Upregulation of PKC genes and isozymes in cardiovascular tissues during early stages of experimental diabetes

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DIABETES MELLITUS COMPRIS E A HOST OF PATHOLOGICAL ALTERATIONS characterized by multiple organ dysfunctions, of which the cardiovascular complications are the major cause of morbidity and mortality. Hyperglycemia is considered the primary etiological factor that initiates circulatory disorders such as cardiomyopathy, angiopathy, and atherosclerosis (15, 28, 38). Several hypotheses have been proposed to explain the adverse effects of elevated blood glucose, including the polyol pathway, nonenzymatic glycation, oxidative stress, and protein kinase C (PKC) activation (15, 28, 38). Of these mechanisms, activation of the PKC signaling pathway has been increasingly recognized as an early and common mechanism leading to cardiovascular dysfunction in hyperglycemia (11, 15, 16, 18, 35, 38).

The PKC family consists of multiple isoforms, categorized into the classic (e.g., α, β1, β2, and γ), the novel (δ, ε, η, θ, μ, and ν), and the atypical (ζ and ι) groups (8, 29, 38). These isozymes show distinct expression patterns and stimulate diverse cell responses (8, 15, 28, 38). Among these isoforms, the classic group, especially PKCα and β, are better characterized, and their potential impacts on the cardiovascular system are emphasized (11, 14–16, 18–19, 25, 28, 38). For example, recent studies reveal a preferential increase of PKCβ synthesis and activity in cardiovascular tissues of diabetic animals (16, 24, 25, 44) as well as in failing hearts of human patients (3, 34). Overexpression of PKCβ in the myocardium of transgenic mice induces myocardial hypertrophy and impairs ventricular function (4, 37). Oral administration of a PKCβ-specific inhibitor normalizes blood flow and vascular barrier function in several organs of diabetic animals (6, 18, 21, 22). The same treatment prevents the impairment of endothelium-dependent vasodilation caused by hyperglycemia in humans (2, 13). In addition to PKCβ, some studies show that elevated glucose activates PKCα (8, 19, 25) and induces vascular endothelial hyperpermeability (14), which is an important mechanism underlying the development of atherosclerotic lesion. Although it is generally agreed that PKC activation contributes to the cardiovascular dysfunction (15, 28, 35), no consensus has been reached regarding the molecular basis of PKC upregulation in hyperglycemia or diabetes. In particular, it is not clear whether the cardiovascular impact of PKC is due to an increased gene/protein expression of the enzyme or merely a posttranslational modulation of its kinase activity.

Streptozotocin (STZ) ablation of the pancreatic β-cells has been used in various animal species for studying type 1 diabetes-related abnormalities. We have previously modified this technique to establish a
swine model of diabetes. We are particularly interested in the swine because of its high comparability to humans in the anatomy and physiology of the cardiovascular systems (20). In addition, the swine is one of the few human-relevant animal models that can develop diabetic atherosclerosis. Our subsequent studies with this model (44) have demonstrated an increase in the enzymatic activity of PKC and its contribution to coronary microvascular barrier dysfunction in the heart. The purpose of this study was to examine the transcriptional and translational regulation of the classic PKC isozymes during the initial stages of diabetes in the same animal model. To quantitatively assess the expression of specific PKC genes, we have cloned and sequenced partial regions of the cDNA for porcine PKCα, β1, and β2, and developed a real-time RT-PCR assay based on the TaqMan methodology (7). The results showed differential upregulation of PKC genes and isozymes in the heart and aorta at both the 4th week and 6th to 8th week of experimental diabetes. Although both PKCα and PKCβ genes were upregulated in the heart, only PKCβ expression was elevated in the aorta. Importantly, there were corresponding increases in the mRNA level and protein expression for PKCβ2 in both tissues, suggesting that PKCβ2 may be a prominent target of diabetic injury in the cardiovascular system.

