Alterations in rat coronary vasoreactivity and vascular protein kinase C isoforms in Type 1 diabetes

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Alterations in rat coronary vasoreactivity and vascular protein kinase C isoforms in Type 1 diabetes. Am J Physiol Heart Circ Physiol 285: H2694–H2703, 2003. First published August 14, 2003; 10.1152/ajpheart.00394.2003.—Vascular complications associated with diabetes mellitus (DM) have been linked to activation of PKC-dependent signaling pathways in both human and animal models of DM. To determine whether aberrant PKC signaling mechanisms impact the coronary circulation, we assessed isolated coronary artery (CA) responses after the induction of Type 1 DM. Male Sprague-Dawley rats were subjected to partial pancreatectomy (DM; n = 23) and compared with age-matched controls (CTL; n = 19). Vasoreactivity was assessed in single CAs (~250 μm internal diameter) after ablationist administration of the Gq-dependent vasoconstrictors endothelin (ET)-1 (10⁻⁶–10⁻⁸ M) and U-44619 (10⁻⁸–10⁻⁵ M) or the voltage-gated Ca²⁺ channel agonist BAY K 8644 (10⁻⁹–10⁻⁵ M) with and without the PKC inhibitor bisindolylmaleimide (Bis; 10⁻⁶ M). Dilator responses to ACh (10⁻⁹–10⁻⁶ M) were also assessed. ET-1 resulted in significantly greater constriction in the DM versus CTL group (50 ± 4% vs. 33 ± 5%, P < 0.0001), whereas responses to U-44619 and BAY K 8644 were similar between groups. Importantly, inhibition of ET-1 and U-44619 in the DM caused by constrictor Bys occurred in the DM but not CTL group (P < 0.05). Western blotting on isolated CAs revealed greater levels of PKC-α, PKC-β₁, and PKC-β₂ by 22%, 15.3%, and 17.6%, respectively, in the DM versus CTL group (P < 0.05), whereas PKC-δ and PKC-ε protein levels were unchanged. DM was also associated with attenuated CA dilations after ACh treatment (P < 0.0566) and reductions in endothelial nitric oxide synthase protein levels versus CTL (P < 0.03). These data suggest that Ca²⁺-dependent PKC signaling pathways, particularly for ET-1, play a greater role in modulating CA vasoconstrictor responses in DM versus CTL. These data further suggest that aberrant CA constrictor and dilator responses are likely to contribute to the coronary vascular pathology associated with DM.

vascular smooth muscle; endothelium; endothelial nitric oxide synthase; endothelium; partial pancreatectomy

However, the mechanisms by which hyperglycemia induces damage to the coronary vasculature and subsequent alterations in cardiac contractile function are poorly understood (44).

Although the vascular complications seen in DM are likely multifactorial in nature, activation of the diacylglycerol (DAG)-PKC-dependent signaling pathway is known to be associated with many of the vascular abnormalities observed in DM (31, 44). In fact, hyperglycemia results in the chronic activation of various PKC isoforms in a variety of tissues (13, 17, 26, 41, 44). Of the PKC isoforms affected by hyperglycemia, there seems to be preferential activation of the β isoforms in the vascular tissues studied to date (31, 44), and there is growing evidence that PKC-β plays a critical role in the development of vascular complications associated with DM. Importantly, PKC-β inhibition has been shown to ameliorate DM retinopathy, nephropathy, neuropathy, and cardiomyopathy (5, 13, 17, 19, 34, 43, 44). One unresolved issue is whether and to what extent alterations in PKC contribute to the aberrant regulation of vascular responsiveness in the coronary circulation.

The extent of coronary vascular tone represents a balance between endothelium-derived vasodilator and vasoconstrictor factors as well as receptor-dependent phenomena localized to vascular smooth muscle. While there is growing evidence that impaired endothelium-dependent vasodilation occurs in a variety of peripheral circulations and contributes to alterations in organ function associated with DM (5, 7, 41), direct studies on the coronary circulation are lacking. Similarly, enhanced vasoconstrictor responses observed in a variety of tissues isolated from DM rats are limited to peripheral circulations (2, 3, 15, 20, 42, 45, 46). In this regard, interactions between voltage-gated calcium channels (VGCCs) and PKC are known to play a critical role in determining the extent of coronary vascular tone under normal physiological conditions (18), and it is possible that alterations in coronary vascular reactivity in DM may be due, in part, to aberrant interactions between VGCCs and PKC (3 62, 15, 42, 45). However, the effects of DM on PKC-VGCC interactions

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and subsequent coronary vascular reactivity remain poorly understood.

