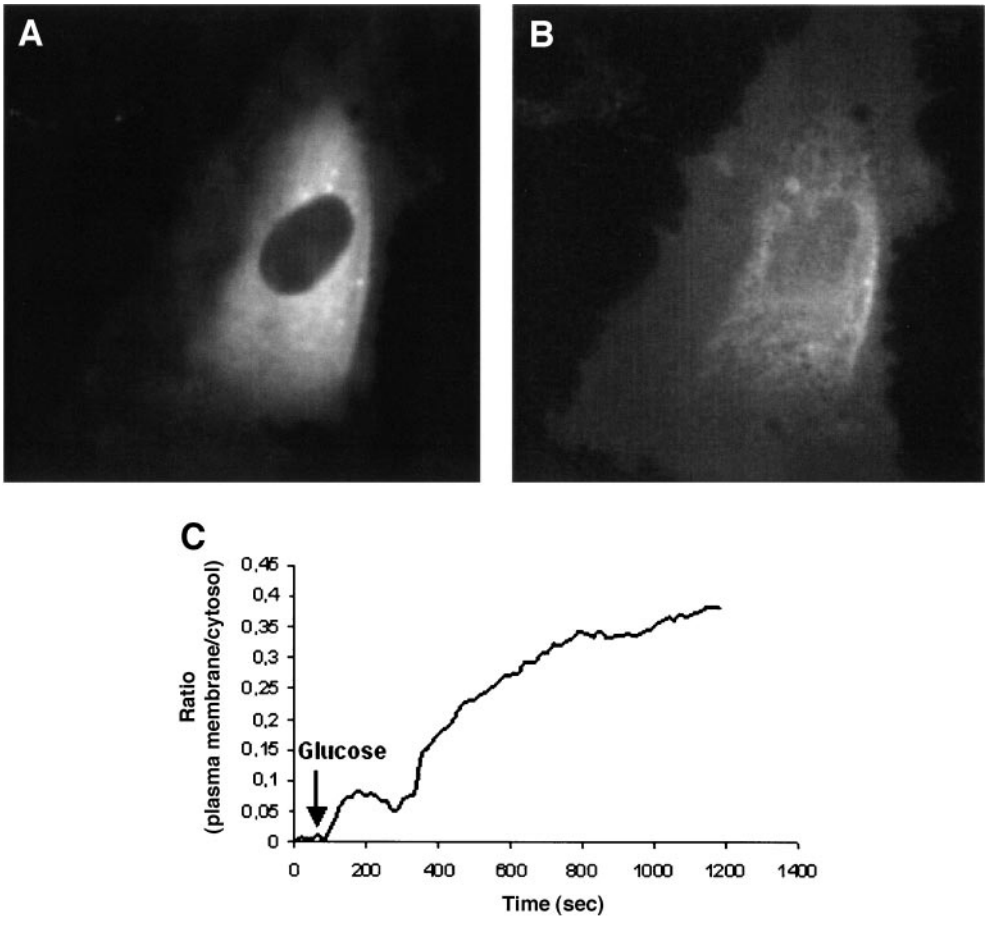

FIG. 2. Effect of acute hyperglycemia (25 mmol/l) on translocation of PKC-β2–GFP in HUVECs grown at 10 mmol/l glucose for 48 h. Images were recorded under basal conditions at 10 mmol/l glucose (*A*) and 20 min after exposure to glucose 25 mmol/l (*B*). *C*: The time course of plasma membrane translocation of PKC-β2–GFP from the cytosol to the cell membrane, as described in Fig. 1. The graph is representative of five similar experiments.

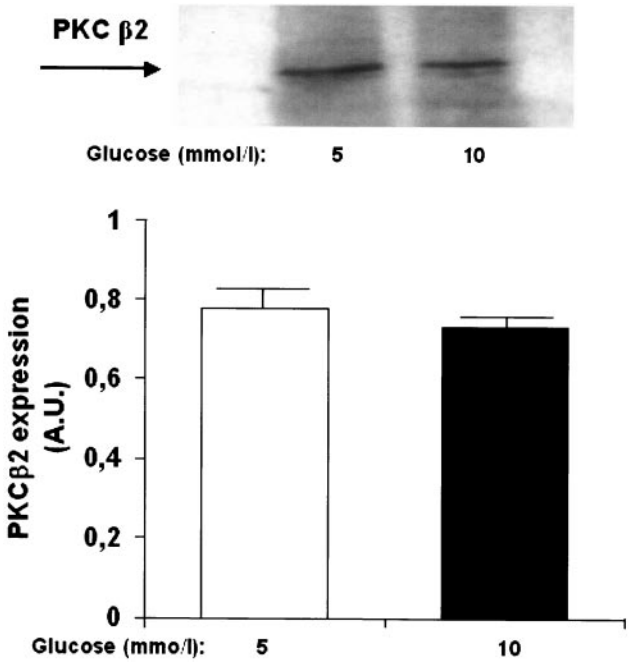

FIG. 3. Total PKC-β2 expression in HUVECs. Cells were grown at 5 and 10 mmol/l glucose for 48 h. Then, the cells were lysed as described in RESEARCH DESIGN AND METHODS. Proteins were separated by electrophoresis and immunoblotted against anti–PKC-β2 and anti–glyceraldehyde-3-phosphate dehydrogenase (as positive control). The upper panel shows a representative immunoblot of total PKC-β2 expression in cells at 5 and 10 mmol/l of glucose. The lower panel shows the PKC expression, calculated as the ratio between PKC-β2 and glyceraldehyde-3-phosphate dehydrogenase protein. The data represent the mean of three experiments.

products, and improves insulin sensitivity (14,15). However, its molecular mechanisms are still poorly defined, although Zhou et al. (16) provided evidence that metformin activates AMP-activated protein kinase (AMPK), a major cellular regulator of lipid and glucose metabolism.

