Expression of constitutively active cGMP-dependent protein kinase inhibits glucose-induced vascular smooth muscle cell proliferation

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Wang S, Li Y. Expression of constitutively active cGMP-dependent protein kinase inhibits glucose-induced vascular smooth muscle cell proliferation. Am J Physiol Heart Circ Physiol 297: H2075–H2083, 2009. First published August 28, 2009; doi:10.1152/ajpheart.00521.2009.—Previously, we have demonstrated that cGMP-dependent protein kinase (PKG) activity is downregulated in vessels from diabetic animals or in vascular smooth muscle cells (VSMCs) exposed to high-glucose conditions, contributing to diabetes-associated vessel dysfunction. However, whether decreased PKG activity plays a role in hyperglycemia-induced proliferation of VSMCs is unknown. In this report, high-glucose-mediated decreased PKG activity in VSMCs was restored by transfection of cells with expression vector for the catalytic domain of PKG-I (PKG-CD, constitutive active PKG). The effect of glucose on cell proliferation was determined. Our data demonstrated that hyperglycemia exposure stimulated VSMC proliferation and G1 to S phase progression of the cell cycle, which was inhibited by restoration of PKG activity. Expression of constitutively active PKG inhibited G1 phase exit in VSMCs under high glucose conditions, which was accompanied by an inhibition of retinoblastoma protein (Rb) phosphorylation (a key switch for G1 to S phase cell cycle progression). Glucose-induced cyclin E expression and cyclin E/cyclin-dependent kinase 2 activity was also reduced by expression of PKG-CD in VSMCs. Moreover, expression of PKG-CD suppressed glucose-induced p27 degradation. These data demonstrate that restoring the high-glucose-mediated decrease in PKG activity in VSMCs inhibits glucose-induced abnormal VSMC proliferation occurring upstream of Rb phosphorylation. Our work provides the first direct evidence linking glucose-induced vascular smooth muscle cell (VSMC) proliferation and cell cycle progression in VSMCs, suggesting that strategies to increase PKG activity might be useful in preventing abnormal VSMC proliferation in diabetic patients and might provide treatments for diabetes-associated proliferative vascular diseases.

guanosine 5’-cyclic monophosphate-dependent protein kinase; vascular smooth muscle cells; cell proliferation; cell cycle; glucose

DIABETES IS ASSOCIATED WITH increased incidence of atherosclerotic macrovascular disease, which is the major cause of morbidity and mortality in people with diabetes (15, 17, 31). Accumulating evidence shows that hyperglycemia/high glucose media stimulated vascular smooth muscle cell (VSMC) proliferation and cell cycle progression (21, 24, 25, 29, 30, 33, 39, 46, 49). Accelerated VSMC proliferation and accumulation in atherosclerotic lesions has also been observed in diabetic animal models (5, 6, 23), suggesting that abnormal VSMC proliferation plays an important role in the progression of diabetes-associated macrovascular diseases. However, the mechanism by which glucose stimulates VSMC proliferation and cell cycle progression is not fully understood.

Normally, arterial smooth muscle cells are contractile and quiescent in the G0/G1 phase of the cell cycle (2, 13, 22). After stimulation, VSMCs can divide by progression through the cell cycle. Cell cycle progression is dependent on the expression of cyclin-dependent kinases (CDKs), which form holoenzymes with their regulatory subunits, the cyclins (28). Progression through the cell cycle requires the cyclin/CDK complexes to phosphorylate the retinoblastoma protein (Rb), which releases the S phase transcription factor E2F to induce gene expression required for DNA synthesis and progression through the cell cycle (47). Activation of CDKs is dependent on their association with specific cyclin regulatory subunits (35). Cyclin D/CDK4/6 and cyclin E/CDK2 complexes phosphorylate Rb (47). In addition, the activity of CDKs is further regulated by association with inhibitory proteins, the cyclin-dependent kinase inhibitors (CKIs) (36). There are two classes of CKIs, the Ink4 family and the Cip/Kip family. The Ink4 family is composed of p15INK4b, p16INK4a, p18INK4c, and p19INK4D and inhibits CDK4 and CDK6 activity. The Cip/Kip family, including p21 (Cip1), p27 (Kip1), and p57 (Kip2), inhibits CDK2 and CDK4 activity. Molecules that target CDKs and CKIs represent a new class of therapeutic agents that influence vasculoproliferative diseases (4, 43).

