Antioxidants Improve Impaired Insulin-Mediated Glucose Uptake and Prevent Migration and Proliferation of Cultured Rabbit Coronary Smooth Muscle Cells Induced by High Glucose

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Background—To explore the role of intracellular oxidative stress in high glucose–induced atherogenesis, we examined the effect of probucol and/or α-tocopherol on the migration and growth characteristics of cultured rabbit coronary vascular smooth muscle cells (VSMCs).

Methods and Results—Chronic high-glucose-medium (22.2 mmol/L) treatment increased platelet-derived growth factor (PDGF)-BB–mediated VSMC migration, [3H]thymidine incorporation, and cell number compared with VSMCs treated with normal-glucose medium (5.6 mmol/L+16.6 mmol/L mannose). Probucol and α-tocopherol significantly suppressed high glucose–induced increase in VSMC migration, cell number, and [3H]thymidine incorporation. Probucol and α-tocopherol suppressed high glucose–induced elevation of the cytosolic ratio of NADH/NAD⁺, phospholipase D, and membrane-bound protein kinase C activation. Probucol, α-tocopherol, and calphostin C improved the high glucose–induced suppression of insulin-mediated [3H]deoxyglucose uptake. Chronic high-glucose treatment increased the oxidative stress, which was significantly suppressed by probucol, α-tocopherol, suramin, and calphostin C.

Conclusions—These findings suggest that probucol and α-tocopherol may suppress high glucose–induced VSMC migration and proliferation via suppression of increases in the cytosolic ratio of free NADH/NAD⁺, phospholipase D, and protein kinase C activation induced by high glucose, which result in reduction in intracellular oxidative stress.

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Key Words: antioxidants ■ muscle, smooth ■ phospholipase ■ protein kinase ■ insulin

Hyperglycemia is an important causative factor in the development of macrovascular complications in diabetes, such as coronary artery disease after percutaneous transmural coronary angioplasty, in which vascular smooth muscle cell (VSMC) migration and proliferation appear to play an important role. However, the mechanisms responsible for accelerated atherosclerosis in diabetes are not clear.

It has been reported that elevated glucose increases oxidative stress, which may play an important role in diabetic vascular complications. Probucol and α-tocopherol have been shown to have potent antioxidant effects. The finding that active oxygen species stimulate VSMC growth suggests that the antioxidants probucol and α-tocopherol might prevent high glucose–induced migration and proliferation of VSMCs, possibly through antioxidative effects.

Insulin resistance and hyperinsulinemia appear to be independent risk factors for ischemic heart disease. In addition to the primary insulin resistance, there is a secondary insulin resistance that results from elevated glucose levels, although this is not the same as what happens in type II diabetes in vivo. VSMCs have been found to have insulin receptors and to exhibit insulin-induced responses. It is not known, however, to what extent the increased oxidative stress and insulin resistance reflect increased VSMC migration and proliferation.

Therefore, the present study examined the effects of probucol and/or α-tocopherol on high glucose–induced coronary VSMC migration and proliferation and high glucose– and insulin-mediated glucose uptake and proliferation by coronary VSMCs. We also designed an experiment to examine intracellular redox state directly by flow cytometry and to examine the role of phospholipase D (PLD) and protein kinase C (PKC) in high glucose–induced oxidative stress, because the involvement of PLD and PKC in high glucose–induced vascular growth has been reported.

Methods

Materials
Suramin and calphostin C were purchased from Sigma Chemical Co. Carboxydichlorofluorescein (CDCFH) diacetate bis-acetoxyethyl
(AM) ester was purchased from Molecular Probe Co. [3H]thymidine, [3H]ethanolamine, and [3H]deoxyglucose ([3H]DOG) were purchased from Amersham Japan Co.

**Cell Culture**

VSMCs were grown from explants of 4-week-old male Japanese White rabbit coronary arteries by the explant method. Cells were identified as VSMCs on the basis of their morphological and growth characteristics as previously reported.12 Briefly, VSMCs exhibited a typical "hill-and-valley" growth pattern and also exhibited positive fluorescence with antibodies against α-smooth muscle actin but no fluorescence with antibodies against factor VIII antigen. VSMCs were grown in DMEM supplemented with 10% FCS.

