Aldose Reductase Inhibitor Improves Insulin-Mediated Glucose Uptake and Prevents Migration of Human Coronary Artery Smooth Muscle Cells Induced by High Glucose

Kenichi Yasunari, Masakazu Kohno, Hiroaki Kano, Mieko Minami, Junichi Yoshikawa

Abstract—We examined involvement of the polyol pathway in high glucose–induced human coronary artery smooth muscle cell (SMC) migration using Boyden’s chamber method. Chronic glucose treatment for 72 hours potentiated, in a concentration-dependent manner (5.6 to 22.2 mol/L), platelet-derived growth factor (PDGF) BB–mediated SMC migration. This potentiation was accompanied by an increase in PDGF BB binding, because of an increased number of PDGF-β receptors, and this potentiation was blocked by the aldose reductase inhibitor epalrestat. Epalrestat at concentrations of 10 and 100 nmol/L inhibited high glucose–potentiated (22.2 mmol/L), PDGF BB–mediated migration. Epalrestat at 100 nmol/L inhibited a high glucose–induced increase in the reduced/oxidized nicotinamide adenine dinucleotide ratio and membrane-bound protein kinase C (PKC) activity in SMCs. PKC inhibitors calphostin C (100 nmol/L) and chelerythrine (1 μmol/L) each inhibited high glucose–induced, PDGF BB–mediated SMC migration. High glucose–induced suppression of insulin-mediated [3H]-deoxyglucose uptake, which was blocked by both calphostin C (100 nmol/L) and chelerythrine (1 μmol/L), was decreased by epalrestat (100 nmol/L). Chronic high glucose treatment for 72 hours increased intracellular oxidative stress, which was directly measured by flow cytometry using carboxydichlorofluorescein diacetate bis-acetoxymethyl ester, and this increase was significantly suppressed by epalrestat (100 nmol/L). Antisense oligonucleotide to PKC-β isoform inhibited high glucose–mediated changes in SMC migration, insulin-mediated [3H]-deoxyglucose uptake, and oxidative stress. These findings suggest that high glucose concentrations potentiate SMC migration in coronary artery and that the aldose reductase inhibitor epalrestat inhibits high glucose–potentiated, PDGF BB–induced SMC migration, possibly through suppression of PKC (PKC-β), impaired insulin-mediated glucose uptake, and oxidative stress. (Hypertension. 2000;35:1092-1098.)

Key Words: pathway, polyol ■ protein kinases ■ oxidative stress ■ insulin resistance ■ atherosclerosis

Hyperglycemia leads to certain metabolic abnormalities, including increased polyol pathway activity.1 Previous studies have suggested that abnormal glucose metabolism resulting in accumulation of intracellular sorbitol may also contribute to late complications of diabetes.2 This type of abnormal metabolism, the occurrence of which has been confirmed in experimental models,3 plays a key role in the development of diabetic complications. Demonstration of a polyol pathway in smooth muscle cells (SMCs)4 and the arterial wall5 suggests that hyperglycemia may lead to accumulation of sorbitol within SMCs, contributing to their dysfunction and remodelling. We recently reported that the aldose reductase inhibitor (ARI) epalrestat prevents high glucose–induced SMC proliferation and hypertrophy.6 This finding suggested that the polyol pathway might play a role in mediating high glucose–potentiated SMC migration.

Insulin resistance and hyperinsulinemia appear to be independent risk factors for ischemic heart disease.7 In addition to the primary insulin resistance that precedes development of type II diabetes mellitus and coronary artery disease, a secondary insulin resistance appears to result from elevated glucose levels.8 SMCs have been found to have insulin receptors and to exhibit insulin-induced responses.9 Moreover, in SMCs, high glucose might lead to increased oxidative stress10 because of sorbitol dehydrogenase–linked changes in the reduced/oxidized nicotinamide adenine dinucleotide (NADH/NAD+) ratio,11 which may contribute to the high glucose–mediated changes in cell function. It is not known, however, to what extent these parameters of impaired insulin response and increased oxidative stress, as resulting from polyol pathway activation, reflect increased SMC migration.

Accordingly, the objectives of the current study were to determine whether elevated glucose concentrations enhance migration of cultured SMCs derived from human coronary artery and, if they do, to examine the effects of ARI on high
glucose–induced enhancement of coronary artery SMC migration. In addition, we examined mechanisms by which the polyol pathway might mediate high glucose–potentiated coronary artery SMC migration.