cGMP-dependent protein kinase (PKG) is a serine/threonine kinase consisting of a regulatory and a catalytic domain within one polypeptide chain. There are two isoforms of PKG: PKG I and PKG II. PKG I is the major isoform existing in VSMCs. PKG I has been shown to modulate VSMC proliferation (9), migration (3), apoptosis (9), and extracellular matrix protein expression (11), suggesting that PKG maintains normal vascular cell function. Decreased PKG expression has been observed in the artery following balloon angioplasty (1). Furthermore, expression of constitutively active PKG reduces neointima formation in a model of in-stent restenosis (37). Decreased PKG levels have also been observed in aorta or VSMCs from both type 1 and type 2 diabetic rat models (19, 51), which were associated with abnormal vasodilation or VSMC migration. We demonstrated that glucose downregulation of PKG-I expression/activity in VSMCs occurs through PKC-dependent activation of the NADPH oxidase-derived reactive oxygen species, contributing to vessel dysfunction (27). These studies suggest that PKG might regulate proliferative responses of VSMCs to high-glucose stimuli.

In the current studies, we asked whether glucose-mediated downregulation of PKG activity is involved in abnormal VSMC proliferation under a high-glucose environment to better understand the mechanisms of diabetes-associated macrovascular diseases. Data from these studies now show that restoration of PKG activity in VSMCs inhibited high-glucose-induced VSMC proliferation and cell cycle progression involving an inhibition of CDK2 activity and Rb phosphorylation.

MATERIALS AND METHODS

Materials. FBS and DMEM, fungizone, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). Elastase was purchased from Elastin Products (Owensville, MO). Collagenase type IV was purchased from Worthington Biochemical (Lakewood, NJ).
Hybond enhanced chemiluminescence (ECL), nitrocellulose membrane, and ECL Western blotting detection reagents were purchased from Amersham Life Science (Piscataway, NJ). Cycloheximide was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies were purchased from the following suppliers: antiphosphorylated Rb (Ser807/Ser811) from Cell Signaling; cyclin D1 from Upstate; cyclin A, cyclin E, CDK2, CDK4, CDK6, p21, and p27 from Santa Cruz; α-smooth muscle actin antibody from Sigma; anti-PKG-I antibody

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from StressGen Biotechnology (Victoria, BC, Canada); anti-his (COOH-terminal) antibody from Invitrogen (Carlsbad, CA); and anti-
vasodilator-stimulated phosphoprotein (VASP) and anti-phosphory-
lated- VASP (Ser 239) antibodies from Calbiochem (San Diego, CA). Secondary antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). Protein G Plus/
Protein A-agarose beads were purchased from Calbiochem. His-
tone H1 was purchased from Boehringer Mannheim (Pleasanton,
CA). Phosphocellulose P81 filter paper was purchased from What-
man (Florham Park, NJ). [32P]ATP was purchased from Amersham
(Piscataway, NJ).

Cell culture. Rat and mouse VSMCs were isolated from the thoracic and abdominal aortas of rats and mice and characterized as
described previously (27). The rats and mice were cared for in
accordance with the National Institutes of Health Guide for the Care
and Use of Laboratory Animals. All protocols were approved by the
institutional animal care and use committee of the University of
Kentucky. Cells were maintained in DMEM containing 10% FBS, 50
µg/ml streptomycin, and 50 U/ml penicillin and subcultured weekly.
Cells of passage 2 and 3 were used in these studies. Primary human
aortic smooth muscle cells were purchased from ATCC and cultured
in complete vascular cell growth media (ATCC). Cells of passage 3
were used in these studies.

Cell growth assay and flow cytometry. VSMCs were cultured in
DMEM media with 10% FBS and then switched to serum-free
DMEM media with 5 mM glucose for 48 h. Cells were treated with
serum-free DMEM media with normal (5 mM) or high (30 mM)
glucose for 48 h. Media was changed every 12 h to maintain glucose
levels. Mannitol (25 mM) + glucose (5 mM) was used as osmolarity
control. After treatment, cells were harvested, and cell proliferation
was measured by both counting the cells in a hematocytometer and
measuring bromodeoxyuridine (BrdU) incorporation during DNA
synthesis in proliferating cells with the commercial cell proliferation
enzyme-linked immunosorbent assay (ELISA) system (Roche
Applied Sciences).