MATERIALS AND METHODS

Animal treatment. Diabetes was induced in juvenile Yorkshire pigs (7 wk of age, weighing 9–12 kg) by intravenous injection of STZ (150 mg/kg) as previously described (20, 44). Age-matched control pigs received vehicle only. All pigs had free access to drinking water and commercial pig diet. The general condition, body weight, and the level of blood glucose were closely monitored in all pigs, and only those that developed sustained hyperglycemia with a blood glucose level >300 mg/dl were included in the diabetes groups. At 2, 4, and 6–8 wk after STZ or vehicle injection, diabetic and age-matched control pigs were anesthetized with pentobarbital sodium (30 mg/kg iv), and their hearts and aortas were surgically removed for RNA or protein extraction.

RNA extraction and RT-PCR. Total RNA was extracted from either the homogenized ventricular myocardium or the aorta segment by using the ToTOLLY RNA Kit (Ambion, Austin, TX) according to the supplier’s instructions. Primers were designed based on known sequences of the human PKCα, β1, and β2 mRNAs (Table 1) and were used to amplify the corresponding cDNAs of porcine PKC isoforms with the TITan One Tube RT-PCR System (Roche Molecular Biochemicals, Mannheim, Germany). The reaction mixture was incubated at 45°C for 60 min, followed by denaturation at 94°C for 3 min. Then 35 cycles of denaturation, annealing, and elongation were performed at 94°C for 30 s, 45°C for 30 s, and 72°C for 90 s, respectively. The PCR products of expected sizes were sequenced directly; sequence analysis and multiple alignments were performed using the Lasergene software (DNASTAR, Madison, WI).

Real-time RT-PCR. The principle of real-time RT-PCR was described in detail in a previous review (7). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an endogenous control for normalization. Primers and TaqMan probes (Table 1) were designed based on the determined sequences of the respective target genes. RT-PCR reactions were detected by an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Each reaction mixture (10 μl) contained 1x TaqMan buffer, 5.5 mmol/l MgCl2, 300 μmol/l of dATP, dGTP, and dCTP, 600 μmol/l dUTP, 900 nmol/l of sense and antisense primers, 100 nmol/l TaqMan probe, 2.5 U MultiScript reverse transcriptase, 0.25 U AmpliGold Taq polymerase, and total RNA (50 ng for detection of PKC α, β1, and β2 mRNAs; 1 ng for GAPDH mRNA detection). The reaction mixture was incubated at 48°C for 30 min and then at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/amplification at 60°C for 1 min for each assay. For each assay, a relative standard curve was obtained through serial dilutions of the total RNA.

Table 1. Sequences of oligonucleotide primers and TaqMan probes used for molecular cloning and quantification of PKCα, β1, and β2 mRNA by real-time RT-PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer/Probe Gene</th>
<th>Target Gene</th>
<th>5’ Position</th>
<th>Sequence (5’–3’)</th>
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<tr>
<td>PKC-A11</td>
<td>antisense</td>
<td>PKCα</td>
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<tr>
<td>PKC-B18</td>
<td>sense</td>
<td>PKCβ1, β2</td>
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<td>GGGCTGGAAAGAAGGCGATT</td>
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<tr>
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<td>PKCβ2</td>
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<tr>
<td>PKC-cF</td>
<td>sense</td>
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<td>6-FAM-TCCCTGATCCAAAGAGGAAAGAAA-TAMRA</td>
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<tr>
<td>PKC-c Probe</td>
<td>probe</td>
<td>PKCα</td>
<td>681</td>
<td>TGTAGAAGCCAGATGCTTGT</td>
</tr>
<tr>
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<td>PKCβ1</td>
<td>2038</td>
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*The TaqMan probe consisted of an oligonucleotide with a 5’ reporter dye (either 6-carboxyfluorescein (FAM), tetrachloro-6-carboxyfluorescein (TET), or fluorescent dye (VIC)), and a 3’ quencher dye (6-carboxytetramethylrhodamine (TAMRA)). The 5’ positions of the primers and probes were based on sequences of human PKCα (GenBank accession no. NM_002737), β1 (NM_002738), β2 (X07109), and GAPDH (NM_002496) mRNAs.

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The target quantity was determined based on the standard curve and then normalized to that of the GAPDH control. The relative expression levels of target genes were presented as percentage of controls.

