Glucose-induced changes in protein kinase C and nitric oxide are prevented by vitamin E

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Ganz, Michael B., and Allen Seftel. Glucose-induced changes in protein kinase C and nitric oxide are prevented by vitamin E. Am. J. Physiol. Endocrinol. Metab. 278: E146–E152, 2000.—Changes in activity or expression of protein kinase C (PKC), reactive oxygen products, and nitric oxide (NO) may account for the alteration in cell behavior seen in diabetes. These changes have been proposed to be part of the pathophysiology of erectile dysfunction. We sought to ascertain if corpus cavernosal vascular smooth muscle cells (CCSMC) grown in a high glucose milieu exhibit changes in the activity and expression of PKC isoforms, NO, and reactive oxygen products and to find out if these changes are prevented by α-tocopherol. Rat CCSMC were grown in 5, 15, and 30 mM glucose concentrations for 3, 7, and 14 days. PKC isoform expression was assayed with isoform-specific antibodies. In CCSMC grown in 30 mM glucose for 2 wk, PKC-β2 isoform was upregulated (n = 4; P < 0.01), whereas the expression of α-, δ-, ε-, and β1-isoforms was unchanged. NO as measured by nitrate-to-nitrite ratio was greatly diminished at 14 days in 30 mM (n = 4; P < 0.002) compared with 5 mM glucose. Reactive oxygen products were upregulated at 14 days when they were assayed by the fluorescent probe dichlorofluorescein diacetate bis(acetoxy-methyl) (DCFH-DA) (n = 5; P < 0.01). When these same cells were exposed to α-tocopherol for 14 days, there was a reduction of PKC-β2 (57.8%; P < 0.01; n = 4) and a reduction in reactive oxygen product formation (71.1%; P < 0.001; n = 4), along with an increase in nitrate-to-nitrite ratio (43.9%; P < 0.01, n = 4). These results suggest that there may be an interrelationship between PKC, NO, and reactive oxygen product formation in CCSMC exposed to a high glucose environment.

Erectile dysfunction (ED) is a cause of major morbidity in the United States (27). It is estimated that 50–70% of all diabetic men will suffer with ED during their lifetime, and the disease is related to pathology in the vascular tree (36, 38). ED appears to strike diabetic men at an earlier age than their nondiabetic cohorts. Whether or not ED is related to glycemic control in non-insulin-dependent diabetes mellitus has not been established.

Although several neurotransmitters have been postulated to play a role in the vasodilatory process, endothelium-derived relaxation factor(s), now established to be nitric oxide (NO) and synthesized by NO synthase (NOS), appears to be the main mediator of penile erection (8, 21, 34). Physiologically, NO release with concomitant penile vascular vasodilation permits blood under systemic pressure to enter into the corpus cavernosal sinusoidal spaces, leading to erection (4–7, 11, 27). It has been proposed that disorders that impair NO synthesis and/or release and changes in protein kinase C (PKC) expression are responsible, in part, for ED (4, 6). However, the responsible subcellular mechanisms remain ill defined.

Several lines of evidence suggest that the enzyme PKC specifically mediates the activation of many cellular events. It has been demonstrated that in diabetes (hyperglycemia) PKC mass is increased (retina, glomeruli, and heart) and NO bioactivity is subsequently altered (20, 22, 23, 24). Recent work (23) has shown that PKC activity and expression may be modulated by the generation of reactive oxygen product formation, and therefore subsequent cellular events regulated by PKC activity are altered. We demonstrate that not only is a specific PKC isoform (β2) upregulated in CCSMC grown under high glucose conditions but that this change can be prevented with exposure to α-tocopherol (vitamin E).

METHODS

Cell Culture

Rat penile tissue was surgically removed. The corpus tissue was isolated and placed in media containing FBS, ITS (insulin, transferrin, and selenium), and HCO3−-buffered saline solution for 2 wk. Rat corpus cavernous vascular smooth muscle cells (CCSMC) were evident as cell outgrowths within 5–7 days. The cells (>95%) stained positive for smooth muscle actin, indicative of smooth muscle cells. Once confluent, the cells were passaged twice before being used for the experiments. The cells were subcultured and plated in 60-mm dishes in varying concentrations of glucose. The standard HCO3− solution contained (in mM): 145 Na+, 5 K+, 1 Mg2+, 1.8 Ca2+, 122 Cl−, 25 HCO3−, 1.0 SO4 2−, 1.0 PO4 3−, and 10 glucose and was buffered to a pH of 7.40 with CO2/HCO3−. Glucose was increased by increments: 5 (control), 15, and 30 mM. The osmolarity (290 mM) of the media was kept the same for each different concentration of glucose through the use of mannitol. CCSMC were exposed to 5, 15, and 30 mM glucose for 3, 7, and 14 days.

