Acute glucose-induced downregulation of PKC-βII accelerates cultured VSMC proliferation

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Yamamoto, Mayumi, Mildred Acevedo-Duncan, Charles E. Chalfant, Niketa A. Patel, James E. Watson, and Denise R. Cooper. Acute glucose-induced downregulation of PKC-βII accelerates cultured VSMC proliferation. Am J Physiol Cell Physiol 279: C587–C595, 2000.—Accelerated vascular smooth muscle cell (VSMC) proliferation contributes to the formation of atherosclerotic lesions. To investigate protein kinase C (PKC)-βII functions with regard to glucose-induced VSMC proliferation, human VSMC from aorta (AoSMC), a clonal VSMC line of rat aorta (A10), and A10 cells overexpressing PKC-βI (βI-A10) and PKC-βII (βII-A10) were studied with the use of three techniques to evaluate glucose effects on aspects affecting proliferation. High glucose (25 mM) increased DNA synthesis and accelerated cell proliferation compared with normal glucose (5.5 mM) in AoSMC and A10 cells, but not in βI-A10 and βII-A10 cells. The PKC-βII-specific inhibitor CGP-53553 inhibited glucose-induced cell proliferation and DNA synthesis in AoSMC and A10 cells. In flow cytometry analysis, high glucose increased the percentage of A10 cells at 12 h after cell cycle initiation but did not increase the percentage of βI-A10 or βII-A10 cells entering S phase. PKC-βII protein levels decreased before the peak of DNA synthesis, and high glucose further decreased PKC-βII mRNA and protein levels in AoSMC and A10 cells. These results suggest that high glucose downregulates endogenous PKC-βII, which then alters the normal inhibitory role of PKC-βII in cell cycle progression, resulting in the stimulation of VSMC proliferation through acceleration of the cell cycle.

atherosclerosis; cell cycle; diabetes mellitus; protein kinase C inhibitor; thymidine

There is an apparent increase in the prevalence of atherosclerosis in diabetic patients (31). Coronary atherosclerotic disease is a primary cause of death in diabetic patients (7), and diabetes mellitus is a well-established risk factor for peripheral vessel disease caused by atherosclerosis (20). Hyperglycemia is probably an important etiologic factor in the development of macrovascular complications in diabetic patients (37, 39). Although the development of atherosclerotic lesions in diabetes mellitus is a complex and dynamic interaction of many factors, the mechanism by which hyperglycemia might cause vascular complications remains unknown.

It is widely recognized that vascular smooth muscle cell (VSMC) proliferation is a key event in the formation of atherosclerotic lesions (29). Abnormal proliferation of VSMC with subsequent formation of intimal thickening is thought to play a role in the development of lesions (34). Prolonged high glucose exposure alters calcium channels (30), growth rates (23, 9), and protein kinase C (PKC) levels (8, 11, 19, 38, 41) in VSMC. High glucose activates PKC in several cell types including VSMC (8, 11, 19, 38, 41).

PKC is a complex family of at least 11 isozymes classified into 3 groups: classic PKCs (PKC-α, -βI, -βII, and γ), which require diacylglycerol (DAG), phospholipids, and Ca2+ for full activity; novel PKCs (PKC-δ, -ε, -η, and -θ), which are phospholipid and DAG dependent but Ca2+ independent; and atypical PKCs (PKC-ζ, -λ, and -μ), which require only phospholipids (12, 25, 35). In VSMC, PKC modulates contraction (28), signal transduction (4, 18, 40), growth rates (23), and DNA synthesis or cell cycle progression (1, 13, 14, 15, 24, 32, 33, 36). Although numerous studies have been performed, little is known about isozyme-specific actions of PKC on VSMC proliferation.

We have shown that PKC-βII has a stimulatory effect and PKC-βI an inhibitory effect on the cell cycle progression in VSMC, because stable overexpression of PKC-βI (βI-A10) and PKC-βII (βII-A10) in A10 cells, a clonal VSMC line derived from rat aorta, altered cell cycle regulation (42). In the present study, we investigated acute effects of extracellular high glucose on AoSMC (primary VSMC cultures of human thoracic aorta) as well as A10, βI-A10, and βII-A10 cell cycle progression.