The purpose of the present study was to examine the regulatory role of PKC on rat coronary artery (CA) vasoconstrictor responses in a setting of chronic DM. It was also the purpose of this investigation to determine whether defects in endothelium-dependent dilation known to occur with DM contribute to enhanced constrictor responses in the rat coronary circulation. A unique aspect of this study was use of the partial pancreatectomy (PPX), an experimental model for the induction of Type 1 DM that is free from nonspecific effects of chemical toxins on peripheral circulations and signal transduction mechanisms. Vasoreactivity was assessed in isolated CAs, as were directional changes in PKC isoform protein levels, endothelial nitric oxide (NO) synthase (eNOS) levels, and endothelin (ET) type A/B (ET_A/B) receptor protein levels.

METHODS

Experimental Animals

All experiments were approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University and were in agreement with the "Guidelines for the Care and Use of Animals" of the American Physiological Society. Male Sprague-Dawley rats were obtained from the breeder (Harlan Sprague Dawley) and randomly divided into DM (PPX; n = 23) and control (CTL; age matched: n = 12; sham surgery: n = 7) groups. All animals were housed in temperature- and humidity-controlled holding facilities with a 12:12-h light-dark cycle and fed ad libitum standard rat chow.

Partial Pancreatectomy

The PPX technique, as first described by Folgia (10), was performed and modified on the basis of previous work in our laboratory (25). Briefly, a microcauterizer was used to eliminate small pancreatic blood vessels and to reduce bleeding during surgery. Sterile conditions were maintained throughout surgery. Rats were anesthetized using isoflurane and were kept on a heated surgical pad. To induce moderate to severe levels of DM, the procedure requires the physical removal of ~90% of the pancreatic tissue from the splenic, duodenal, and pyloric regions while the major blood vessels are left intact. The ablation of tissue was accomplished using sterile cotton Q-tips. At the conclusion of surgery, rats were given ampicillin (5 mg/100 g body wt sc) as an antimicrobial agent, 1 ml saline, and 0.02 mg/kg buprenorphine, an analgesic. Exact procedures were followed for sham-operated rats with the exception of removal of the pancreatic tissue. Two weeks after the induction of DM, a tail vein blood sample was obtained from PPX animals in the fed state to determine plasma glucose concentrations, and rats with a plasma glucose concentration of <250 mg/dl were excluded from the study. While the purpose of PPX is to eliminate the pancreatic tissue, thereby eliminating the β-cells of the pancreas that secrete insulin and subsequently induce DM, the pancreas is also responsible for secreting a variety of digestive enzymes. Therefore, the possible confounding effects of the absence of these digestive enzymes are hereby noted. It should be mentioned, however, that despite reductions in digestive enzyme production, rats grow, do not need daily insulin, and do not display any significant digestive problems such as chronic diarrhea.

Isolation of Coronary Arteries

After ~6 wk of DM, animals were weighed and anesthetized after a 5-h fast. The hearts of the animals were quickly excised and placed in ice-cold saline. Blood (1 ml) was taken for the determination of plasma glucose. Proximal sections of the abdominal aorta were also dissected and quick frozen in liquid nitrogen. After the removal of the right ventricular wall and atria, the heart was weighed and moved to a dissecting chamber containing cold physiological saline solution (PSS; 4°C). The PSS used in all experiments contained (in mM) 145 NaCl, 4.7 KCl, 2.0 CaCl2, 1.17 MgSO4, 1.2 NaH2PO4, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, and 3.0 MOPS and was adjusted to a pH of 7.4 and filtered through 0.22-μm filters (Fisher Scientific, Pittsburgh, PA). Segments of the septal artery [first-order (1A) branches] of ~250-μm internal diameter (ID) and 0.5–1.0 mm in length were dissected with the aid of an Olympus dissecting microscope. Isolated vessels were cannulated at each end with glass micropipettes, secured with 11-0 nylon ligatures, and viewed through an inverted microscope (×25 magnification, WPI). The vessels were pressurized at 100 cmH2O by two independent fluid-filled reservoirs, which were attached to the micropipettes. Vessels that displayed leaks were discarded and excluded from the study. Importantly, endothelium-intact vessels were used for all protocols associated with the present study. The microscope was coupled to a videocamera (CUE) and television monitor (Panasonic). A video tracking system (Colorado Video Calipers, Texas A&M University) was utilized to continuously monitor vessel ID throughout a given experiment in association with PowerLab data-acquisition software.