To perform BrdU incorporation assay, cells were seeded in 96-well
culture plates. After normal or high glucose treatment, medium was
removed, and the cells were incubated with a BrdU-labeling solution
(provided by the kit) for 3 h. After labelling, FixDenat solution
(provided by the kit) was added and incubated for 30 min. Then,
anti-BrdU-POD solution was added to cells and incubated for 90
min. Finally, substrate was added to cells to allow color develop-
ment. After color developed, the reaction was stopped, and the
absorbance of the samples was measured in an ELISA plate reader
at 450 nM. Culture medium alone was used as a control for
nonspecific binding.

For cell cycle analysis, cells were harvested after 48 h of treatment.
DNA was stained with 100 µg/ml propidium iodide for 30 min at 4°C,
and 1 × 10⁶ cells were then analyzed with a fluorescence-activated
cell sorter (FACS) (Becton-Dickinson).

Transient transfection of VSMC. The cDNA of bovine catalytic
domain of PKG Iα (PKG-CD) was inserted in expression vector of
cDNA4/TTO/myc-His C/CD as described previously (45). The empty
vector pcDNA4/TTO/myc-His C was used as control. VSMCs were
transiently transfected with PKG-CD expression vector or empty
vector using Nucleofector reagent and electroporator (AMAXA, San
Diego, CA). A green fluorescent protein (GFP) expression vector was
cotransfected in cells to determine the transfection efficiency. In
addition, transfection efficiency was confirmed by immunoblotting to
detect the expression of PKG-CD or His-tagged PKG-CD in cell
lysates recognized by anti-PKG-I and anti-his (COOH-terminal)
antibodies, respectively.

PKG activity assay. Cells were harvested and homogenized.
Supernatants were assayed for PKG activity by measuring the incorpo-
ration of p32 from [γ-32P]ATP in a specific PKG substrate, the
BPDeTide: RKISASEFDRPL (AnaSpec) as described previously (3).
Briefly, 10 µl of extract was assayed for PKG activity in 100 µl assay
buffer with 0.2 mM [γ-32P] ATP, 100 µM of a BPDeTide, and 1µM
protein kinase A (PKA) inhibitor. Assays were conducted at 30°C
for 5 min in the presence or absence of 10 µM cGMP. In the absence
of cGMP, the constitutive PKG activity is measured; the addition
of cGMP allows measuring cGMP-stimulated PKG activity. The reac-
tion was terminated by aliquoting samples to P81 phosphocellulose
paper and washing in 75 mM phosphoric acid. Next, the paper was
dried and counted in 10 ml scintillation fluid. PKG activity was
expressed as nanomoles of peptide phosphorylated per minute per
milligram of cell extract protein.

Western blot analysis. VSMCs were transiently transfected with
empty vector or expression vector for PKG-CD using an electropo-
roration method and were seeded in six-well plates at a density of 3 ×
10⁴ cells/ml. Cells were grown in growth medium containing 10% FBS during
which cells reached 80% confluence and then rendered quiescent
by culturing in serum and insulin-free DMEM containing 5 mM
for 48 h. Cells were treated with serum-free medium contain-
ing either 5 (normal glucose) or 30 (high glucose) mM glucose for
48 h. Media was changed every 12 h to maintain glucose levels.
Cells were harvested, and cell lysates were prepared. The protein levels
of PKG-CD, His-tagged PKG-CD, VASP, phosphorylated-VASP
(Ser 239), cyclins, CDKs, p21, and p27 were determined by immuno-
blotting.

In vitro kinase assay. The activities of cyclin E-CDK 2 were
measured using an in vitro kinase assay as described previously
(32). VSMCs were cultured. After treatment, cell lysates were
prepared. Cell lysates (100–200 µg of total protein) were pre-
cloned with Protein G Plus/Protein A-agarose beads for 30 min at
4°C. The extracts were then incubated with anti-cyclin E antibody
for 4°C at 12 h and then for 1 h with 25 µl of Protein G
Plus/Protein A-agarose beads. Immunocomplexes were washed
times with lysis buffer and two times with kinase buffer,
including 40 mM Tris-HCl (pH 7.5), 150 mM NaCl, 20 mM
MgCl₂, and 1 mM dithiothreitol. Subsequently, the beads were
resuspended in 30 µl kinase buffer containing 2 µg of histone H1,
7 µM ATP, and 5 µCi of [γ-32P]ATP and incubated at 37°C for 30
min. The kinase reaction was stopped by addition of 100 mM
EDTA. The reaction products were spotted on phosphocellulose
P81 paper, rinsed in 75 mM phosphoric acid and 96% ethanol,
and air-dried. The radioactivity of the reaction products on the paper
was determined using a liquid scintillation counter.