**GenBank accession number.** The GenBank accession numbers for the partial mRNA sequences of pig PKCα, β1, and β2 are AY093442, AY093443, and AY093444, respectively.

**Immunoblotting.** A trunk of ventricular tissue or a segment of aorta was homogenized and lysed in 50 mmol/l Tris-HCl, pH 7.5, containing 1% Triton X-100 and a cocktail of protease inhibitors. The clarified lysate was fractionated with SDS-PAGE on 8% gel and then transferred to nitrocellulose membrane. The membrane was incubated overnight at 4°C with a primary polyclonal antibody to a PKC isoform (either α, β1, or β2) (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with a secondary antibody conjugated to horseradish peroxidase. The immunoreactive bands were detected with LumiGLO chemiluminescent substrate (Cell Signaling, Beverly, MA), scanned by reflectance-scanning densitometry and quantified using NIH Image software. Protein concentrations of the supernatant were measured according to the Bradford method.

**Data analysis.** In each experiment, the levels of mRNA and protein expression in diabetic pigs were compared with those obtained from age-matched control pigs and presented as percentages of controls. All data are expressed as means ± SE, with n representing the number of pigs used in the study. Analysis of variance was used to evaluate the significance of intergroup differences. *P < 0.05 was considered significant for the comparisons.

**RESULTS**

Eighteen of 20 pigs receiving STZ treatment developed sustained hyperglycemia with an average blood glucose level of 432 ± 23 mg/dl, significantly higher than that in control pigs (92 ± 7 mg/dl) (Table 2). The diabetic pigs generally appeared healthy and their eating pattern and behavior were not significantly different from the normal pigs. They gained weight over the experimental period at a rate slower than that of age-matched controls. Histopathological studies revealed that STZ treatment induced a selective destruction of the pancreatic β-cells, but did not cause recognizable pathological changes in vital organs up to the 8th week of hyperglycemia.

Sequence analysis indicated that both the porcine and human PKC genes shared high nucleotide and (predicted) amino acid sequence identity. In particular, the porcine PKCα gene was 92% in nucleotides and 99% in amino acids identical to human PKCα in the 5’ regions (830 bp). The porcine PKCβ1 and β2 mRNAs had an identical nucleotide sequence in the 5’ region from 1703 to 2000 (based on human PKCβ1 numbering) but comprised distinct 3’ terminal coding and nontranslated regions. In the sequenced 3’ region (771 bp) of PKCβ1, the swine shared 92% nucleotide and 100% amino acid sequence identity with the humans. Similarly, the nucleotide and amino acid sequences were 91% and 100% identical between the pigs and humans in the partial 3’ region of PKCβ2.

In the real-time RT-PCR assay, all the relative standard curves showed a strong linear relationship between the Ct values and the RNA concentrations. The slopes of relative standard curves for the target genes were −2.692 for PKCα (r = 0.9966; Ct standard deviation 0.0426 to 0.268), −3.4107 for PKCβ1 (r = 0.9977, Ct standard deviation 0.0535 to 0.309), −3.5338 for PKCβ2 (r = 0.9973, Ct standard deviation 0.0861 to 0.1911), and −3.1385 for GAPDH (r = 0.9963, Ct standard deviation 0.0017 to 0.0234), respectively.

As shown in Figs. 1 – 3, significant increases in the mRNA levels of PKCα (Fig. 1A), PKCβ1 (Fig. 2A), and PKCβ2 (Fig. 3A) were observed in the pig heart during the progress of diabetes. The upregulation of PKCα and PKCβ2 genes appeared to start as early as the second week but was most significantly seen at 4 wk after STZ treatment. In the aorta, the expression of PKCα mRNA was not altered after induction of diabetes (Fig. 4A). However, both the PKCβ1 and β2 genes were expressed at a higher level at 4 wk and 6 – 8 wk of diabetes (see Figs. 5A and 6A).

