Hyperglycemia-Induced Protein Kinase C β2 Activation Induces Diastolic Cardiac Dysfunction in Diabetic Rats by Impairing Caveolin-3 Expression and Akt/eNOS Signaling

Shaoqing Lei,1 Haobo Li,1 Jinjin Xu,1 Yanan Liu,1 Xia Gao,1 Junwen Wang,2,3 Kwok F.J. Ng,1,3 Wayne Bond Lau,4 Xin-liang Ma,1 Brian Rodrigues,5 Michael G. Irwin,1,3 and Zhengyuan Xia1,3

Protein kinase C (PKC)β2 is preferably overexpressed in the diabetic myocardium, which induces cardiomyocyte hypertrophy and contributes to diabetic cardiomyopathy, but the underlying mechanisms are incompletely understood. Caveolae are critical in signal transduction of PKC isoforms in cardiomyocytes. Caveolin (Cav)-3, the cardiomyocyte-specific caveolar structural protein isoform, is decreased in the diabetic heart. The current study determined whether PKCβ2 activation affects caveola and Cav-3 expression. Immunoprecipitation and immunofluorescence analysis revealed that high glucose (HG) increased the association and colocalization of PKCβ2 and Cav-3 in isolated cardiomyocytes. Disruption of caveola by methyl-β-cyclodextrin or Cav-3 small interfering (si)RNA transfection prevented HG-induced PKCβ2 phosphorylation. Inhibition of PKCβ2 activation by compound CGP53353 or knockdown of PKCβ2 expression via siRNA attenuated the reductions of Cav-3 expression and Akt/endothelial nitric oxide synthase (eNOS) phosphorylation in cardiomyocytes exposed to HG. LY333531 suppressed the decreased expression of myocardial NO, Cav-3, phosphorylated (p)-Akt, and p-eNOS and also mitigated the augmentation of O2•−, nitrotyrosine, Cav-1, and iNOS expression. In conclusion, hyperglycemia-induced PKCβ2 activation requires caveola and is associated with reduced Cav-3 expression in the diabetic heart. Prevention of excessive PKCβ2 activation attenuated cardiac diastolic dysfunction by restoring Cav-3 expression and subsequently rescuing Akt/eNOS/NO signaling. Diabetes 62:2318–2328, 2013

Cardiovascular disease is the leading cause of diabetes-related death (1). While most diabetic heart failure etiology concerns coronary disease associated with atherosclerosis, a diabetes-associated cardiomyopathy has been reported in humans (2) and animal models of type 1 (3) and type 2 (4) diabetes. Numerous studies by our group (5,6) and others (7,8) have demonstrated that high glucose (HG) increases the association and colocalization of PKCβ2 and Cav-3 in isolated cardiomyocytes. Disruption of caveola by methyl-β-cyclodextrin or Cav-3 small interfering (si)RNA transfection prevented HG-induced PKCβ2 phosphorylation. Inhibition of PKCβ2 activation by compound CGP53353 or knockdown of PKCβ2 expression via siRNA attenuated the reductions of Cav-3 expression and Akt/endothelial nitric oxide synthase (eNOS) phosphorylation in cardiomyocytes exposed to HG. LY333531 suppressed the decreased expression of myocardial NO, Cav-3, phosphorylated (p)-Akt, and p-eNOS and also mitigated the augmentation of O2•−, nitrotyrosine, Cav-1, and iNOS expression. In conclusion, hyperglycemia-induced PKCβ2 activation requires caveola and is associated with reduced Cav-3 expression in the diabetic heart. Prevention of excessive PKCβ2 activation attenuated cardiac diastolic dysfunction by restoring Cav-3 expression and subsequently rescuing Akt/eNOS/NO signaling.

From the 1Department of Anesthesiology, University of Hong Kong, Hong Kong, China; the 2Department of Biochemistry, University of Hong Kong, Hong Kong, China; the 3Shenzhen Institute of Research & Innovation, University of Hong Kong, Shenzhen, China; the 4Department of Emergency Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania; and the 5Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada.

Corresponding author: Zhengyuan Xia, zyxia@hku.hk.
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Reprint requests: Zhengyuan Xia, zyxia@hku.hk.

