Requirement of Aldose Reductase for the Hyperglycemic Activation of Protein Kinase C and Formation of Diacylglycerol in Vascular Smooth Muscle Cells

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Activation of protein kinase C (PKC) has been linked to the development of secondary diabetes complications. However, the underlying molecular mechanisms remain unclear. We examined the contribution of aldose reductase, which catalyzes the first, and the rate-limiting, step of the polyol pathway of glucose metabolism, to PKC activation in vascular smooth muscle cells (VSMCs) isolated from rat aorta and exposed to high glucose in culture. Exposure of VSMCs to high glucose (25 mmol/l), but not iso-osmotic mannitol, led to an increase in membrane-associated PKC activity, which was prevented by the aldose reductase inhibitors tolrestat or sorbinil or by the inhibition of aldose reductase by small interfering RNA (siRNA). The VSMCs were found to express low levels of sorbitol dehydrogenase, and treatment with sorbinil decreased the levels of reactive oxygen species (ROS) and novel (δ and ε) isoforms of PKC. Inhibition of aldose reductase prevented membrane translocation of PKC-β2 and -δ and delayed the activation of PKC-β1 and -ε, whereas membrane translocation of PKC-α and -γ was not affected. Treatment with tolrestat prevented phosphorylation of PKC-β2 and -δ. High glucose increased the formation of diacylglycerol (DAG) and enhanced phosphorylation of phospholipase C-γ1 (PLC-γ1). Inhibition of aldose reductase prevented high glucose–induced DAG formation and phosphorylation of PLC-γ1 and PLC-β2 and -δ. Inhibition of phospholipid hydrolysis by D609, but not by the synthetic alkyl-1-lysophospholipid 1-O-octadecyl-2-O-methyl-rac-glycero-phosphocholine, or edelfosine, prevented DAG formation. Treatment with sorbinil decreased the levels of reactive oxygen species in high-glucose–stimulated VSMCs.

Hence, inhibition of aldose reductase, independent of sorbitol dehydrogenase, appears to be effective in diminishing oxidative stress and hyperglycemic changes in signaling events upstream to the activation of multiple PKC isoforms and PLC-γ1 and may represent a useful approach for preventing the development of secondary vascular complications of diabetes. Diabetes 54: 818–829, 2005

Cardiovascular complications are the leading cause of morbidity and mortality associated with diabetes (1,2). Diabetes accelerates atherosclerotic lesion formation (1,3,4), and it is an important predictor of restenosis after percutaneous transluminal coronary angioplasty (5–7). Although multiple factors contribute to diabetic changes in cardiovascular physiology, chronic and persistent hyperglycemia has been recognized as one causative factor (1,8). Hyperglycemia-induced oxidative stress (9) and the formation of advanced glycosylation end products (AGEs) (8,10) are associated with long-term diabetes, and it has been shown that administration of the soluble extracellular domain of the AGE receptor diminishes accelerated lesion formation in atherosclerosis-prone mice (11). Nevertheless, the mechanisms by which hyperglycemia affects atherogenesis and promotes restenosis of injured arteries remain poorly understood. In culture, high glucose increases vascular smooth muscle cell (VSMC) growth (12–15) and stimulates abnormal intracellular signaling (8). One of the key signaling events affected by glucose is activation of the protein kinase C (PKC) family of protein kinases (1,8,16). Diabetes leads to tissue-specific activation of specific PKC isoforms, and inhibition of PKC prevents multiple vascular abnormalities in diabetic rats and mesangial expansion and glomerular dysfunction in db/db mice (16), suggesting that the PKC enzymes are important mediators of biochemical and functional changes in diabetic vessels.