METHODS

Materials. A10 (ATCC CRL 1476) were obtained from American Type Culture Collection, and AoSMC were obtained from Clonetics (San Diego, CA). Dulbecco's modified

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Eagle’s medium (DMEM), 0.04% trypsin-EDTA, fetal bovine serum (FBS), Dulbecco’s phosphate-buffered saline (DPBS), penicillin G, and streptomycin sulfate were purchased from Gibco BRL (Gaithersburg, MD). Smooth muscle growth medium (SMGM), human recombinant epidermal growth factor (hEGF), human recombinant fibroblast growth factor (hFGF), dexamethasone, gentamycin, and amphotericin-B were purchased from Clontech. Culture materials including 75- and 25-cm² flasks, 6-, 12-, 24-, and 96-well plates, and 100-mm culture dishes were purchased from Costar (Cambridge, MA). EDTA and neomycin derivative G418 (Geneticin) were purchased from Gibco (Grand Island, NY).

-p-Glucose, mannitol, propidium iodide (PI), phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, and nonylphenoxypolyethoxy ethanol (NP-40) were purchased from Sigma (St. Louis, MO). The cell proliferation assay kit was purchased from Promega (Madison, WI). [3H]thyminidine (dThd; 1 mCi) was purchased from Du Pont NEN (Boston, MA). The protein assay kit with bovine serum albumin (BSA) for a standard and goat anti-rabbit γ-globulin coupled to hors eradish peroxidase (HRP) were purchased from Bio-Rad (Hercules, CA). Specific antibodies for PKC-β I and -II were purchased from Santa Cruz (Santa Cruz, CA). The enhanced chemiluminescence (ECL) system was purchased from Amersham (Arlington Heights, IL). Specific PKC inhibitors, CGP-53353 and CGP-41251, were kindly provided by Dr. D. Fabbro (Novartis Pharma, Basel, Switzerland). CGP-53353 inhibits PKC-β II with an IC₅₀ of 0.41 mM and PKC-β I with an IC₅₀ of 3.8 mM. CGP-41251 is a staurosporine derivative that inhibits cPKC (IC₅₀ = 0.05 µM) (7a).

Cell culture. The clonal VSMC line A10 derived from fetal rat aorta has been characterized as a model for the investigation of various drug and hormone effects on biochemical changes (16). Cells were grown in DMEM with 5.5 mM glucose containing 10% FBS, 10 U/ml penicillin G, and 50 µg/ml streptomycin sulfate at 37°C in a humidified, 5% CO₂–95% air atmosphere in either 25-cm² flasks (for cell cycle analysis), 6-well plates (for Western blot analysis), 24-well plates (for [3H]dThd incorporation study), or 96-well plates (for cell proliferation assay). Early passages. Less than five following plating from frozen stocks, of cells were used here. To evaluate A10 as a model for examining glucose effects on cell cycle progression, we used primary cultures of AoSMC. These cells were grown in SMGM containing 5.5 mM glucose, 5% FBS, 10 ng/ml hEGF, 2 ng/ml hFGF, 390 ng/ml dexamethasone, 50 mg/ml gentamycin, and 50 ng/ml amphotericin-B at 37°C in a humidified atmosphere. 5% CO₂–95% air atmosphere according to the company recommendations. Cells were grown to 90% confluence, and media were changed every 5 days during growth.

Overexpression of PKC-β I and -II isoforms in A10 cells. The rat cDNA encoding PKC-β I in the expression vector pMVT (10) and PKC-β II in pMTH (22) were used to transfect A10 using calcium phosphate coprecipitation. The PKC expression vectors were kind gifts from Dr. Harald Mischak (GSF, Munich, Germany) (22). A10 cells maintained in DMEM containing 10% FBS with 5.5 mM glucose were trypanblueized with 0.1% trypsin-0.4% EDTA, seeded on 100-mm culture dishes in the same medium, and grown to confluence. Cells were then incubated for 4 h with calcium phosphate-DNA coprecipitates containing 15 µg of the expression vector or the empty vector as a control. After 18 h, cells were switched to fresh medium. Within 48 h, cells were trypsinized and replaced in DMEM supplemented with 10% FBS with 600 µg/ml of the Geneticin to select for cells with neomycin resistance as described previously (42). After 10–16 days in selection medium, cells were examined for the presence of PKC-β I and -II isoform overexpression by Western blot analysis. Overexpression was defined for these cells as a four- to fivefold increase in PKC content measured by Western blot analysis compared with A10 or empty vector-transfected control cells as described previously (42).