The aims of the present study were to determine *1*) whether acute hyperglycemia induces the translocation of PKC-β2 isoform in human umbilical endothelial vein cells (HUVECs), *2*) whether metformin has the potential to inhibit glucose-induced PKC-β2 translocation from the cytosol to the cell membrane, and *3*) the mechanism(s) of these effects.

RESEARCH DESIGN AND METHODS

HUVECs were obtained from Clonetics (San Diego, CA). Cells were cultured in MCDB 131 medium (Sigma Aldrich, St. Louis, MO), pH 7.4, supplemented with 10% FCS (Eurobio), 1 μg/ml hydrocortisone (Sigma Aldrich), 5 μl/ml endothelial cell growth factor 100 × (Sigma Aldrich), 100 units/ml penicillin, 100 μg/ml streptomycin (Eurobio), and 2 mmol/l L-glutamine (Eurobio). They were maintained in a humidified 5% CO_2 incubator at 37°C. Thereafter, the cells were subcultured for 48 h in the presence of either normal (5 mmol/l) or elevated (10 mmol/l) glucose concentration in the presence or absence of metformin (20 mmol/l). All of the experiments were carried out after 4–10 passages.

Adenoviral generation and infection. We fused in-frame the green fluorescent protein (GFP) cDNA to a cDNA coding for the PKC-β2 isoform to examine the function and fate of the resulting chimera in living cells. The cells were infected with PKC-β2–GFP hybrid using recombinant adenovirus technique. Adenoviruses were constructed and amplified using the pAdEasy system as previously described. Adenoviral generation from the recombinant shuttle vectors was performed as previously described (17). At 70% confluence the HUVECs were exposed to adenovirus for the transfection of PKC-β2–GFP. After 48 h cells were expressing the chimera PKC-β2–GFP and were ready to be visualized (18).

Intracellular monitoring of PKC-β2 translocation. HUVECs grown at 5 or 10 mmol/l glucose serum media with and without metformin (20 μmol/l) were placed in an observing camera with 2 ml saline solution (135 mmol/l NaCl, 5 mmol/l KCl, 0.4 mmol/l KH_2PO_4, 1 mmol/l $MgSO_4$, 20 mmol/l HEPES, 1 mmol/l CaCl, and 5 or 10 mmol/l glucose, pH 7.4). They were held at 37°C on the heated microscope plane and then exposed to two different acute stimuli: hyperglycemia (25 mmol/l glucose for 2 h) and hydrogen peroxide (500 μmol/l H_2O_2 for 2 h). Fluorescence was monitored by the quantification of changes to the plasma membrane–to–cytosol ratio fluorescence intensity of PKC-β2–GFP chimera over time at an excitation wavelength of 494 nm and an emission wavelength of 518 nm with a fluorescein isothiocyanate filter.

Measurement of reactive oxygen species. Intracellular reactive oxygen species (ROS) generation was measured with the fluoroprobe 4-((9-acridinecarbonyl)amino)-2,2,6,6-tetramethylpiperidin-oxyl, free radical (TEMPO-9-AC), which is permeable to cells and produces a specific fluorescent signal after its oxidation by ROS (19). Cells were loaded with TEMPO-9-AC (10 μmol/l) for 10 min at 37°C in a physiological buffer at 5 or 10 mmol/l of glucose in the presence or absence of metformin (20 μmol/l).

After the removal of external TEMPO-9-AC, cells were placed on the stage of an inverted Zeiss Axiovert 200 motorized microscope, as previously described. Fluorescence was monitored by the quantification of the changes in fluorescence intensity over time at an excitation wavelength of 358 nm and an emission wavelength of 461 nm with a DAPI filter. The fluorescence rate was calculated as the slope of the linear least squares fitting of fluorescence intensity. In some experiments the cells were pretreated with polyethylene glycol (PEG) superoxide dismutase (250 units/ml for 24 h), PEG catalase (250 units/ml for 24 h), diphenyleneiodonium chloride (DPI; 50 μmol/l, for 1 h), apocynine (100 μmol/l, for 1 h), and rotenone (100 nmol/l, for 1 h).

Determination of xanthine oxidase and catalase activities. HUVECs were grown in culture with 5 and 10 mmol/l glucose with and without metformin (20 μmol/l) for 48 h and then washed, resuspended in PBS, and added to scintillation vials containing licigenin (326 μg/ml), xanthine (100 μg/ml), and Krebs-HEPES buffer and counted every 30 s for 5 min. Quantification of O_2^- production was performed by measuring chemiluminescence of lucigenin in the presence of xanthine oxidase reaction, using a liquid scintillation counter (Packard Tri-Carb 1600TR) in out-of-coincidence, single-photon count mode, as previously described (20).

Catalase activity was determined according to Rapoport et al. (21) with slight modifications. The rate of decomposition of H_2O_2 was measured at 240 nm in a spectrophotometer (Lambda 1; Perkin-Elmer). The cells were homogenized in lysis buffer (12.5 mmol/l Tris, 2 mmol/l EGTA, 25 mmol/l β-glycerophosphate, 2 mmol/l Na_3VO_4, 10 μmol/l phenylmethylsulfonyl fluoride, 1 μmol/l leupeptin, and 5 μmol/l aprotinin). Protein concentrations were determined with Lowry et al.'s (22) method, using BSA as standard and 500–1,000 μg protein transferred to a cuvette containing H_2O_2 (25 mmol/l). Absorbance was recorded every 30 s for 5 min. In this study, 1 unit of catalase activity equaled the amount of protein that converts 1 μmol H_2O_2/min at 25°C. All samples were measured in duplicate.

