Selective Inhibition of Protein Kinase Cβ2 Prevents Acute Effects of High Glucose on Vascular Cell Adhesion Molecule-1 Expression in Human Endothelial Cells

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Background—Enhanced expression of adhesion molecules by the endothelium may account for vascular damage in diabetics and nondiabetic patients who develop stress hyperglycemia during acute myocardial infarction. We analyzed the phosphorylation of protein kinase Cβ2 (PKCβ2) at serine/threonine residues, which may contribute to the endothelial dysfunction during acute hyperglycemia. Furthermore, this study was designed to investigate whether selective blockade of this regulatory mechanism may prevent the development of endothelial hyperadhesiveness.

Methods and Results—Incubation of the human aortic endothelial cells with high glucose (22.2 mmol/L) resulted in significant increase of vascular cell adhesion molecule (VCAM)-1 protein expression (172±15% versus control; P<0.01). Phorbol 12-myristate 13-acetate, a potent activator of PKC, mimicked the effect of high glucose on VCAM-1 expression. High glucose led to a rapid increase (181±22% versus control; P<0.01) of membrane-bound PKCβ2, reflecting activation of this enzyme. The nonselective inhibitor of PKCβ1 and PKCβ2 isoforms LY379196, as well as CGP53353, a highly selective inhibitor of PKCβ2, prevented in a dose-dependent manner upregulation of VCAM-1. Incubation with high glucose was associated with increased PKCβ2 phosphorylation at the Ser-660 residue, and both LY379196 and CGP53353 prevented this event. Exposure of the cells to high glucose also reduced the protein level of the inhibitory subunit of nuclear factor-κB, IkBα, leading to its enhanced binding activity. Selective inhibition of PKCβ abolished IkBα degradation.

Conclusions—Our findings demonstrate for the first time that phosphorylation of Ser-660 represents a selective regulatory mechanism by which hyperglycemia regulates adhesion molecule expression and activation in endothelial cells. Such conditions, endothelial cells become a target for cytokines secreted by adherent leukocytes that in turn result in inflammation and activation of the coagulation cascade. Subsequent induction of tissue factor expression is one of the crucial steps in thrombus formation. However, the precise molecular mechanism by which hyperglycemia regulates adhesion molecule transcription is still unclear.

Key Words: diabetes mellitus • cell adhesion molecules • endothelium • inhibitors

Stress hyperglycemia during myocardial infarction is associated with an increased risk of in-hospital mortality in patients with and without diabetes.1,2 Furthermore, after stroke, the severity of hyperglycemia correlates strongly with mortality and morbidity in diabetic and nondiabetic patients.3 One of the possible mechanisms that could explain the worse outcomes in acute hyperglycemia is glucose-induced upregulation of adhesion molecules in endothelial cells.4,5 Expression of adhesion molecules leads to rolling and migration of activated white blood cells into the vessel wall. Under such conditions, endothelial cells become a target for cytokines secreted by adherent leukocytes that in turn result in inflammation and activation of the coagulation cascade. Subsequent induction of tissue factor expression is one of the crucial steps in thrombus formation. However, the precise molecular mechanism by which hyperglycemia regulates adhesion molecule transcription is still unclear.

Glucose upregulates protein kinase C (PKC).6,7 PKC comprises several structurally related serine/threonine kinases classified in 3 groups. The “conventional” or “classical” PKCs include PKCα, β1, β2, γ, and δ. These isoforms can be activated by Ca2+ and/or by diacylglycerol (DAG) as well as phorbol esters. The “novel” PKC δ, ε, θ can also be activated by DAG and phorbol esters but are Ca2+ independent. The “atypical” PKCs, which include PKCζ and PKCe, are unresponsive to Ca2+/DAG and phorbol esters.8 Three mechanisms of activation of PKC exist: (1) phosphorylation, (2) ligand binding (DAG, phospholipids and Ca2+), and (3) pairing with PKC-binding proteins, which leads to subcellular localization of this enzyme.9 Particularly interesting is the modulation of PKC activity by phosphorylation of serine and threonine residues.