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RESEARCH DESIGN AND METHODS

Induction of diabetes and drug treatment. Male Sprague-Dawley rats (aged 8 weeks) weighing 260 ± 10 g equilibrated to surroundings for 3 days before experiments. Diabetes was induced via single tail vein injection of STZ (60 mg/kg; Sigma, St. Louis, MO) dissolved in citrate buffer (0.1 mol/L, pH 4.5), while control rats were injected with an equal volume of citrate buffer alone. One
One week after STZ injection, rats exhibiting hyperglycemia (blood glucose ≥16.7 mmol/L) were considered diabetic and were subjected to outlined experiments. One week after diabetes induction, rats were treated with vehicle or PKCβ inhibitor LY333531 (also named ruboxistaurin, a drug that has been approved by the U.S. Food and Drug Administration for the prevention of vision loss in patients with diabetic retinopathy [22]) by oral gavage for 4 weeks at a dose of 1 mg/kg/day (demonstrated to adequately inhibit PKCβ activation in rat heart and vasculature [23,24]). This model was chosen based on our most recent study (25) and a study of others (26) showing that STZ diabetic rats developed cardiac dysfunction 35 days after STZ injection, with concomitant cardiomyocyte hypertrophy and cardiac fibrosis formation (25)—two major features of diabetic cardiomyopathy. After 4 weeks’ treatment, cardiac functions were determined; the rats were then deeply anesthetized with sodium pentobarbital (65 mg/kg), and hearts were either rapidly excised for cardiomyocyte isolation or frozen in liquid nitrogen for later analysis. Subgroups of control and untreated diabetic rats were terminated at 8 weeks of STZ-induced diabetes, and heart tissue samples were processed to analyze changes of cardiac PKCβ2 and Cav-3 antibodies (see RESEARCH DESIGN AND METHODS).

Echocardiography. At the conclusion of 4 weeks’ treatments, transthoracic echocardiography was performed at experiment termination via a 17.5-MHz linear array transducer system (Vevo 770, High Resolution Imaging System; VisualSonics, Toronto, Ontario, Canada), and left ventricular (LV) dimensions and LV diastolic and systolic function were assessed by M-mode and Doppler echocardiography as we previously described (25). LV internal dimensions at end systole (LVIDs) and diastole (LVIDd) were used to calculate fractional shortening (FS) by the following formula: FS (%) = (LVIDd − LVIDs)/LVIDd × 100%. LV posterior wall dimensions at end diastole (LVPWd) and systole (LVPWs) were used to calculate fractional LV posterior wall thickening (FLVPW) by the following formula: FLVPW(%) = LVPWs − LVPWd/LVPWd × 100%. The peak velocity of early (E) and late (A) diastolic filling was used to calculate the ratio of E and A (E/A). LV end diastolic volume (LVEDV) and end systolic volume (LVESV) were used to calculate ejection fraction (EF) by the following formula: EF (%) = LVEDV − LVESV/LVESV × 100%. The heart rate (HR), systolic interventricular septal thickness, diastolic interventricular septal thickness, LV isovolumic relaxation time (IVRT), and stroke volume (SV) were also monitored. All echocardiographically derived measures were obtained by averaging the readings of three consecutive beats.

Measurement of cardiomyocytes cross-sectional area. After the completion of 4 weeks’ treatment, cardiomyocyte cross-sectional diameters were assessed by hematoxylin-eosin-stained paraffin-embedded sections of left ventricles (1–2 μm) longitudinally oriented to the muscle fibers in the sub-endocardium and subepicardium as previously described (6). Cross-sectional areas were randomly selected in five fields that visualized capillary profiles and nuclei. Images of the left ventricle sections were captured by an Axiosplas image-capturing system (Zeiss) and analyzed by Axiovision Rel. 4.5 image-analyzing software. A minimum of 150 cells per animal was chosen for analysis.

Preparation of isolated rat ventricular cardiomyocytes. Calcium-tolerant cardiomyocytes were prepared from rat ventricles via a modified method as

![Diagram A](image1.png)

**FIG. 1. Expression of PKCβ2 and Cav-3 in cardiomyocytes isolated from control and STZ-induced diabetic rats (8 weeks).** A: Representative Western blot demonstrating p-PKCβ2 (Ser660) and total PKCβ2 expression. B: Representative Western blot demonstrating Cav-3 expression with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control. C: Cell lysates containing equal amounts of total protein were subjected to immunoprecipitation (IP) with anti-Cav-3 antibody (Ab) and analyzed by immunoblot (IB) with PKCβ2 and Cav-3 antibody. All results are expressed as means ± SEM. n = 6–8 per group. *P < 0.05 vs. control. D: Confocal laser microscopic image of adult rat cardiomyocytes in response to HG. Isolated cardiomyocytes from nondiabetic rats were incubated with LG or HG for 36 h and underwent standard immunofluorescent staining with PKCβ2 and Cav-3 antibodies (see RESEARCH DESIGN AND METHODS).
previously described (27). Cells isolated from a single rat heart were plated on Matrigel-coated culture dishes and allowed to recover for 3 h. Cultured ventricular cardiomyocytes were incubated in low glucose (LG) (5.5 mmol/L), high glucose (HG) (25 mmol/L), or mannitol/glucose (19.5 mmol/L mannitol plus 5.5 mmol/L glucose) at 37°C in Medium 199 (Gibco, Grand Island, NY) containing various treatments and then snap-frozen in liquid nitrogen for future analysis. Lactate dehydrogenase (LDH) release (a measure of cell injury) in culture medium was detected via a commercial LDH kit (Roche, Mannheim, Germany).

**Immunoprecipitation.** Isolated cardiomyocytes were homogenized in lysis buffer. A total of 500 μg cell extracts were subjected to immunoprecipitation with 2 μg Cav-3 primary antibody in the presence of 20 μL protein A/G plus-agarose. After extensive PBS washes, the immunoprecipitates were denatured with 1× sodium dodecyl sulfate loading buffer and subjected to analysis for PKCβ2 expression by Western blot as described below.