Methods

Materials

Platelet-derived growth factor (PDGF) (recombinant BB), calphostin C, and chelerythrine chloride were purchased from Sigma Chemical Co. SMC basal medium (SmBM) and human coronary SMCs were purchased from Clonetics Corp. Trypsin EDTA (Versine) and fetal calf serum (FCS) were purchased from Gibco Laboratories. Multi-well pipettes and flasks were purchased from Becton Dickinson and Co. Microchemotaxis chambers and polycarbonate filters were purchased from Neuro Probe Inc, and Nucleopore Corp, respectively. Diff-Quick staining solution was purchased from Green-Cross Corp. The protein kinase C (PKC) assay system, [125 I]-PDGF BB, and [3 H]-deoxyglucose ([3 H]-DOG) were purchased from Amersham Pharmacia Biotech. Carboxydichlorofluorescein (CDCFH) diacetate bis-acetoxymethyl (AM) ester was purchased from Molecular Probe Co.

Cell Culture

Human coronary artery SMCs were cultured in SmBM containing human epidermal growth factor (0.5 ng/mL), human fibroblast growth factor (2 ng/mL), 5% FCS, 50 mg/mL gentamicin sulfate, and 50 mg/mL amphotericin B.12 Cells were identified as SMCs on the basis of morphological and immunohistochemical characteristics as previously reported.13 Subconfluent SMCs between the fourth and eighth passages were used for experiments.

Experimental Protocol

For the migration, metabolic and biochemical assay, PDGF-β receptor-binding assay, [1 H]-DOG uptake, flow cytometry and fluorescence microscopy, and PKC activity experiment, cells were allowed to grow for 72 hours in high (22.2 mmol/L glucose) or normal (5.6 mmol/L glucose+16.6 mmol/L mannose) glucose medium with 10% FCS in the presence or absence of epalrestat, calphostin C, or chelerythrine. Mannose was used to control osmolality. Migration (Figures 1 through 3), PKC assay (Figure 4), and oxidative stress (Figure 5) experiments were performed in the presence or absence of PDGF BB (10 ng/mL). Antisense and sense oligonucleotides to PKC-β isof orm were used to examine involvement of this isof orm (Table).

Migration Assay

Migration of SMCs was assayed using a modified version of Boyden’s chamber method with microchemotaxis chambers and polycarbonate filters, as previously reported.14 In this experiment, polycarbonate filters with pores 12 μm in diameter were used. Cultured SMCs were trypsinized and suspended at a concentration of ~5.0×10^5 cells/mL in SmBM supplemented with 0.5% FCS. Cell numbers were counted with an electronic cell counter to correct for the number of cells in migration. A 200-μL SMC suspension was placed in the upper chamber, and 40 μL of medium (0.4% BSA containing 10 ng/mL PDGF BB or vehicle) was placed in the lower chamber. The chamber was incubated at 37°C under 5% CO₂ in air for 6 hours. After incubation, SMCs on the upper side of the filter were scraped off and the filter was removed. The SMCs that had migrated to the lower side of the filter were washed with Diff-Quick staining solution, and counted under a microscope.
Glucose Transport Analyses
SMCs were cultured for 72 hours in normal or high glucose SmBM in the absence or presence of epalrestat, calphostin C, or chelerythrine. For glucose transport studies, SMCs were grown to confluence and, on the day of the experiment, were incubated with physiological salt solution (PSS) containing (in mmol/L) 145 NaCl, 5 KCl, 10 HEPES, 1 MgSO₄, 0.5 Na₂HPO₄, and 1.5 CaCl₂. Cells were acclimatized in 2 mL of PSS for 1 hour, after which the buffer was replaced with 2 mL of PSS containing 2 mL of vehicle (0.01 mol/L HCl) or 100 μmol/mL (0.7 mmol/L) human insulin. After 20 minutes of this pretreatment, solutions were replaced with identical solutions containing trace amounts (0.7 nmol/L) of [³H]-DOG. Transport of [³H]-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 (using 3 washes with PSS) to scintillation vials.¹⁵

Assay of Intracellular Oxidative Stress
Intracellular oxidative stress was measured using a fluorescent dye, CDFCF diacetate bis-AM ester. CDFCF diacetate bis-AM ester is a nonpolar compound that is converted to a nonfluorescent polar derivative (CDFCF) by cellular esterases after incorporation into cells. CDFCF is membrane-impermeable and rapidly oxidized to highly fluorescent carboxydichlorofluorescein in the presence of intracellular hydroperoxide and peroxides. For assays, medium was replaced with Hanks’ solution containing 5 mmol/L CDFCF diacetate bis-AM ester at appropriate times after stimulation. After 5 minutes of incubation at room temperature, the fluorescence intensity of each point was measured by flow cytometry as previously reported.¹⁴ The excitation wavelength was 510 to 530 nm. Relative fluorescence intensities were calculated using untreated control cells as a standard.