Cell proliferation assay by MTT method. The effects of glucose on cell proliferation were examined in AoSMC, A10, βI-A10, and βII-A10 cells. A10, βI-A10, and βII-A10 cells in secondary cultures were trypsinized and seeded in 96-well plates at 1 × 10⁵ cells/well in 100 µl of DMEM containing 10% FBS with various concentrations (5.5–40 mM) of d-glucose and 5.5 mM d-glucose plus 34.5 mM mannitol as an osmotic control. To determine the contribution of PKC-β II function in glucose-induced cell proliferation, cells were also treated with CGP-53353, a PKC-β II-specific inhibitor (27), and CGP-41251, a classic PKC inhibitor (7a). Cell proliferation assay was performed using the cell proliferation assay kit as follows. After incubation at 37°C for 48–96 h, 15 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added (final concentration 0.5 µg/ml). After 4 h incubation, 100 µl of 20% sodium dodecyl sulfate (SDS) were added to dissolve the formazan crystals formed. Optical density was measured with an ELISA plate reader using test and reference wavelengths of 570 and 630 nm, respectively.

AoSMC were also examined under the same conditions but with SMGM to determine whether glucose effects were similar in AoSMC. For the A10 and AoSMC, we established a linear relationship between the MTT reduction assay and viable cell number. Expression of the empty vector in A10 cells had no effect on the response to glucose reported here. Measurement of [3H]dThd incorporation into DNA. The effect of glucose on DNA synthesis in A10, βI-A10, and βII-A10 cells was determined using [3H]dThd incorporation in 5.5 mM (normal) and 25 mM (high) glucose as described previously (42). Cells were seeded on 24-well plates, and G₁/early G₂ synchronization was achieved by serum deprivation. Media were switched to DMEM containing 10% FBS with normal or high glucose for 48 h. Every 6 h, cells were incubated with 1 ml of the same medium containing 2.0 µCi/ml [3H]dThd for 1 h at 37°C. Cells were rinsed with ice-cold DPBS buffer, and acid-soluble radioactivity was removed by treatment with 10% trichloroacetic acid. Cells were then dissolved with 1 N NaOH and neutralized with 1 N HCl. The [3H] radioactivity in acid-soluble pools was determined, and DNA synthesis was estimated [counts per minute (cpm)/well]. Expression of the empty vector had no effect on DNA synthesis.

Cell cycle analysis using flow cytometry. The effects of glucose on cell cycle progression were analyzed in A10, βI-A10, and βII-A10 cells using flow cytometry as described previously (42). Cells were seeded, and G₁/early G₂ synchronization was achieved by serum deprivation. Media were switched to DMEM containing 10% FBS with 5.5 mM (normal) or 25 mM (high) glucose to initiate the cell cycle. Cells were sampled at the indicated time after initiation of the cell cycle. Cells were washed with ice-cold DPBS and fixed with ice ethanol. Cells were then removed from ethanol and stained with 1 ml of DPBS containing 50 µg/ml PI. Analysis of the stained nuclei was performed by using a fluorescence-activated cell analyzer with cell cycle-analyzing computer software (FACSStarPlus, Becton Dickinson, San Jose, CA) in 1×10⁶ total cells. The expression of empty vector had no effect on cell cycle progression reported here.