Corresponding to the gene upregulation, there was an increase in the protein expression of certain PKC isozymes. In particular, PKCα and β2 were significantly elevated in the diabetic heart at 4 and 6 – 8 wk (Figs. 1B and 3B). In the aorta, only the β2 isozyme was significantly increased in the same period (Fig. 6B). Although PKCα was detected in abundance in control vessels, its protein level was not significantly altered by diabetes at the early time points (Fig. 4B), consistent with the detection of unaltered mRNA expression of this isofrom shown in Fig. 4A. Interestingly, the PKCβ1 protein expression showed no evident change in both the heart and aorta (Figs. 2B and 5B).

**DISCUSSION**

We have previously reported a short-term (4 – 8 wk) effect of STZ-induced diabetes on the activation of PKC and PKCβ3-associated coronary vascular barrier dysfunction in the same porcine model (44). This study further assessed the PKC mechanism at the transcriptional and translational levels. The results indicated that the gene and protein expression of PKC isozymes were differentially upregulated in cardiovascular tissues of diabetic pigs. In particular, the mRNA and protein levels of PKCβ2 were elevated in both the heart and aorta most significantly at 4 – 8 wk after experimental induction of diabetes. The current data in combination with our previous finding that PKC inhibitors
corrected hyperglycemia-induced coronary vascular leakage provide new evidence linking the PKC pathway to diabetic heart and vascular diseases. We suggest that the preferential upregulation of PKCβ2 may contribute to the initiation of cardiovascular dysfunction in association with diabetic mellitus. The β2 isozyme may be a common effector of hyperglycemic injury and thus represents a prominent therapeutic target.

Numerous clinical trials and animal experiments support a role for PKCs in the initiation and progression of diabetic complications. A sustained elevation of PKC activity is observed in various cardiac and vascular tissues during diabetes or hyperglycemia (9, 11, 16, 18, 24, 25, 33, 41, 42), correlating with the development of angiopathy, retinopathy, and nephropathy (15, 28, 38). In the heart, a causal relationship has been established between PKC activation and diabetic cardiomyopathy (3, 11, 27, 34–37). Treatment with selective PKC inhibitors normalizes the circulatory disturbance and prevents glucose-induced vascular leakage induced by hyperglycemia and diabetes (6, 18, 21, 22).

The signaling pathways leading to PKC activation have been the subject of extensive investigation. Recent evidence indicates that hyperglycemia induces de novo synthesis of diacylglycerol (DAG) (9, 16–18, 24, 28, 33, 40, 41), which stimulates PKC kinase activity by causing its subcellular translocation from the cytosol to the plasma membrane (15, 28, 30, 38). Although the translocation serves as an indication of functional activation of the enzyme, the process per se is insufficient to explain the overall increase in PKC contents during diabetes (8, 9, 11, 16, 19, 25, 30, 41). In addition, DAG is known to activate multiple PKC isoforms, yet only a limited number of isoforms are detected with increased kinase activities in diabetic vascular tissues (8, 15, 28, 30, 38). Therefore, it is likely that other mechanisms are involved in the upregulation of the kinase. Our data suggest that hyperglycemia may increase both the abundance and activity of PKC by upregulating its gene and protein expression. To our knowledge, this is the first report providing direct evidence for diabetes-associated PKC abnormality at both the transcriptional and translational levels.

![Figure 1](http://physiolgenomics.physiology.org/)

**Fig. 1.** Time-dependent effect of hyperglycemia on mRNA (A) and protein (B) expression of PKCα isozyme in the heart of diabetic pigs. Animals were killed at 2, 4, and 6–8 wk after STZ injection. Total RNA was extracted from hearts for real-time RT-PCR quantification, and the relative quantity of PKCα mRNA was normalized to that of GAPDH mRNA as described in MATERIALS AND METHODS. The protein content of the PKC isozyme was determined by SDS-PAGE followed by immunoblotting analysis of homogenized hearts. Results are expressed as percentages of controls and shown as means ± SE (*P < 0.05 vs. control). Control represents age-matched and vehicle-treated for 6 wk. Representative immunoblots are shown at *top in B*. Numbers in parentheses indicate the number of hearts examined.