Antisense Oligonucleotide
Phosphothioate-modified oligodeoxynucleotides for PKC-β isoform were designed as reported by Li et al.¹⁹ and purified by high-performance liquid chromatography by Japan Bio Service Co. The PKC-β sequence was selected from the area of conserved sequence between PKC-βI and PKC-βII: antisense PKC-β, 5'-AGC GCA CGG TGC TCT CCT CG-3'; and sense PKC-β, 5'-CGA GGA GAC CAG CGT GCG CT-3'. These oligodeoxynucleotides were added to serum-free SmBM 24 hours before the start of PDGF BB stimulation with transfection using cationic compound; lipofectin reagent (Gibco) and oligonucleotides were effectively taken up by SMCs.

Statistical Methods
Statistical analysis was performed by analysis of variance and Scheffe’s modified t test.³⁰ Values of P<0.05 were considered significant.

Results
Effect of High Glucose on PDGF BB-Induced SMC Migration
Figure 1 shows the dose-dependent effects of various concentrations of glucose on human coronary artery SMC migration in the absence (left) or presence (right) of PDGF BB. Chronic high glucose treatment for 72 hours potentiated 10 ng/mL PDGF BB-stimulated cell migration in a concentration-dependent manner between 5.6 and 22.2 mmol/L. SMC migration increased during the initial 6 hours of incubation, after which the rate of increase declined slightly. Subsequent migration experiments were therefore performed with cells incubated for 6 hours. Mannose alone did not affect SMC migration between 5.6 and 22.2 mmol/L (data not shown).
Effect of Epalrestat on PDGF BB–Induced SMC Migration

Figure 2 shows effects of the ARI epalrestat at 10 and 100 nmol/L on high glucose–potentiated SMC migration induced in the absence (left) or presence (right) of PDGF BB (10 ng/mL) for 6 hours as described in Methods. Relative fluorescence intensity was measured by flow cytometry as follows: Mean Fluorescence Intensity = $\frac{\text{Fluorescence of Each Channel} \times \text{Cell Number of Channel}}{\text{Total Cell Number}}$. Values are mean±SD fluorescence intensity (n=8). *P<0.05. B, Representative findings of the effects of epalrestat on the oxidative stress of cultured human coronary SMCs incubated with normal or high glucose medium for 72 hours. Oxidative stress was measured in the absence (A through C) or presence (D through F) of PDGF BB for 6 hours by flow cytometry and expressed as fluorescence intensity. The area below the flow cytometric curve is the same (3000 cells). Therefore, the decrease and increase in number of cells at low and high fluorescence intensity, respectively, indicate the increase in mean fluorescence intensity (mean oxidative stress).

Effect of Epalrestat on Glucose-Induced Increase in Fructose and Cytosolic NADH/NAD+ in SMCs

Fructose levels were significantly increased from 13±3 (normal glucose) to 27±2 μmol/L (high glucose) (n=8, P<0.05). Epalrestat (100 nmol/L) significantly decreased the glucose-induced increase in fructose level from 27±2 to 15±3 μmol/L (n=8, P<0.05).
PKC-β Isoform and High Glucose–Mediated Changes in SMCs*