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threonine amino acid residues within its catalytic and regulatory domains.10 For some proteins, it is known that phosphorylation of distinct amino acid residues can not only dramatically increase the catalytic activity of the enzyme but also even change substrate specificity.11 The main problem in the development of drugs targeting PKC is a ubiquitous participation of PKC in different signaling cascades from a wide array of receptors (convergence), resulting in multiple cellular effects (ie, proliferation, apoptosis, gene expression).12 Thus, nonspecific blockade of PKC would be associated with severe side effects for whole organism.13 Several studies have strongly implicated activation of PKCβ in the pathogenesis of the vascular complications of diabetes.14,15 However, a precise characterization of the regulatory mechanism PKCβ isoform involved is still missing. This prompted us to investigate the regulation of PKCβ isoform in human aortic endothelial cells (HAECs) exposed to high glucose. Use of selective inhibitors specifically interfering with this subtype is challenging. In this study, we addressed serine/threonine phosphorylation of PKCβ to define selective pharmacological tools against endothelial hyperadhesiveness under high-glucose conditions.

Methods

Cell Culture
HAECs were obtained from Clonetics and grown in gelatin-coated flasks in optimized endothelial growth medium (Clonetics) supplemented with 10% FCS and without hydrocortisone. The cells were detached by exposure to trypsin/EDTA for ~120 seconds in HEPES-buffered saline and reseeded in collagen-coated 6-cm cell-culture dishes or 24-multilwell plates. Cells were first grown to confluence in humidified air, 5% CO2 at 37°C. Confluent cells were maintained in endothelial growth medium containing 2% FCS. They were incubated with control (5.5 mmol/L) and at an elevated glucose concentration (22.2 mmol/L). Cells between the third and sixth subpassages were used.

Drugs
The selective inhibitor of PKCβ, CGPS335316 was kindly provided by Dr Doriano Fabbro (Novartis Pharma AG, Basel, Switzerland); the PKC inhibitor LY379196 (specific for β1 and β2 isoforms) was provided by Eli Lilly.17 Calphostin C and phorbol 12-myristate 13-acetate (PMA) were purchased from Calbiochem.

Western Blotting
HAECs were washed twice with PBS and harvested in the extraction buffer (120 mmol/L sodium chloride, 50 mmol/L Tris, 20 mmol/L sodium fluoride, 1 mmol/L benzamidine, 1 mmol/L DTI, 1 mmol/L EDTA, 6 mmol/L EGTA, 15 mmol/L sodium pyrophosphate, 0.8 μg/mL leupeptin, 30 mmol/L p-nitrophenyl phosphate, 0.1 mmol/L PMSF, and 1% NP-40) for immunoblotting. All cell debris was removed by centrifugation at 12 000 g for 10 minutes at 4°C. The samples (20 μg) were treated with 5% Laemmli’s SDS-PAGE sample buffer (0.35 mol/L Tris-Cl, pH 6.8, 15% SDS, 56.5% glycerol, 0.0075% bromophenol blue), followed by heating at 95°C for 3 minutes, and then subjected to 8% SDS-PAGE gel for electrophoresis. The proteins were then transferred onto Immobilon-P filter papers (Millipore AG) with a semidy transfer unit (Hoefer Scientific). The membranes were then blocked by use of 5% skim milk in PBS-Tween buffer (0.1% Tween 20; pH 7.5) for 1 hour and incubated with the antibody anti-human vascular cell adhesion molecule (VCAM-1) (R&D), anti-PKCβ (Gibco), anti-IκBα, anti-phospho-PKCα/β (Thr641) (all from Cell Signaling Technology), and anti-phospho-PKCβ (Santa Cruz). The immunoreactive bands were detected by an enhanced chemiluminescence system (Amersham).

Figure 1. Expression of vascular cell adhesion molecule-1 (VCAM-1) in HAECs exposed to control (5.5 mmol/L) as well as high concentration of glucose (22.2 mmol/L; A) and PMA (1 μmol/L; B). Effect of high glucose concentration on PKCβ accumulation in particulate fraction at 60 minutes (C). Representative Western blots and densitometric quantification are shown. C, Data are mean±SEM (n=5 to 6). *P<0.05 vs control; **P<0.05 vs glucose.