Determination of PKC Isoform Expression

PKC isoforms in CCSMC were detected with PKC isoform-specific antibodies. Polyclonal antibodies against β1-, β2-, α-,
δ-, and ε-isoforms were purchased from Santa Cruz (Santa Cruz, CA). Antibody specificity was determined by immunoblotting in the presence of 10 µg/ml competing peptide as we have done previously (13). The competing peptide for PKC-β2 did not block β1 staining, and the same was found for δ- and ε-isoforms, thereby defining the specificity of the antibodies.

Determination of PKC-β2 Activity

We isolated cytosolic and membrane fractions, as reported previously (13), of CCSMC grown in 5, 15, and 30 mM glucose. This technique is directly correlated with PKC activation, as it measures crude membrane preparations (all organelle, nuclear, and plasma membranes). We measured enrichment of plasma membrane enzyme activity to ensure purity of our membrane preparation. Translocation is determined by a comparison of scan analysis of the PKC band in both the cytosol and membrane fractions and is a standard way for determining activity (13). The linear relationship between amount of antibody bound and amount of PKC present has been determined with known amounts of purified PKC isoforms from neuroblastoma cells.

NO Analysis

We have sought to determine whether there is a change in NO release as measured by standard nitrate-to-nitrite ratio (nitrite/nitrate) measurements in these same cells grown in 5, 15, and 30 mM glucose. With identical conditions to the experiments above, we incubated cultured CCSMC and then measured for nitrate/nitrite as has been performed previously (13) at time 0, 3, 7, and 14 days (3).

Reactive Oxygen Product Formation

Intracellular reactive oxygen species in cultured cells may be detected by using the fluorescent probe dichlorofluorescein diacetate bis(acetoxy-methyl) (9). Third-to-fifth passages CCSMC were grown on glass coverslips (9 × 50 mm) in 5, 15, and 30 mM glucose. Twenty-four hours before all studies, the medium was changed from 10% FBS to 0.5% FBS to halt cell growth. We examined cells subcultured in the varying glucose concentrations for 3, 7, and 14 days. Fluorescent measurements are made with an LS-5B spectrofluorometer (Norwalk, CT) with the coverslip mounted in a temperature-controlled flow through cuvette at an angle of 60° to the incident beam and calculations made every 5 s. Intracellular dye was alternately excited at wavelengths of 492 and 440 nm and emission at 525 nm. By using conventional fluorescent ratio-metric analysis, one is able to calculate the precise change in reactive oxygen product formation.

Vitamin E Experiments

α-Tocopherol was diluted in solution as previously reported (25) and added to the cells in 50-µg amounts each day. The α-tocopherol was added immediately after the cells were exposed to either 5, 15, and/or 30 mM glucose. The α-tocopherol was replenished daily (added as a 100-µl amount). Control experiments received the vehicle that was used to deliver the α-tocopherol (1% alcohol solution) as a 100-µl amount. PKC immunoblotting was then executed after 0 and then 3, 7, and 14 days after exposure.

Solutions

The standard HCO₃⁻ solution contained (in mM): 145 Na⁺, 5 K⁺, 1 Mg²⁺, 1.8 Ca²⁺, 122 Cl⁻, 25 HCO₃⁻, 1.0 SO₄²⁻, 1.0 PO₄³⁻, and 5 glucose and was buffered to a pH of 7.40 with CO₂/HCO₃⁻ for all experiments. The only change was that of glucose as stated above. α-Tocopherol was dissolved in a 1% alcohol solution on the day of the experiment to the appropriate concentration.

Materials

Dichlorofluorescein diacetate bis(acetoxy-methyl) was obtained from Molecular Probes (Eugene, OR). DMEM, FBS, penicillin, streptomycin, and phosphate buffered saline (PBS) solution were purchased from Gibco Laboratories (Grand Island, NY). Fibroblast growth factor, insulin, transferrin, and selenium were obtained from Collaborative Research (Bedford, MA). All PKC antibodies were purchased from Santa Cruz. Plastic cuvettes, phorbol 12-myristate, and other laboratory chemicals (phorbol esters) were purchased from Sigma (St. Louis, MO). Tissue culture flasks and petri dishes were obtained from Falcon (Lincoln Park, NJ).