<table>
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<tr>
<th>PKC Activity, pmol/L per min per 10⁶ cells</th>
<th>Normal Glucose</th>
<th>High Glucose</th>
<th>High Glucose + AS</th>
<th>High Glucose + S</th>
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<tr>
<td>–PDGF BB</td>
<td>12 ± 3</td>
<td>21 ± 4</td>
<td>15 ± 2†</td>
<td>19 ± 2</td>
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<tr>
<td>+PDGF BB</td>
<td>28 ± 4</td>
<td>52 ± 6</td>
<td>35 ± 4†</td>
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<th>SMC migration, cells per 4 HPFs</th>
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<th>High Glucose</th>
<th>High Glucose + AS</th>
<th>High Glucose + S</th>
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<tr>
<td>–PDGF BB</td>
<td>7 ± 2</td>
<td>13 ± 2</td>
<td>9 ± 2†</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>+PDGF BB</td>
<td>18 ± 2</td>
<td>32 ± 4</td>
<td>22 ± 4†</td>
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<th>PDGF-β receptor, sites per cell</th>
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<th>High Glucose + AS</th>
<th>High Glucose + S</th>
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<tr>
<td>28 000 ± 3000</td>
<td>54 000 ± 4000</td>
<td>32 000 ± 4000†</td>
<td>52 000 ± 6000</td>
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<th>Oxidative stress, arbitrary units</th>
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<th>High Glucose + AS</th>
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<tr>
<td>–PDGF BB</td>
<td>25 ± 3</td>
<td>52 ± 4</td>
<td>31 ± 4†</td>
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<tr>
<td>+PDGF BB</td>
<td>60 ± 5</td>
<td>112 ± 12</td>
<td>70 ± 6†</td>
<td>108 ± 10</td>
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</table>

*Effect of PKC-β isoform antisense (AS) and sense (S) oligonucleotides on normal glucose–induced (5.6 mol/L glucose + 16.6 mol/L mannose) or high glucose–induced (22.2 mol/L glucose) changes in PKC activity, SMC migration, PDGF-β receptor number, insulin-mediated [¹H]-DOG uptake, and oxidative stress in SMCs in the presence (+) or absence (−) of PDGF BB (10 ng/mL). Data are mean ± SD of 4 determinations of a single representative experiment.†P<0.05 vs high glucose–treated cells.

For more reliable assessment of the human coronary SMC cytosolic NADH/NAD⁺ ratio, lactate/pyruvate ratios were measured in SMCs. The ratio was higher with high glucose medium than with normal glucose medium after 72 hours of incubation (normal glucose 3.2 ± 0.5, high glucose 6.2 ± 0.6; n = 8, P<0.05). Epalrestat (100 nmol/L) completely prevented this increase in the lactate/pyruvate ratio (high glucose + epalrestat 3.1 ± 0.6; n = 8, not significantly different from normal glucose but significantly different from high glucose, P<0.05).

Involvement of PKC in PDGF BB–Induced SMC Migration

To examine the involvement of PKC in high glucose–potiated SMC migration, we examined the effects of the PKC inhibitors calphostin C and chelerythrine on PDGF BB–induced SMC migration. Inhibition of SMC migration by 100 nmol/L calphostin C or 1 μmol/L chelerythrine was observed in the absence (left) or presence (right) of PDGF BB (Figure 3). Involvement of PKC was also confirmed by measurement of particulate PKC activity. Particulate PKC activity was increased by high glucose treatment, and this increase was significantly reduced by 100 nmol/L epalrestat in the absence (left) or presence (right) of PDGF BB (Figure 4). Calphostin C (100 nmol/L) and chelerythrine (1 μmol/L) each completely blocked activation of particulate PKC induced by high glucose in the absence (left) or presence (right) of PDGF BB (Figure 4). Calphostin C and chelerythrine at these concentrations did not cause loss of cells in the confluent state. After incubation, <5% of cells were found in the supernatant media. Cell viability was also checked by trypan blue staining, which confirmed that >95% of cells were alive.

Effect of Epalrestat on PDGF BB Binding to SMCs Cultured With Normal and High Glucose Concentrations

The binding capacity of PDGF BB to SMCs increased in a dose-dependent manner, reaching a plateau at a concentration of 20 ng/mL. Scatchard analysis showed that the PDGF receptor had similar affinities to PDGF BB in all experimental conditions (normal glucose alone 0.98 nmol/L, with 100 nmol/L epalrestat 1.02 nmol/L, with 100 nmol/L calphostin C 0.98 nmol/L, or with 1 μmol/L chelerythrine 1.02 nmol/L; high glucose alone 1.02 nmol/L, with 100 nmol/L epalrestat 1.04 nmol/L, with 100 nmol/L calphostin C 1.02 nmol/L, or with 1 μmol/L chelerythrine 1.02 nmol/L) and that high glucose increased receptor number, which was decreased by epalrestat, calphostin C, and chelerythrine (normal glucose 2.88×10⁴/cell, with 100 nmol/L epalrestat 2.86×10³/cell, with 100 nmol/L calphostin C 2.81×10³/cell, or with 1 μmol/L chelerythrine 2.83×10²/cell; high glucose alone 5.40×10³/cell, with 100 nmol/L epalrestat 3.01×10²/cell, with 100 nmol/L calphostin C 2.82×10² cell, or with 1 μmol/L chelerythrine 2.84×10²/cell).