PKC-β2 expression. HUVECs were lysed in buffer (12.5 mmol/l Tris, 2 mmol/l EGTA, 25 mmol/l β-glycerophosphate, 2 mmol/l Na_3VO_4, 10 μmol/l phenylmethylsulfonyl fluoride, 1 μmol/l leupeptin, 5 μmol/l aprotinin, and 1% NP-40) and centrifuged for 10 min at 10,000 rpm at 4°C. Supernatants were solubilized with SDS-PAGE sample buffer. Total cell lysates (30 μg) were resolved on 10% SDS-PAGE gel and electroblotted onto nitrocellulose membrane (Hybond enhanced chemiluminescence; Amersham) in blotting buffer containing 48 mmol/l Tris, 39 mmol/l glycine, 0.037% SDS, 20% methanol (vol/vol) for 3 h at 100 V in the cold, using a Transblot cell (Elettrofor, Padova, Italy). The membranes were blocked overnight at 4°C in Tween-PBS, containing PBS and 0.05% (vol/vol) Tween, and 5% BSA. Membranes were exposed with anti–PKC-β2 antibody overnight at 4°C. Membranes were washed (four times for 20 min) with the same buffer and then incubated with 1:5,000 goat anti-rabbit antibody conjugate to horseradish peroxidase. Detection was performed using the enhanced chemiluminescence system. The density of the resulting protein bands was analyzed by a Versa Doc 1000 imaging system (Bio-Rad, Hercules, CA), and the results were expressed relative to the control(s) in the same blot, set at 100%, as the PKC-β2–to–glyceraldehyde-3-phosphate dehydrogenase densitometric ratio. Protein concentration of the supernatant was determined with Lowry et al.'s method (22).

Statistical analysis. All of the experiments were performed at least in triplicate. The results are expressed as the means ± SE of at least three individual experiments. Comparisons were performed using nonparametric two-tailed tests for unpaired data with SPSS, Microsoft Excel, or Origin 7 (OriginLab, Northampton, MA) software.

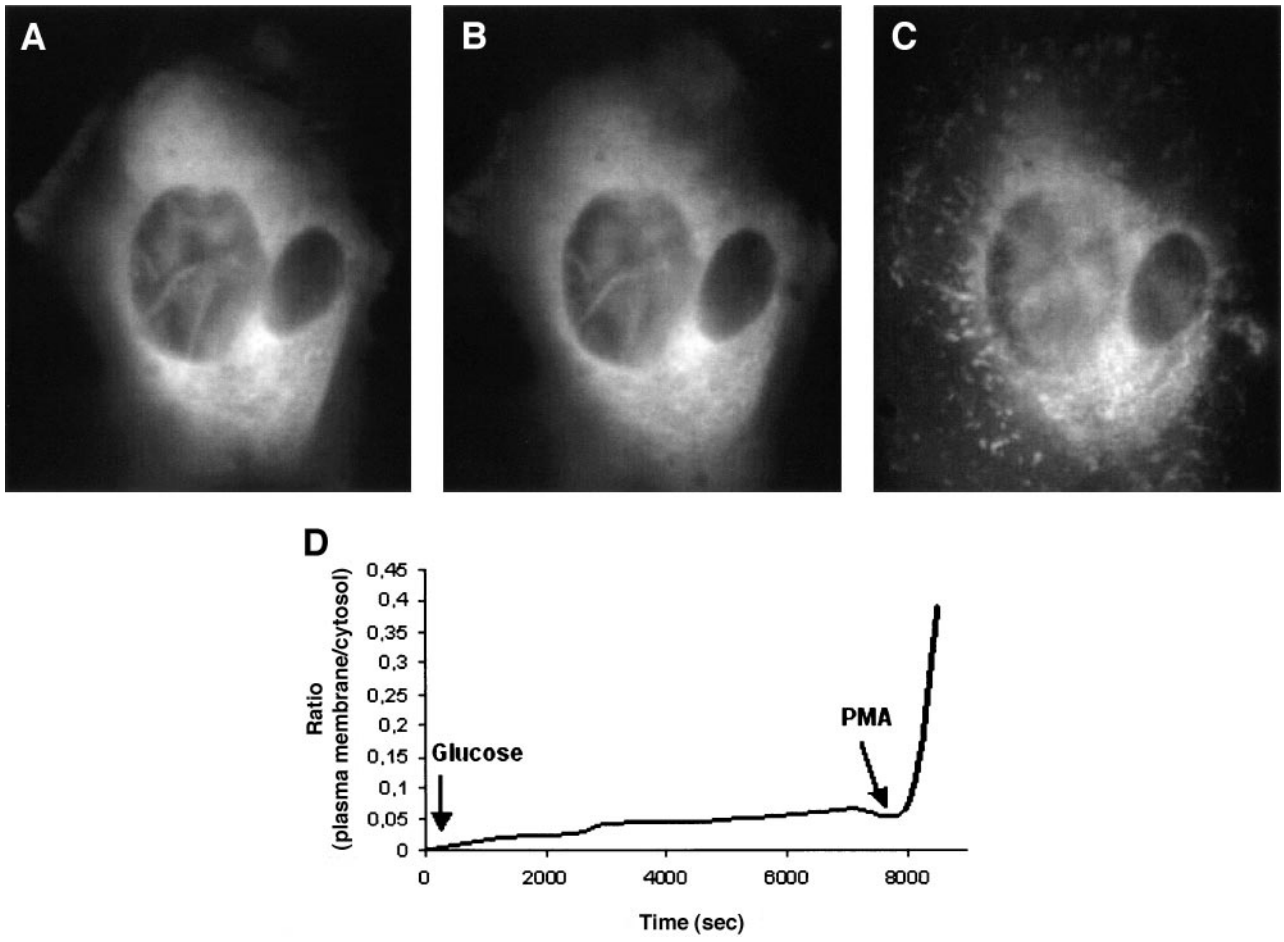

FIG. 4. Effect of metformin on PKC-β2 translocation in HUVECs after acute hyperglycemia. The images represent cells grown with 10 mmol/l glucose and 20 μmol/l metformin for 48 h before (*A*) and 2 h after (*B*) the addition of 25 mmol/l glucose. *C*: The same cell after the addition of PMA (300 nmol/l) for 5 min. *D*: The time course of plasma membrane translocation of PKC-β2–GFP from the cytosol to the cell membrane. The graph is representative of five experiments.