Western blot analysis. The changes in immunoreactive PKC-β I and -II after 5.5 mM (normal) and 25 mM (high) glucose exposures were measured in AoSMC, A10, βI-A10, and βII-A10 cells by Western blot analysis using specific PKC-β I and -II antibodies. Cells in secondary cultures maintained in DMEM containing 10% FBS or SMGM con-
taining 5% FBS with 5.5 mM glucose were trypsinized and seeded on 6-well plates at a density of 1\times10^6 cells/well in 3 ml of the same medium. When cells were grown to 80% confluency, G_0/early G_1 synchronization was achieved by serum deprivation for 72 h. The cell cycle was then initiated, and cells were incubated for the indicated times at 37°C with 5.5 mM (normal) or 25 mM (high) glucose. Subsequently, cells were washed with ice-cold DPBS, scraped from dishes with a rubber policeman, and centrifuged at 2,000 rpm for 5 min, and the cell pellet was lysed in a buffer containing 20 mM Tris·HCl (pH 7.5), 145 mM NaCl, 10% (wt/vol) glycerol, 5 mM EDTA, 0.2 mM Na_2VO_4, 0.1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotonin, and 1% (wt/vol) NP-40. The suspension was sonicated for 5 s and centrifuged at 2,000 rpm for 5 min, and the resultant supernatant was used as the whole cell lysate fraction. The protein concentration was determined by the method of Bradford (2) with BSA as a standard. All of the procedures were performed at 4°C. The samples were dissolved in Laemmli’s sample buffer containing 1% SDS (17) and resolved by SDS-PAGE on 4.5% (wt/vol) stacking gels and 10% (wt/vol) resolving gels in a Mini-PROTEIN II dual-slab cell (Bio-Rad) and electrophotographically transferred to nitrocellulose membranes (Bio-Rad). Membranes were equilibrated in Tris-buffered saline (TBS; 20 mM Tris·HCl and 50 mM NaCl, pH 7.40), and nonspecific binding sites were blocked by 10% dried milk in TBS containing 0.1% Tween 20 (TBS-T) at room temperature for 1–2 h. Membranes were then incubated in TBS containing antibodies to PKC-βI and -βII (Santa Cruz) at room temperature for 3 h. After being washed with TBS-T, membranes were incubated in TBS-T containing a goat anti-rabbit γ-globulin coupled to HRP (Bio-Rad) for 30 min. The blots were developed with the ECL system (Amersham) and visualized by exposure to Hybond-C extra ECL film (Amersham). The developed films were analyzed by a densitometric scanner linked to a Macintosh computer (Apple, Cupertino, CA). Less than five consecutive passages of A10 cells were used for determination of PKC-βII levels, because it has been reported that PKC isozyme expression varies with multiple passages of VSMC (8). Expression of empty vector had no effect on the PKC-βII expression reported here, as described previously (42).

Data analysis. Experiments were repeated at least three times to ensure reproducible results. Data are expressed as means ± SE of the number of observations. The statistical significance was assessed by one-way analysis of variance (ANOVA) or Student’s t-test. P < 0.05 was considered statistically significant. Significance was determined after three or more separate experiments.

RESULTS

A10 cells as a model for human VSMC to demonstrate high glucose effects on cell proliferation and DNA synthesis. To establish A10 cells as an appropriate model to demonstrate acute effects of high glucose on VSMC proliferation, we examined cell proliferation in both human AoSMC and rat A10 cells using a dye-based assay that measured relative cell number. Human aortic smooth muscle cells (AoSMC), primary human vascular smooth muscle cells (VSMC), and A10 cells, a clonal VSMC cell line from rat aorta. To validate A10 as a model for studying glucose effects on human VSMC proliferation, effects of glucose on cell proliferation (A) and DNA synthesis (B) were compared between AoSMC and A10 cells. A: cells were plated in 96-well plates in appropriate media with 5.5–40 mM glucose and 5.5 mM glucose + 34.5 mM mannitol as an osmotic control. Cell proliferation of AoSMC and A10 cells was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described in METHODS. Data are expressed as the percentage of control (5.5 mM glucose exposure). Cell proliferation was stimulated by increasing glucose concentrations in A10 and AoSMC. Data are means ± SE for quadruplicate determinations (n = 4) repeated in 3 separate experiments. *P < 0.05, **P < 0.01 vs. 5.5 mM glucose. B: cells were plated in the 24-well plates and synchronized, and then the cell cycle was initiated with appropriate media with normal (5.5 mM) or high (25 mM) glucose. The [3H]thymidine (dThd) incorporation was measured as described in METHODS. Data demonstrate the percentage of control (before cell cycle initiation) in radioactivity (counts per minute (cpm)/well) with 5.5 mM (normal) or 25 mM (high) glucose. In AoSMC (top) and A10 cells (bottom), high glucose increased [3H]dThd incorporation compared with normal glucose, although the maximum peak of [3H]dThd incorporation occurred at different times. Data are means ± SE for triplicate determinations (n = 3) repeated in 3 separate experiments. *P < 0.05, **P < 0.01 vs. normal (5.5 mM) glucose.
added to reinitiate the cell cycle in synchronized cell cultures, DNA synthesis was calculated as [3H]dThd activity (cpm/well). In AoSMC, [3H]dThd incorporation increased following serum addition to a maximum level of 729.8 ± 77.1% of control by 30 h with normal (5.5 mM) glucose. With high (25 mM) glucose, [3H]dThd incorporation increased to a maximum level of 985.0 ± 14.0% of control after 24 h, which demonstrated that high glucose stimulated and accelerated DNA synthesis in AoSMC. In A10 cells, DNA synthesis increased to 704.3 ± 78.0% of control by 24 h with normal glucose. With high glucose, DNA synthesis increased to 927.1 ± 71.0% of control 12 h after reinitiation of the cell cycle (Fig. 1B). Although the peak of [3H]dThd incorporation into DNA occurred at slightly different times in A10 and AoSMC, high glucose increased and accelerated DNA synthesis in both A10 and AoSMC. From these results, A10 cells were determined to be an analogous model for elucidating glucose-induced cell proliferation mechanisms in VSMC.