**Immunofluorescence.** Isolated cardiomyocytes were plated on Matrigel precoated glass coverslips, incubated either in LG or HG in Medium 199 for 36 h, and fixed in ice-cold acetone for 5 min. The fixed cells were blocked in PBS with Tween 20 (PBST) with 10% goat serum and 1% BSA for 30 min and further incubated with a mixture of mouse against rat Cav-3 antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit against rat PKCβ2 antibody (1:100; Santa Cruz Biotechnology) in 1% BSA in PBST in a humidified chamber for 1 h at room temperature. After three PBST washings, the cells were incubated for 1 h with a mixture of Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG (1:2,000; Invitrogen, Carlsbad, CA). Cells were washed three times and prepared for confocal laser scanning microscopic imaging with mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

**PKCβ2 siRNA and Cav-3 siRNA studies in H9C2 cells.** Embryonic rat cardiac H9C2 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS in a humidified atmosphere (5% CO2) at 37°C. Commercial PKCβ2 siRNA and Cav-3 siRNA (Santa Cruz Biotechnology) were used for inhibition of both PKCβ2 and Cav-3 expression per the manufacturer’s protocol. After transfection with control, PKCβ2 or Cav-3 siRNA, cells were incubated in either LG or HG in Dulbecco’s modified Eagle’s medium for 36 h and snap-frozen in liquid nitrogen.

**Determination of myocardial levels of NO, O2−, and nitrotyrosine.** Frozen heart tissues were pulverized separately with mortar and pestle in liquid nitrogen, homogenized in ice-cold PBS, and centrifuged at 3,000 × g for 15 min at 4°C for supernatant collection. The supernatant protein concentration was determined via a Lowry assay kit (Bio-Rad, Hercules, CA). Concentrations of nitrates (NO2−) and nitrates (NO3−), the stable end products of NO, were determined by the Griess reaction as previously described (28). NO levels were expressed as nanomoles per microgram of protein. Myocardial O2− production was determined via lucigenin chemiluminescence method (29,30). The supernatant samples were loaded with dark-adapted lucigenin (5 μmol/L) and read in 96-well microplates by luminometer (GloMax; Promega) with and without pretreatment with the NOS inhibitor L-Nω-nitroarginine methyl ester (l-NAME) (100 μmol/L [15]) for 30 min at room temperature. Light emission, expressed as mean light units/min/100 μg protein, was recorded for 5 min. Myocardial nitrotyrosine levels (micrograms per milligram protein) in the collected supernatant were determined by chemiluminescence detection via the Nitrotyrosine Assay kit per the manufacturer’s protocol (Millipore).

**Separation of cytosol and membrane fractions of heart tissues.** For characterization of subcellular distributions of targeted proteins, cytosol and membrane fractions of cardiac tissue lysates were separated by ultracentrifugation as previously described (5). Cytosol and membrane fractions were denatured by 5× sodium dodecyl sulfate loading buffer and subjected to analysis for PKCβ2 and PKCβ3 expression by Western blot as described below.

**Isolation of Cav-rich fractions.** Caveolae were isolated by discontinuous sucrose gradient centrifugation as previously described (21). Each heart sample gradient was separated into 12 fractions. Fractions 4–6 were considered the lipid raft fractions, and fractions 8–12 were considered the heavier fractions. Equal protein amounts were loaded for Western blot analysis.

**Western blot analysis.** Equal protein amounts from isolated cardiomyocytes, H9C2 cells, and rat heart homogenate were resolved by 7.5–12.5% SDS-PAGE
and subsequently transferred to polyvinylidene fluoride membrane for immunoblot analysis as previously described (31).

**Statistical analysis.** Densitometry was obtained by image analysis software (Bio-Rad). All values are presented as means ± SEM. Comparisons between multiple groups were made by one-way ANOVA followed by Tukey test for multiple comparisons. Statistical analysis was performed by GraphPad Prism (GraphPad Software, San Diego, CA). P values <0.05 were considered significant.

**RESULTS**

**Expression and association of PKCβ2 and Cav-3 in cardiomyocytes isolated from diabetic rats.** We previously reported activation of the PKCβ2, but not PKCβ1, isoform in the diabetic heart (6). In the current study, we examined whether PKCβ2 activation was associated with abnormal Cav-3 expression, a muscle-specific marker of caveolae (16). Diabetes moderately increased PKCβ2 phosphorylation on Thr642 residue (data not shown), but the increase in phosphorylation of PKCβ2 was most profound at Ser660, without influencing total PKCβ2, resulting in a markedly increased ratio of phosphorylated PKCβ2 to total PKCβ2 (Fig. 1A). Decreased Cav-3 expression was observed in cardiomyocytes isolated from 8-week diabetic rat hearts compared with age-matched controls (Fig. 1B). We next examined the relationship between Cav-3 and PKCβ2 by immunoprecipitation experiments in isolated cardiomyocytes. While a small amount of PKCβ2 remained constitutively associated with Cav-3 during basal conditions, the diabetic condition increased its association with Cav-3 (Fig. 1C). To confirm our findings, we used confocal immunofluorescence staining. Limited PKCβ2 was present during basal conditions in association with Cav-3 in the cell membrane (indicated by scant yellow punctate staining of the cell periphery); 36 h of HG stimulation significantly increased regions of colocalization between PKCβ2 and Cav-3 compared with LG stimulation (Fig. 1D).