**Experimental Protocol**

In the migration and [3H]DOG uptake experiment, plated cells were allowed to grow for 72 hours in normal-glucose (5.6 mmol/L glucose +16.6 mmol/L mannose) or high-glucose (22.2 mmol/L) medium with 10% FCS in the absence or presence of probucol or α-tocopherol. Plate cells were used in the [3H]DOG experiment. Plated cells were trypsinized and suspended in DMEM with 0.1% FCS. Then cells were used in the migration experiment. In growth and signal transduction experiments, plated cells were allowed to grow for 72 hours in normal- or high-glucose medium with 10% FCS in the absence or presence of probucol and/or α-tocopherol. Then cells were cultured in DMEM with 0.1% FCS for 48 hours to try to induce quiescence. Glucose and antioxidants were included for this period. Plated cells were used in cell count experiments, [3H]thymidine incorporation experiments, and measurement of PLD and PKC. In migration experiments, PDGF-BB was used.

**Migration Assay**

The migration of VSMCs was assayed by a modification of Boyden’s chamber method using microchemotaxis chambers (Neuro Probe Inc) and polycarbonate filters (Nucleopore Corp) with pores 5.0 μm in diameter, as previously reported.13 A volume of 200 μL of VSMC suspension (3.0×10^4 cells) was placed in the upper chamber, and 40 μL of DMEM containing a migration factor such as PDGF-BB was placed in the lower chamber. The chamber was incubated at 37°C under 5% CO2 in air for 4 hours. After incubation, the filter was removed and the VSMCs on the upper side of the filter were scrapped off. The VSMCs that had migrated to the lower side of the filter were fixed in methanol, stained with Diff-Quick staining solution, and counted under a microscope for quantification of VSMC migration. Migration activity was expressed as the number of cells that had migrated per high-power field (×400).

**Effect of Elevated Glucose on Cell Numbers**

VSMCs were placed in 6-well culture dishes and grown in high- or normal-glucose DMEM containing 10% FCS for 72 hours. After the medium was aspirated, the same medium with 0.1% FCS was applied for 48 hours. Cultures were washed with a calcium- and magnesium-free PBS (PBS(−)) and resuspended with trypsin EDTA solution. Counts were performed with an electronic cell counter.13

**Determination of DNA Synthesis**

Relative rates of DNA synthesis were assessed by determination of [3H]thymidine incorporation into trichloroacetic acid–precipitable material.12 After VSMCs were treated as described in the experimental protocol, cells grown in 24-well culture dishes were pulsed 4 hours with [3H]thymidine (10 μCi/mL), washed with cold PBS(−), and incubated with 5% trichloroacetic acid at 4°C for 10 minutes. Cells were dissolved in 1N NaOH at 37°C for 30 minutes and then neutralized. Radioactivity was determined by liquid scintillation counting.

![Graph](image-url)
Flow Cytometric Analysis of Cell Cycle Stage

After VSMCs were treated as described in the experimental protocol, VSMCs grown in flasks were detached with 0.25% trypsin at 37°C for 5 minutes and then pelleted by centrifugation (1000 rpm for 55 minutes). The cells were resuspended in 200 μL of solution A [trypsin 30 mg/L, citric acid 3.4 mmol/L, spermine 1.5 mmol/L, Tris/HCl 0.5 mmol/L, Nonidet P-40 2 mL/L]. Ten minutes later, 150 μL of solution B (trypsin inhibitor 500 mg/L, RNase 100 mg/L, citric acid 3.4 mmol/L, spermine 1.5 mmol/L, Tris/HCl 0.5 mmol/L, Nonidet P-40 2 mL/L) was added, and the mixture was left to stand for 10 minutes. All cell cycle samples were analyzed within 3 hours with a flow cytometer (EPICS Profile).

Metabolic and Biochemical Assays

VSMCs were incubated in normal or high glucose with or without probucol or α-tocopherol 100 nmol/L for 24 to 120 hours. Incubations were terminated by rapid addition of 3N perchloric acid to the culture medium with shaking. The tubes were then centrifuged, and the supernatant was removed and assayed for fructose by standard enzymatic methods. The effect of elevated glucose levels on the cytosolic lactate/pyruvate ratio was measured as an indicator of the cytosolic NADH/NAD⁺ ratio. The concentration of lactate or pyruvate was measured by the enzymatic method using lactate oxidase or pyruvate oxidase, respectively.