Experimental Protocol for Assessment of Vascular Reactivity

Vessels were allowed to equilibrate for 1 h, during which time the temperature of the chamber was warmed to and maintained at 37°C with a circulating water bath. The vessels were bathed in 2 ml warm PSS plus 1 g/100 ml albumin (U.S. Biochemical), and the PSS-albumin was replaced every 15 min. All drugs were obtained from Sigma (St. Louis, MO) unless otherwise specified. Drugs were administered abuminally.

Endothelium-dependent dilation was evaluated by examining responses to ACh (10–3–10–5 M). Sensitivity to receptor-mediated vasoconstrictor agonists was evaluated by examining responses to ET-1 (10–10–10–8 M, Peninsula Laboratories) and the prostanoid receptor agonist U-44619 (10–9–10–5 M, Cayman Chemical), receptors known to signal through PKC. To examine contractions mediated by VGCCs, responses to the VGCC agonist BAY K 8644 (10–9–10–5 M) were examined. Concentration-response curves were obtained by adding increasing concentrations of the drugs directly into the bath. Importantly, only one experimental drug (inhibitor) was used on a given vessel, and drug concentrations for ET-1, U-44619, or BAY K 8644 were increased after the response to the preceding dose was maximal (~5 min/dose). Only vessels that exhibited ~30% spontaneous tone were used for ACh concentration-response curves. Spontaneous tone was not used as a criterion for any other protocols. Vessels that did not respond to ET-1, U-44619, or BAY K 8644 were excluded from the study. To determine the role of PKC on vasoconstrictor responses, separate sets of experiments with ET-1, U-44619, or BAY K 8644 were performed in the presence of a specific PKC inhibitor, bisindolylmaleimide.
(Bis; 10−6 M). This inhibitor displays a greater relative specificity for Ca2+-dependent conventional PKCs (εPKC; α, β1, β2, and γ) compared with novel PKCs (nPKC; δ, ε, η, and θ), which are Ca2+ independent (30). Vessels were incubated in Bis for 30 min before the start of concentration-response curve determination.

Finally, maximal diameter was obtained at the conclusion of each experiment by bathing the vessels in Ca2+-free PSS to obtain the maximal passive diameter at 100 cmH2O. Diameters were normalized to this measurement for the purpose of comparison, as previously described by Muller et al. (33) (see below).

**Protein Expression in Isolated CAs and the Abdominal Aorta**

Segments of the left CA (1A branch, ~250 μm ID and ~1,200 μm long, n = 3) were dissected in cold PSS solution (4°C). Vessel pieces (n = 2–3 pieces/tube) were snap frozen and stored at −70°C until ready for use. Whole vessel pieces were solubilized as previously described (22). Briefly, a modified lysis buffer (30 μl) containing 250 mM Tris (pH 8.8), 6 M urea, 160 mM DTT, 2% SDS, and 0.001% bromophenol blue was added to each sample. Vessel pieces were subjected to boiling (2 min), vortexing, and centrifugation (15,000 rpm, 30 s). This cycle was repeated three times. Protein determination was assessed using NanoOrange (Molecular Probes). For proximal segments of the abdominal aorta, vessel pieces were homogenized in 1 ml cold lysis buffer [20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 0.05% β-mercaptoethanol, 30 μg/ml leupetin, 3 μg/ml pepstatin A, 1 mM PMSF, and 1% Triton X-100] using the TH hand-held homogenizer. Homogenates were subjected to centrifugation for 60 min at 100,000 g (4°C). Protein concentrations of the resulting supernatant were determined by the method of Bradford (15).