Statistics

Data are reported as means ± SE in all the tables. Statistical significance was judged by the unpaired Student's t-test. In experiments in which glucose concentrations were used (see Tables 1–4), the data were compared with control (normal glucose).

Fig. 1. Western blots of corpus cavernosal vascular smooth muscle cells (CCSMC) exposed to 5, 15, and 30 mM glucose after 14 days. A: α-protein kinase C (PKC); B: β₁-PKC; C: ε-PKC; D: δ-PKC; E: β₂-PKC.
Table 1. PKC isoform expression

<table>
<thead>
<tr>
<th>PKC Isoform</th>
<th>5 mM (total)</th>
<th>15 mM (total)</th>
<th>30 mM (total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>35.1 ± 8.2</td>
<td>41.1 ± 6.7</td>
<td>42.1 ± 8.1</td>
</tr>
<tr>
<td>β₂</td>
<td>2.2 ± 1.9</td>
<td>8.2 ± 4.2</td>
<td>27.1 ± 7.2*</td>
</tr>
<tr>
<td>β₁</td>
<td>23.0 ± 5.1</td>
<td>23.7 ± 4.1</td>
<td>28.1 ± 6.7</td>
</tr>
<tr>
<td>ε</td>
<td>17.2 ± 3.1</td>
<td>12.2 ± 4.4</td>
<td>19.0 ± 8.1</td>
</tr>
<tr>
<td>δ</td>
<td>13.2 ± 6.4</td>
<td>19.1 ± 8.1</td>
<td>12.7 ± 6.9</td>
</tr>
</tbody>
</table>

Values are means ± SE. PKC, protein kinase C. Values reflect densitometric analysis of 4 experiments for each isoform after 14 days in their respective glucose concentrations. All analyses are made in comparison to 5 mM glucose for the respective isoform after 14 days. *P < 0.01.

RESULTS

Expression of PKC Isoforms in Culture

Cells were grown under 5, 15, and 30 mM glucose. The cells were then harvested, and with specific PKC isoform antibodies, we performed Western blot analysis (Fig. 1; n = 4 for each glucose concentration). Changes in isoform expression are summarized in Table 1. There was no statistical difference in the expression for α, β₁, δ-, and ε-isoforms after days 3, 7, and 14 whether exposed to 15 (data not shown) or 30 mM glucose (Table 1). However, changes were evident in 15 mM glucose for β₂-isoform at 14 days. There was an even greater increase in PKC-β₂ in 30 mM at 7 days, from 2.2 ± 0.9 to 12.1 ± 4.2 (n = 5; P < 0.03), and this increased by 14 days in 30 mM (Table 1). The data clearly demonstrate that PKC isoform expression is modulated in the cell culture model of hyperglycemia; PKC-β₂ becomes evident after chronic exposure to that of 30 mM after 7 days and is most pronounced after 14 days.

To determine if PKC-β₂ is activated (translocated), we isolated membrane and cytosolic fractions. Activity is determined by how much isoform in bound to the membrane. As shown in Table 2, when CCSMCs grown in 30 mM glucose for 14 days are acutely exposed to 100 nM phorbol 12-myristate, there is a statistical increase in the membrane-bound fraction that is maximal within 60 min. Interestingly, there is a significant amount of PKC β₂ bound to the membrane after the cells have been in 30 mM glucose for 14 days. This suggests that the isoform is active in the basal state (before phorbol exposure).

Production of Nitrate/ Nitrite

Using the standard assay as a surrogate for NO production, we measured nitrate/nitrite and examined whether there is a change under hyperglycemic conditions. As is shown (Table 3), it appears that cells grown in the highest glucose concentration readily express the least amount of nitrate/nitrite. This decrease, a reflection of NO release, occurs after prolonged exposure to hyperglycemia and also is concentration dependent. This is again further evidence supporting the existence of changes induced in high glucose media that may be responsible for the pathobiology seen in ED.