PKC-βII was involved in high glucose-induced A10 cell proliferation. Cell proliferation as determined by cell number was significantly increased in A10 and AoSMC as media glucose concentrations increased (Fig. 1A). Glucose-induced cell proliferation in A10 cells was blocked by CGP-53353, a PKC-βII-specific inhibitor, at 1 μM (Fig. 2A). High glucose concentrations did not significantly alter βI-A10 and βII-A10 cell proliferation rates, although PKC-βI overexpression increased and PKC-βII overexpression decreased cell proliferation in normal glucose as described previously (42). Stable overexpression of PKC-βI and -βII blocked the effect of glucose concentrations on cell proliferation. Although CGP-53353 did not have a significant block on βI-A10 cell proliferation (Fig. 2B), CGP-53353 increased βII-A10 cell proliferation slightly (Fig. 2C) as anticipated, demonstrating specific inhibition for PKC-βII by CGP-53353. Longer treatment periods (1 wk) with CGP-53353 increased βII-A10 cell proliferation more than that shown in Fig. 2, but cell viability of βI-A10 cells was decreased 72–96 h postincubation (data not shown). CGP-41251, a cPKC inhibitor, inhibited A10, βI-A10, and βII-A10 cell proliferation by 60–80% without regard for glucose concentrations (data not shown). Mannitol had no stimulatory effect on A10, βI-A10, or βII-A10 cell proliferation.

Glucose-stimulated [3H]dThd incorporation was blocked by CGP-53353 in A10 but not in βI-A10 and βII-A10 cells. DNA synthesis was examined by dThd incorporation following serum addition to reinitiate the cell cycle in synchronized cells. DNA synthesis occurred between 6 and 30 h after cell cycle initiation in A10 cells (Fig. 3A). [3H]dThd incorporation was observed only at the 6-h time point in βI-A10 cells (Fig. 3B), but incorporation occurred between 12 and 42 h in βII-A10 cells with normal glucose levels (Fig. 3C). This alteration in DNA synthesis induced by PKC-βI and -βII overexpression supports our previous demonstration that PKC-βI overexpression accelerated and PKC-βII overexpression delayed the peak of [3H]dThd incorporation (42). In A10 cells, high glucose significantly increased the incorporation of [3H]dThd from 48.6 ± 8.2 × 10⁻⁴ cpm/well at 24 h to 65.0 ± 4.9 × 10⁻⁴ cpm/well at 6 h after cell cycle initiation (P < 0.05) that was inhibited by CGP-53353 in A10 cells (Fig. 3A). However, no significant effects of high glucose were found
Glucose, or high glucose exposure increased and accelerated the \[^{3}\text{H}\]dThd incorporation compared with normal glucose exposure. The downregulation of PKC-\(\beta\)II levels following normal glucose exposure (Fig. 5). Levels of PKC-\(\beta\)I did not change significantly (from 75 ± 5.5% to 120 ± 6.5% of control) compared with the control levels during normal and high glucose exposure in AoSMC, A10, and \(\beta\)-A10 cells. The overexpression of PKC-\(\beta\)I is not evident in this blot because exposure time with the film was shortened to stay in the linear range of the ECL reagent.