In addition to PKC activation, hyperglycemia also leads to stimulation of the polyol pathway (17,18). In this pathway, glucose is reduced to sorbitol, which is subsequently oxidized to fructose (17,18). The first, and the rate-limiting, step of this pathway is catalyzed by aldose reductase, a cytosolic monomeric enzyme that reduces glucose with low affinity. At normal glucose concentration, aldose reductase–catalyzed reduction represents a minor source of glucose utilization; however, the contribution of aldose reductase to glucose metabolism increases mark-
edly during hyperglycemia, leading to the depletion of reducing equivalents and the accumulation of osmotically active sorbitol (8,10,16–18). That aldose reductase–dependent changes in the cell osmolality and redox state contribute to hyperglycemic tissue injury is supported by extensive work showing that inhibition of aldose reductase prevents, delays, or even reverses diabetic changes in the lens, kidney, and nerves (17–21). Nonetheless, the role of aldose reductase in mediating hyperglycemic changes in vascular tissue remains poorly understood, and the contribution of the polyol pathway to high-glucose–induced PKC activation has not been assessed. Our previous studies show that inhibition of aldose reductase prevents the activation of PKC and nuclear factor-κB (NF-κB) in VSMCs treated with TNF-α (22) or high glucose (23). However, it remains unclear which specific isoforms of PKC are affected by inhibiting aldose reductase and whether inhibition of aldose reductase interferes with signaling events upstream to PKC activation. Accordingly, we examined the effects of inhibiting aldose reductase on the activation of both classical and novel isoforms of PKC. Our results show that inhibition of aldose reductase prevents membrane translocation and phosphorylation of multiple PKC enzymes by inhibiting the generation of diacylglycerol (DAG) from phospholipid hydrolysis and abrogates the activation of phospholipase C-γ (PLC-γ).

RESEARCH DESIGN AND METHODS

PBS, penicillin/streptomycin solution, trypsin, fetal bovine serum (FBS), and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Life Technologies. Anti-α, β1, β2, γ, δ, and ε PKC antibodies; α-glucose; and δ-mannitol were purchased from Sigma. Phospho–PKC-β (Thr505) and phospho–PLC-γ1 (Thr783) antibodies were obtained from Cell Signaling. Phospho–PLC-γ1 (Thr505) and phospho–PLC-δ (Tyr783) antibodies were obtained from Abcam. Aldose reductase small interfering RNA (siRNA) was designed and synthesized as described elsewhere (23). Control siRNA and RNAiFect transfection reagent were obtained from Qiagen. Sorbinil and tolrestat were obtained as gifts from Pfizer and Amshar Pharmacia, Arlington Heights, IL). Briefly, the growth-arrested VSMCs (1 × 10⁶ cells/well) in 6-well dishes were preincubated without or with (10 μmol/l) aldose reductase inhibitors for 24 h, followed by incubation with high glucose or mannitol (19.5 mmol/l mannitol + 5.5 mmol/l normal glucose) for different time intervals. Total cell lysates were extracted with chloroform: methanol (1:2 vol/vol) after centrifugation at 5,000 rpm for 2 min. The lower chloroform phase was evaporated under nitrogen and was analyzed for DAG by the addition of DAG kinase in the presence of [γ-32P]ATP. After 30 min, the reaction was stopped, and the [32P]-labeled phosphatidic acid (PA) was separated using Amprep mini-columns and quantified by determining radioactivity using a liquid scintillation counter. Each experiment was repeated four times.

**Measurement of PLC-γ1 activation.** To determine the PLC-γ1 activation on high-glucose treatment, Western blot analysis was carried out using antibodies against phospho–PLC-γ1. Equal amounts of cytoplasmic extracts were subjected to 10% SDS-PAGE. The proteins were transferred to nitrocellulose membrane and probed with the indicated antibodies, and the antigen–antibody complex was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

**Measurement of reactive oxygen species.** VSMCs were plated in a 24-well plate at a density of 1.5 × 10⁶ cells/well in DMEM and growth-arrested at 60–70% confluence in 0.1% FBS in the presence and absence of 10 μmol/l sorbinil. After 24 h of serum starvation, the cells were stimulated with high glucose. At indicated times, the cells were treated with 50 μmol/l DCFH diacetate and incubated in the dark for 10 min. Changes in fluorescence intensity were measured using an Fx-800 microplate fluorescence reader (BioTek Instruments) at excitation/emission wavelengths of 485/528 nm (26).

**Measurement of aldose reductase and sorbitol dehydrogenase.** The VSMCs were homogenized in 50 mmol/l phosphate, pH 7.4, containing 5 mmol/l dihydrothreitol, 0.5 mmol/l NaCl, and 1:1,000 protease inhibitor cocktail (Sigma). The cell extract was centrifuged at 13,000 g for 15 min at 4°C, and the supernatant was used for the measurements of aldose reductase and sorbitol dehydrogenase activity spectrophotometrically, as described elsewhere (27).