**FIG. 3.** Expression of p-PKCβ2 and Cav-3 in cultured cardiomyocytes and H9C2 cells after various treatments in LG (5.5 mmol/L) or HG (25 mmol/L) conditions for 36 h. A: Representative Western blot demonstrating p-PKCβ2 (Ser660) in comparison with total PKCβ2 in cardiomyocytes exposed to HG in the presence of a selective PKCβ2 inhibitor, CGP (1 μmol/L), or CD (10 μmol/L). B: Representative Western blot demonstrating p-PKCβ2 and Cav-3 expression in H9C2 cells transfected with Cav-3 siRNA exposed to LG or HG. C: Representative Western blot demonstrating Cav-3 expression in cultured cardiomyocytes exposed to HG in the presence of CGP (1 μmol/L). D: Representative Western blot demonstrating p-PKCβ2, total PKCβ2 expression, and Cav-3 expression in H9C2 cells transfected with PKCβ2 siRNA in LG or HG conditions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading control. All results are expressed as means ± SEM. n = 7. *P < 0.05 vs. all other groups, #P < 0.05 vs. control and siRNA-treated groups.
Effect of HG on expression and association of p-PKC\(_{\beta2}\) and Cav-3 in isolated cardiomyocytes over time. HG conditions significantly increased the ratio of p-PKC\(_{\beta2}\) to total PKC\(_{\beta2}\) (indicating PKC\(_{\beta2}\) activation) in cardiomyocytes within 1 h for up to 48 h (Fig. 2A). Peak increase in the ratio of p-PKC\(_{\beta2}\) to total PKC\(_{\beta2}\) occurred after 12 h HG exposure. The osmotic control mannitol exerted no effects upon p-PKC\(_{\beta2}\) to total PKC\(_{\beta2}\) and Cav-3 expression (data not shown). In contrast to the quick increase of phosphorylated (p)-PKC\(_{\beta2}\)/total PKC\(_{\beta2}\) as early as 1 h after HG exposure, Cav-3 expression did not significantly increase until 6–12 h after HG exposure, reduced to basal levels within 24 h, and significantly decreased 36–48 h after initial HG exposure (Fig. 2B). Cardiomyocyte LDH release significantly increased 24 h after HG exposure, with a rising tendency continuing 36–48 h after initial HG exposure (Fig. 2C).

Hyperglycemia-induced PKC\(_{\beta2}\) activation involves caveolae and is associated with reduced Cav-3 expression. We next investigate the interplay between PKC\(_{\beta2}\) activation and caveolae (and Cav-3) under hyperglycemic conditions. Given that PKC\(_{\beta1}\) activation induced by HG requires caveolae in primary mesangial cells (32), we determined whether caveolae are crucial in HG-induced PKC\(_{\beta2}\) activation in isolated cardiomyocytes from nondiabetic rats. As shown in Fig. 3A, phosphorylation of PKC\(_{\beta2}\) induced by HG was prevented by either the selective PKC\(_{\beta2}\) inhibitor CGP-53353 (1 \(\mu\)mol/L, Sigma-Aldrich

![FIG. 4. Expression of p-Akt (Ser 473) and p-eNOS (Ser 1177) in cultured cardiomyocytes and H9C2 cells in various treatments in LG (□) (5.5 mmol/L) or HG (■) (25 mmol/L) conditions for 36 h. Representative Western blot demonstrating p-Akt in comparison with total Akt (A) and p-eNOS in comparison with total eNOS in cardiomyocytes exposed to HG in the presence of selective PKC\(_{\beta2}\), CGP (1 \(\mu\)mol/L), CD (10 \(\mu\)mol/L), or CGP+CD combination (B). Representative Western blot demonstrating p-Akt and p-eNOS expression in H9C2 cells transfected with PKC\(_{\beta2}\) siRNA (C) or Cav-3 siRNA (D) in LG or HG conditions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading control. All results are expressed as means ± SEM. \(n = 7\). *\(P < 0.05\) vs. all other groups, #\(P < 0.05\) vs. control and siRNA-treated groups.](diabetes.diabetesjournals.org)
[IC50 values are 0.41 and 3.8 μmol/L, respectively, for PKCβ2 and PKCβ1] or methyl-β-cyclodextrin (CD) (50 μmol/L), a disrupter of cholesterol-rich caveolae (33). To determine whether Cav-3 is required for PKCβ2 activation, we subjected H9C2 cells treated with rat-specific Cav3 small interfering (si)RNA to both LG and HG conditions. siRNA-mediated reduction of Cav-3 expression by ~60% (Fig. 3B) prevented augmented phosphorylation of PKCβ2 in HG conditions. No effects upon PKCβ2 phosphorylation were observed in cells exposed to LG (Fig. 3B). We also determined whether excessive PKCβ2 activation induced by HG is associated with reduced Cav-3 expression. Selective inhibition of PKCβ2 activation by CGP reversed the reduction of Cav-3 expression in primary cardiomyocytes exposed to HG (Fig. 3C). Similarly, in H9C2 cells, knockdown of PKCβ2 by siRNA reduced PKCβ2 phosphorylation in cells incubated in LG and HG conditions (Fig. 3D) and attenuated decreased Cav-3 expression in cells exposed to HG, with no impact upon Cav-3 expression in cells exposed to LG (Fig. 3E).