**Data Analysis**

Vasomotor responses of septal CAs are presented as percent dilation/constriction and were expressed by the following equation: \[D_{SS} - D_{B} \times D_{SS} = 100\], where \(D_{SS}\) is the steady-state diameter in response to the drug and \(D_{B}\) is the baseline diameter right before the start of the concentration-response curve. Group comparisons for concentration-response curves to pharmacological agents were analyzed with two-way ANOVA for repeated measures using the PROC Mixed General Linear Models program associated with Statistical Analysis Software (SAS). Diameter measures at each dose were compared between groups by ANOVA. From the concentration-response curves of vasoactive agents, the half-maximal effective concentrations (EC50) were calculated, presumably reflecting the affinity of the agonist for its receptor. For the calculation of EC50, each concentration-response curve was fitted by regression analysis to a four-parameter sigmoidal equation using Prism software. Because EC50 is normally distributed according to a log scale rather than a linear scale, log EC50 values were used to make statistical comparisons. Statistical differences in log EC50 values were determined using two-way ANOVA. Presumably, differences

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**Fig. 1. Display of optimization methods.** Homogenates were diluted to equate 5, 10, and 15 μg of protein, respectively, for PKC isoforms [PKC-βI (A) and PKC-βII (B)] and endothelial nitric oxide synthase [eNOS (C)]. These immunoblots represent samples obtained from control (CTL) rats.
in EC50 with no change in the maximum response would suggest a defect in receptor coupling to downstream signaling targets, whereas differences in maximal responses may or may not reflect differences in receptor number. For immunoblotting, an unpaired, two-tailed Student’s t-test was used to determine group differences between mean values of protein levels. Significance of differences between mean values for the physical and physiological characteristics of animals was also determined by unpaired, two-tailed Student’s t-test. The least-significant-difference method was employed for all post hoc comparisons. All variables are reported as means ± SE. Significance was defined as P < 0.05.

RESULTS

Physical and Physiological Characteristics of Animals

The physical and physiological characteristics of the groups of rats are shown in Table 1. As expected, DM rats weighed less and had higher plasma glucose concentrations versus CTL rats. Body weight, heart weight, heart weight-to-body weight ratio, and plasma glucose concentrations were all significantly different in DM versus CTL rats (P < 0.05). Mean maximum vessel diameter measured at 100 cmH2O in Ca2+-free PSS was similar in CAs isolated from DM (262.5 ± 11 μm) and CTL (284.5 ± 11 μm) rats.

Reactivity of CAs

Endothelium-mediated vasodilator responses. ACh produced concentration-related increases in ID in CAs from both DM and CTL rats (Fig. 2). ANOVA indicated that the curves were different between DM and CTL CAs (P = 0.0566), with arteries from CTL rats exhibiting significantly greater relaxation (P < 0.05) in the dose range from 1 × 10−6 to 1 × 10−8 M ACh than CAs from DM rats. Maximal dilation was greater in CAs from CTL versus DM rats. The sensitivity to ACh was not significantly different (P = 0.1442) between DM and CTL CAs, as indicated by EC50 values (DM = 262.5 ± 11 μm; CTL = 284.5 ± 11 μm) rats.

Vasoconstrictor responses. ET-1 produced dose-dependent constriction in isolated CAs from all groups (Fig. 3). ANOVA revealed a significant group × drug interaction (P < 0.0001) in concentration-response curves. CAs from DM rats exhibited significantly greater constriction (P < 0.05) than CAs isolated from CTL rats in the dose-response range from 2.6 × 10−9 to 3.0 × 10−9 M for ET-1. Inhibition of PKC with the specific antagonist Bis significantly attenuated ET-1-mediated constriction in CAs from DM but not CTL rats. Sensitivity to PKC inhibition, however, was not statistically different between groups (P = 0.84), as indicated by the EC50 values (DM = 1.2 × 10−9 ± 5.1 × 10−11 M; CTL = 1.2 × 10−9 ± 1.8 × 10−10 M; DM + Bis = 1.1 × 10−9 ± 1.6 × 10−10 M; CTL + Bis = 1.0 × 10−9 ± 3.1 × 10−10 M).

The specific prostanoid receptor agonist U-44619 also produced dose-dependent constriction in isolated arteries from all groups (Fig. 4). ANOVA revealed a significant group × drug interaction (P < 0.02) in concentration-response curves. Bis attenuated U-44619-mediated constriction in CAs from DM but not CTL rats. This difference in sensitivity was further reflected by the significant difference (P < 0.05) in EC50 values of CAs from DM and CTL rats in the presence of the PKC inhibitor Bis. Taken together, these data suggest that alterations in PKC contribute, at least in part, to enhanced vasoconstrictor responses to ET-1 and U-44619 in CAs isolated from DM rats.