**High glucose increased the percentage of A10 cells, but not \(\beta\)-A10 and \(\beta\)II-A10 cells, that entered S phase.** To further characterize the effects of glucose on the cell cycle, flow cytometry was performed. In contrast to \[^{3}\text{H}\]dThd incorporation, flow cytometry reveals the percentage of cells that have completed DNA synthesis and the percentage of cells undergoing G2/M phase. Flow cytometric analysis demonstrated that the duration of S phase was ~8 h (from 8 to 16 h), and the peak of S phase occurred 12 h after cell cycle initiation in A10 cells (Fig. 4A). In A10 cells, high glucose exposure increased the percentage of S-phase cells at 12 h compared with normal glucose exposure (Fig. 4A), and CGP-53353 blocked the glucose-induced increase of S-phase cell percentage (data not shown). However, no significant effect of high glucose on phase distribution was noted in \(\beta\)-A10 and \(\beta\)II-A10 cells (Fig. 4, B and C) even though overexpression of PKC-\(\beta\)I and -\(\beta\)II altered the duration of S phase as described previously (42). PKC-\(\beta\)I overexpression accelerated and shortened S phase, and PKC-\(\beta\)II overexpression slowed and prolonged S phase. The flow cytometry data supported the results of \[^{3}\text{H}\]dThd incorporation into DNA. In this case, however, cells that had completed DNA synthesis were sorted later than those reflecting active DNA synthesis.

**High glucose did not affect PKC-\(\beta\)I expression in AoSMC, A10, or \(\beta\)-A10 cells.** During cell cycle progression in AoSMC, A10, and \(\beta\)-A10 cells, contents of PKC-\(\beta\)I were analyzed by Western blot (Fig. 5). Levels of PKC-\(\beta\)I did not change significantly (from 75 ± 5.5% to 120 ± 6.5% of control) compared with the control levels during normal and high glucose exposure in AoSMC, A10, and \(\beta\)-A10 cells. The overexpression of PKC-\(\beta\)I is not evident in this blot because exposure time with the film was shortened to stay in the linear range of the ECL reagent.

**High glucose exposure-induced downregulation of PKC-\(\beta\)II in AoSMC and A10 but not in \(\beta\)-A10 cells.** During cell cycle progression in AoSMC, A10, and \(\beta\)-A10 cells, PKC-\(\beta\)II immunoreactivities were demonstrated by Western blot analysis (Fig. 6). Peaks of DNA synthesis as measured by \[^{3}\text{H}\]dThd incorporation correlated to changes in PKC-\(\beta\)II levels following normal and high glucose exposure as follows. With normal glucose, PKC-\(\beta\)II levels in AoSMC decreased to 68.0 ± 5.1% of control before the peak of DNA synthesis, and PKC-\(\beta\)II levels in A10 cells decreased to 49.0 ± 3.5% of control before the peak of DNA synthesis, similarly. With high glucose, PKC-\(\beta\)II levels in AoSMC decreased to 25.0 ± 1.9% of control before the peak of DNA synthesis, and PKC-\(\beta\)II levels in A10 cells decreased to 20.0 ± 1.8% of control. Decreases in PKC-\(\beta\)II levels were observed at ~10–12 h before DNA synthesis in both AoSMC and A10 cells and with high glucose exposure. Greater decreases in PKC-\(\beta\)II levels occurred for longer periods of time in high glucose than with normal glucose exposure. The downregulation of

![Fig. 3. Glucose effects on incorporation of \[^{3}\text{H}\]dThd into DNA and effect of PKC-\(\beta\)II-specific inhibitor (CGP-53353) in A10 (A), \(\beta\)-A10 (B), and \(\beta\)II-A10 cells (C). Cells were grown in 24-well plates and synchronized before initiation into the cell cycle with DMEM containing normal (5.5 mM) or high (25 mM) glucose. The \[^{3}\text{H}\]dThd incorporation was measured as described in METHODS. Data are expressed as \[^{3}\text{H}\] radioactivity (× 10 ^{-4} \text{ cpm/well}) with normal (5.5 mM) glucose, high (25 mM) glucose, or high glucose + CGP-53353. In A10 cells, high glucose exposure increased and accelerated the \[^{3}\text{H}\]dThd incorporation compared with normal glucose, and CGP-53353 inhibited the glucose-induced increase and acceleration of DNA synthesis (A). Although PKC-\(\beta\)I overexpression accelerated and PKC-\(\beta\)II overexpression slowed dThd incorporation, high glucose and CGP-53353 exposure had no significant stimulatory effects on the \[^{3}\text{H}\]dThd incorporation in \(\beta\)-A10 (B) or \(\beta\)II-A10 cells (C). Data in A are percentages of control shown in Fig. 1B, bottom, to compare data for AoSMC. Data are means ± SE for triplicate determinations (n = 3) repeated in 3 separate experiments. **P < 0.01 vs. normal (5.5 mM) glucose.
PKC-β II in A10 cells presumably reflects the glucose-induced activation and turnover of enzyme as well as the destabilization of PKC-β II mRNA (26). In βII-A10 cells, PKC-β II levels were sustained in normal glucose (74.0 ± 4.3–85 ± 7.3% of control) and in high glucose (105 ± 7.3–120 ± 10.3% of control) during the cell cycle and increased somewhat with high glucose compared with normal glucose. The development of the blots was