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay
HAECs cultured in 60-mm plastic dishes were washed twice with PBS and harvested into 1.5-mL tubes by scraping in 1 mL of ice-cold hypotonic buffer (mmol/L: 10 KCl, 10 HEPES, 0.1 EDTA, 0.1 EGTA, 1.0 DTT, 1.0 PMSF) + 6 mL of Nonidet P40 (a final concentration of 0.6%) and incubated on ice for 20 minutes. Cells were homogenized by vigorous vortexing for 10 seconds. The homogenate was centrifuged (3000 rpm) at 4°C for 10 minutes to
obtain a pellet of nuclei. The isolated nuclei in the pellet were resuspended in 40 mL of ice-cold hypertonic buffer (mmol/L: 400 NaCl, 20 HEPES, 1.0 EDTA, 1.0 EGTA, 1.0 DTT, 1.0 PMSF). Nuclear proteins were extracted by incubation of the homogenate on ice for 20 minutes with intermittent vortexing. The supernatant containing nuclear proteins was collected after centrifugation at 8000 rpm, 4°C for 10 minutes. The nuclear protein was then transferred into a new precooled tube and stored at −70°C until use. Protein concentration was determined by Bio-Rad Protein Assay.

Double-stranded synthetic oligonucleotide to consensus sequence for NF-κB (5'-AGTTGAGGGGACTTTCCCAGG-3') (Promega) was 5’ end-labeled with [γ-32P]ATP and T4 polynucleotide kinase (Promega). The mixture of 2 mL [γ-32P]ATP, 4 mL oligonucleotide probe, 2 mL 10× kinase buffer (Promega), and 10 mL H2O was incubated at 37°C for 20 minutes, and then 2 mL of 0.5 mol/L EDTA was added to inactivate kinase. In addition, 178 mL of TE buffer (10 mmol/L Tris-HCl, pH 8.0, and 1 mmol/L EDTA) was added. Protein/DNA binding reaction was carried out in 20 mL of mixture containing a binding buffer (Promega) (50 mmol/L NaCl, 1 mmol/L MgCl2, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 10 mmol/L Tris-HCl, pH 7.5, and 0.05 g/L poly[dIdC] in 4% glycerol), nuclear protein (5 to 10 mg), and 32P-labeled oligonucleotide (2 mL). The mixture was incubated for 30 minutes at room temperature. After the incubation, the sample was loaded onto 7.5% denaturing polyacrylamide gel (5 mL, 30% acrylamide/bis acrylamide, 2.5 mL 5× TBE, 25 mL TEMED, 12.3 mL H2O, 200 mL 10% ammonium persulfate) at 200 V for 1 hour in an electrophoresis buffer (0.5× TBE) containing 45 mmol/L Tris, 45 mmol/L boric acid, and 1.0 mL/mol EDTA. The gel was subjected to autoradiography at −70°C for 24 hours.

Cell Fractionation
PKCβ translocation was measured by Western blotting after cell fractionation into a cytosolic and a particulate fraction. Cells were harvested and sonicated, and samples were centrifuged at 100 000 g for 1 hour at 4°C. The supernatant was used as the cytosolic fraction. The pellet was resuspended in 40 mL of buffer containing 1% Triton X-100 and 0.1% SDS. Cell debris was separated by centrifugation, and the supernatant was used as the particulate detergent-soluble fraction.

Statistical Analysis
Results are expressed as mean±SEM, and n indicates number of experiments. Statistical evaluation of the data was performed with Student’s t test for simple comparison between 2 values when appropriate. For multiple comparisons, results were analyzed by ANOVA followed by Fisher’s test. A value of P<0.05 was considered statistically significant.

Results
Effect of Glucose and Phorbol Ester on VCAM-1 Expression
Incubation of HAECs with medium containing high glucose (22.2 mmol/L) resulted in a significant increase of VCAM-1 protein expression (172±15% versus control; n=6; P<0.01). The nonselective PKC inhibitor calphostin C (300 nmol/L) prevented such VCAM-1 upregulation, suggesting an involvement of PKC (Figure 1A). PMA (1 μmol/L), a potent activator of “classic” and “novel” isoforms of PKC, mimicked the effect of high glucose on VCAM-1 expression (352±52% versus control; n=5; P<0.01, Figure 1B).

