Enhanced Thromboxane Receptor-Mediated Responses and Impaired Endothelium-Dependent Relaxation in Human Corpus Cavernosum from Diabetic Impotent Men: Role of Protein Kinase C Activity

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Received May 29, 2006; accepted August 2, 2006

ABSTRACT

We have evaluated the influence of protein kinase C (PKC) activity on penile smooth muscle tone in tissues from diabetic and nondiabetic men with erectile dysfunction. Human corpus cavernous (HCC) strips were obtained from impotent diabetic and nondiabetic men at the time of penile prosthesis implantation and studied in organ chambers. Contractility responses to a prostanoid precursor, to prostanoids, and to the endothelium-dependent vasodilator acetylcholine were studied. Arachidonic acid (AA; 100 \mu M) caused cyclooxygenase-dependent relaxation of HCC. This relaxation was impaired in diabetic tissues and normalized by blocking thromboxane (TP) receptors with 20 nM [1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ29548). Diabetes did not affect prostaglandin (PG)E₁, induced relaxation, but it reduced relaxation induced by the PGF₁α metabolite PGF₀. This effect was related to an interaction of PGF₀ with TP receptors. Diabetic tissues had reduced endothelium-dependent relaxation, which was partially improved by SQ29548 and completely normalized by the PKC inhibitor 3-[1-[(dimethylaminopropyl)-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrole-2,5-dione monohydrochloride (GF109203X; 1 \mu M). In HCC from nondiabetic patients, treatment with the PKC activator phorbol-12,13-dibutyrate (0.3 \mu M) significantly attenuated endothelium-dependent relaxation, an effect prevented by coadministration of GF109203X. Tissues from diabetic patients had enhanced sensitivity to the contractile effects of the TP receptor agonist 9,11-dideoxy-9α,11α-epoxyethano PGF₂α (U46619) (EC₉₀ = 0.65 ± 0.42 and 6.01 ± 2.28 nM in diabetic and nondiabetic patients, respectively). Inhibition of PKC with 1 \mu M GF109203X prevented diabetes-induced hypersensitivity to U46619-induced contractions (EC₉₀ = 8.55 ± 3.12 \mu M). Overactivity of PKC in diabetes is responsible for enhanced contraction and reduced endothelium-dependent relaxation of HCC smooth muscle. Such alterations can result in erectile dysfunction.

Diabetic men are at higher risk for suffering from erectile dysfunction than the general population (Feldman et al., 1994; Martín-Morales et al., 2001). Erectile function depends on the relaxant capacity of penile smooth muscle, which is required for vasodilation and cavernosal expansion leading to blood accumulation and penile erection (Sáenz de Tejada et al., 1991). Human penile smooth muscle tone is regulated by a tight balance between contractile and relaxant mediators. Alteration of the physiological mechanisms of tone regulation leading to a disbalance that favors contractile pathways and/or reduces relaxation could cause the inability to achieve an adequate erection. Prostanoids participate in the regulation of penile smooth muscle tone. EP receptors mediate relaxation, whereas the TP receptors mediate contraction in human corpus cavernosum (HCC) tissue (Angulo et al., 2002). Prostaglandin (PG)E₁...
has been shown to produce trabecular smooth muscle relaxation and penile erection and has been widely used as intracavernosal therapy for impotence (Porst, 1996). PGE1 has a short half-life, but it may be converted to PGE2, which is an active metabolite with similar properties to PGE1, but with a longer half-life (Ney et al., 1991).

Prostanoid-driven pathways can be altered by diabetes. Indeed, excessive production of contractile prostanoids (Koltai et al., 1990; Davi et al., 1997) or enhanced contractile responses to prostanoids have been described previously (McCarty, 1998; Hattori et al., 1999).

Several molecular mechanisms have been proposed to be responsible for the vascular alterations associated to diabetic men with erectile dysfunction and to evaluate the role of PKC activity of PKC is known to be elevated in diabetes (Koya and Hercules, CA) with bovine serum albumin as standard.

Materials and Methods

Human Corpus Cavernosum Tissues. Human corpus cavernosum specimens were obtained from impotent diabetic and nondiabetic men at the time of penile prosthesis insertion after giving informed consent. Some specimens were also collected from organ donors. Protocols were approved by the local Ethics Committee. Tissues were maintained at 4–6°C in M-400 solution (composition per 100 ml: 4.19 g of mannitol, 0.205 g of KH2PO4, 0.97 g of K2HPO4·3H2O, 0.112 g of KCl, and 0.084 g of NaHCO3) until used, which was between 2 and 16 h from extraction (Angulo et al., 2002).