Table 2. PKC β₂ response to phorbol ester exposure

<table>
<thead>
<tr>
<th>Cells</th>
<th>Fraction at Time0</th>
<th>Fraction at Time30</th>
<th>Fraction at Time60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosolic</td>
<td>Membrane</td>
<td>Cytosolic</td>
</tr>
<tr>
<td>Corpus cells in 30 mM glucose</td>
<td>12.1 ± 1.1</td>
<td>16.2 ± 3.1</td>
<td>8.2 ± 2.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. Corpus cavernosal vascular smooth muscle cells were exposed to phorbol 12-myristate, and we measured translocation at 30 and 60 min. In a paired t-test we demonstrated that *P < 0.002 (vs. 5 mM glucose at time 0).

Reactive Oxygen Products

We then sought to assess whether we can affect the production of reactive oxygen products in 5, 15, and 30 mM glucose. Reactive oxygen products increased when the cells were grown in a high glucose (30 mM) vs. a low glucose (5 mM) concentration. Reactive oxygen product formation increase was not evident at 24, or at 48 h (data not shown), but was statistically evident at 72 h and persisted for 2 wk (Table 4). There is an increase in reactive oxygen products with prolonged incubation in high glucose conditions.

Response to α-Tocopherol

Expression of PKC isoforms in culture. We sought to ascertain if, by blocking reactive oxygen production with α-tocopherol, we could alter PKC β₂ production. Cells were grown as described above, but 50 µg of α-tocopherol were added daily (every day) for 14 days. We used 50 µg of α-tocopherol because maximal effect was evident at this dose (75 and 100 µg did not inhibit cell PKC expression any further; data not shown). The cells were then harvested and, using PKC isoform antibodies to β₂, we performed Western blot analysis. Cells grown in 30 mM glucose were exposed to 50 µg of daily α-tocopherol for 7 and 14 days. As shown in Fig. 2, PKC β₂, while still present, was greatly reduced after prolong exposure to α-tocopherol.

Production of nitrate/ nitrite. We then measured the effect of the daily administration of 50 µg of α-tocopherol on the production of nitrate/nitrite in cells exposed to 30 mM glucose. As is shown in Fig. 3, when cells incubated in 30 mM were exposed to daily α-tocopherol, the cells had a significant increase in nitrate/nitrite compared with those cells not exposed to this reactive oxygen scavenger. This effect was initially seen but was not statistically evident at 7 days. Therefore, cells continuously exposed to α-tocopherol even in the presence of 30 mM glucose are able to restore, in part, NO bioactivity.
Reactive oxygen product. We measured changes in reactive oxygen product when the cells were exposed to daily 50 μg of α-tocopherol. Reactive oxygen product was decreased after prolonged exposure to α-tocopherol even in the face of 30 mM glucose. This effect was seen at 3 days and maintained in the continued presence of α-tocopherol after 2 wk (Fig. 4).

DISCUSSION

There is a growing body of evidence that suggests that hyperglycemic-induced diabetic injury modulates cell biological behavior and that this is reflected in changes in interrelated changes in PKC, NO, and reactive oxygen species (18, 28). We have demonstrated that in these cells subcultured in hyperglycemia there is an increase in PKC-β₂ and reactive oxygen species with concomitant reduced NO production. These effects are prevented, in part, by exposure to α-tocopherol.

PKC has demonstrated participation in the cellular response to injury, in the inflammatory process, and in the immune response, and that signaling through PKC is essential for the activation of neutrophils, exocytosis, cytokine release, and cellular proliferation (1, 16, 41). The role of glucose and alterations in PKC-β has been the focus of intensive investigations. Elevation of blood hexose level in diabetes of experimental galactosemia has been found to induce abnormalities of metabolism, which may play a role in the development of diabetic complications (25, 40). Activity of PKC has been found to be elevated in retina, glomeruli, and heart of diabetic animals in purified microvessels from diabetic rats and in endothelial and mesangial cells cultured in high glucose or galactose (12, 24). Moreover, recent work (M. B. Ganz and T. Kern, unpublished observations) has established that the rat non-insulin-dependent diabetes mellitus heart is more specifically associated with a PKC-β distribution defect. The elevated enzyme activity has been attributed, in part, to excessive production of diacylglycerol from glucose or galactose (19, 20, 39). Normalization of the diabetes-induced abnormality of PKC activity (β-isoform and other PKC isoforms) corrects diabetes-induced abnormalities in metabolism and physiology (blood flow in retina and kidney and peripheral nerve conduction). Finally, diabetes-induced metabolic abnormalities in some tissues are normalization of PKC activity with specific inhibitors (18, 24).