Insulin-Stimulated [¹H]-DOG Uptake

The rate of uptake of [¹H]-DOG into cells was linear between 0 and 15 minutes of incubation, regardless of glucose concentration. Insulin-stimulated [¹H]-DOG uptake was significantly decreased from 203±5 (normal glucose) to 133±6 (high glucose) pmol/10⁶ cells per 5 minutes (n = 8, P<0.05) after 72 hours of incubation with 22.2 mmol/L glucose. This decrease was prevented by coincubation with 100 nmol/L epalrestat (195±7 pmol/10⁶ cells per 5 minutes, n = 8, NS versus normal glucose), calphostin C (193±7 pmol/10⁶ cells per 5 minutes, n = 8, NS versus normal glucose), or 1 μmol/L chelerythrine (193±7 pmol/10⁶ cells per 5 minutes, n = 8, NS versus normal glucose) (Figure 4). Thus, epalrestat enhanced...
high glucose–induced, insulin-mediated glucose efflux by suppression of PKC. Glucose treatment did not change basal [1H]-DOG transport activity (127±5 pmol/10⁶ cells per 5 minutes). Mannose had no effect on basal or insulin-stimulated [1H]-DOG uptake.

**Effects of Epalrestat on SMC Intracellular Oxidative Stress**

Chronic high glucose treatment for 72 hours increased oxidative stress in the presence or absence of PDGF BB. Epalrestat (100 nmol/L) decreased intracellular oxidative stress. The PKC inhibitors calphostin C (100 nmol/L) and chelerythrine (1 μmol/L) also decreased intracellular oxidative stress in the presence or absence of PDGF BB (Figure 5A). Figure 5B shows representative effects on oxidative stress in coronary SMCs as measured by flow cytometry.

**Inhibition of PKC-β Isoform Activation by Antisense Oligonucleotide**

To determine whether activation of PKC-β isoform is associated with high glucose–induced changes in migration, PDGF-β receptor expression, glucose uptake, and oxidative stress, the effects of antisense oligonucleotide to PKC-β isoform were examined. Antisense oligonucleotide to PKC-β isoform at 10 μmol/L significantly inhibited high glucose–induced changes (Table). However, sense oligonucleotide to PKC-β isoform (10 μmol/L) had no effects (Table).

**Discussion**

We found that epalrestat inhibited high glucose–potentiating, PDGF BB–mediated migration of human coronary SMCs in a concentration-dependent manner. A glucose-induced increase in migration of coronary SMCs was observed for concentrations of 11.1 to 22.2 mmol/L glucose, which are equivalent to blood glucose levels present in diabetic patients. The concentration of epalrestat tested in the present study is within the range of circulating levels after in vivo administration. Therefore, our findings suggest that the ARI epalrestat may prevent coronary SMC migration in vivo.

The mechanisms by which epalrestat exerts its antimigratory effect remain to be determined. However, we have shown that the polyol pathway probably plays a role in mediating high glucose–induced PKC activation, possibly through increased fructose formation and NADH/NAD⁺. We have also demonstrated the involvement of PKC in cell growth and proliferation. In the present study, epalrestat inhibited high glucose–potentiating SMC migration and PKC activation. Epalrestat also inhibited high glucose–potentiating PDGF BB binding. Our findings thus suggest that high glucose increases PKC activity, possibly through the polyol pathway in coronary SMCs, which may upregulate PDGF-β receptors and may potentiate PDGF BB–mediated SMC migration. However, we cannot completely rule out the possibility that mechanisms other than PKC activation may underlie this process, because PDGF has been reported to act independently of PKC mechanisms.

As troglitazone enhanced the insulin-mediated glucose efflux, epalrestat enhanced insulin-mediated glucose efflux in SMCs in the present study. This insulin-sensitizing action may be due to suppression of increased PKC activity, because activation of PKC impairs insulin-mediated glucose uptake.

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**References**

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