Organ Chamber Studies. Strips of corpus cavernosum tissue (3 x 3 x 7 mm) were immersed in 8-ml organ chambers containing physiological salt solution (PSS) of the following composition: 119 mM NaCl, 4.6 mM KCl, 1.5 mM CaCl2, 1.2 mM MgCl2, 24.9 mM NaHCO3, 11 mM glucose, 1.2 mM KH2PO4, and 0.027 mM EDTA at 37°C continuously bubbled with 95% O2, 5% CO2 mixture to maintain a pH of 7.4. Each tissue strip was incrementally stretched to optimal isometric tension, as determined by maximal contractile response to 125 mM KCl solution

Measurement of Cyclic AMP in Human Corpus Cavernosum Tissue. Corpus cavernosum strips were immersed in 8-ml organ chambers containing PSS, maintained at 37°C, and aerated with 5% CO2, 95% O2, pH 7.4. Each tissue strip was incrementally stretched to optimal isometric tension, as determined by maximal contractile response to 1 µM phenylephrine (Kim et al., 1991; Azadzoi et al., 1992). Contractile responses were evaluated by adding increasing concentration of compounds on unstimulated strips. For the relaxation studies, tissues were contracted with 0.5 to 3 µM phenylephrine, and relaxation responses were evaluated by cumulative additions of compounds to the chambers.

Protein Determinations. Proteins were determined using the Bio-Rad Protein Assay kit microtiter plate assay procedure (Bio-Rad, Hercules, CA) with bovine serum albumin as standard.

Drugs and Materials. Arachidonic acid, indomethacin, phenylephrine, acetylcholine chloride, U46619, 3-isobutyl-1-methylxanthine, and phorbol-12,13-dibutyrate (PDBu) were obtained from Sigma-Aldrich (St. Louis, MO). Prostaglandin E1 (alprostadil) was obtained from Pharmacia (Barcelona, Spain). Prostaglandin E2 (13,14-dehydro-PGE2) was purchased from Cayman Chemical. SQ29548 was obtained from Sigma/RBI (Natick, MA). Bisindoxymaleimide I (GF109203X) was obtained from Alexis Corporation (Lausanne, Switzerland). Prostanoid derivatives were dissolved at 10 mM concentration in ethanol, and GF109203X was dissolved in dimethyl sulfoxide at 10 mM concentration (final concentration of dimethyl sulfoxide was 0.001%). Dilutions were made in distilled water at the time of the experiment. Nonprostanoid drugs were dissolved in distilled water. Indomethacin was dissolved in 1.5 mM Na2CO3.

Data Analysis. Contractile effects produced by drugs are expressed as the percentage of maximal contraction to the agent or as the percentage of contraction elicited by 125 mM K+ (equimolar substitution of NaCl for KCl in PSS). Relaxation responses are expressed as percentage of total relaxation (loss in tone) induced by the addition of 0.1 mM papaverine HCl to the chambers at the end of the experiment. All data are expressed as mean ± S.E. Complete concentration-response curves were compared by a two-factor analysis of variance (ANOVA) test using StatView software for Apple computers (SAS Institute, Cary, NC). Cyclic AMP determinations were compared by a one-factor ANOVA followed by a Student-Newman-Keuls test using GraphPad software (GraphPad Software Inc., San Diego, CA) for Apple computers.

Results

Specimens of corpus cavernosum tissue from 62 impotent patients (31 nondiabetic and 31 diabetic patients) were obtained for this study. The demographic data and medical comorbidities of these patients are shown in Table 1. No significant differences in age or comorbidities were observed among diabetic and nondiabetic populations. Although the percentage of patients with arterial hypertension was higher among diabetic patients, this difference did not reach statistical significance (Table 1). The data were insufficient to accurately differentiate noninsulin-dependent diabetic men treated with insulin from those who were insulin-dependent diabetic patients, and, of course, treated with insulin. However, endothelium-dependent relaxation was similar in HCC from diabetic patients treated with insulin or not [Emax for acetylcholine (ACH): 50.0 ± 5.8% in diabetic patients not receiving insulin, n = 13; and 49.9 ± 3.7% in diabetic patients treated with insulin, n = 18; not significantly different].