RESULTS

PKC-β2 translocation. In HUVECs grown at 5 mmol/l glucose for 48 h, acute exposure to 25 mmol/l glucose did not induce PKC-β2 translocation (Fig. 1). On the contrary, in cells grown at 10 mmol/l glucose, we observed a significant PKC-β2 translocation from the cytosol to the membrane in response to acute exposure to 25 mmol/l glucose (Fig. 2). The basal membrane-to-cytosol fluorescence ratio of PKC-β2–GFP chimera was similar in cells grown in 5 and 10 mmol/l glucose (0.43 ± 0.02 vs. 0.37 ± 0.1 arbitrary units [AU]), as was the total PKC-β2 expression (Fig. 3).

The treatment of cells grown at 10 mmol/l glucose with metformin (20 μmol/l for 48 h) induced a 10-fold reduction of PKC-β2 translocation induced by acute hyperglycemia (25 mmol/l), with a similar half-life (20 min) (Fig. 4). However, metformin did not affect the translocation of PKC-β2 induced by phorbol myristate acid (PMA; 300 nmol/l) (Fig. 4).

To verify whether hyperglycemia-induced oxidative stress is involved in PKC-β2 translocation, we determined the effects of H_2O_2 (500 μmol/l for 20 min). As shown in Fig. 5, the exposure of cells to H_2O_2 induced a sigmoidal activation of PKC-β2 translocation, with a half time of 10 min. On the contrary, in metformin-treated cells, PKC-β2 translocation in response to 500 μmol/l H_2O_2 was very modest and prolonged (half-life 3 h), whereas the PMA response was unaffected (Fig. 6).

Measurement of ROS. Intracellular ROS generation was measured with the fluoroprobe TEMPO-9-AC, and the specificity of the probe was determined in cells grown at 10 mmol/l glucose and pretreated with PEG-catalase (250 units/ml for 24 h) or PEG–superoxide dismutase (250 units/ml for 24 h). As shown in Fig. 7, ROS production induced by 10 mmol/l glucose was significantly attenuated by catalase ($P < 0.001$) and not by superoxide dismutase. These results suggest that the probe specifically reacts with H_2O_2.

Next, we evaluated ROS production in cells grown in normal (5 mmol/l) and high (10 mmol/l) glucose. As shown in Fig. 8*A*, there was no significant basal ROS generation in cells grown in 5 mmol/l glucose, whereas ROS production was increased in cells cultured in 10 mmol/l glucose (Fig. 8*B*). Pretreatment with 20 μmol/l metformin markedly blunted ROS generation in HUVECs cultured in 10 mmol/l glucose (Fig. 8*C*). Basal ROS production in cells grown in 10 mmol/l glucose was significantly higher than in cells grown in 5 mmol/l glucose (26 ± 2 vs. 16 ± 2 fluorescence rate, AU, $P < 0.01$) (Fig. 8*D*). The exposure to metformin (20 μmol/l) in cells cultured in 10 mmol/l glucose signifi-

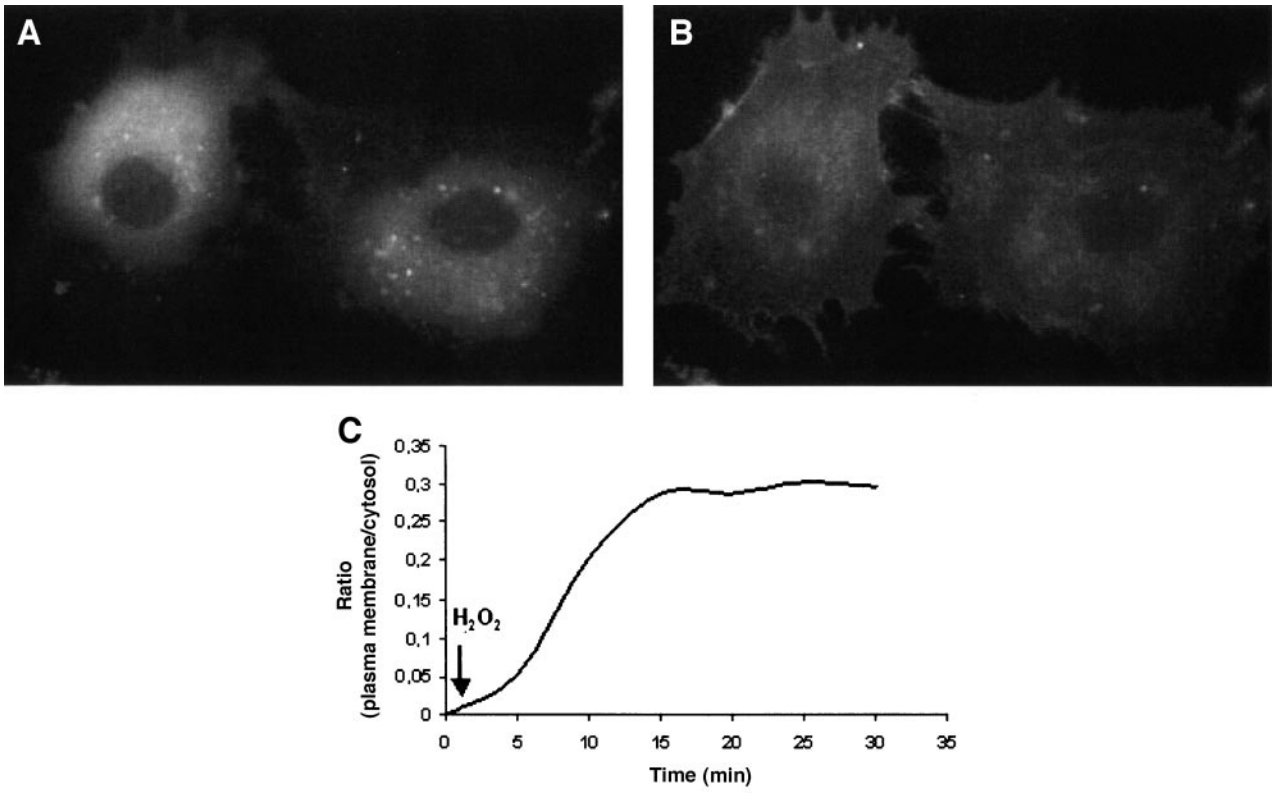

FIG. 5. Effect of H_2O_2 on PKC-β2–GFP translocation. HUVECs are shown before (*A*) and after (*B*) the addition of 500 μmol/l H_2O_2. *C*: The time course of plasma membrane translocation of PKC-β2–GFP from the cytosol to the cell membrane, as previously described, and is representative of five experiments.