Finally, the selective L-type Ca2+ channel agonist BAY K 8644 produced dose-dependent constriction in arteries from all groups (Fig. 5). BAY K 8644 sensitivity and maximal BAY K 8644-induced constriction of arteries isolated from DM and CTL rats were similar. Also, BAY K 8644 sensitivity and dose-dependent contraction of arteries isolated from DM and CTL rats were similar in the presence of Bis. The EC50 values were not significantly different (P = 0.36) between any

Table 1. Physical and physiological characteristics of DM and CTL animals

<table>
<thead>
<tr>
<th></th>
<th>Heart Weight, g</th>
<th>Body Weight, kg</th>
<th>Heart Weight/Body Weight, g/kg</th>
<th>Maximum Vessel Diameter, μm</th>
<th>Plasma Glucose, mg/dl</th>
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<tbody>
<tr>
<td>DM</td>
<td>0.8478 ± 0.03*</td>
<td>0.2847 ± 0.01*</td>
<td>2.928 ± 0.06*</td>
<td>262.5 ± 11</td>
<td>445.4 ± 35*</td>
</tr>
<tr>
<td>CTL</td>
<td>0.9571 ± 0.02</td>
<td>0.3555 ± 0.01</td>
<td>2.719 ± 0.08</td>
<td>284.5 ± 11</td>
<td>232.6 ± 12</td>
</tr>
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Values are means ± SE; n = 23 diabetes mellitus (DM) and 19 control (CTL) rats. *Significantly different between DM and CTL values, P < 0.05.
of the groups (DM = 6.8 × 10⁻⁸ ± 3.1 × 10⁻⁸ M; CTL = 8.9 × 10⁻⁸ ± 3.5 × 10⁻⁸ M; DM + Bis = 4.0 × 10⁻⁷ ± 3.6 × 10⁻⁷ M; CTL + Bis = 3.7 × 10⁻⁸ ± 8.2 × 10⁻⁹ M). BAY K 8644 and Bis were used to examine the role of PKC modulation in VGCC-mediated constriction. These results indicate that VGCC-PKC interaction is not responsible for aberrant vasoreactivity in DM.

Expression of eNOS and PKC Isoforms in CAs

Figure 6 shows the results of immunoblot analysis of eNOS content in isolated CAs from DM and CTL rats. Arteries from DM rats had significantly decreased (P < 0.03) eNOS levels compared with arteries from CTL rats. In isolated CAs from DM rats, eNOS was de-
creased by 24.5% versus CTL rats. Interestingly, abdominal aortas from DM rats also had significantly decreased ($P < 0.002$) eNOS levels compared with abdominal aortas from CTL rats. In isolated abdominal aortas from DM rats, eNOS was decreased by 22.3% versus CTL rats.

Figure 7 shows the results of immunoblot analysis of PKC-α, PKC-βI, PKC-βII, PKC-δ, and PKC-ε. PKC-α levels were elevated in CAs from DM rats and were significantly increased by 21.6% ($P < 0.03$) in CAs from DM compared with CTL rats. PKC-βI expression was also significantly elevated ($P = 0.059$) in CAs from DM rats, as was PKC-βII expression ($P < 0.02$). PKC-βI and PKC-βII levels were increased by 15.3% and 17.6%, respectively. In contrast, PKC-ε and PKC-δ levels were similar ($P > 0.05$) in CAs from DM and CTL rats. Interestingly, in isolated abdominal aortas, there were no significant differences in any of the PKC isoform protein levels from DM and CTL rats (data not shown). These results suggest a significant increase in cPKC levels compared with nPKC levels in isolated CAs from DM versus CTL rats. Finally, Fig. 8 shows immunoblot analysis results for ETA/ETB receptor protein levels assessed in CAs isolated from DM and CTL rats. Importantly, group differences were not observed in ETA/ETB receptor protein levels ($P > 0.05$).

**DISCUSSION**

The purpose of this investigation was to determine whether defects in vasoreactivity in DM were present in the rat coronary vasculature. We examined the hypothesis that DM results in enhanced vasoconstrictor responses to the specific receptor agonists ET-1 and U-44619 as well as the calcium channel agonist BAY K 8644. To determine the relative role of PKC on constrictor responses, a specific PKC inhibitor, Bis, was used in a separate series of experiments in conjunction with the vasoconstrictor stimuli. We also examined the hypothesis that DM results in attenuated vasodilator responses to the endothelium-dependent vasodilator ACh. An in vitro approach was used to evaluate the effects of DM on the vasoreactivity of CAs from DM and CTL rats. Protein levels for eNOS, PKC-βI, PKC-βII, PKC-α, PKC-δ, and PKC-ε were also determined in CAs and abdominal aortas isolated from DM and CTL rats, respectively. Our findings indicate that vasoconstrictor responses were enhanced in DM and were, in part, mediated by PKC. Our study also demonstrates attenuated NO-mediated vasodilatation with concurrent reductions in eNOS protein levels in DM. This study provides the first evidence for significant alterations in coronary vasoreactivity and eNOS/PKC isoform pro-