**Statistical analysis.** Data are presented as the means ± SE, and P values were determined using unpaired Student’s t test.

**RESULTS**

**Inhibition of aldose reductase prevents high-glucose–induced PKC activation.** To examine the contribution of the polyol pathway to high-glucose–induced activation of PKC, we incubated growth-arrested VSMCs without or with sorbinil or tolrestat for 24 h followed by incubation with high glucose or mannitol (19.5 mmol/l mannitol + 5.5 mmol/l normal glucose). At 19.5 mmol/l glucose or 19.5 mmol/l mannitol to the culture and continuation of the incubation for another 3 h. Total membrane-bound PKC activity increased on stimulation with glucose but not with mannitol. The high-glucose–induced PKC activity was markedly prevented by sorbinil as well as tolrestat (Fig. 1A). However, the total cytosolic PKC activity was unaffected by aldose reductase inhibitors or by mannitol (Fig. 1A). Based on these results, we conclude that aldose reductase or products of aldose reductase catalysis are required for high-glucose–induced PKC activation.

**RNA interference ablation of aldose reductase prevents high-glucose–induced PKC activation.** Although...
sorbitol dehydrogenase. It has been speculated that hyperglycemic injury and dysfunction is in part due to redox imbalance caused by activation of the polyol pathway (16). Activation of this pathway could lead to increased oxidation of NADPH (by aldose reductase) and the generation of NADH (by sorbitol dehydrogenase), resulting in a hypoxia-like distribution of pyridine coenzymes. To assess the contribution of sorbitol dehydrogenase to high glucose-induced PKC activation, we measured the expression and activity of sorbitol dehydrogenase in VSMCs. As shown in Fig. 2A, the immunoreactivity of the VSMC extract with anti-sorbitol dehydrogenase antibody was minimal (<10-fold lower than that of an equal concentration of liver extract). In contrast, 15-fold higher levels of aldose reductase protein were present in VSMCs than liver. These observations suggest that as compared with aldose reductase,
sorbitol dehydrogenase is poorly expressed in VSMCs. Moreover, we were unable to detect sorbitol dehydrogenase activity in VSMCs, although robust activity could be measured in liver extracts. In contrast to the liver, the smooth muscle cells displayed sixfold higher activity in the VSMCs (Fig. 2A), indicating that VSMCs express much higher levels of aldose reductase than sorbitol dehydrogenase. Despite low expression levels, sorbitol dehydrogenase may be important in regulating high-glucose–mediated activation of PKC. To test this possibility, serum-starved VSMCs were incubated with 50 μmol/l of the sorbitol dehydrogenase inhibitor CP-166572 or 10 μmol/l sorbinil for 24 h and then stimulated with high glucose for 3 h, and membrane translocation of PKC was measured. As shown in Fig. 2B, treatment with CP-166572 did not affect high glucose–induced PKC activation, although, as before, treatment with sorbinil prevented high-glucose–induced PKC activation. Together, these observations suggest that the levels of sorbitol dehydrogenase in VSMCs are minimal and that sorbitol dehydrogenase does not regulate the involvement of aldose reductase in mediating high-glucose–induced PKC activation.

Identification of PKC isozymes activated by high glucose in VSMCs. To further study the effects of aldose reductase on PKC activation, we examined glucose-induced changes in both classical and novel isoforms of the PKC family of kinases. For these experiments, cells were either left untreated or stimulated with high glucose. After different times of exposure, cells were lysed and their membrane and cytoplasmic fractions separated as described in Research Design and Methods. Western blots using isoform-specific antibodies were developed to identify translocation of PKC enzymes (α, β1, β2, γ, δ, and ε) from the cytosol to the membrane. As shown in Fig. 3, the cellular localization of the PKC isoforms examined was variably affected on exposure to high glucose. As reported previously (16), most dramatic shifts were observed in the PKC-β isoforms β1 and β2, both of which nearly disappeared from the cytosol and were translocated to the membrane within 120 min of stimulation with high glucose. In addition to PKC-β, the distribution of PKC-γ and δ were also affected by high glucose. After 120 min of stimulation, the membrane fraction was considerably enriched in these isoforms. In contrast, distribution of PKC-α was not significantly altered by high glucose, and only marginal gains in the membrane fraction were observed. No significant change in the distribution of PKC-ε was observed for the first 60 min of stimulation with high glucose, although after 120 min a significant increase in the membrane abundance of the protein was noted.