Statistical analysis. Data are means ± SE. Differences between
groups were determined by ANOVA or by t-test as appropriate.
The significance levels were P < 0.05.
RESULTS

Expression of constitutively active PKG to restore PKG activity inhibits glucose-induced VSMC proliferation and cell cycle progression. Previously, we demonstrated that PKG activity is downregulated in vessels from diabetic animals or in VSMCs exposed to high-glucose media, contributing to diabetes-associated vessel dysfunction (27). In the current studies, we determined whether decreased PKG activity is involved in glucose stimulation of VSMC proliferation and cell cycle progression.

PKG activity was restored in VSMCs by transfection of primary rat aortic smooth muscle cells with expression vector for constitutively active PKG (PKG-CD), and the effect of glucose on cell proliferation and cell cycle progression was determined. Mannitol was used as osmolality control. The results showed that expression of the constitutively active PKG inhibited glucose stimulation of VSMC proliferation demonstrated by both cell counts (Fig. 4A) and BrdU incorporation assay (Fig. 4B). High-glucose exposure stimulated an increase in the percentage of cells entering the S phase was also blocked by the expression of constitutively active PKG (Fig. 1C). Transfection efficiency in these cells was determined by co-transfection of an expression vector for GFP and reached about 80–85%. Moreover, expression of PKG-CD and His-tagged PKG-CD in cell lysates was analyzed to confirm the transfection efficiency. The anti-PKG-I antibody can recognize both endogenous PKG-I and expressed PKG-CD. As shown in Fig. 1, D and E, endogenous PKG-I was downregulated by high-glucose treatment, which confirmed our previous studies (27). In addition, both PKG-CD (recognized by anti-PKG-I antibody) and His-tagged PKG-CD (recognized by anti-His (COOH-terminal) antibody) were observed in cell lysates under both normal and high-glucose conditions, indicating the efficient transfection of PKG-CD expression vector in VSMCs.

PKG activity assay was also analyzed in these cells. As shown in Fig. 1F, there was no significant difference of basal PKG activity (in the absence of cGMP) between the high-glucose group and normal glucose group. Transfection of KG-CD similarly increased basal PKG activity in both the normal glucose and high-glucose groups. However, high-glucose concentrations significantly decreased maximal endogenous PKG activity (in the presence of cGMP). Expression of PKG-CD overcame glucose-mediated downregulation of PKG activity (Fig. 1F). The effect of PKG-CD expression on VASP protein levels and phosphorylation levels of VASP at Ser239 in VSMCs treated with normal or high-glucose media was also determined by immunoblotting. VASP is a ubiquitously expressed endogenous substrate for PKG, and phosphorylation of VASP at Ser239 has been used as a biomarker for activation of PKG (38). High-glucose treatment did not affect the expression of total VASP protein levels. However, phosphorylated VASP at Ser239 was significantly decreased in cells treated with high glucose, which was overcome by expression of PKG-CD (Fig. 1G). Together, these data indicate that restoration of PKG activity in VSMCs inhibits high-glucose-induced VSMC proliferation and S phase entry.

To exclude the possibility of a species-specific effect, the obtained results for rat VSMCs were compared with VSMCs derived from murine and human VSMCs. As shown in Fig. 2, high-glucose treatment stimulated both murine and human VSMC proliferation (demonstrated by both cell counts and BrdU incorporation assay) and increased in the percentage of cells entering the S phase (FACS analysis), which was inhibited by expression of PKG-CD. The efficient transfection for PKG-CD expression vector was confirmed by immunoblotting as described above (data not shown). This result excludes the possibility of a species-specific effect of our finding.

Expression of constitutively active PKG inhibits glucose-induced phosphorylation of Rb. In the following studies, we determined the mechanism by which restoration of PKG activity inhibits high glucose-induced G1 to S phase progression in VSMCs. We examined the effect of expression of PKG-CD on Rb phosphorylation at specific phosphorylation sites (Ser807/811). It is known that cyclin/CDK-dependent Rb phosphorylation is necessary for cells to exit G1 phase and enter S phase (47). As shown in Fig. 3, VSMCs exposed to high glucose media exhibited increased levels of phosphorylated Rb at Ser807/811, which was inhibited by expression of PKG-CD. This data suggest that expression of PKG-CD inhibits glucose-induced VSMC proliferation and cell cycle progression, at least in part, through an inhibition of Rb phosphorylation.