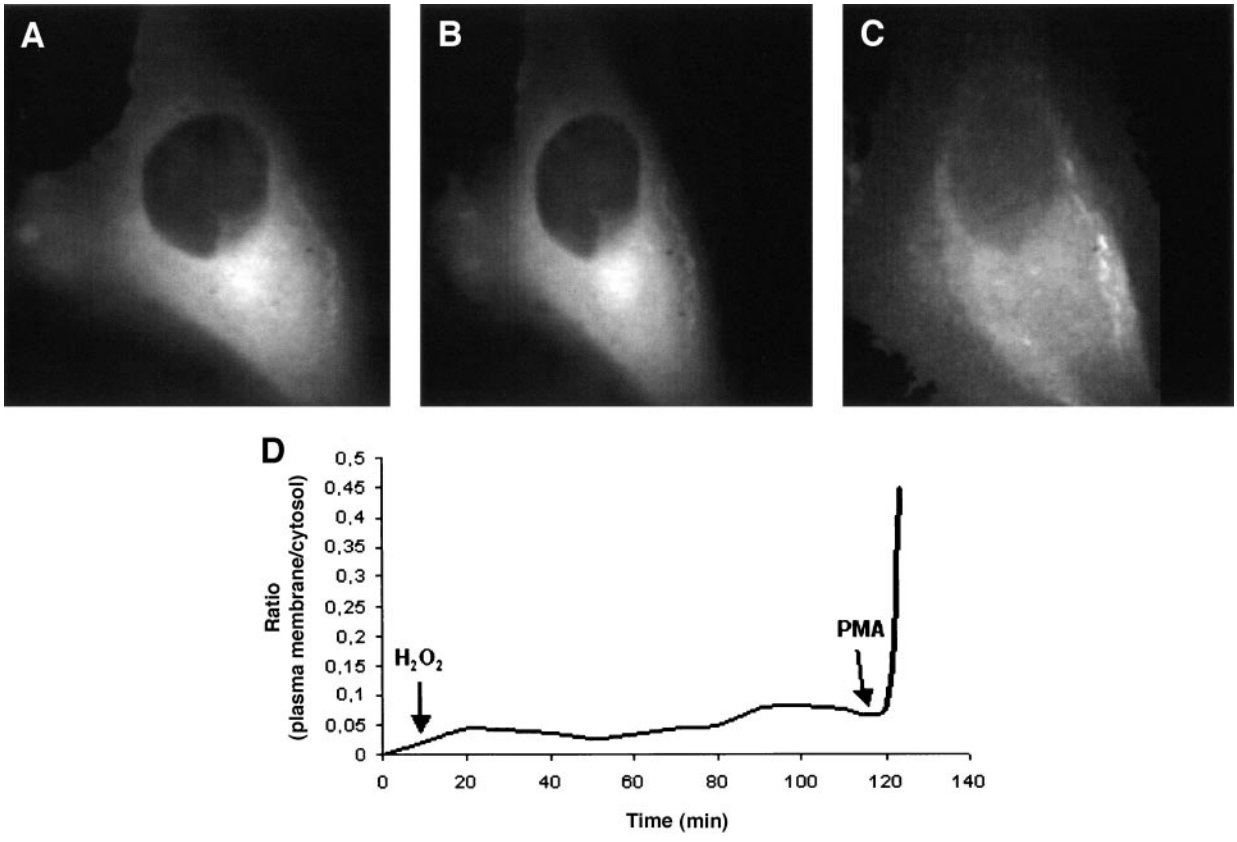

FIG. 6. Effect of metformin on PKC-β2 translocation in HUVECs after H_2O_2 addition. The images represent cells grown with normal glucose (5 mmol/l) and 20 μmol/l metformin for 48 h before (*A*) and 2 h after (*B*) the addition of 500 μmol/l H_2O_2. *C*: The same cell after the addition of PMA (300 nmol/l) for 5 min. *D*: The time course of plasma membrane translocation of PKC-β2–GFP from the cytosol to the cell membrane. The graph is representative of five experiments.

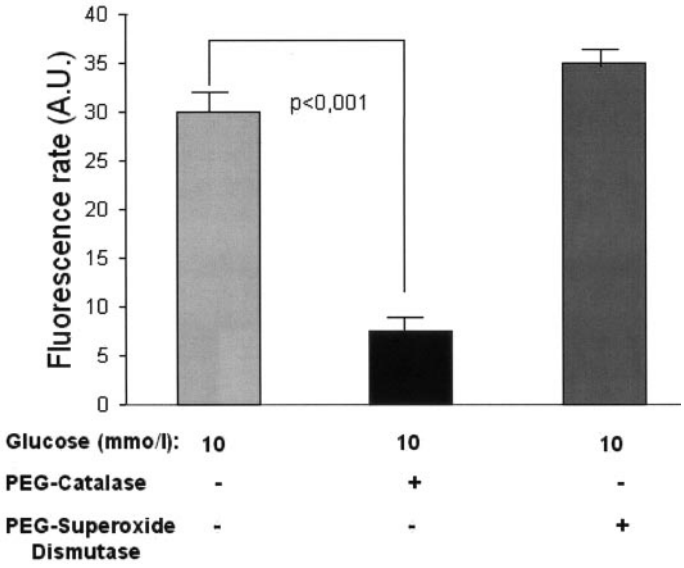

FIG. 7. TEMPO-9-AC specificity. HUVECs were treated with PEG superoxide dismutase (250 units/ml, for 24 h) and poliethilglicole catalase (250 units/ml, for 24 h). Cells were loaded with TEMPO-9-AC (10 μmol/l) for 10 min at 37°C in physiological buffer. The fluorescence was monitored at an excitation wavelength of 358 nm and an emission wavelength of 461 nm with a DAPI (4,6-diamidino-2-phenylindole) filter.