![Figure 2](http://physiolgenomics.physiology.org/)

**Fig. 2.** Time-dependent effect of hyperglycemia on mRNA (A) and protein (B) expression of PKCβ1 isozyme in the heart of diabetic pigs. Total RNA was extracted from hearts for real-time RT-PCR quantification, and the relative quantity of PKCβ1 mRNA was normalized to that of GAPDH mRNA as described in MATERIALS AND METHODS. The protein content of the PKC isozyme was determined by SDS-PAGE followed by immunoblotting analysis of homogenized hearts from control pigs and diabetic pigs at 2, 4, and 6–8 wk of diabetes. Results are expressed as percentages of controls and shown as means ± SE (*P < 0.05 vs. control). Control represents age-matched and vehicle-treated for 6 wk. Representative immunoblots are shown at *top in B*. Numbers in parentheses indicate the number of hearts examined.
The finding of PKC gene upregulation may open an avenue for therapeutic targeting of selective PKC isozymes. However, the molecular mechanism by which hyperglycemia induces differential upregulation of PKC genes and proteins remains elusive. Multiple factors and their complex interactions may be involved in the regulatory pathway, and potential PKC regulators include DAG and other phospholipids, PDK (phosphoinositide-dependent kinase), RACKs (C kinase receptors), and tyrosine and serine/threonine kinases (29, 30, 32). Within this context, blood glucose is a potent stimulator of de novo synthesis of DAG and it also induces lipid synthesis and free fatty acid (FFA) production as indicated by a parallel increase in the levels of plasma FFA and blood glucose in diabetic patients (10, 26, 39). FFAs in turn further stimulate DAG synthesis (1, 10, 26, 39, 43) propagating the PKC signaling. Thus it is possible that hyperglycemia and/or hyperglycemia-increased FFA activate PKC by stimulating the synthesis of DAG, which acts as a positive regulatory feedback mechanism to increase the transcription and translation of PKC genes. Furthermore, FFAs or glucose metabolites may enhance the expression of RACKs at specific subcellular structures, resulting in selective recruitment of PKC isozymes to membrane compartments (5, 10, 29, 30, 32, 39). Sustained membrane translocation may cause a relative reduction of cytosolic PKC, which signals a further PKC synthesis. This hypothesis could partially explain the preferential upregulation of PKC isozymes in diabetic vascular tissues. Another potential mechanism involved in PKC regulation is the hyperglycemia-induced oxidative stress and subsequent activation of stress-sensitive signaling pathways including nuclear factor-kB, p38 MAP kinase, and NH_{2}-terminal Jun kinase (5, 10).

PKCα is implicated in the mediation of vascular dysfunction in diabetes (11, 14, 19, 25). It has been shown that d-glucose treatment increases the permeability of cultured aortic endothelial cells via a PKC-dependent mechanism (14). Interestingly, we detected a significant increase in the PKCα expression in the heart but not in the aorta. This result is consistent with previous studies in diabetic rats (11, 16, 19, 25). It is likely that the regulation of specific PKC isoforms varies across species and tissues. In this regard, our analysis was performed in the pig, a human-relevant animal model that develops diabetic cardiovascular complications characteristic of human disease. To date we have not found any literature reports regarding

![Fig. 3. Time-dependent effect of hyperglycemia on mRNA (A) and protein (B) expression of PKCβ2 isozyme in the heart of diabetic pigs. For descriptions of real-time RT-PCR quantification, SDS-PAGE, immunoblotting analysis, significance, and other details, legend to Fig. 1.](image)

![Fig. 4. Time-dependent effect of hyperglycemia on mRNA (A) and protein (B) expression of PKCα isozyme in the aorta of diabetic pigs. Total RNA was extracted from aorta for real-time RT-PCR quantification, and the relative quantity of PKCα mRNA was normalized to that of GAPDH mRNA. The protein content of the PKC isoforms was determined by SDS-PAGE followed by immunoblotting analysis of homogenized aortas. Results are expressed as percentages of controls and shown as means ± SE (*P < 0.05 vs. control). Control represents age-matched and vehicle-treated for 6 wk. Representative immunoblots are shown at top in B. Numbers in parentheses indicate the number of aortas examined.](image)
PKCα expression in diabetic vessels of either patients or pigs.