Expression of constitutively active PKG regulates cyclin expression and CDK activity in VSMCs. To delineate whether the upstream signaling that mediates Rb phosphorylation in VSMCs after high-glucose exposure is altered and affected by expression of PKG-CD, first, we performed Western blotting to look at the protein levels of cyclin A, E, and D1 and CDK 2, 4, and 6 in cell lysates. Cyclin D binds to CDK4/6, leading to activation of CDK4/6, whereas cyclin E binds to CDK2 and enhances CDK2 activity. Both CDK2 and CDK4/6 activation results in the phosphorylation of Rb protein. Our results showed that high-glucose exposure increased cyclin A, E, and D1 protein levels. However, CDK2, 4, and 6 protein levels in VSMCs were not altered by high-glucose treatment. In addition, expression of PKG-CD inhibited only glucose-induced cyclin E expression, but not cyclin A and cyclin D1 levels (Fig. 4). Next, we determined whether cyclin E-associated CDK2 activity was changed in VSMCs after high-glucose exposure. We measured CDK2 activity. As shown in Fig. 5, high-glucose treatment significantly increased CDK2 activity, which was inhibited by expression of PKG-CD. Together, these data indicate that expression of PKG-CD inhibits glucose-induced cyclin E expression and cyclin E-CDK2 activity in VSMCs.

Expression of constitutively active PKG prevents glucose-induced p27 degradation. In addition to be regulated by cyclin/CDKs, CDKs can also be regulated by a family of CKIs. CDK2 activity can be inhibited by CKIs such as p21\textsuperscript{waf1}, p27\textsuperscript{kip1}, and p57\textsuperscript{kip2} (44). Therefore, first, we analyzed the effect of PKG-CD on p21\textsuperscript{waf1} and p27\textsuperscript{kip1} mRNA levels. As shown in Fig. 6A, high-glucose treatment significantly downregulated p27 protein levels in VSMCs, which was inhibited by expression of PKG-CD. p21 levels were not significantly changed by high-glucose treatment.

Next, to determine the mechanisms by which restoration of PKG activity prevents glucose-induced degradation of p27, we examined the effect of PKG-CD on p27 mRNA levels and protein stability. The mRNA levels of p27 did not change with glucose treatment or with expression of PKG-CD (data not shown). We then determined the effect of PKG-CD on
p27 turnover. VSMCs were transfected with expression vector for PKG-CD or control vector. Next, cycloheximide (10 μg/ml) was added to inhibit the de novo protein synthesis in the presence of high-glucose media. The rate of p27 degradation was significantly decreased in cells with expression of PKG-CD (Fig. 6B). Expression of PKG-CD had no significant effect on p27 turnover under normal glucose media (data not shown). These data indicate that PKG-CD regulates p27 mainly through posttranscriptional mechanisms by stabilizing glucose-induced p27 degradation.

**DISCUSSION**

Previously, we demonstrated that high-glucose treatment downregulated PKG activity in VSMCs in vitro as well as in the vessels of a diabetic mouse model, contributing to diabetes-associated vessel dysfunction (27). Here, we provide the first direct evidence linking decreased PKG activity to high-glucose-induced proliferation and cell cycle progression in VSMCs. Our data demonstrated that restoration of PKG activity in VSMCs inhibited high-glucose-induced VSMC proliferation and G1 to S cell cycle progression. The mechanisms by which restoration of PKG activity suppresses glucose-induced VSMC proliferation involve an inhibition of glucose-induced p27 degradation, glucose-induced expression of cyclin E and cyclin E-CDK2 activity, and glucose-induced Rb phosphorylation.

The effect of glucose on VSMC proliferation has been studied by many investigators. Most studies showed increased...
proliferation of VSMCs after high-glucose exposure (21, 24, 25, 29, 30, 33, 39, 46, 49) while others failed to observe the mitogenic effect of glucose on VSMCs (18, 41). The reason for such discrepancy is unclear and may relate to the different concentrations of glucose or serum in the culture media or different time periods of glucose treatment. Nevertheless, our current studies using primary rat, murine, and human aortic smooth muscle cells support the mitogenic effect of glucose on VSMC growth, which has been observed by other investigators using different origins of VSMCs, including bovine, porcine, and human (20, 30, 39, 46). Moreover, smooth muscle cells from diabetic individuals exhibited increased proliferation (12). Taken together, our work and that of others supports the pathophysiological importance of glucose-mediated VSMC proliferation.