<table>
<thead>
<tr>
<th>TABLE 1</th>
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| Demographic data and comorbidities of patients from whom tissues were collected for this study
|
| Values in parentheses are percentages. |

<table>
<thead>
<tr>
<th></th>
<th>Nondiabetic Patients</th>
<th>Diabetic Patients</th>
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<tr>
<td>n</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>Age (year)</td>
<td>58.2 ± 1.6</td>
<td>59.5 ± 1.4</td>
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<tr>
<td>Treatment for diabetes</td>
<td>Hypoglycemics</td>
<td>9 (29.0)</td>
</tr>
<tr>
<td>Insulin</td>
<td>18 (58.1)</td>
<td></td>
</tr>
<tr>
<td>Diet control</td>
<td>4 (12.9)</td>
<td></td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>7 (22.6)</td>
<td>7 (22.6)</td>
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<tr>
<td>Hypertension</td>
<td>5 (16.1)</td>
<td>10 (32.2)</td>
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<tr>
<td>Smoking habit</td>
<td>21 (67.7)</td>
<td>18 (58.1)</td>
</tr>
<tr>
<td>Neurological alterations</td>
<td>6 (19.3)</td>
<td>8 (25.8)</td>
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<tr>
<td>Hormonal alterations</td>
<td>1 (3.2)</td>
<td>2 (6.4)</td>
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Relaxation of Human Corpus Cavernosum Tissue Induced by Arachidonic Acid. Addition of 100 μM arachidonic acid (AA) produced a relaxant response in human corpus cavernosum strips that was prevented by incubation with the cyclooxygenase inhibitor indomethacin (5 μM), in accordance with previous observations (Angulo et al., 2002). This relaxation was significantly impaired in tissues from diabetic patients. Treatment of diabetic tissues with the TP receptor blocker SQ29548 (20 nM) caused a full recovery of AA-induced relaxation (Fig. 1).

Relaxant Responses Elicited by PGE1 and PGE0 in Human Corpus Cavernosum Tissue. The active metabolite of PGE1, PGE0, relaxed corpus cavernous tissue, but this relaxant response was reduced compared with that induced by its parent molecule (Fig. 2A). PGE0 (1 μM), however, produced an increase of cAMP content in human cavernosal tissue similar to that obtained with PGE1 at the same concentration (Fig. 2B). The treatment of cavernosal tissue with 20 nM SQ29548 did not modify relaxation induced by PGE1 (data not shown), but it significantly improved the relaxant responses evoked by PGE0 (Fig. 2C). Indeed, treatment with SQ29548 abolished the differences between PGE1- and PGE0-induced relaxations (Fig. 2D).

Influence of Diabetes on the Relaxation of Human Corpus Cavernosum to PGE1 and PGE0. The relaxations induced by PGE1 in corpus cavernosum tissues from diabetic patients were not different from those obtained in tissues from nondiabetic patients (Fig. 3A). In contrast, the responses to PGE0 were significantly impaired in tissues from diabetic patients (Fig. 3B). This impairment was reversed by treating the tissues with the TP receptor blocker SQ29548 (20 nM) (Fig. 3C).

Effect of Diabetes and TP Receptor Blockade on Endothelium-Dependent Relaxation of Human Corpus Cavernosum. Human corpus cavernosum strips were relaxed by the cumulative addition of 1 nM to 10 μM ACh. The ACh-induced relaxation in this tissue was significantly impaired by diabetes. Treatment with 20 nM SQ29548 did not alter endothelium-dependent relaxation of cavernosal strips from nondiabetic patients, but it significantly improved ACh-induced relaxation in corpus cavernosum from diabetic patients. However, SQ29548 was not able to completely recover endothelium-dependent relaxation in diabetic tissues (Fig. 4; in nondiabetic patients, precontraction values were 2.40 ± 0.74 and 2.43 ± 1.01 g for control and SQ29548, respectively; in diabetic patients, precontraction values were 2.14 ± 0.67 and 2.41 ± 0.75 g for control and SQ29548, respectively; not significant).

Effects of Modulation of PKC Activity on Endothelium-Dependent Relaxation of Corpus Cavernosum from Diabetic and Nondiabetic Patients. The inhibition of PKC activity with 1 μM GF109203X significantly potentiated ACh-induced relaxation of corpus cavernosum from diabetic patients (Fig. 5; in nondiabetic patients, precontraction values were 3.44 ± 0.46 and 3.05 ± 0.68 g for control and SQ29548, respectively; in diabetic patients, precontraction values were 3.00 ± 0.91 and 2.86 ± 0.81 g for control and SQ29548, respectively; not significant). After treating with GF109203X, endothelium-dependent relaxation of diabetic corpus cavernosum was not different from that of tissues from nondiabetic patients. Conversely, the stimulation of PKC activity with the phorbol ester PDBu (0.3 μM) drastically reduced endothelium-dependent relaxation to ACh in human corpus cavernosum from nondiabetic patients (Fig. 6A; precontraction values were 2.29 ± 0.68 and 1.68 ± 0.41 g for control and PDBu, respectively; not significant). This inhibitory effect of PDBu on ACh-induced relaxation was prevented by cotreating cavernosal strips with the PKC inhibitor GF109203X (1 μM) (Fig. 6B; precontraction values were 2.60 ± 1.02 and 2.76 ± 0.95 g for control and PDBu plus GF109203X, respectively; not significant).