Pretreatment with tolrestat prevented the high-glucose–induced subcellular redistribution of PKC isoforms. The most dramatic effects were observed with PKC-β2 and -δ, although the redistribution of PKC-β1 and -ε was also affected. PKC-α, which was minimally affected by high glucose, was relatively insensitive to inhibition of aldose reductase by tolrestat, which also only marginally affected the membrane translocation of PKC-γ. In the presence of tolrestat, there was no discernable membrane translocation of PKC-β1 for 30 min, and the increase thereafter was slight. Similarly, a marked decrease in the membrane translocation of PKC-β1 was observed at all times examined. Slight inhibition in the translocation of PKC-ε was also observed in the presence of tolrestat on stimulation with high glucose for 10–60 min, although the extent of translocation after 120 min was minimally affected (Fig. 3). In contrast, the PKC-δ isoform was uniformly affected at each time point examined, with significant suppression of membrane translocation even after 120 min of stimulation with high glucose. Together, these data demonstrate that inhibition of aldose reductase variably inhibits membrane translocation of PKC isoforms in cells stimulated with high glucose.

![FIG. 2. Role of sorbitol dehydrogenase in high-glucose–induced PKC activation. A: Expression and activity of sorbitol dehydrogenase (left panel) and aldose reductase (right panel) in VSMCs and liver. Cytosolic extracts of liver and VSMCs were prepared and separated by 12% SDS-PAGE, and Western blots were developed by using antibodies raised against either sorbitol dehydrogenase or aldose reductase as indicated. Bar graph shows activities of aldose reductase and sorbitol dehydrogenase determined spectrophotometrically as described before (27). N.D, not detectable; SMC, smooth muscle cells. B: Effect of sorbitol dehydrogenase inhibition (SDI) and aldose reductase inhibition (ARI) on high-glucose (HG)-induced PKC activity. Serum-starved VSMCs were preincubated with or without 10 μmol/l sorbinil or 50 μmol/l CP-166572 (sorbitol dehydrogenase inhibition) for 30 min and stimulated with high glucose for 3 h. After incubation, membrane fractions were isolated, and PKC activity associated with each of these fractions was measured. Bars represent the means ± SE (n = 3–4). #P < 0.001 vs. normal glucose (NG); **P < 0.001 vs. high glucose. NS, nonsignificant.](image)
glucose. The most pronounced inhibition was observed in the translocation of PKC-β2 and -δ, followed by PKC-β1, and -ε, with little changes in PKC-α or -γ.

**Aldose reductase inhibition prevents phosphorylation of PKC-β2 and -δ induced by high glucose.** Because high-glucose–induced membrane translocation of PKC-β2 and -δ was prevented by aldose reductase inhibitors, we next measured whether inhibition of aldose reductase also prevents phosphorylation of PKC-β2 and -δ by using phospho-specific antibodies in the total cell extracts. As shown in Fig. 4, stimulation of VSMCs with high glucose caused phosphorylation of PKC-β2 and -δ, which was prevented in the presence of tolrestat. However, the total PKC expression is not affected by high glucose in the absence or the presence of aldose reductase inhibitors.

**Inhibition/ablation of aldose reductase prevents DAG formation.** Because the activation of classical and atypical PKC isoforms is dependent on DAG formation (28,29), we tested the hypothesis that inhibition of PKC translocation by inhibition of aldose reductase is caused by a decrease in DAG formation in response to stimulation with high glucose. Under basal conditions, when the cells were cultured in normal growth medium containing either 5.5 mmol/l glucose or 5.5 mmol/l glucose + 19.5 mmol/l mannitol, the intracellular concentration of DAG in VSMCs was 290 ± 45 and 282 ± 15 pmol/10⁶ cells, respectively.