Role of PKCβ Inhibition on Glucose- and PMA-Induced VCAM-1 Expression
Replacement of the medium from 5.5 to 22.2 mmol/L glucose led to a fast and significant increase (181±22% versus control; n=5; P<0.01) of detergent-soluble membrane-bound PKCβ, reflecting activation of this enzyme (Figure 1C). Treatment of the cells with the inhibitor of β2, and β1 isoforms LY379196 as well as with the selective PKCβ2 inhibitor CGP53353 prevented glucose-induced VCAM-1 upregulation in a dose-dependent manner (Figure 2A). Hence, activation of PKCβ2 regulates VCAM-1 expression in human endothelial cells acutely exposed to elevated glucose. Furthermore, LY379196 and CGP53353 were able to blunt the PMA-stimulated VCAM-1 expression (Figure 2B). Both inhibitors were also able to diminish VCAM-1 expression in control cells (data not shown), indicating that PKCβ2 maintains the basal level of VCAM-1 in HAECs.

Role of Ser-660 and Thr-641 Phosphorylation in PKCβ2-Mediated VCAM-1 Expression
Western blotting with antibodies against phosphorylated PKCβ2 at specific amino residues revealed that incubation of the cells with high glucose increased Ser-660 phosphorylation within the catalytic domain of this molecule (242±60% versus control; n=5; P<0.01; Figure 3). Treatment of the cells with PMA also elicited similar phosphorylation of Ser-660 (355±38% versus control; n=5; P<0.01; Figure 3). The time course of glucose-induced Ser-660 phosphorylation was consistent with the rapid accumulation of membrane-bound PKCβ. The inhibitor of both PKCβ isoforms LY379196 as well as the selective PKCβ2 inhibitor CGP53353 blunted PMA-induced Ser-660 phosphorylation in dose-dependent manner (Figure 4), suggesting that phosphorylation of PKCβ2 at Ser-660 may thus represent a selective regulatory mechanism for glucose-induced upregulation of VCAM-1.
Interestingly enough, phosphorylation at the other amino acid residue, Thr-641, was not affected by high glucose (data not shown). Incubation of glucose-treated cells with the PKC\(_\beta\) inhibitors did not affect the phosphorylation of Thr-641 (data not shown).

**Effect of High Glucose and PMA on NF-κB Activation and IκB\(_\alpha\) Degradation**

We also determined the effect of high glucose on NF-κB and compared the results with the effect of PMA. Incubation with elevated glucose increased NF-κB binding activity compared with control cells. The increase in NF-κB activity was a relatively early event, occurring within 1 hour after addition of glucose. Maximal increase in NF-κB activity appeared at 2 and 4 hours, and this effect was reversed at 24 hours (data not shown). Quantitatively, the increase in NF-κB activity after exposure to elevated glucose for 2 hours was 297±71% versus control cells (n=3, P<0.05, Figure 5A). High glucose–induced activation of NF-κB was inhibited by calphostin C.

Accordingly, incubation of the cells with high glucose or treatment with PMA led to decreased protein levels of the inhibitory subunit of NF-κB, IκB\(_\alpha\), because of increased degradation of this factor (Figure 5B). Both nonselective and selective inhibitors of PKC\(_\beta\) abolished PMA-induced IκB\(_\alpha\) degradation (Figure 6).

**Discussion**

The cell culture model used in this study reflects endothelial dysfunction in clinical settings of acute hyperglycemia, which occurs in both nondiabetic and diabetic patients after myocardial infarction or stroke.\(^{18}\) Transient changes in the expression of adhesion molecules in response to acute hyperglycemia trigger local inflammation and in turn contribute to clinical events.

In this study, we demonstrated for the first time that incubation of HAECs with high glucose leads to PKC\(_\beta\)-dependent upregulation of VCAM-1 expression. Several lines of evidence support this conclusion. First, stimulation of the cells with glucose induced “translocation” of PKC\(_\beta\) from the cytosolic cellular fraction to the particulate detergent-soluble fraction. Such compartmentalization is typical for activation of classic PKC. Second, we observed a selective Ser-660 phosphorylation within the catalytic domain of PKC\(_\beta\). Third, selective inhibition of PKC\(_\beta\) was able to abolish phosphorylation of Ser-660 as well as upregulation of VCAM-1.