PLD Activity Measured by Ethanolamine Release

VSMCs were allowed to grow for 24 to 72 hours in high- or normal-glucose DMEM with 10% FCS in the absence or presence of antioxidants. Then cells were cultured in high- or normal-glucose DMEM with 0.1% FCS for 48 hours. VSMCs in 35-mm dishes were cultured in medium containing [³H]ethanolamine (5 μCi · mL⁻¹ · dish⁻¹) for 24 hours (the latter half of the 48-hour period with 0.1% FCS) to label cellular phosphatidylethanolamine. Glucose and antioxidants were included for this period. After removal of the labeling medium, the cells were washed twice with buffer A [20 mmol/L HEPES (pH 7.4), 120 mmol/L NaCl, 5.6 mmol/L glucose 16.6 mmol/L mannose, or 22.2 mmol/L glucose]. After 0.5 to 1 hour of incubation with buffer A, the reaction was terminated by removing buffer A and adding 0.75 mL methanol. The cells were harvested by gentle scraping. Ethanolamine metabolites from the aqueous phase were fractionated on Dowex 50 w (H⁺) packed columns as previously described.

Cell Fractionation and Assay of PKC

VSMCs were cultured for 24 to 72 hours in normal- or high-glucose DMEM with 10% FCS in the absence or presence of antioxidants. Thereafter, cells were cultured in normal- or high-glucose medium with 0.1% FCS for 48 hours. Then VSMCs were washed twice with an ice-cold assay buffer [50 mmol/L Tris/HCl (pH 7.5) buffer containing 2 mmol/L EDTA, 2 mmol/L EGTA, 0.25 mol/L sucrose, 10 mmol/L 2-mercaptoethanol, 0.21 mmol/L leupeptin, and 0.23 mmol/L phenylmethylsulfonyl fluoride]. Cells were then scraped and sonicated with three 10-second bursts. The homogenates were centrifuged at 100 000 g for 60 minutes at 4°C to separate the cytosolic and particulate fractions. The pellet resuspended in the assay buffer containing 1% Nonidet P-40 was stirred on ice for 1 hour and was then centrifuged at 100 000g for 30 minutes. PKC activity was measured by a modification of a method previously reported using the Amersham PKC assay system.
Glucose Transport Analyses

VSMCs were cultured for 72 hours in normal- or high-glucose DMEM in the absence or presence of antioxidant, suramin, or calphostin C. For glucose transport studies, VSMCs were grown to confluence and on the day of the experiment were incubated with physiological salt solution (PSS) containing 145 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L HEPES, 1 mmol/L MgSO₄, 0.5 mmol/L Na₂HPO₄, and 1.5 mmol/L CaCl₂. Cells were acclimatized in PSS for 1 hour, after which the buffer was replaced with PSS containing vehicle (0.01 mol/L HCl) or 100 μU/mL porcine insulin. After 20 minutes of this pretreatment, solutions were replaced with identical solutions containing tracer amounts (700 pmol/L) of [3 H]DOG. Transport of [3 H]DOG was allowed to proceed for 5 minutes. Wells were then aspirated and washed 3 times with ice-cold PSS. Cells were solubilized with 0.5 mol/L NaOH and neutralized with HCl, and the mixture was quantitatively transferred to scintillation vials. Radioactivity was then determined.

Assay of Intracellular Redox State

Intracellular redox state levels were measured with a fluorescent dye, CDCFH diacetate bis-AM ester, a nonpolar compound that is converted into a nonfluorescent polar derivative (CDCF) by cellular esterases after incorporation into cells. CDCF is membrane-impermeable and rapidly oxidized to the highly fluorescent carboxydichlorofluorescein (CDCF) in the presence of intracellular hydrogen peroxide and peroxidases. For assays, medium was replaced with Hank’s solution containing 3 mmol/L NaCl, 15 mmol/L KCl, 10 mmol/L HEPES, 1 mmol/L MgSO₄, 0.5 mmol/L Na₂HPO₄, and 1.5 mmol/L CaCl₂. Cells were incubated for 20 minutes at room temperature with 20 μM CDCFH diacetate bis-AM ester, washed four times with ice-cold PSS, and resuspended in 1 ml of PSS. The excitation wavelength was 510 to 530 nm. Relative fluorescence intensities were calculated with untreated control cells as a standard.

Statistical Methods

Statistical analysis was performed by ANOVA and Scheffe’s modified t test. Values of P < 0.05 were considered significant.