**FIG. 3.** Inhibition of aldose reductase abrogates membrane translocation of PKC isoforms. Serum-starved VSMCs were preincubated without or with 10 μmol/l tolrestat for 24 h and then incubated in high glucose (HG) for the indicated times. The cytosol and membrane fractions were prepared, pooled extracts from three independent experiments were separated by SDS-PAGE, and Western blots were developed using antibodies to the indicated PKC isoforms.

**FIG. 4.** Inhibition of aldose reductase abrogates phosphorylation of PKC-β2 and -δ isoforms. Serum-starved VSMCs were preincubated without or with 10 μmol/l tolrestat for 24 h and then incubated in high glucose (HG) for the indicated times. The total cell extracts were prepared, pooled extracts from three independent experiments were separated by SDS-PAGE, and Western blots were developed using phospho-specific and total PKC-β2 and -δ antibodies.
When the cells were stimulated with high glucose, a rapid, time-dependent increase in DAG formation was observed that peaked within 1 h after stimulation, at which time a maximal increase (threefold) in DAG concentration was observed. Thereafter, even with the continued presence of glucose, the DAG concentration diminished steadily, although even after 24 h the levels of DAG in high-glucose–treated cells remained higher than in cells cultured in the normal growth medium (5.5 mmol/l glucose). Preincubation of cells with the aldose reductase inhibitors sorbinil or tolrestat (10 μmol/l each) for 24 h blocked the hyperglycemia-induced increase in DAG levels (Fig. 5A). Preincubation of the cells with these inhibitors alone did not affect the basal levels of DAG in VSMCs grown in normal glucose without inhibitors (data not shown). Although markedly attenuated, DAG levels in the presence of high glucose and aldose reductase inhibitors were still somewhat higher than those in unstimulated cells. Similarly, transfection of VSMCs with aldose reductase siRNA also attenuated the increase in DAG in cells stimulated with high glucose (Fig. 5B). No change in DAG was observed in cells transfected with control siRNA or the transfection reagent. These observations support the view that inhibition of aldose reductase prevents the activation of PKC isoforms by decreasing DAG.

Aldose reductase inhibition prevents phosphorylation of PLC-γ1 induced by high glucose. Because DAG synthesis involves activation of PLC (29,30), we examined the effect of aldose reductase inhibition on phosphorylation of PLC-γ1. PLC-γ1 is activated by growth factors (31,32) and oxidative stress (33), and is thought to mediate mitogenic signaling in smooth muscle cells (34,35). As shown in Fig. 6, incubation of VSMCs with high glucose led to a large increase in phosphorylated PLC-γ1 as detected by anti–phospho-PLC-γ1 antibodies. An appreciable increase in PLC phosphorylation was observed within 10 min, and a maximal increase (sixfold) was observed within 30 min. Thereafter, a gradual decrease in the extent of phosphorylation was observed, and after 120 min a less than twofold increase in phosphorylation remained. Only basal levels of PLC-γ1 phosphorylation were observed in cells cultured in 5.5 mmol/l glucose. Moreover, the addition of 19.5 mmol/l mannitol did not result in an increase in the phosphorylation of PLC-γ1. Pretreatment with 10 μmol/l tolrestat prevented a high-glucose–induced increase in phospho–PLC-γ1. Only minimal (less than two-
fold) increases in the phosphorylation of PLC-γ1 was observed in tolrestat-treated cells, indicating that inhibition of aldose reductase prevents high-glucose–induced phosphorylation of PLC-γ1.

**High glucose–induced DAG formation is prevented by inhibiting PLC.** The formation of DAG in response to stimulation with growth factors and cytokines is mediated by PLC-dependent hydrolysis of membrane phospholipids (28,31,32). To elucidate the role of phosphatidylincholine (PC) in high-glucose–induced DAG synthesis, we examined whether inhibition of PLC would prevent DAG formation. DAG formation was completely inhibited in cells treated with D609 at a concentration of 100 μmol/l (Fig. 7). This concentration was approximately half of that used to completely inhibit tumor necrosis factor–α–induced PC-PLC activation (36) and one fourth of that required to block tumor necrosis factor–α–induced NF-κB activation (37), indicating that PC hydrolysis is a significant source of DAG formation in cells treated with high glucose. In comparison, DAG formation was only minimally affected on pretreatment with the alkyl phospholipid analog ET-18-OCH3, which inhibits PKC (38). Treatment with 25 μmol/l ET-18-OCH3, more than six times that required to completely inhibit TPA (12-O-tetradecanoylphorbol-13-acetate)-induced PKC-β (39), did not significantly affect DAG formation (Fig. 7). Neither D609 nor ET-OCH3 decreased DAG levels in cells maintained in 5.5 mmol/l glucose. Collectively, these results indicate that in cells treated with high glucose, a significant proportion of DAG is derived from the activity of PC-PLC, with a minimal contribution from the PKC-phosphatidylinositol (PI) pathway.