It has been proposed that oxidative stress may play a role in ED through the generation of free radicals that, in turn, lead to modulation of PKC activity with eventual disordered NO production. Many tissues in the diabetic show significant evidence of oxidative stress, including supranormal levels of reactive oxygen species (26). It has now been demonstrated that reactive oxygen species can directly increase the activity of PKC independent of diacylglycerol production and that inhibition of PKC activity can inhibit the hyperglycemia-induced increase in free radical production (26). Moreover, phosphorylation of phospholipase D and PKC-α in fibroblasts is induced by reactive oxygen species along with other changes in PKC (31, 32). These results demonstrate that the expression of reactive oxygen species occurs before PKC isoform expression changes are evident. In addition, we have demonstrated the presence of the specific reactive oxygen species marker, nitrotyrosine, in the diabetic human penile corpus cavernosum (Seftel, personal communication).

Hyperglycemia-induced decreased NO content has been demonstrated in a number of cells (10, 33). The direction of change (increase or decrease in NO) is dependent on the cell type (14, 15). We have demonstrated the constitutive expression of eNOS in primary human CCSMC in culture and have demonstrated that when these cells are exposed to 15 and 30 mM glucose, they show a significant decrease in eNOS expression (Seftel, unpublished observations). Thus the CCSMC

Table 3. Nitrate/nitrite cell culture data

<table>
<thead>
<tr>
<th>Cells</th>
<th>Ratio for 3 Days</th>
<th>Ratio for 7 Days</th>
<th>Ratio for 14 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corpus Cells (5 mM glucose)</td>
<td>1.11±0.27</td>
<td>1.23±0.16</td>
<td>1.07±0.27</td>
</tr>
<tr>
<td></td>
<td>(n=9)</td>
<td>(n=6)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>Corpus Cells (15 mM glucose)</td>
<td>1.51±0.14*</td>
<td>1.58±0.24*</td>
<td>1.48±0.21*</td>
</tr>
<tr>
<td></td>
<td>(n=12)</td>
<td>(n=5)</td>
<td>(n=4)</td>
</tr>
<tr>
<td>Corpus Cells (30 mM glucose)</td>
<td>2.27±0.30†</td>
<td>2.01±0.23†</td>
<td>1.92±0.22†</td>
</tr>
<tr>
<td></td>
<td>(n=8)</td>
<td>(n=4)</td>
<td>(n=3)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4. Nitrate/nitrite, ratio of nitrate to nitrite. In a paired t-test we demonstrated that *P < 0.02 and †P < 0.001 (vs. 5 mM concentration of glucose).

Fig. 2. Western blot demonstrating effect of α-tocopherol on PKC-β₂ protein expression after 14 days. A: CCSMC in 30 mM glucose for 14 days [control]. B: CCSMC in 30 mM glucose and α-tocopherol vehicle for 14 days. C: CCSMC in 30 mM glucose and α-tocopherol for 14 days.
appear to be a source of NO production, which may be negatively affected by hyperglycemia at the level of eNOS.

Investigators have demonstrated that the activation of PKC increases venular permeability that requires the production of NO. These data indicate that NO may be a target protein of a specific PKC isoform (29, 32, 35). Pharmacological inhibition studies of total PKC activity have demonstrated an alteration of NO mRNA and a change in the release of NO. In addition, the hyperpermeability of effect of PKC-activating agents could be blocked by NO inhibitors, supporting the concept that PKC displays its signaling effect by modulating the activity of NO synthase (30, 35, 37). Coincubation of endothelial cells with inhibitors of PKC increased the accumulation of nitrite but did not restore it to the levels obtained when cells were cultured in 5 mmol/l glucose. It is conceivable that reactive oxygen species formation may lead to a direct decrease in eNOS expression as an early event in hyperglycemia-mediated cavernosal disease as some have reported (2, 10).

Reactive oxygen species and peroxides can cause protein cross-linking or fragmentation, DNA breaks, lipid peroxidation, and membrane damage and might be responsible for some of the sequelae of hyperglycemia (20). Administration of antioxidants including α-tocopherol has been found to prevent oxidative stress in the retina and heart of the diabetic rats and to partially inhibit the development of retinopathy in those rats (28). The link to PKC has not been definitively demonstrated. Information on the interrelationship of hyperglycemia-induced abnormalities of penile metabolism provides new insight on the role that reactive oxygen species coupled with NO has in the pathogenesis of ED in diabetes.

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