Results

Inhibition of High Glucose–Induced Cell Migration by Probucol and/or α-Tocopherol

The effects of probucol and α-tocopherol on the migration of VSMCs treated with PDGF-BB 1 ng/mL are shown in Figure 1. Probucol and/or α-tocopherol significantly inhibited high glucose–potentiated VSMC migration in the presence of 1 ng/mL PDGF-BB in a dose-dependent manner. The PLD inhibitor suramin or the PKC inhibitor calphostin C also significantly inhibited VSMC migration. VSMCs cultured in normal glucose were unaffected by probucol and/or α-tocopherol 100 nmol/L.

Inhibition of High Glucose–Induced Cell Proliferation by Probucol and/or α-Tocopherol

As shown in Figure 2, cell proliferation in high-glucose medium was more accelerated than that in normal-glucose medium. Probucol and α-tocopherol individually and to-
Coronary VSMCs were plated in T-25 flasks and cultured in DMEM with 5.6 mmol/L glucose (G) + 16.6 mmol/L mannose (M), 22.2 mmol/L glucose, or 22.2 mmol/L glucose + probucol (P) 100 nmol/L, α-tocopherol (VE) 100 nmol/L, suramin (S) 100 μmol/L, or calphostin C (C) 100 nmol/L until confluent. Cells were maintained in 0.1% FCS for 48 hours to try to induce quiescence. Cell cycle stage was determined by flow cytometry as described in Methods. Each value represents the mean±SD percent area of 12 determinations for 3 or 4 different cell preparations.

*P<0.05 vs corresponding control in glucose 5.6 mmol/L + mannose 16.6 mmol/L group.
†P=NS: not significantly different vs corresponding control in glucose 5.6 mmol/L + mannose 16.6 mmol/L group.

Protein kinase C (PKC) activities with normal and high glucose

As shown in Figure 5, PLD activities in high glucose–treated cells were greater than those in normal glucose–treated cells. With normal-glucose medium was coincubated with probucol and/or α-tocopherol for 24 to 120 hours at 100 mmol/L, glucose-induced PLD activation was significantly reduced (Figure 5A).

PLD Activities With Normal- and High-Glucose Media

As shown in Figure 5, PLD activities in high glucose–treated cells were greater than those in normal glucose–treated cells. When high-glucose medium was coincubated with probucol and/or α-tocopherol for 24 to 120 hours at 100 mmol/L, glucose-induced PLD activation was significantly reduced (Figure 5A).

PKC Activities With Normal and High Glucose

As shown in Figure 6, membrane-bound (particulate) PKC activities in high glucose–treated cells were greater than those in normal glucose–treated cells. With normal-glucose medium, neither probucol nor α-tocopherol reduced basal PKC activity. When high-glucose medium was coincubated with probucol or α-tocopherol 100 mmol/L, or suramin 100 μmol/L for 24 to 120 hours, glucose-induced PKC activation was significantly reduced (Figure 6A).

Insulin-Stimulated [³H]DOG Uptake and VSMC Proliferation

After 72 hours of incubation with 22.2 mmol/L glucose, insulin-stimulated [³H]DOG uptake was significantly decreased. This decrease was significantly prevented by coincubation with 10 μmol/L suramin, 100 mmol/L calphostin C, probucol, or α-tocopherol. [³H]thymidine incorporation was measured to study the effect of high glucose on insulin-mediated VSMC growth. High-glucose treatment for 72
hours enhanced [3H]thymidine incorporation. This change was significantly prevented by coincubation with suramin, calphostin C, probucol, or a-tocopherol (Figure 7).

**Effects on VSMC Intracellular Redox State of Probucol and/or a-Tocopherol**

High-glucose treatment for 24 to 120 hours increased oxidative stress 78% to 104% (Figure 8A). Figure 8B shows representative effects on results of oxidative stress of coronary VSMCs as measured by flow cytometry. Probucol and/or a-tocopherol, suramin, and calphostin C significantly decreased intracellular oxidative stress.

**Discussion**

We demonstrated in the present study that high-glucose medium potentiated PDGF-BB–mediated migration of coronary VSMCs and that probucol and a-tocopherol dose-dependently inhibited this increase in migration. It has been reported that high-glucose medium increases the production of active oxygen species and that the suppression of increased active oxygen species by catalase and by the antioxidant N-acetylcysteine results in suppression of migration of VSMCs. These findings suggest that increased oxidative stress by high glucose may cause increased migration of VSMCs and that the antioxidants probucol and a-tocopherol may prevent this increase. We also showed in the study that PLD activity was increased in high glucose–treated cells and that this increase was prevented by probucol and a-tocopherol. Because PLD increases formation of diacylglycerol, which may play a role in migration of VSMCs (Figure 1), suppression of PLD by probucol and/or a-tocopherol may in turn suppress the increase in VSMC migration induced by high glucose.