Effects of Diabetes and PKC Inhibition on the Contraction of Human Corpus Cavernosum Mediated by TP Receptors. The thromboxane analog U46619 produced concentration-dependent contractions of HCC smooth muscle. Tissues from diabetic patients showed enhanced sensitivity to U46619 compared with tissues from nondiabetic patients (Fig. 7A) as demonstrated by lower EC50 values for the TP receptor agonist (0.65 ± 0.42 and 6.01 ± 2.28 nM for diabetic and nondiabetic tissue, respectively; p < 0.05). Treatment with the PKC inhibitor GF109203X (1 μM) did not alter U46619-induced contractions in corpus cavernosum tissue from nondiabetic patients, but it significantly inhibited these responses in cavernosal tissue from diabetic patients. In fact, treatment with GF109203X completely prevented the hypersensitive contractile responses to U46619 of diabetic tissues (Fig. 7A). Neither diabetes nor PKC inhibition significantly affected maximal contractile response to U46619 (Fig. 7B).

Discussion

The changes in tone following the addition of AA, which are prevented by indomethacin, show the capacity of HCC to
synthesize cyclooxygenase products that affect contractility of penile smooth muscle. In this tissue, arachidonic acid promotes the synthesis of relaxant and constrictor prostanoids. EP₂/EP₄ receptors and TP receptors for relaxation and contraction, respectively, mediate responses to prostanoids in HCC (Angulo et al., 2002). The reduction of AA-induced relaxation in diabetic HCC could be due to an impairment of synthesis/activity of relaxant prostanoids or to an enhance-
were collected for the experiments. Human cavernosal tissue similar to that induced by PGE1 at 0.1 mM papaverine.

data are expressed as mean ± S.E.M. of the percentage of total relaxation induced by 0.1 mM papaverine. n indicates the number of patients from whom the tissues were collected for the experiments. †††, p < 0.001 versus no diabetes; ††, p < 0.01 versus diabetes by a two-factor ANOVA.

Fig. 4. Effects of diabetes and blockade of TP receptors with 20 nM SQ29548 on endothelium-dependent relaxation of human corpus cavernosum strips contracted with phenylephrine. Data are expressed as mean ± S.E.M. of the percentage of total relaxation induced by 0.1 mM papaverine. n indicates the number of patients from whom the tissues were collected for the experiments. †††, p < 0.001 versus no diabetes; ††, p < 0.01 versus diabetes by a two-factor ANOVA.

Fig. 5. Effects of diabetes and treatment with the PKC inhibitor GF109203X (1 μM) on endothelium-dependent relaxation of human corpus cavernosum strips contracted with phenylephrine. Data are expressed as mean ± S.E.M. of the percentage of total relaxation induced by 0.1 mM papaverine. n indicates the number of patients from whom the tissues were collected for the experiments. †††, p < 0.001 versus no diabetes; ††, p < 0.01 versus diabetes by a two-factor ANOVA.

ment of synthesis/activity of contractile prostanoids. Our results favor the later explanation, because blockade of TP receptors with SQ29548 normalized relaxation to AA in HCC from diabetic patients.

Although PGE_0 caused an increase of cAMP content in human cavernosal tissue similar to that induced by PGE_1 at the same concentration, indicating comparable capacity of the molecules to activate adenyl cyclase, its capacity to relax HCC was reduced compared with that of its parental molecule. Since SQ29548 eliminated the differences between PGE_0 and PGE_1, it is likely that PGE_0, but not PGE_1, interacts with TP receptors. Furthermore, because diabetes impaired the relaxant capacity of PGE_0 in human trabecular tissue, but not PGE_1-induced relaxations and the blockade of TP receptors made comparable responses to PGE_0 between comparable diabetic and nondiabetic tissues, an increased TP receptor-mediated response seems to be responsible for diabetes-induced reduction of relaxation to PGE_0. Diabetes-induced alteration of the TP receptor-mediated pathway is demonstrated by the hypersensitivity to the contractile activity of the thromboxane analog U46619 observed in diabetic tissues. Furthermore, hypersensitivity of TP receptor-mediated responses may also be involved in the impairment of endothelium-dependent relaxation of HCC associated to diabetes, because blockade of these receptors partially improved ACh-induced relaxation in HCC from diabetic men.