cantly reduced ROS production (from 26 ± 2 to 10 ± 2 AU, $P < 0.01$) (Fig. 8*D*).

To understand the source of glucose-induced ROS production, we determined the effects of apocynin and DPI, inhibitors of plasma membrane NAD(P)H oxidase, and of rotenone, an inhibitor of mitochondrial electron transport. As shown in Fig. 9, the effect of hyperglycemia on ROS production was completely abolished by the treatment of cells with apocynin and DPI (16 ± 2 and 19 ± 2 vs. 31 ± 2 AU, $P < 0.005$) but not by rotenone. The effect of metformin on ROS production was not affected by the presence of rotenone (data not shown). We then evaluated the effects of metformin on xanthine oxidase and catalase activity in HUVECs grown at 5 and 10 mmol/l glucose. As shown in Fig. 10, xanthine oxidase activity was 3.3-fold increased at 10 mmol/l glucose with respect to 5 mmol/l glucose ($P < 0.001$), but it was not modified by metformin treatment.

Catalase activity was increased by 26% under hyperglycemic conditions in comparison to euglycemic conditions (0.038 ± 0.001 vs. 0.030 ± 0.0002 units/mg, $P < 0.029$). The treatment of cells with metformin induced a further increase of catalase activity at both 5 mmol/l glucose (0.041 ± 0.0003 vs. 0.030 ± 0.0001 units/mg, $P < 0.002$) and 10 mmol/l glucose (0.046 ± 0.0002 vs. 0.038 ± 0.0002 units/mg, $P < 0.041$) (Fig. 11).

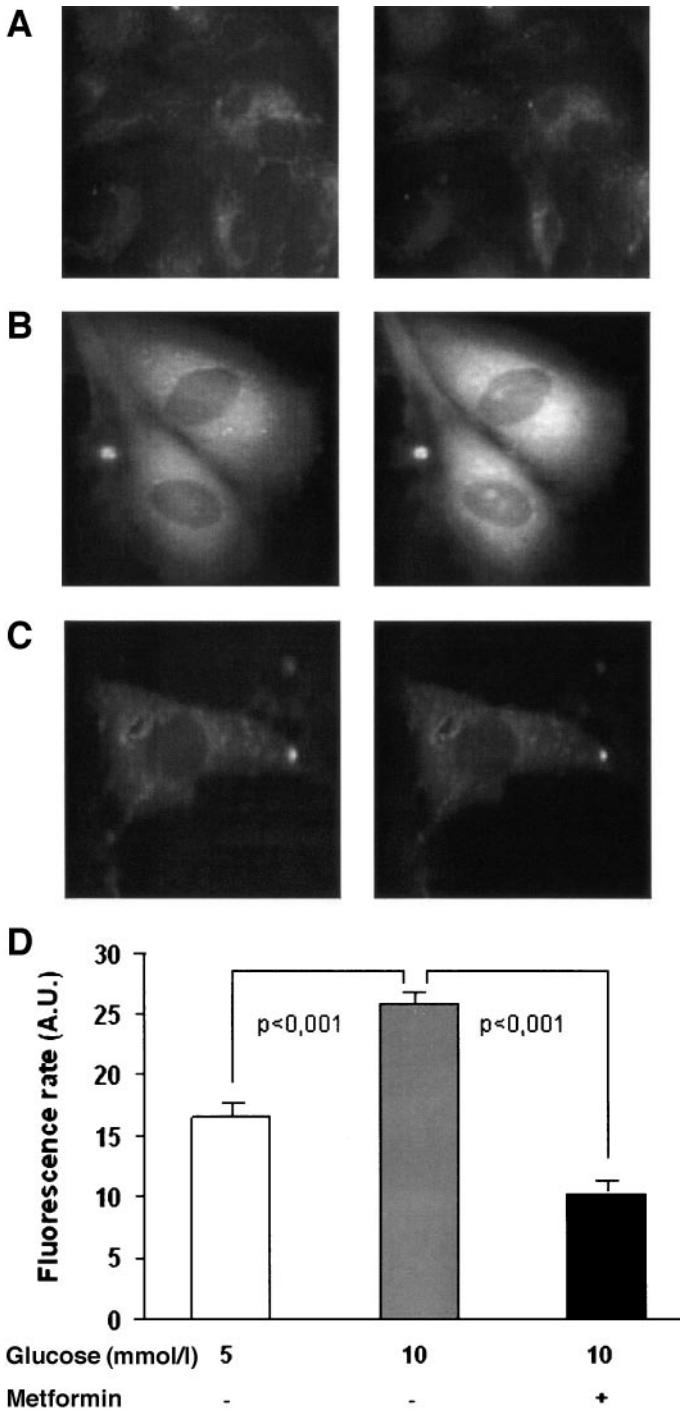

FIG. 8. Measurement of ROS. Intracellular ROS generation was measured in HUVECs with the fluoroprobe TEMPO-9-AC. *A*: The fluorescence intensity measured in HUVECs grown at 5 mmol/l glucose, observed at time 0 and after 10 min. *B*: The fluorescence intensity in HUVECs grown at 10 mmol/l glucose, at time 0 and after 10 min. *C*: The fluorescence intensity in the HUVECs grown at 10 mmol/l glucose and 20 μmol/l metformin for 48 h, observed at time 0 and after 10 min. *D*: The fluorescence rate, calculated as the slope of the linear least squares fitting of fluorescence intensity for 10 min. Data are the means ± SE of 10 experiments.