There are at least 3 possible mechanisms of inhibition of high glucose–induced proliferation of coronary VSMCs by probucol and a-tocopherol. The first is suppression of oxidative modification of LDLs, as already reported. The second is suppression of the polyol pathway. In the present study, we showed that the increase in fructose and NADH/NAD⁺ ratio resulting from sorbitol oxidation in the second step of the polyol pathway induced by high glucose was suppressed by probucol and a-tocopherol, suggesting that antioxidants may block this pathway. In fact, the antioxidant vitamin C is reported to suppress the polyol pathway activity induced by high glucose. The third possible mechanism is suppression of the intracellular oxidative stress induced by high glucose, because production of active oxygen species can occur as a result of glycoxidation and increased PKC activity, and 2 different antioxidants were found to suppress VSMC migration and proliferation.

In the present study, probucol and a-tocopherol suppress the high glucose–induced increase in membrane-bound PKC.
activity. Because activated PKC produces active oxygen species, and active oxygen species stimulate VSMC growth, it is reasonable to assume that this suppression of increase in PKC activity by probucol and \( \alpha \)-tocopherol plays a role in suppression of high glucose–mediated VSMC proliferation. It has been reported that the specific PKC inhibitors PKC (19–36) and H-7 prevent high glucose–induced proliferation through PKC suppression. Because PLD is reported to activate PKC in VSMCs, and PKC activation is suppressed by suramin (Figure 6), it is possible

**Figure 6.** Bar graph shows distribution of PKC activities in particulate fractions of VSMCs. Results are mean±SD of 12 determinations. VSMCs were grown to confluence and then kept in DMEM containing indicated concentration of glucose with or without probucol (P) and/or \( \alpha \)-tocopherol (VE) or suramin (S) for 24 to 120 hours (A) or 72 hours (B) before PKC activity was measured as described in Methods. SDs of each group were 5% to 18% and were not significantly different from normal-glucose (NG) group, except high-glucose (HG) group. *P<0.05.

**Figure 7.** Insulin-stimulated \(^{3}H\)-DOG uptake and VSMC proliferation. VSMCs were grown to confluence and kept in DMEM containing indicated concentration of glucose (mol/L; M) with or without probucol and/or \( \alpha \)-tocopherol at 100 nmol/L for 72 hours before insulin-stimulated \(^{3}H\)-DOG uptake and \(^{3}H\)-thymidine incorporation measurement as described in Methods. Shaded bars represent insulin-stimulated condition. Results are mean±SD of 12 determinations. *P<0.05.
that antioxidants inhibit PLD, in turn suppressing PKC activity.

Probucol and α-tocopherol inhibited increases in the percentage of VSMCs in the S and G2-M stages induced by high glucose. Because probucol and α-tocopherol decreased PKC activity and activation of PKC is required for cell cycle progression and S-phase entry of VSMCs,11 it may be that glucose-induced cell cycle progression is due to PKC activation and that this change is blocked by antioxidants.

This study examined the effect of high glucose on insulin-mediated [3H]DOG uptake. Chronic glucose treatment decreased insulin-mediated [3H]DOG uptake. This insulin resistance may be due to increased PKC activity, because activation of PKC increases insulin resistance.13 However, PKC activation with phorbol esters is well known to increase glucose uptake.24,25 These findings suggest that PKC activation by high glucose differs from that by phorbol ester. In fact, it has been reported that PKC isoforms activated by high
glucose are different from those activated by phorbol ester.\textsuperscript{26} Probulc and α-tocopherol improved the insulin resistance in VSMCs induced by high glucose. Because troglitazone was shown to prevent the glucose-induced inhibition of insulin receptor,\textsuperscript{9,13} it may be that probucol and α-tocopherol restore high glucose–induced insulin resistance by their antioxidative effects. Normalization of PKC activity by probucol and α-tocopherol may play some role in this effect.

In conclusion, our findings suggest that oxidative stress may play a role in the vascular migration and proliferation induced by high-glucose medium. The intracellular mechanism of action of antioxidations may include the suppression of PLD activation, PKC activation, and increase in percentage of cells in the S phase in the cell cycle induced by high glucose, and this suppression may be the result of the intracellular antioxidative effects. PKC may thus be a link between high glucose, oxidative stress, impaired insulin-mediated glucose uptake, and increased insulin-mediated VSMC growth.

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References

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