In contrast to the α isoform, the PKCβ isoforms (predominantly β2) were expressed at a higher level in both the heart and aorta under the diabetic condition, suggesting that PKCβ may be a prominent target of hyperglycemic injury in the cardiovascular system. In support of this notion, a preferential increase in PKCβ activity was observed in the diabetic heart and aorta (16, 24, 25, 44) as well as in cultured aortic endothelial and smooth muscle cells exposed to high glucose (16, 24). The importance of the β isozyme in the pathological regulation of cardiovascular function is substantiated by studies in which oral administration of LY333531, a PKCβ-specific inhibitor, ameliorates abnormal hemodynamics in multiple organs of diabetic animals (2, 6, 18, 21, 22). The similar inhibitors also correct coronary vascular leakage induced by high concentrations of d-glucose and experimental diabetes (44). Furthermore, overexpression of PKCβ2 in the heart induces ventricular hypertrophy, myocardial fibrosis, and impaired cardiac performance, which are characteristics of diabetic cardiomyopathy (37).

While a correlated upregulation of mRNA expression and protein synthesis was observed for both the α and β isoforms at 4 wk of diabetes, there was an evident, but not statistically significant, increase in the cardiac PKCβ2 protein content at 2 wk, when its respective mRNA level showed no apparent changes. Although the precise molecular reactions leading to such a phenomenon were not revealed, multiple factors could play a role in the increased protein levels. For example, hyperglycemia might exert an acute effect on the cardiomyocytes to augment mRNA translatability or protein stability of particular PKC isoforms. This mechanism could also contribute to the observed preferential activation of PKCβ2 in the diabetic hearts and aortas. In addition, we noted that the increase in PKCβ2 protein content was concomitant with an unchanged or slightly decreased PKCβ1 protein expression. A possible explanation for this is that the synthesis of the β1 isozyme may be differently regulated at the posttranscriptional level. It is known that the β1 and β2 mRNAs are generated through alternative splicing from a single transcript, rendering the isoforms different from each other only in a very short (50 amino acids) sequence at the carboxy terminus (23, 31). Again, mRNA translatability, protein stability, posttranslational modification and degradation may alternatively influence the protein expression of the isoforms in diabetes. It has been shown that in colon tumors both PKCβ1 and β2 are expressed with a high mRNA level, but only β2 shows an increased protein expression, in contrast with a dramatic decrease in β1 protein levels (12).

Quantification of mRNA expression of specific genes has always been essential to the studies of gene regu-
lation. Current methods used for mRNA quantification include Northern blotting, dot hybridization, quantitative in situ hybridization, RNase protection assay, RT-PCR, competitive RT-PCR, and cDNA arrays (7). Most of these methods have inherent limitations such as low sensitivity and the requirement of extensive optimization with exogenous competitors or post-assay manipulations and thus are less desirable for quantification of low-abundance mRNAs. Real-time RT-PCR detects targets at the geometric phase of amplification, in contrast to the linear phase in competitive RT-PCR, thence ensuring the assay precision and accuracy. In this study, the use of gene-specific TaqMan probes conferred the assay specificity, allowing differential quantification of the alternative splice variants of PKCβ transcript. We found that the method was rapid, exquisitely sensitive, reproducible, reliable, and of high throughput. To our understanding, this is the first report on real-time RT-PCR quantification of PKCα and PKCβ mRNA expression.

In summary, this study reports molecular analyses of PKCα, β1, and β2 genes and isoforms in the porcine cardiovascular tissues during early stages of experimental diabetes. The results reveal a preferential increase in the mRNA expression and protein content of PKCβ2, suggesting that PKCβ2 upregulation may contribute to cardiovascular dysfunction in the disease. The significance of the current study resides with the quantitative assessment of PKC gene profiles in a human-relevant animal model, which provides an association between hyperglycemia and PKC abnormality. Further studies are necessary to establish the causal role and precise mechanisms of hyperglycemia in the regulation of PKC synthesis and function.

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REFERENCES