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Fig. 7. Effects of DM on various PKC isoforms in CAs. A: PKC isoform content from representative immunoblots prepared from CAs as described in the text. Equal amounts of protein were loaded per lane for CAs (7 µg/lane), PKC-βI and βII (7–8 µg/lane), PKC-δ (8 µg/lane), and PKC-ε (8 µg/lane). B: average PKC isoform protein content of CAs from DM and CTL rats for PKC-α ($n = 12/12$), PKC-βI ($n = 12/12$), PKC-βII ($n = 16/16$), PKC-δ ($n = 10/13$), and PKC-ε ($n = 19/19$). Values are means ± SE (normalized by expressing all values relative to CTL). *$P < 0.03$, DM vs. CTL for PKC-α; **$P < 0.02$, DM vs. CTL for PKC-βI; ***$P = 0.059$, DM vs. CTL for PKC-βII.

Fig. 8. Effects of DM on ETA/ETB receptor protein levels in CAs. A: ETA/ETB receptor content results from immunoblots prepared from CA samples as described in the text. Equal amounts of protein were loaded per lane for CAs (7 µg/lane). B: average ET-1 receptor protein content of CAs from 8 different DM rats and 8 different CTL rats. Values are means ± SE (normalized by expressing all values relative to CTL). There was no statistical difference between DM and CTL.
tein levels in DM using the PPX rat model that may contribute to the increased coronary vascular resistance and diminished contractile reserve capacity associated with diabetic cardiomyopathy.

Effects of DM on Endothelial-Dependent Vasodilation

Studies (5–7, 41) have reported impaired endothelium-dependent vasodilation in DM in a variety of circumstances, and we now extend these findings to the rodent coronary circulation. Our results indicate that DM results in the attenuation of NO-mediated vasodilation (ACh induced) in isolated CAs from rats. Specifically, the maximal dilatatory responses to ACh were decreased in CAs isolated from DM versus CTL rats. Furthermore, deficits in endothelium-mediated dilation observed in our study were supported by parallel findings of reduced eNOS protein levels in the coronary vasculature of DM versus CTL rats. Previously, indirect assessment of vasodilator responses in DM versus non-DM mongrel dogs suggested that impaired dilation of CAs occurs after administration of ACh (4, 24). Our results, combined with those in the literature, support the concept that endothelium-mediated vasodilation is attenuated in the coronary vasculature of DM rats and demonstrate, for the first time, directional changes in coronary eNOS protein levels.

While reports of DM-associated increases in eNOS levels of cultured endothelial cells and vessels exist (5, 17, 39), evidence also suggests that reductions in eNOS levels occur in DM (9). Recent evidence suggests that eNOS levels in cultured bovine retinal endothelial cells (BRECs) are decreased in response to high glucose levels (9) and that PKC can negatively influence the expression of eNOS mRNA in cultured bovine aortic endothelial cells (35). Consistent with these results, in our study, decreased coronary eNOS protein levels in DM compared with CTL rats were associated with increased levels of specific cPKC isoforms. It is tempting to speculate that the impaired endothelium-dependent dilation in DM animals in the present study is due, in part, to PKC-mediated reductions in eNOS protein levels and coordinate reductions in NO synthesis. Future experiments are needed to address this important issue.

Effects of DM on Vasoconstrictor Responses

In the present study, we examined the effects of intracellular signaling pathways involving PKC on enhanced coronary vasoconstrictor responses in DM. Two Gq-coupled receptor-mediated vasoconstrictors were employed: ET-1, an ETα/ETβ receptor agonist, and U-44619, a prostanoid receptor agonist. Both ET-1 and U-44619 are thought to induce potent vasoconstriction through activation of PKC. Our findings of enhanced coronary constrictor responses to ET-1 in DM are consistent with previous studies demonstrating enhanced norepinephrine-mediated vasoconstriction in mesenteric, aortic, caudal, and renal vasculatures of DM rats (2, 3, 20, 45) as well as greater constrictor responses to phorbol esters in first-order arterioles of skeletal muscles of DM rats (42). Furthermore, Hattori and colleagues (15) found enhanced vasoconstriction after both ET-1 and U-44619 treatment in the aorta of DM rats, which was effectively diminished to a greater extent by PKC inhibition in DM versus CTL rats. These findings are consistent with the observation that enhanced vasoconstrictor responses present in DM are, in part, mediated by PKC.