**Hyperglycemia-induced activation of PKCβ2 is associated with caveola-modulated Akt/eNOS signaling.** Next, we investigated the impact of PKCβ2 activation by HG in the downstream signaling molecules Akt and eNOS, both of which are modulated by Cavs (34). Cardiomyocytes incubated in HG exhibited decreased phosphorylation of Akt at Ser473 and eNOS at Ser1177, and these decreases were reversed by CGP treatment (Fig. 4A and B). Caveolar disruption by CD further exaggerated HG-mediated reduction of Akt phosphorylation (Fig. 4A) but did not further exacerbate HG-induced reduction of p-eNOS expression (Fig. 4B). However, CGP-mediated restoration of eNOS phosphorylation in HG-treated cardiomyocytes was abolished during concomitant CD treatment (Fig. 4B). For confirmation of the relative effects of PKCβ2 and Cav-3 upon HG-mediated changes in p-AKT and p-eNOS, H9C2 cells were subject to both PKCβ2 and Cav-3 knockdown by siRNA. PKCβ2 knockdown significantly increased the phosphorylation of Akt and eNOS in HG-treated cells—effects that were not observed in LG-treated cells (Fig. 4C). Knockdown of Cav-3 resulted in further reduced expression of both p-Akt and p-eNOS in both LG- and HG-treated cells (Fig. 4D).

**Inhibition of PKCβ2 activation by LY333531 attenuates cardiac caveolar dysfunction in diabetic rats.** To further investigate the role of PKCβ2 activation in diabetes-induced abnormalities, we treated STZ-induced diabetic rats with the PKCβ inhibitor LY333531 for 4 weeks. PKCβ2, but not PKCβ1, isoform was excessively activated in the diabetic heart, as demonstrated by increased membrane translocation of PKCβ2 but not PKCβ1—a phenomenon inhibited by LY333531 (Fig. 5A). PKCβ2 inhibitor LY333531 administration suppressed augmented whole-heart Cav-1 expression (Fig. 5B) and prevented whole-heart decreased Cav-3 expression (Fig. 5C). Caveolae fractions were isolated via sucrose gradient centrifugation isolated caveolae-enriched fractions. All samples containing equal amounts of protein or a volume equal to that of the fraction with the least detectable amount of protein for “protein-free” fractions (1,2) and unfractonated samples were probed for Cav-1, Cav-3, and PKCβ2 immunoreactivity. E: Cav-1 and Cav-3 and PKCβ2 expression in all the fractions (1–12) were calculated by relative densitometric values and expressed as percentage of control. All results are expressed as means ± SEM. n = 7. *P < 0.05 vs. all other groups.

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![Image](diabetes.diabetesjournals.org)
discontinuous sucrose gradient centrifugation of whole cell lysates. Cav-1 was found predominantly in fractions 8–10, whereas Cav-3 was located within both lipid fractions (4–6) and heavier fractions (8–12). PKCβ2 was predominantly coexpressed within Cav-3–rich fractions (Fig. 5D). Densitometric analysis of all fractions (1–12) demonstrated that PKCβ2-inhibitor LY333531 significantly reduced augmented Cav-1 and PKCβ2 expression and suppressed decreased Cav-3 expression in diabetes (Fig. 5E).