Vascular smooth muscle contraction induced by ligands of TP receptor involves G protein-mediated activation of phospholipase C, which promotes inositol-1,4,5-trisphosphate generation and subsequent release of calcium from intracellular stores, leading to the activation of contractile machinery (Hirata et al., 1991; Coleman et al., 1994). The activity of phospholipase C also yields diacylglycerol, which is an activator of PKC. In addition, the increase of intracellular calcium concentration could facilitate the activation of some PKC isoforms. Activated PKC could activate Ca^{2+}-channels that would contribute to increases in intracellular calcium concentration, participating in agonist-induced contraction (Fish et al., 1988; Navedo et al., 2005). PKC activity could also potentiate contraction by enhancing calcium sensitivity of contractile mechanisms (Gokina et al., 1999; Ding and Murray, 2005), an effect also demonstrated in human arteries (Martinez et al., 2000). In fact, PKC participates in calcium-sensitizing pathways of contraction of rabbit corpus cavernosum smooth muscle (Takahashi et al., 2003).

Despite the above-mentioned findings, the relevance of PKC activation in physiological contraction mediated by TP receptors remains controversial. In rat mesenteric artery, PKC inhibitors have been shown to significantly reduce U46619-mediated contractions (Tasaki et al., 2003), whereas no alteration in mesenteric artery contraction to U46619 by PKC inhibitors has also been reported in in vivo studies (Bauer et al., 1999). In addition, inhibition of PKC did not modify U46619-mediated contraction of pulmonary circulation in rats and cats (Kaye et al., 1995). Consistent with these findings, we find in this study that contraction of HCC from nondiabetic patients to U46619 was not influenced by PKC inhibition. This suggests that the cellular signaling pathway triggered by TP receptor activation in HCC, at least in nondiabetic conditions, does not involve the participation of PKC.

Diabetes is associated with an increase in PKC activity in vascular tissue, probably related to increased glucose-induced de novo formation of DAG. Increase of PKC activity under hyperglycemic conditions has been previously observed in cultured human cavernosal cells (Ganz and Seftel, 2000). Hypersensitivity to TP receptor activation in diabetic HCC is likely mediated by a PKC-dependent mechanism, because inhibition of PKC completely reversed the hypersen-
sitive response to U46619. The observation that PKC inhibition reverses diabetes-induced potentiation of TP receptor-mediated contraction and that TP receptor blockade improves endothelium-dependent relaxation in diabetic HCC would suggest that the beneficial effects of PKC inhibition on endothelial function in HCC from diabetic patients could be attributed to its influence on TP-mediated responses. But, although the improvement in endothelium-dependent relaxation by TP receptor blockade was only partial, PKC inhibition completely reversed endothelial dysfunction, suggesting that PKC overactivity is influencing other components of endothelium-dependent relaxation in diabetic tissues. The specific PKC isoform involved cannot be determined in our study, since GF109203X, at the concentration used in this study, has been shown to inhibit the PKC isoforms α, βI, βII, γ, δ, and ε (Toullec et al., 1991; Martiny-Baron et al., 1993).

Overactivity of PKC could impair endothelial function through different pathophysiological mechanisms. PKC activation increases NADPH oxidase activity (Gorlach et al., 2000) and induces the uncoupling endothelial NO synthase activity (Hink et al., 2001). These actions lead to generation of superoxide anion that reduces the bioavailability of NO for causing endothelial relaxation. In addition, PKC activity has also been suggested to inhibit post-translational activation of endothelial NO synthase (Michell et al., 2001), compromising NO production. Thus, the overactivity of PKC associated to diabetes could, in addition to potentiating TP receptor-mediated responses, inhibit endothelium-dependent relaxation by affecting the NO-mediated responses.

In our study, further support for the pathophysiological role of excessive activity of PKC was demonstrated by provoking impairment of endothelium-dependent relaxation by inducing overactivity of PKC with a phorbol ester in HCC from nondiabetic patients. Nangle and collaborators previously reported an improvement of endothelium-dependent...
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and neurogenic dysfunction of corpus cavernosum from diabetic mice after chronic PKC inhibition (Nangle et al., 2003). We demonstrate that diabetes causes hypersensitivity to the contractile effects of prostanoids in HCC by a mechanism involving overactivity of PKC. This overactivity of PKC is also involved in the impairment of endothelium-dependent relaxation in tissue from diabetic impotent men. Thus, TP receptor blockade and PKC inhibition are therapeutic targets for the treatment of erectile dysfunction associated with diabetes.

Acknowledgments

We thank Maite Guerricabeitia and M. Victoria Martínez for excellent technical assistance in cAMP determinations.

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