Fig. 4. Effects of glucose on the cell cycle analysis using flow cytometry in A10 (A), βI-A10 (B), and βII-A10 cells (C). Cells were grown in 25-mm² flasks and synchronized, and then the cell cycle was initiated by incubation with DMEM containing 10% fetal bovine serum (FBS) with normal (5.5 mM) or high (25 mM) glucose for 0–24 h. The distribution of the cell cycle was examined as described in METHODS. Data are expressed as the percent distribution of cell cycle phases G0/G1, S, and G2/M in normal and high glucose. In A10 cells, high glucose exposure increased the percentage of cells entering S phase (DNA synthesis phase) by 17% (*P < 0.05) and accelerated cell proliferation compared with normal glucose exposure (A). No significant glucose effects were found in cell cycle distribution of βI-A10 (B) and βII-A10 (C) cells, although PKC-β1 overexpression accelerated and PKC-βII slowed and prolonged S phase. Similar results were obtained in 3 separate experiments. *P < 0.05 compared with normal (5.5 mM) glucose.

Fig. 5. PKC-β1 immunoreactivity following glucose treatment in AoSMC, A10, and βI-A10 cells. Cells were grown in 6-well plates and synchronized, and then the cell cycle was initiated by switching medium to smooth muscle growth medium (SMGM) or DMEM containing FBS in the presence of normal (5.5 mM) or high (25 mM) glucose. After incubation for indicated times, PKC-β1 immunoreactivity of whole cell lysates was analyzed by Western blot using anti-PKC-β1 antibody (Santa Cruz) (A) and analyzed by densitometric scanning (B) as described in METHODS. Data are expressed as percentage of control, before cell cycle initiation, in optical densitometry. PKC-β1 levels were not significantly changed during cell cycle, and there were no significant glucose effects on PKC-β1 levels in AoSMC, A10, and βI-A10 cells. Because each enhanced chemiluminescence (ECL) development time of immunobotted nitrocellulose membrane was different to demonstrate a clear band, it is impossible to compare PKC-β1 basal expression levels among individual cells. Similar results were obtained in 3 separate experiments. Data are means ± SE for 3 separate experiments.
cells probably reflects the high level of heterogeneous promoter-driven PKC-βII overexpression that is resistant to glucose regulation at transcriptional and post-transcriptional levels (26). Vector controls were identical to wild-type A10 cells.

DISCUSSION

In this report, we used three methods to evaluate cell proliferation in response to normal and high glucose levels. Each method reveals a different aspect of cell proliferation. The uptake of [3H]thymidine reflects active DNA synthesis at specific times. The G1/S flow cytometry data reflect the total percentage of cells relative to DNA content. The MTT dye method reflects cell number. When the methods are examined together, they present a cohesive description of glucose effects in vascular smooth muscle cells. This study showed that human and rat VSMC proliferation was increased by high (25 mM) glucose exposure compared with normal (5.5 mM) glucose, and proliferation was dependent on increasing glucose concentrations. High glucose-induced stimulatory effects were not due to changes in osmolarity. The high glucose effect on cell proliferation is through increased DNA synthesis, resulting in acceleration of the cell cycle. High glucose increased and accelerated DNA synthesis and the entry of cells into S phase and accelerated G1/M phase as shown in Fig. 4. The agreement of glucose effects in both AoSMC and A10 cells suggests that A10 cells are a suitable model for further examining glucose-stimulated cell proliferation mechanisms in VSMC. The stimulatory effects of high glucose on cell proliferation as shown by all three methods did not occur in βI-A10 and βII-A10 cells, where PKC-βI and -βII expression was controlled by a promoter-driven mechanism introduced by the expression vector. The report that high glucose stimulates the progression of cells from G1 to S/M phases in rat primary cultured VSMC and A10 cells (9) is in keeping with our results, because glucose increased the percentage of S phase cells as shown by flow cytometry.