**Changes in reactive oxygen species generation.** Several of the effects of hyperglycemia have been linked to increased reactive oxygen species (ROS) generation (8). Hence, to determine whether inhibition of aldose reductase affects high-glucose–induced changes in ROS generated by VSMCs, serum-starved cells were either left untreated or treated with 10 μmol/l sorbinil for 24 h and then stimulated with high glucose for 15, 30, and 60 min. At the end of incubation, the cells were loaded with DCHF, and their ROS concentration was determined by changes in DCHF fluorescence. As shown in Fig. 8, sorbinil-treated cells displayed less DCHF fluorescence than untreated cells, suggesting that inhibition of aldose reductase decreases ROS generation in VSMCs. There was no change in ROS generation in cells treated with sorbinil in normal glucose. However, the addition of mannitol (M) to normal glucose (NG) significantly increased ROS generation, while the addition of tolrestat (HG + tolrestat) decreased ROS generation. These data suggest that inhibition of aldose reductase decreases ROS generation in VSMCs.
PKC-ε with previous studies showing that high glucose activates activation of several PKC isoforms. This is in agreement found that exposing VSMCs to high glucose results in the inhibition of aldose reductase during diabetes (16). We reported in retina, aorta, heart, and kidney, although in neuronal tissues PKC is inhibited during diabetes (16). We in hyperglycemic signaling in addition to PKC activation. inhibition of aldose reductase could prevent multiple changes of diabetes (1,4,8,16). Inhibition of aldose reductase, linked to the development of vascular and renal complications of diabetes (43). Selective inhibition of PKC-ε, high glucose also led to membrane translocation of PKC-δ. Although activation of PKC-δ in high-glucose–treated VSMCs (40) and mesangial cells (48) has been reported before, its significance to the development of vascular complications of diabetes has not been assessed. Overexpression of PKC-δ in smooth muscle cells increases the activity of p38 kinase (40), which could contribute to growth arrest and apoptosis. Indeed, PKC-δ null mice have a higher number of smooth muscle cells and exacerbated vein-graft arteriosclerosis compared with wild-type animals (49), indicating that the activation of PKC-δ may be associated with smooth muscle cell apoptosis. Given that diabetic atherosclerotic lesions have fewer smooth muscle cells (50), it is tempting to speculate that activation of PKC-δ contributes to the instability of diabetic plaques, and that inhibition of aldose reductase leading to decreased activation of PKC-δ could enhance the stability of arterial lesions in diabetic individuals. The minimal effect of aldose reductase inhibition on PKC-ε activation may be an additional protective feature because PKC-ε stimulation prevents against ischemic and oxidative injury (51). However, the role of PKC-γ activation is unclear. Although PKC-γ is predominantly a neuronal isoform of PKC, it is also expressed in VSMCs (52,53) and is activated on adenosine (52) or fibroblast growth factor stimulation (53), suggesting that it may be involved in modulating responses to hypoxia or cell growth. Additional investigations are required to examine its role in the development of diabetes complications.