Whereas maximal ET-1-induced vasoconstriction was significantly greater in DM, we observed no differences in U-44619-induced coronary vasoconstriction in DM versus CTL rats. However, our results do provide evidence for an important regulatory role of PKC on coronary vasoconstrictor responses seen in DM after administration of either ET-1 or U-44619. Specifically, administration of a PKC inhibitor caused a significant reduction in constrictor responses induced by both ET-1 and U-44619 in CAs isolated from DM but not CTL rats. These results demonstrate that enhanced vasoconstriction in DM is mediated, at least in part, by alterations in PKC. A possible explanation for the difference in the maximal constrictor responses observed in CAs from DM rats to ET-1 versus U-44619 may be due to differences in the responsiveness of vascular smooth muscle to circulating constrictor factors. Indeed, studies (31, 36) have reported that the expression of ET-1 is increased in the retina of DM rats and in cultured BRECs exposed to high (25 mM) glucose levels. Moreover, Bis inhibited glucose-induced ET-1 overexpression in cultured BRECs (36). Therefore, it is possible that increased levels of circulating ET-1 and/or ET-1 receptors, combined with enhanced levels of PKC, converge to produce a contractile hypersensitivity of vascular smooth muscle. In the present study, however, we were unable to detect differences in ETα/ETβ receptor protein levels in isolated coronary arteries from DM compared with CTL rats. Thus, while we cannot rule out the possibility that alterations in circulating ET-1 levels contribute to aberrations in vascular responsiveness under conditions of DM, our data do support a primary role for PKC in mediating enhanced coronary vasoconstrictor responses present in DM.

Interestingly, PKC inhibition by Bis had no effect on vasoconstrictor responses to either ET-1 or U-44619 in CAs isolated from CTL compared with DM rats. As previously mentioned, this inhibitor has a greater relative specificity for cPKC over nPKC isoforms. Therefore, a logical conclusion would be to assume that nPKCs play a greater role in generating coronary constrictor responses in CTL rats, whereas cPKCs mediate constrictor responses to a greater extent in CAs isolated from DM rats. While the contribution of individual PKC isoforms to the regulation of vascular smooth muscle contraction are incompletely understood (18, 28), our findings of increasing cPKC levels in isolated CAs from DM versus CTL rats and therefore increases in the ratio of cPKCs to nPKCs support a more dominant role for cPKCs in mediating enhanced vasoconstrictor responses in DM.
Finally, the results of the present study indicate that vasoconstrictor responses mediated by VGCCs were similar in CAs isolated from DM and CTL rats. Furthermore, there was no significant effect of PKC inhibition on BAY K 8644 coronary responsiveness in either DM or CTL rats, further suggesting that VGCC-PKC interactions do not account for DM-related changes in contractile responsiveness. These findings are seemingly at odds with others in the literature, whereby enhanced vasoconstriction in DM after BAY K 8644 was observed (3, 20, 42). This may be due to differences in the vasculature circulation employed. Previous studies demonstrating enhanced vasoconstriction in response to BAY K 8644 examined responses in mesenteric, renal, and skeletal muscle vessels, whereas we examined responses specific to the coronary vasculature. While our results suggest that the underlying cellular mechanisms responsible for enhanced vasoactivity in DM do not involve VGCCs, they do suggest a role for receptor-second messenger signaling systems in CAs of DM rats. Specifically, our functional data suggest that both attenuated endothelium-mediated dilation and enhanced vasoconstrictor responses are present in the coronary vasculature of DM compared with CTL rats, and these alterations in vasoreactivity in DM may contribute to increased coronary vascular resistance and potentially decreased contractile reserve capacity in diabetic cardiomyopathy.