PKG-I is a major downstream target molecule for nitric oxide and the cGMP signaling pathway in VSMCs. Accumulating evidence supports the anti-proliferative effect of nitric oxide/cGMP on VSMCs (26). The anti-proliferative effect of cGMP on VSMCs has been shown to be mediated by activation of either PKG (50) or PKA (10). Studies from Yu et al. (50) suggest that agents increasing cGMP inhibited epidermal growth factor-stimulated VSMC proliferation through activation of PKG (50), whereas Cornwell et al. (10) showed that cGMP inhibited platelet-derived growth factor (PDGF)-stimulated VSMC proliferation through activation of PKA, but not PKG. The effect of PKG itself on VSMC proliferation has also been studied. Boerth et al. (3) showed that increased PKG activity in VSMCs by expression of PKG I (adding cGMP to activate it) or expression of the PKG-CD had no effect on PDGF-stimulated VSMC proliferation. Moreover, smooth muscle cells from diabetic individuals exhibited increased proliferation (12). Taken together, our work and that of others supports the pathophysiological importance of glucose-mediated VSMC proliferation.

In agreement with our previous studies (27), our present data confirmed high-glucose exposure mediated downregulation of PKG-I expression/activity in VSMCs. When VSMCs were transfected with constitutively active PKG to restore PKG activity to normal glucose levels, these cells were unresponsive to high-glucose-stimulated proliferation. These data suggest that decreased PKG activity may mediate glucose stimulation of VSMC proliferation. However, under normal glucose conditions, increased PKG activity did not have effects on VSMC growth. This is in contrast to the study from Chiche et al. (9) in which they showed that increased PKG activity in VSMCs inhibited serum-stimulated VSMC proliferation. This discrepancy may be explained by different mitogenic stimuli to VSMCs (PDGF vs. serum). In our current studies, we determined whether PKG regulates glucose-stimulated VSMC proliferation.

Fig. 4. Effect of expression of PKG-CD on the expression of cyclin-dependent kinases (CDKs) in VSMCs under both normal and high-glucose conditions. VSMCs were transiently transfected with empty vector or expression vector for PKG-CD and made quiescent in serum-free media. Cells were then treated with normal (5 mM) or high (30 mM) glucose for 48 h. The protein levels of cyclins or CDKs in cell lysates were assayed by Western blotting. β-Actin was used as a loading control. The blot shown is the representative of 3 separate experiments. The relative levels of cyclins or CDKs were determined by scanning densitometry of immunoblots. Results are means ± SE. P < 0.05 vs. NG (*), vs. NG + PKG-CD (#), and vs. HG (^).

Fig. 5. Expression of PKG-CD inhibits glucose-induced cyclinE-CDK2 activity in VSMCs. VSMCs were transfected with constitutively active PKG to restore PKG activity to normal glucose levels, these cells were unresponsive to high-glucose-stimulated proliferation. These data suggest that decreased PKG activity may mediate glucose stimulation of VSMC proliferation. However, under normal glucose conditions, increased PKG activity did not have effects on VSMC growth. This is in contrast to the study from Chiche et al. (9) in which they showed that increased PKG activity in VSMCs inhibited serum-stimulated proliferation under normal glucose
media. This difference may be attributable to different components in culture media used to treat cells: serum-free DMEM media in our studies vs. 4% serum-DMEM media in studies from Chiche et al.