This study provides evidence that high glucose decreased PKC-βII levels during early S phase in AoSMC and A10 cells as determined by [3H]dThd incorporation and Western blot analysis. Although PKC-βII levels were decreased somewhat in early S phase with normal glucose, decreases in PKC-βII levels were more prominent and were accompanied by acceleration of DNA synthesis with high glucose exposure. However, in βII-A10 cells, PKC-βII levels were sustained, suggesting that PKC-βII overexpression made cells resistant to glucose-induced changes before S phase. Significant changes in PKC-βI levels were not observed before S phase in AoSMC, A10, or βI-A10 cells.

PKC was activated by high glucose in VSMC (23). Several studies suggested that prolonged high glucose exposure leads to the downregulation of PKC activity following the glucose-induced PKC activation in rat nerves (6) and soleus muscles (5). We previously demonstrated that overnight glucose exposure downregulated PKC-β (βI + βII) levels in A10 cells, although no
distinction between PKC-βI or -βII was made (4). In this study, the specific downregulation of only PKC-βII by acute high glucose exposure during early S phase was demonstrated. This decrease might result from PKC-βII activation by acute high glucose exposure; however, regulation of PKC-βII gene expression may also be occurring (26).

Other studies suggested that prolonged or chronic high glucose exposure leads to a sustained elevation of PKC levels in vascular tissues (8, 11, 19, 38, 41). These findings may seem to be at odds with the present study; however, several differences between our experiments and those studies exist. First, we examined PKC-βI and -βII immunoreactivities, whereas previous studies demonstrated total PKC activity. Second, we synchronized proliferating cells before high glucose exposure to demonstrate glucose effects during the first cell cycle following synchronization. The period from 0 to 18 h and proceeding to 48 h after cell cycle initiation was observed for acute glucose effects on PKC-βI and -βII expression in the first cell cycle postsynchronization. Previous studies examined PKC levels after chronic high glucose exposure (7–14 days) of nonsynchronized (quiescent) cell cultures in vitro (8, 11, 19, 38, 41) or diabetic animals in vivo (11, 19). Finally, we demonstrated PKC-βI and -βII immunoreactivity in whole cell lysates to show changes of total PKC-βI and -βII expression during cell cycle progression, whereas previous studies demonstrated subcellular glucose-induced PKC activity translocation (8, 11, 19, 38, 41) or PKC immunoreactivity (8, 11) in cytosol and membrane fractions. PKC activation occurs unavoidably with various homogenization techniques used in PKC assay studies, and chromatographic purification of PKC activity may not actually correlate with PKC protein levels (3). Thus PKC-βII downregulation induced by acute high glucose exposure preceding early S phase as demonstrated here is a different phenomenon from chronic high glucose-induced effects previously reported (8, 11, 19, 38, 41).

Although previous studies demonstrated involvement of PKC in VSMC proliferation using PKC inhibitors, phorbol esters, and various growth factors (1, 13–15, 18, 24, 32, 33, 36, 40), little is known about the roles of specific PKC isoforms. In this study, we extended our previous report, which demonstrated that PKC-βI and -βII might have regulatory roles in the G1/S transition of A10 cell cycle progression (16) to primary cultures of human VSMC. In AoSMC, like in A10 cells, decreases in PKC-βII levels were significantly sustained before early S phase in high glucose compared with normal glucose conditions. Some quenching of PKC-βII expression occurred normally in G1/S or early S phase during progression of the cell cycle. PKC-βII levels were unaltered in βII-A10 cells overexpressing the isoform in both high and normal glucose conditions. This suggests that the vector-driven overexpression of PKC-βII overrides glucose effects on gene expression during the cell cycle. This is anticipated because the PKC-βII cDNA encodes only translated regions of PKC-βII, and cis-elements regulating transcriptional and posttranscriptional process-