Activation of PKC isoforms by high glucose appears to be related to an increase in DAG synthesis because both DAG formation and PKC activation were prevented in cells treated with tolrestat, sorbinil, or aldose reductase siRNA. Although chronic elevation of DAG in cells cultured in high glucose or tissues isolated from diabetic animals has been demonstrated before (16), the role of DAG in mediating diabetic changes has not been elucidated. Increased DAG formation stimulates classical and novel forms of PKC; however, under oxidative stress these kinases could also be tyrosine phosphorylated and activated in a DAG-independent manner (54). Because high glucose increases ROS generation (44,48), it is possible that one mechanism.
by which hyperglycemia activates PKC could be by inducing direct tyrosine phosphorylation. Hence, the observation that inhibition of aldose reductase simultaneously prevents both DAG formation and PKC activation supports the view that the activation of PKC-αι, -ε, and -ε is indeed caused by increased DAG formation. However, the inability of aldose reductase inhibitors to completely prevent PKC-αι, -ε, or -ε activation, despite marked suppression of DAG levels, suggests that either different PKC isoforms have different DAG affinities or that PKC-αι, -ε, and -ε are activated by DAG-independent tyrosine phosphorylation due to residual oxidative stress in cells treated with aldose reductase inhibitors. Further experiments are required to distinguish between these possibilities.

It has been proposed that during hyperglycemia, DAG is synthesized de novo from glycolysis (55). However, our data suggest that most of the DAG formed in high glucose is derived from phospholipids because it was inhibited by D609, which prevents PC-PLC hydrolysis (36), and to some extent phospholipase D activity (56). Reasons for this difference remain unclear. In previous studies with VSMCs, an increase in de novo DAG synthesis (measured by the incorporation of [3H]glucose) was measured between 1 and 3 days of culturing in high glucose (56). Although DAG levels were significantly elevated after 24 h, no significant incorporation of [3H]glucose was noted until 2 days (55). In our experiments, DAG was increased within 1 h after stimulation with high glucose. A similar time course of increase in DAG formation within 30 min of exposure to high glucose has been reported for mesangial cells (57). Because agonist-stimulated DAG formation in VSMCs is a multistep process with both positive and negative feedback mechanisms (58), one possible explanation may be that the early increase in DAG is caused by phospholipid hydrolysis, which then chronically elevates DAG by stimulating de novo synthesis. Additional studies are required to test this hypothesis; however, the PC origin of DAG is further supported by the lack of inhibition by ET-OCH₃, which inhibits the PKC-PI-PLC pathway (38,59), indicating that DAG is not derived from PI hydrolysis or PKC-mediated inhibition of DAG kinase, which could lead to the accumulation of DAG by preventing the formation of phosphatidic acid. This is consistent also with previous observations that PKC inhibition does not prevent DAG synthesis (60), and therefore inhibition of DAG kinase is unlikely to contribute to the accumulation of DAG during hyperglycemia.

The observations that high glucose stimulates PLC-γ phosphorylation and that this is prevented by inhibiting aldose reductase apparently support the view that high-glucose–induced DAG formation is caused by PLC hydrolysis. However, PLC-γ selectively hydrolyzes phosphoinositol phospholipids (32), and inositol phosphates are not increased in aortic cells cultured in high glucose (55). Furthermore, the lack of inhibition of DAG formation by ET-18-OCH₃ argues against PI hydrolysis as a significant source of DAG under these conditions because activation of PKC in VSMCs inhibits PI hydrolysis by PLC (61). Nevertheless, the role of PLC-γ1 in hyperglycemic injury cannot be ruled out. During angiogenesis, the activation of PKC-αι is dependent on PLC-γ1 phosphorylation (62), and activation of PKC by oxidative stress is abolished in PLC-γ1–deficient cells (63), suggesting that PLC-γ1 is an upstream regulator of PKC and a potential modulator of VMSC growth and apoptosis in high glucose. Significantly, this role of PLC-γ1 could be sustained even in the absence of phosphoinositide hydrolysis, which is not stimulated by high glucose (55), because it has been shown that the mitogenic effects of PLC-γ1 depend on its ability to act as a guanine nucleotide exchange factor for PI 3-kinase enhancer (64), independent of PI hydrolysis. Hence, the observation that inhibition of aldose reductase prevents
PLC-γ1 phosphorylation may be related to the antimitogenic effects of aldose reductase inhibitors, and it suggests that in high glucose PLC-γ1 is phosphorylated because of oxidative stress induced by activation of aldose reductase. Therefore, one potential mechanism by which inhibition of aldose reductase is able to simultaneously prevent DAG formation, PKC activation, and PLC-γ1 phosphorylation may be the preservation of glucose-induced changes in the redox state of the cell (13,26). Even though aldose reductase by itself is an antioxidant enzyme that detoxifies aldehydes derived from lipid peroxidation (24,25,65,66), adventitious glucose reduction by aldose reductase during hyperglycemia depletes reducing equivalents (16–18), and it has been shown that inhibition of aldose reductase prevents changes in glutathione and pyridine nucleotides in VSMCs cultured in high glucose (13,15). This view is further supported by our observation that inhibition of aldose reductase decreases ROS levels in VSMCs cultured in high glucose.