It is known that cell proliferation is dependent on the cell cycle progression, in which cells transit through the G0/G1 phase to the S phase, and eventually to the G2-M phase. Progression through G1 and entry in the S phase is rate-limiting and is regulated by formation and activation of cyclin/CDK complexes, and endogenous CKIs inactivate these complexes and lead to G1 arrest (42). Cyclin D/CDK2, -4, or -6 controls early G1, and at this stage p21 and/or p27 can bind and exert cell cycle inhibition. Cyclin E/CDK2 accumulates during late G1 and triggers passage into S phase. Cyclin D/CDK4/6 and cyclin E/CDK2 complexes participate in the phosphorylation of Rb protein, resulting in release of the transcription factor E2F, which is required for cells to enter S phase (47). Our results from this study demonstrated that high-glucose treatment significantly increased protein levels of cyclin A, E, and D1. In contrast, recent studies from Chan et al. (8) only showed elevated cyclin A and D1 levels by glucose, but not cyclin E levels. This discordance may be due to different types of VSMCs: primary VSMCs in our studies vs. VSMC cell line A7r5 in their studies. In addition, our data also demonstrated that increased PKG activity did not inhibit glucose-induced cyclin D1 levels, suggesting that the cyclin D/CDK4/6 complex that activated in the early G1 phase is not influenced by increased PKG activity (34). Inhibition of glucose-induced cyclin E- and cyclin E-associated CDK2 activity by increased PKG activity in our studies further supports that PKG affects late G1 events occurred in VSMCs when exposed to high-glucose media. Consistently, studies from Hanada et al. (16) exhibited that overexpression of PKG inhibited cyclin E transcription and resulted in inhibition of serum-stimulated mesangial cell cycle progression. Together, our studies suggest that the inhibition of glucose-induced Rb phosphorylation in VSMCs by increased PKG activity may be partially due to reduced cyclinE/CDK2 activity.

In addition to regulation of cyclin/Cdk, we also demonstrated that the mechanism of increased PKG activity-mediated growth inhibition in VSMCs under high-glucose conditions involves p27-mediated cyclin/CDK inhibition. In this study, p27 was abundantly expressed in VSMCs cultured in serum-

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Fig. 6. Expression of PKG-CD prevents glucose-induced protein degradation of CDK inhibitory protein p27. VSMCs were transiently transfected with empty vector or expression vector for PKG-CD and made quiescent in serum-free media. A: cells were then treated with normal (5 mM) or high (30 mM) glucose for 48 h. The protein levels of p21 or p27 in cell lysates were assayed by Western blotting. ß-Actin was used as a loading control. The blot shown is the representative of 3 separate experiments. The relative levels of p21 or p27 were determined by scanning densitometry of immunoblots. Results are means ± SE. P < 0.05 vs. NG (A) and vs. HG (#). ns, No significant difference. B: cycloheximide (10 µg/ml) was added, and the levels of p27 were determined by Western blotting at various time points.

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Fig. 7. Model for the role of PKG in regulation of glucose-induced cell cycle progression in VSMCs. High-glucose concentrations stimulate G1 to S phase cell cycle progression and VSMC proliferation, contributing to the development of diabetes-associated proliferative vascular disease. High-glucose concentrations downregulate PKG activity. Expression of constitutively active PKG-I (PKG-CD) to restore PKG activity inhibits glucose-induced cyclin E expression and increases p27 protein stability, resulting in decreased CDK 2 activity and reduced Rb phosphorylation, leading to cell cycle arrest and cell growth inhibition.
free DMEM media with normal glucose concentration, and its level was downregulated by high-glucose treatment as previously described (8). We also showed that increased PKG activity inhibited glucose-mediated p27 degradation. Similar results that cAMP is associated with p27 upregulation and cGMP with a transient increase in p27 levels have been reported in human VSMCs (14). Moreover, we demonstrated that increased PKG activity regulates p27 mainly through posttranscriptional mechanisms by stabilizing glucose-induced p27 degradation. This result is in contrast from studies from Cen et al. (7), in which they showed that activation of PKG increased p27 expression through a transcriptional mechanism in human SW480 colon cancer cells, suggesting that the mechanisms by which PKG regulates p27 expression may be cell type specific. In addition, studies have demonstrated that S phase kinase-associated protein-2 (skp2), a component of the skp1-cul5-F-box ubiquitin ligase complex, is responsible for regulation of p27 degradation (40). A recent study showed that cGMP or cAMP through activation of PKA inhibited skp2 expression and VSMC proliferation (48). Whether increased PKG activity regulates skp2 and contributes to stabilization of p27 in our system needs to be investigated.

In conclusion, our studies provide the first direct evidence linking decreased PKG activity to high-glucose-induced proliferation and cell cycle progression in VSMCs. The possible mechanism of the effect of PKG on the regulation of glucose-induced cell proliferation was shown in Fig. 7. Our studies indicate that restoration of PKG activity inhibits high-glucose-induced VSMC proliferation. Increased PKG activity also inhibits glucose-induced G1 to S phase cell cycle progression by inhibiting glucose-induced cyclin E expression, cyclin E-CDK 2 activity, glucose-induced p27 degradation, and phosphorylation of Rb in VSMCs.

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REFERENCES