**Inhibition of PKCβ2 activation by LY333531 attenuates diastolic dysfunction in diabetic rats.** At the end of the treatment period, untreated diabetic rats had significantly elevated blood glucose and reduced body weight and heart weight compared with control rats, which were not altered by LY333531 treatment (Table 1). However, the ratio of heart weight to body weight, an indirect index of myocardial hypertrophy, in the untreated diabetic rats was significantly higher than that in the control rats, which was significantly attenuated by LY333531 treatment (Table 1). Further, the LV cardiomyocyte cross-sectional areas as assessed in hematoxylin-eosin–stained cardiac sections in the untreated diabetic rats (478.8 ± 58.9 μm²) were significantly bigger than in the control rats (256.7 ± 37.9 μm²) and significantly attenuated by LY333531 treatment (329.5 ± 47.7 μm²), showing that LY333531 can attenuate cardiomyocyte hypertrophy in diabetes. We further determined rat cardiac function via echocardiography. As shown in Table 1, no significant change in FS, FLVPW, or EF was observed among all experimental groups (although nonsignificant decreased values were recorded in the untreated diabetic group). Such data suggest preserved systolic function at 5 weeks in diabetic rats used in our model. However, diastolic dysfunction was manifested. The HR and the E/A ratio were significantly decreased in diabetic rats as a consequence of glycemia-induced cardiac PKCβ2 activation (329.5 ± 37.9 vs. C; #P < 0.01 vs. D). The diabetic condition significantly increased myocardial iNOS (an adverse marker of myocardial hypertrophy and fibrosis) (Fig. 6A) and increased O2⁻ (Fig. 6B) and nitrotyrosine production (Fig. 6C) in cardiac tissues. LY333531 suppressed all of these derangements. Further studies revealed that the diabetes-induced augmented O2⁻ levels could be blocked by the NOS inhibitor L-NAME (100 μmol/L) (Fig. 6B), suggesting an NOS-dependent mechanism for O2⁻ accumulation. We next investigated related signaling molecules, including Akt, eNOS, and inducible NOS (iNOS). The diabetic condition did not affect total cardiac Akt and eNOS expression but significantly decreased p-Akt (Ser473) and p-eNOS (Ser1177) expression, both of which were reversed by LY333531 (Fig. 6D and E). Consistent with a recent study (36), our results demonstrate that diabetes increased myocardial iNOS (an adverse marker mediating nitrative stress [23]), which was reversed by LY333531 (Fig. 6F).

**DISCUSSION**

In the current study, we have demonstrated that hyperglycemia-induced cardiac PKCβ2 activation requires caveolae. We provide evidence that excessive PKCβ2 activation is associated with reduced Cav-3 expression, contributing to LV IVRT and reductions in LVVd and SV. Four weeks' treatment with LY333531 restored the values of E/A, IVRT, LVVd, and SV to levels that were comparable with those in the control rats but without significant effect upon HR.

**LY333531 ameliorated diabetes-induced derangements of myocardial NO, O₂⁻, and nitrotyrosine content and reversed changes in cardiac Akt, eNOS, and inducible NOS.** Diabetes is associated with decreased NO levels and increased O₂⁻ and nitrotyrosine production (14,35), agents of oxidative and nitrative stress. For determination of whether LY333531 conferred cardioprotection in part by reducing oxidative and nitrative stress in diabetes, the levels of NO, O₂⁻, and nitrotyrosine in diabetic heart tissues were assessed. The diabetic condition significantly increased NO levels (Fig. 6A) and increased O₂⁻ (Fig. 6B) and nitrotyrosine production (Fig. 6C) in cardiac tissues. LY333531 suppressed all of these derangements. Further studies revealed that the diabetes-induced augmented O₂⁻ levels could be blocked by the NOS inhibitor L-NAME (100 μmol/L) (Fig. 6B), suggesting an NOS-dependent mechanism for O₂⁻ accumulation. We next investigated related signaling molecules, including Akt, eNOS, and inducible NOS (iNOS). The diabetic condition did not affect total cardiac Akt and eNOS expression but significantly decreased p-Akt (Ser473) and p-eNOS (Ser1177) expression, both of which were reversed by LY333531 (Fig. 6D and E). Consistent with a recent study (36), our results demonstrate that diabetes increased myocardial iNOS (an adverse marker mediating nitrative stress [23]), which was reversed by LY333531 (Fig. 6F).

**TABLE 1**

| General characteristics and echocardiographic assessment of left ventricle dimensions and functions in rats |
|---------------------------------------------------------------|
| Control | Diabetes | Diabetes plus LY333531 |
|-----------------|---------|-----------------------------|
| Blood glucose (mmol/L) | 6.26 ± 0.53 | 28.24 ± 4.64** | 27.32 ± 3.43** |
| Body weight (g) | 490.1 ± 14.3 | 295.4 ± 28.8* | 371.1 ± 17.4* |
| Heart weight (g) | 1.66 ± 0.15 | 1.25 ± 0.18* | 1.91 ± 0.16* |
| Heart weight/body weight (mg/g) | 3.39 ± 0.07 | 4.23 ± 0.06* | 3.53 ± 0.08# |
| HR (bpm) | 332 ± 7.8 | 270 ± 10.8* | 286 ± 9.2# |
| LVDDs (mm) | 4.75 ± 0.43 | 4.68 ± 0.41 | 4.74 ± 0.49 |
| LVPwD (mm) | 8.29 ± 0.53 | 7.61 ± 0.62 | 8.13 ± 0.49 |
| FS (%) | 42.70 ± 4.13 | 38.50 ± 5.42 | 41.53 ± 3.84 |
| IVSDs (mm) | 2.33 ± 0.16 | 2.28 ± 0.07 | 2.27 ± 0.13 |
| LVDDs (mm) | 1.72 ± 0.05 | 1.75 ± 0.05 | 1.73 ± 0.06 |
| LVDPWs (mm) | 2.90 ± 0.08 | 2.92 ± 0.07 | 2.91 ± 0.06 |
| LVDPwD (mm) | 1.85 ± 0.06 | 1.91 ± 0.09 | 1.87 ± 0.08 |
| LVVP (%) | 56.76 ± 7.50 | 52.88 ± 10.03 | 55.61 ± 4.81 |
| LVSs (μL) | 107.6 ± 15.8 | 109.8 ± 13.7 | 114.5 ± 11.9 |
| LVVis (μL) | 378.7 ± 25.6 | 315.4 ± 21.8* | 380.4 ± 19.7# |
| EF (%) | 71.6 ± 4.7 | 65.2 ± 5.7 | 69.9 ± 3.9 |
| IVRT (ms) | 21.75 ± 1.25 | 27.83 ± 1.80* | 20.63 ± 1.38# |
| E velocity (cm/s) | 1,340.9 ± 44.6 | 1,217.8 ± 33.7* | 1,331.0 ± 40.8# |
| A velocity (cm/s) | 890.1 ± 37.1 | 1,023.4 ± 34.7* | 917.9 ± 28.9# |
| E/A ratio | 1.49 ± 0.09 | 1.19 ± 0.05* | 1.45 ± 0.085# |
| SV (μL) | 285.7 ± 19.1 | 230.8 ± 17.9* | 279.4 ± 20.8# |