Effects of DM on PKC Isoform Expression

A number of studies (11, 12, 21, 23, 27, 29, 36, 40) have demonstrated that DM-associated hyperglycemia results in the activation of various PKC isoforms in a variety of tissues. Moreover, preferential activation of the β-isozymes apparently occurs in some vascular tissues (31, 44), and PKC-β inhibition can ameliorate many of the pathophysiological changes seen in the DM vasculature. In the coronary circulation, we observed significant increases in PKC-βI, PKC-βII, and PKC-α protein levels with DM. Alternatively, PKC-δ and PKC-ε protein levels were similar in CAs from DM versus CTL rats. While seemingly at odds with the cardioprotective nature of some PKC isoforms in a variety of experimental models, there is growing evidence for the role of the PKC-α and PKC-β isoforms in the development of DM-associated vascular complications. Specifically, PKC-α has been implicated in the mediation of increased vascular permeability in endothelial cells exposed to high glucose (16). Furthermore, a recent study (14) demonstrated that in hyperglycemia, PKC-α is involved in the regulation of apoptosis of vascular smooth muscle cells. Moreover, activation of PKC-α has been identified as a major signal transducer required for VEGF-induced proliferation, which is a common abnormality in the DM vasculature leading to vascular dysfunction (38). Thus, while our CA results are in agreement with, and extend previous findings of, increased levels of PKC-α and PKC-β in left ventricular myocardium and the aorta isolated from DM rats and pigs (11, 12, 23, 29), we did not observe group differences in PKC isoforms in the abdominal aorta isolated from DM and CTL rats, respectively.

The preferential activation of certain PKC isoforms in DM such as the PKC-α and PKC-β isoforms remains poorly understood. Clearly, PKC isoforms exert different functional responses depending on cellular location, in part due to differences in cellular compartmentalization mediated by receptors for activated C kinase (RACKs) (32). The effect of DM on RACKs, however, has not been investigated. Another possible mechanism for the preferential activation of cPKCs in DM includes enhanced sensitivity of DAG to Ca2+, whereby cPKCs may be more sensitive to DAG activation than nPKCs (31, 44). Other possibilities that merit further investigation include differences in the rates of protein synthesis and degradation of various PKC isoforms as well as PKC isoform-specific sensitivity to posttranslational modification such as phosphorylation and/or glycosylation.

Limitations

In the present study, an endothelium-intact whole artery preparation was utilized to assess vascular reactivity in conjunction with changes in protein levels for PKC and eNOS levels in a rodent model of DM. Therefore, we cannot distinguish between possible DM-related defects specific to endothelial versus vascular smooth muscle cells in our experimental paradigm. However, based on well-characterized findings that alterations in NO-mediated dilation of vascular smooth muscle are primarily related to defects in endothelium-mediated production of NO (18, 34, 41), we conclude that directional changes in eNOS levels and diminished Ach-mediated dilation in isolated coronary arteries of DM rats are likely to reside in the endothelium. Similarly, because the regulatory role of PKC on vascular smooth muscle persists in the absence of endothelium (24, 28, 39), observed changes in PKC protein levels and vascular responsiveness after the administration of Gq-coupled agonists in the presence and absence of PKC inhibition as described herein likely reflect defects specific to vascular smooth muscle.

However, future studies are indicated to address the potential significant interactive effects among NO, PKC, and ET-1 signal transduction pathways, respectively, on the vascular dysregulation associated with DM. Finally, the relative contributions of hyperglycemia versus hypoinsulinemia in mediating alterations in PKC-related signal transduction mechanisms were not assessed in the present study. Future studies designed to tease out the singular and combined effects of DM-associated metabolic derangements on vascular responsiveness and associated signal transduction are warranted.

In summary, this study reports attenuated NO-mediated vasodilation with concurrent reductions in eNOS levels in isolated CAs from DM versus CTL rats. This study also reports enhanced cPKC-mediated vasoconstriction (ET-1 induced) but no effect of DM on...
VGCC-induced vasoconstriction in isolated CAs, suggesting that enhanced vasoconstrictor responses may be receptor dependent in DM. Taken together, the data suggest that attenuated vasodilation and enhanced vasoconstriction in the coronary vasculature, in part mediated by PKC, underlie increases in coronary vascular resistance in DM. These results were further supported by immunoblot analyses revealing, for the first time, increased PKC-β1, PKC-βII, and PKC-α in isolated CAs of DM versus CTL rats. Future studies examining PKC translocation and activation, PKC mRNA expression, and the use of specific PKC isoform inhibitors in isolated CAs of DM rats are needed to determine the exact role and function of specific PKC isoforms in cardiovascular dysfunction associated with DM milieu.

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DISCLOSURES

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