Several pioneering studies on the role of different PKC isoforms in development of diabetic microangiopathies and macroangiopathies have demonstrated an involvement of PKC\(_\beta\).\(^{14,15}\) A putative mechanism for the preferential activation of PKC\(_\beta\) is the accumulation of DAG.\(^{19}\) Indeed, PKC\(_\beta\) isoforms are more sensitive to DAG than other isoforms, especially in the presence of lower concentrations of Ca\(^{2+}\).\(^{20}\)

The principal biochemical pathway leading to accumulation of DAG in the hyperglycemic condition is its de novo synthesis.\(^{19}\) De novo synthesis of DAG is not accompanied by elevation of intracellular Ca\(^{2+}\), in contrast to the hydrolysis of phosphatidylinositol, which simultaneously produces DAG.
and inositol 1,4,5-trisphosphate. Therefore, diacylglycerol generated from the de novo pathway in the absence of a parallel rise in Ca$^{2+}$ may preferentially activate the PKCβ rather than the PKCa isoform.21

Of particular interest is the fact that in our study, both PKC inhibitors did not affect the phosphorylation of Thr-641. These findings not only rule out phosphorylation of Thr-641 as a regulatory mechanism for glucose-induced upregulation of VCAM-1 expression but also may indicate that the catalytic activity of PKCβ2 is not completely shut down even with very high concentrations of these drugs. Therefore, PKCβ2 may still participate in physiological life-supporting processes.22 Indeed, we did not observe either an increase of cell death rate or changes of the cell shape by use of LY379196 as well as CGP53353 (data not shown). The absence of apparent cytotoxic effect together with favorable inhibition of glucose-induced VCAM-1 upregulation in the endothelium makes the PKCβ2 inhibitor a promising drug for treatment of endothelial dysfunction during acute hyperglycemia.

The molecular link between activation of PKC and upregulation of VCAM-1 expression under high-glucose conditions might involve NF-κB activation.23 The human gene encoding VCAM-1 contains in its promoter region 2 binding sites for NF-κB.24 As already reported, cells exposed to high glucose as well as vascular tissue from diabetic patients show increased binding activity of NF-κB to promoter regions of many inflammatory genes.4,25,26 However, which PKC isoform was responsible for glucose-induced NF-κB activation in arterial endothelial cells remained unclear.

Distinct PKCs stimulate NF-κB in a different manner, because the activation of NF-κB comprises several different crucial steps, including degradation of its cytoplasmic inhibitor, IκB.27 Currently, 5 distinct IκB proteins have been shown to functionally retain NF-κB in the cytoplasm and render it inactive. Of the different IκB proteins, the best-described is IκBα.28 IκBα is phosphorylated by serine kinases after stimulation such as oxidative stress29 or cytokines.30 Phosphorylation of IκBα targets the IκBα for ubiquitination and rapid degradation by 26S proteasomes.31 The degradation of IκBα then allows the unbound NF-κB to translocate into the nucleus, where it can transactivate the enhancer elements of many proinflammatory genes.32

According to our results, glucose-induced Ser-660 phosphorylation of PKCβ leads to an IκBα-dependent mechanism of NF-κB activation and hence, upregulation of VCAM-1 expression (Figure 7). Indeed, treatments with both high glucose and PMA exerted a degradation of IκBα protein, which was completely prevented by selective inhibition of PKCβ2.

Furthermore, treatment with PKCβ inhibitor improves endothelium-dependent NO-mediated vasodilatation.33 Thus, an additional link between inhibition of PKCβ2 and prevention of IκBα degradation observed in our study may be provided by the finding that NO itself is able to inhibit NF-κB activation and VCAM-1 expression by stabilization of IκBα protein.28

In conclusion, our findings demonstrate for the first time that phosphorylation of Ser-660 represents a selective regulatory mechanism for glucose-induced upregulation of VCAM-1. These results are relevant in understanding the intracellular signaling associated with pathological hyperadhesiveness of arterial endothelium in acute hyperglycemia and provide a new pharmacological target to protect the vessels in periods of transient increase of blood sugar.

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Schematic representation of intracellular signaling that modulates upregulation of VCAM-1 expression in HAEs exposed to high glucose. LY379196 and CGP53353 abolish glucose-induced Ser-660 phosphorylation of PKCβ, and hence IκBα degradation, preventing activation of NF-κB and VCAM-1 expression. IκBα inhibitory subunit of NF-κB. After IκBα degradation, NF-κB heterodimers (p50:p65) translocate into nucleus, where they upregulate transcription of target genes.

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