DISCUSSION

In this study we demonstrated that *1*) acute hyperglycemia induces the translocation of PKC-β2 from the cytosol to the membrane only in cells previously exposed to chronic hyperglycemia, *2*) metformin inhibits hyperglycemia-induced PKC-β2 translocation, *3*) chronic hyperglycemia produces oxidative stress that is likely mediated by the plasma membrane NAD(P)H oxidase mechanism and is blunted by metformin, and *4*) catalase activity is increased in cells exposed to chronic hyperglycemia and to metformin, whereas xanthine oxidase activity is increased by hyperglycemia but unaffected by metformin treatment. These findings suggest that oxidative stress induced by chronic hyperglycemia plays an important role in pro-

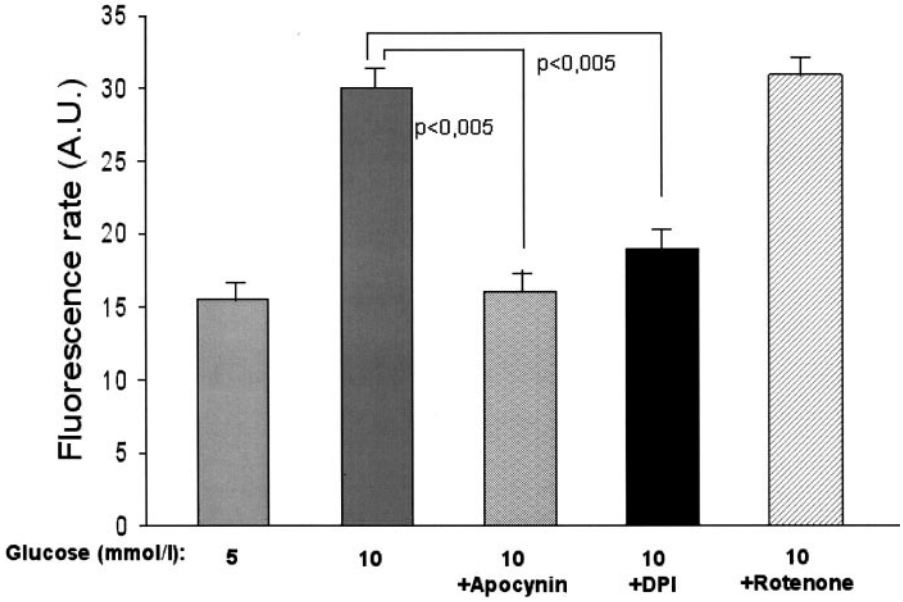

FIG. 9. Analysis of cell ROS generation in the presence of different inhibitors. The cells were cultured in 10 mmol/l glucose in the presence of DPI (50 μmol/l, for 1 h), apocynine (100 μmol/l, for 1 h), rotenone (100 nmol/l, for 1 h), and oxypurinol (10 μmol/l, for 1 h). The fluorescence rate was calculated as the slope of the linear least squares fitting of fluorescence intensity for 10 min. Data are the means ± SE of five experiments.

moting the activation of PKC-β2 triggered by acute hyperglycemia.

These in vitro findings have a strong analogy with postprandial hyperglycemia, a condition that can directly promote atherogenesis, thus predisposing both diabetic and nondiabetic individuals to cardiovascular complications (10). Our study in living endothelial cells demonstrates that elevated glucose concentrations cause complex and dynamic changes in the localization of the glucose-sensitive PKC-β2 isoform. Interestingly, we show that an acute glucose challenge is able to induce the PKC translocation only in cells that were previously exposed to chronic hyperglycemia. This suggests that acute hyperglycemia is necessary but not sufficient to activate PKC-β2 and that it may be related to the intracellular content of diacylglycerol (6), as clearly shown by Ido et al. (23).

The activation of PKC in diabetes is crucial for the development and progression of long-term complications. Therefore, several clinical trials are evaluating the effects of specific inhibitors with considerable selectivity and reversibility, recently synthesized (24). The UKPDS demonstrated a lower incidence of death caused by coronary artery disease in obese type 2 diabetic patients treated with metformin than in patients treated with other therapies but with the same glycemic control. This suggests that metformin may possess a protective effect against diabetes-related cardiovascular complications and that it may improve endothelial function in vivo in diabetic humans (25) and in rats by direct mechanisms (26). Our data support these hypotheses and demonstrate that in vitro metformin attenuates the translocation of PKC-β2 from the cytosol to the membrane induced by acute hyperglycemia in cells previously exposed to chronic hyperglycemia, independent of the intracellular PKC-β2 protein concentration.

Several studies have reported that high glucose may play an important role in the activation of PKC through ROS production (7,8). It is known that oxidative stress activates PKC through the regulatory role of calcium-independent phospholipases A2 (9), and, on the other hand, PKC itself may induce oxidative stress through the phosphorylation of p47phox subunit of NAD(P)H oxidase (27); indeed, antioxidants, such as vitamin E, inhibit hyperglycemic PKC activation in vascular tissues (28,29).