Data are means ± SEM. Control or STZ-induced diabetic rats were either untreated or treated with PKCβ inhibitor LY333531 (1 mg/kg/day) by oral gavage for 4 weeks. n = 8. IVSD, diastolic interventricular septal thickness; IVSs, systolic interventricular septal thickness. *P < 0.05, **P < 0.01 vs. C; #P < 0.05 vs. D.
to abnormal Akt/eNOS signaling during hyperglycemia. Inhibition of excessive activation of PKC\(\beta_2\) by compound LY333531 improves cardiac diastolic function, possibly via attenuation of caveolar dysfunction and rescuing Akt/eNOS/NO function in the diabetic heart. To our best knowledge, this is the first study examining the relationship between PKC\(\beta_2\) and Cav-3 in cardiomyocytes subjected to hyperglycemic conditions.

It is well established that chronic hyperglycemia induces abnormal activation of PKC, which contributes to diabetic cardiovascular complications (37,38). However, the PKC-signaling pathway is complicated by numerous isoforms, each with varying cellular distribution and opposing function at times (39). The PKC\(\beta_2\) isoform is most frequently implicated in diabetic cardiovascular complications (5–8). Our current study further confirmed that PKC\(\beta_2\), but not PKC\(\beta_1\), is excessively activated in the diabetic heart. Although the precise mechanisms by which hyperglycemia induces PKC\(\beta_2\) activation in cardiomyocytes are not fully understood, evidence supports the vital role of caveolae (the specialized plasma membrane microdomains modulating signaling transduction pathways of molecules docked within them [16]) in hyperglycemia-induced PKC\(\beta_2\) activation. This is well supported by our immunoprecipitation and immunofluorescence studies demonstrating that hyperglycemia increased the association and colocalization of PKC\(\beta_2\) and Cav-3. Caveolar disruption by methyl-\(\beta\)-cyclodextrin (33) suppressed hyperglycemia-induced PKC\(\beta_2\) activation in isolated cardiomyocytes. Cav-3 knockdown by siRNA prevented augmented PKC\(\beta_2\) phosphorylation in H9C2 cells exposed to HG, suggesting that Cav-3 is required specifically for hyperglycemia-induced PKC\(\beta_2\) activation in cardiomyocytes.

Cav-3 is the predominant cardiomyocyte Cav isoform essential for caveolar function. In the current study, we demonstrated that cardiomyocyte Cav-3 expression increased 6–12 h after HG exposure but reduced to basal levels within 24 h and progressively further reduced to lower than basal levels after 36–48 h of HG exposure. The initial increase in Cav-3 expression after HG exposure observed in our study is an acute response to the calorie surplus similar to that reported by other researchers (40). The significant reduction of Cav-3 expression in cardiomyocytes after prolonged HG exposure is consistent with our results from the intact rats, which showed that Cav-3 expression was decreased in isolated cardiomyocytes from rats of 8- and 5-week duration of diabetes. Loss of Cav-3 expression results in cardiomyopathy (41), and reduction in cardiac Cav3 protein expression is highly correlated with reduction in LV FS in mice with constitutive overexpression of A1-adenosine receptor–induced cardiac dilatation and dysfunction and with makers of heart failure phenotype in humans (42). Our study results suggest that excess PKC\(\beta_2\) activation contributes to attenuated Cav-3 expression in the diabetic heart, as inhibition of PKC\(\beta_2\) activation by CGP53353 or siRNA-mediated PKC\(\beta_2\) expression knockdown prevented the decline of Cav-3 expression in cells exposed to HG. LY333531 treatment ameliorated diabetic heart caveolae dysfunction.
PKC_\beta_2 activation likely exerts adverse effects in the diabetic heart via alteration of the Akt/eNOS signaling pathway, which is modulated by Cavs (34). Although Cav-1 negatively regulates eNOS in cardiovascular tissues (43,44), the colocalization of Cav-3 and eNOS may facilitate eNOS activation by both cell surface receptors and cellular surface NO release for intercellular signaling in cardiomyocytes (17). This is supported by our findings that disruption of caveolea function by methyl-\beta-cyclodextrin or Cav-3 siRNA prevented Akt phosphorylation and suppressed eNOS phosphorylation in cardiomyocytes. Additionally, hyperglycemia decreased phosphorylated Akt and eNOS both in isolated cardiomyocytes and in diabetic heart tissues, leading to decreased myocardial NO levels, reduced Cav-3 levels, and increased Cav-1 cardiac levels. Inhibition of PKC_\beta_2 activation suppressed or abrogated these alterations. Therefore, inhibition of PKC_\beta_2 activation may rescue proper Akt/eNOS/NO signaling in the diabetic heart via Cav regulation.