It has been suggested that inhibition of aldose reductase could prevent DAG formation by normalizing pseudo-hypoxia established due to redox imbalance imposed by high glucose (15). According to this hypothesis, high glucose increases sorbitol synthesis via aldose reductase, which depletes NADPH. Moreover, subsequent metabolism of sorbitol to fructose via sorbitol dehydrogenase increases the NADH-to-NAD⁺ ratio, which accelerates the formation of phosphatidic acid from dihydroxyacetone phosphate, leading to increased DAG formation. However, our data are inconsistent with this model for two reasons: 1) we found no evidence of sorbitol dehydrogenase activity in VSMCs, and treatment with a sorbitol dehydrogenase inhibitor did not prevent PKC activation; and 2) DAG formation was prevented by inhibiting PC-PLC, indirectly suggesting a minimal contribution of dihydroxyacetone-derived DAG and de novo DAG synthesis to high glucose—induced PKC activation (vide supra). Hence, it appears unlikely that DAG synthesis in high glucose is driven by NADH generated by sorbitol dehydrogenase. Nevertheless, changes in the redox state of pyridine nucleotides may be primary determinants of DAG formation and may be a response to aldose reductase–mediated depletion of NADPH. Our results suggest that increased ROS generation in high glucose is sustained in part by aldose reductase and that DAG formation in high glucose is caused by aldose reductase–imposed oxidative stress, which activates PLC and generates DAG. This is consistent with the observation that inhibition of aldose reductase decreases ROS formation, DAG synthesis, and PKC activation. However, we cannot rule out the possibility that the decrease in ROS formation in sorbinil-treated cells is caused in part by inhibition of PKC-dependent activation of NAD(P)H oxidase (Fig. 9) Clearly, additional experiments will be required to elucidate the precise mechanism by which aldose reductase regulates DAG synthesis.

Although the mechanisms by which inhibition of aldose reductase prevents high-glucose–induced redox changes remain unclear, our current observations suggest that aldose reductase is upstream of phospholipid-derived DAG formation (Fig. 9). Thus, aldose reductase inhibitors, which could simultaneously prevent activation of PKC isoforms involved in high glucose–induced inflammation (PKC-β1), abnormal cell growth (PKC-β1 and -β2), free radical generation (PKC-β2), and apoptosis (PKC-δ), while minimally affecting PKC isoforms involved in protections against hypoxia and oxidative stress (PKC-ε), may be more beneficial in preventing vascular complications of diabetes than isoform-selective PKC inhibitors. Moreover, inhibition of aldose reductase may provide benefits in addition to those expected from preventing the activation of individual PKC isoforms. Inhibition of DAG formation could prevent DAG-induced PKC-independent activation of NADPH oxidase (67), and inhibition of PLC-γ1 could diminish the independent effects of this enzyme on PI 3-kinase enhancer and mitogenesis (64), changes that will not be abolished by inhibiting PKC isoforms alone.

In summary, the results of this study show that inhibition of aldose reductase prevents the formation of DAG in VSMCs stimulated with high glucose. This results in the inhibition of selective PKC isoforms. Based on these observations, we speculate that increased metabolism of glucose via the polyol pathway is essential for the stimulation of DAG synthesis and the activation of several PKC isoforms and PLC-γ1. The multiple beneficial effects of aldose reductase inhibition suggest that aldose reductase inhibitors may be of value in treating the vascular complications of diabetes.

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REFERENCES


DIABETES, VOL. 54, MARCH 2005 827


38. Lassagne B, Alexander RW, Clark M, Akers M, Griendling KK: Phosphati-
Dylcholine is a major source of phosphatidic acid and diacylglycerol in angiotensin II-stimulated vascular smooth-muscle cells. *Biochem J* 292:509–517, 1993