In this study, we demonstrated that hydrogen peroxide induced a marked PKC-β2 activation and that this effect was inhibited by the treatment of cells with metformin. Therefore, our findings suggest that mild chronic hyperglycemia may cause a condition of cellular oxidative stress, which is the major factor responsible for PKC-β2 activation in HUVECs. To confirm this, we demonstrated,

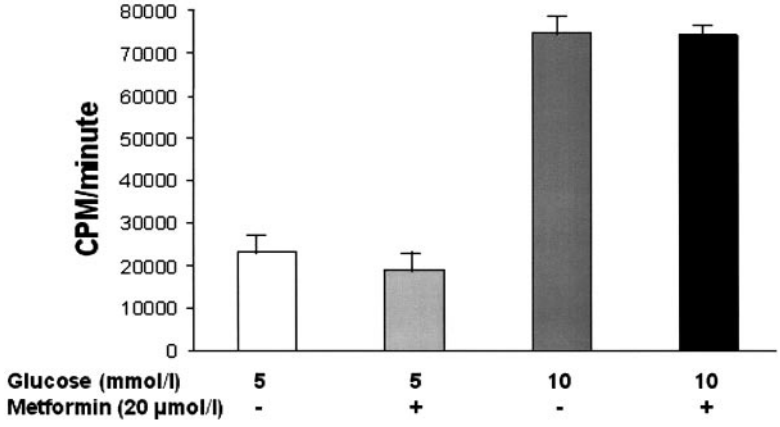

FIG. 10. Xanthine oxidase. Cells grown at 5 and 10 mmol/l glucose were treated for 48 h with metformin (20 μmol/l). Data are the means ± SE of six experiments.

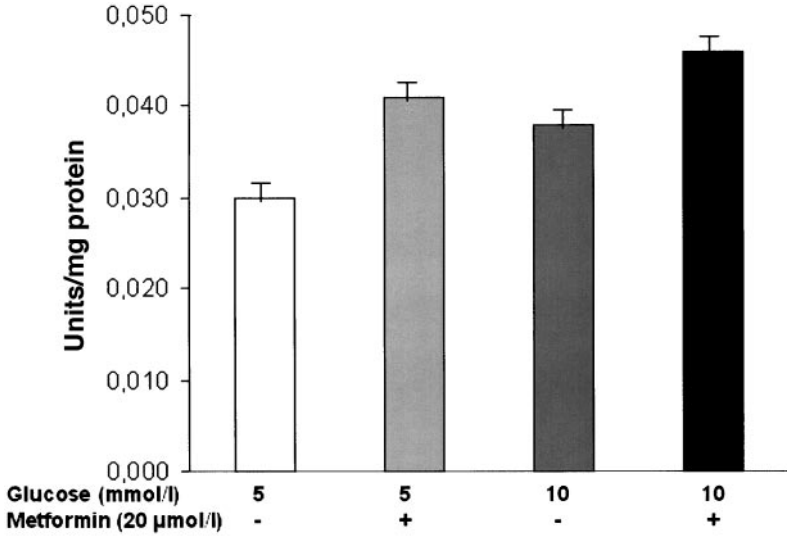

FIG. 11. Catalase activity. Cells grown at 5 and 10 mmol/l glucose were treated for 48 h with metformin (20 μmol/l). All experiments were performed in duplicate. Results are the means of six experiments.

by using a hydrogen peroxide–specific probe, an increase in the oxidative stress level that was blunted by metformin treatment. This effect was also abolished by DPI and apocynin, NAD(P)H inhibitors, whereas we did not find any effects of rotenone on H_2O_2 production. Although it has been demonstrated that hyperglycemia may increase the production of ROS from the mitochondrial electron transport chain (5), this was observed at higher glucose concentrations (30 mmol/l) than those used in this study. Thus, under our experimental conditions, NAD(P)H oxidase represents the main pathway responsible for the overproduction of H_2O_2 in mild chronic hyperglycemia, and it may be a target of the action of metformin.

We also tested whether metformin could exert its antioxidant activity by modifying other oxidases. We found that hyperglycemia significantly increases the activity of xanthine oxidase in HUVECs. However, we did not observe any effect of metformin on this enzyme. We further tested the hypothesis that metformin could influence the activity of catalase, which converts hydrogen peroxide to water. We thus tested the activity of catalase in HUVECs cultured in 5 and 10 mmol/l glucose with and without metformin (20 μmol/l). We found that mild hyperglycemia induces a slight increase in catalase activity, and we also observed a slight but significant upregulation of this enzyme in the presence of metformin, under conditions of both eu- and hyperglycemia. Therefore, our data demonstrate that metformin reduces the production of ROS also by its ability to stimulate endogenous scavenging mechanisms such as catalase. To date, the exact molecular mechanisms of metformin action are still poorly understood, although it has been recently shown that this compound can act through an indirect activation of AMPK, which is impaired in type 2 diabetes (30).

Recently, He et al. (17) offered evidence that the elusive target of metformin actions is AMPK, and they demonstrated that metformin, at a concentration of 10 to 40 μmol/l, is required for time-dependent AMPK activation in rat hepatocytes in vitro. In a recent study, Zou et al. (31) found that elevated concentrations may generate mitochondrial $O_2^{\bullet -}$; however, our current data would suggest that metformin stimulates AMPK on one site; whereas, it inhibits PKC on the other site. These effects appear relevant not only for the treatment of hyperglycemia but also for preventing long-term complications beyond its antihyperglycemic action. This integrated regulation by PKC and AMPK has also been recently confirmed for endothelial nitric oxide synthase (32).

We conclude that in living endothelial cells, hyperglycemia causes translocation of the PKC-β2 isoform and that metformin inhibits this effect because of a direct antioxidant effect mediated by inhibiting NAD(P)H oxidase and stimulating catalase activity. These findings not only substantiate the beneficial effects of metformin shown in large intervention studies in type 2 diabetic patients but also offer a clear explanation for the positive effects that these drugs have on the vascular system.

ACKNOWLEDGMENTS

We thank Telethon-Italy (grants 1285 and GTF01011), the Italian Association for Cancer Research (AIRC), the Human Frontier Science Program, the Italian Ministry of University and Research (MIUR), the Italian Space Agency (ASI), and the National Research Council (CNR) for financial support. G.A.R. was supported by a research leave fellowship and program grant (067081/Z/02/Z) from the Welcome Trust and project grants from the Medical Research Council and Juvenile Diabetes Research Foundation International.

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