Previous studies have demonstrated involvement of increased iNOS expression with cardiovascular abnormalities in STZ-induced diabetic rats (23,45). Our study confirms increased iNOS cardiac content in diabetic rats, an adverse mediator of nitrative stress (23), and increased nitrotyrosine was also demonstrated in diabetic heart tissue. The NOS inhibitor L-NAME blocked diabetes-induced augmentation of O_2^- levels, indicating eNOS uncoupling, which is in line with a recent study in STZ-induced diabetic mice (46). Furthermore, treatment of diabetic rats with LY333531 inhibited cardiac iNOS expression and reduced both nitrotyrosine and O_2^- production. Diabetes is associated with decreased NO levels and increased O_2^- and nitrotyrosine production (14,35), which are implicated with oxidative/nitrative stress and eNOS uncoupling. Our study provides direct evidence showing that inhibition of PKC_\beta_2 activation can mitigate oxidative/nitrative stress and eNOS uncoupling.

Initial LV diastolic dysfunction, reduced contractility, and prolonged diastole are the hallmarks of diabetic cardiomyopathy (47,48). In the current study, diabetes significantly reduced E/A ratio, which was concomitant with significantly increased IVRT and decreased LVd and SV but did not alter FS, FLVPW, or EF. Such data indicate that myocardial diastolic (but not systolic) dysfunction occurs in 5-week STZ-induced diabetes rats, which can be ameliorated by LY333531. Our findings are in general agreement with the findings of Mihm et al. (26), who conducted a series study in similar STZ diabetic rats and showed that diastolic dysfunction occurred early during the course of the disease, which progressed to LV dilation reflected as increases in LVIDd and LVIDs with concomitant increase in LV luminal area and systolic dysfunction reflected as reduction in LV FS 35 days after STZ injection and onward. It should be noted that the E/A ratio derived from the conventional Doppler echocardiography as used in our current study is not a load-independent parameter and may have inherent limitations, e.g., the peak E wave velocity can be highly dependent upon HR (26), while the HR in the diabetic rats was lower than that in the control group (Table 1). The newly developed Doppler tissue echocardiography (DTE) can acquire myocardial wall and mitral annular velocity online, and the early diastolic annular velocity measured using DTE has been reported to be a preload independent index for evaluating LV diastolic function. A combination of

FIG. 7. Schematic depicting hyperglycemia-induced PKC_\beta_2 activation effects upon Cav-3–modulated Akt/eNOS signaling pathway. Cardiomyocyte caveolae are required for hyperglycemia-induced PKC_\beta_2 activation (translocation from cytosol to caveolea membrane). Excessive PKC_\beta_2 activation decreased Cav-3 expression, impairing the Akt/eNOS signaling pathway. Solid arrows depict stimulation, while transverse “T” shape indicates inhibition. PI3K, phosphatidylinositol 3-kinase.
DTE-derived E’/A’ ratio and the mitral inflow patterns (E/A ratio) obtained by conventional Doppler echocardiography in future studies should help to provide better estimations of diastolic dysfunction. However, the significant reduction of E/A ratio as a consequence of significant reduction of E velocity and significant enhancement of A velocity in combination with concomitant increase in IVRT and reductions in LVDD and SV in the diabetic group could jointly suggest diastolic dysfunction in the current study. LY333531 treatment in diabetic rats did not affect HR but corrected all of the above changes, suggesting that LY333531 treatment prevented the development of diastolic dysfunction. Future functional study with cardiomyocytes isolated from rodents in various stages of diabetes will help establish the relative contribution of the slowing in cardiomyocyte relaxation time and LV stiffness (due to fibrosis) to the development of diastolic dysfunction.

In summary, our study demonstrates that hyperglycemia-induced PKCβ2 activation is associated with caveolar dysfunction and, consequently, deranged Akt/eNOS signaling (Fig. 7). Inhibition of PKCβ2 activation attenuated cardiac diastolic dysfunction by restoring Cav-3 expression and subsequently rescuing Akt/eNOS/NO signaling. PKCβ2 blockade may therefore represent a novel therapeutic avenue in the treatment of diabetic cardiomyopathy.

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S.L. performed the study and wrote the manuscript. H.L., J.X., Y.L., and X.G. performed the study. J.W., K.F.J.N., W.B.L., X.-l.M., and B.R. contributed to data analysis and interpretation. M.G.I. reviewed and approved the research protocol. Z.X. reviewed and approved the research protocol and wrote the manuscript. Z.X. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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