Inactivation of 14-3-3 Protein Exacerbates Cardiac Hypertrophy and Fibrosis through Enhanced Expression of Protein Kinase C β2 in Experimental Diabetes

Narasimman Gurusamy,[a] Kenichi Watanabe,*[,a] Meilei Ma,[a] Shaosong Zhang,[b] Anthony J. Muslin,[b] Makoto Kodama,[c] and Yoshifusa Aizawa[c]

[a] *Department of Clinical Pharmacology, Niigata University of Pharmacy and Applied Life Sciences; 5–13–2 Kamishineicho, Niigata 950–2081, Japan:* [b] *Departments of Cell Biology and Physiology, Center for Cardiovascular Research, Washington University School of Medicine; St Louis, MO, 63110, U.S.A. and* [c] *First Department of Medicine, Niigata University School of Medicine; Niigata 951–8510, Japan.*

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Diabetic cardiomyopathy is associated with cardiac hypertrophy and fibrosis. Activation of protein kinase C (PKC) has been implicated in the diabetes-induced cardiovascular complications. PKCβ2 isoform is preferentially found to be activated in the diabetic myocardium. However, the role of PKCβ2 in diabetic cardiomyopathy is not clear. 14-3-3 family members are dimeric phosphoserine-binding proteins that regulate signal transduction, apoptotic and checkpoint control pathways, and have been shown to bind with PKC isozymes and negatively regulate their enzymatic activities. The present study tests whether 14-3-3 protein regulates cardiac hypertrophy and fibrosis in streptozotocin (STZ)-induced diabetic mice, using transgenic mice with cardiac specific over-expression of dominant negative (DN) 14-3-3 protein. In addition, we examined the relationship between 14-3-3 protein and PKCβ2 in the diabetic myocardium. Cardiac myocyte diameter, content of cardiac fibrosis, left ventricular tissue expressions of atrial natriuretic peptide, transforming growth factor β1, collagen III and PKCβ2 were significantly elevated 28 and 56 d after STZ injection in transgenic DN-14-3-3 mice, when compared to their non-transgenic counterparts. These results clearly demonstrate that the functional inactivation of 14-3-3 protein in DN-14-3-3 mice exacerbates diabetes-induced cardiac hypertrophy and fibrosis. The exacerbations of cardiac hypertrophy and fibrosis were significantly and positively correlated with the enhanced expression of PKCβ2 in DN-14-3-3 mice. Our results indicate for the first time that 14-3-3 protein negatively regulates cardiac hypertrophy and fibrosis, possibly through controlling the expression of PKCβ2 in the diabetic myocardium.

Key words 14-3-3 protein; cardiac hypertrophy; fibrosis; protein kinase C; diabetes mellitus; streptozotocin

Cardiac hypertrophy and interstitial fibrosis are adaptive responses of the heart to several pathophysiological stimuli, and are the major determinant of morbidity and mortality from cardiovascular disease.[1,2] 14-3-3 proteins are intracellular, dimeric, phosphoserine binding molecules that play a critical role in signal transduction, apoptotic and checkpoint control pathways.[3,4] Even though 14-3-3 protein has been shown to bind to various protein kinases, transcription factors, receptor proteins, and proteins involved in apoptosis,[5—7] the known biological functions of 14-3-3 protein are limited. Previously, we have demonstrated the involvement of 14-3-3 protein in pressure overload-induced myocardial apoptosis[8] and in diabetes-induced myocardial apoptosis,[9] and in the present study, we tested whether 14-3-3 protein regulates cardiac hypertrophy and fibrosis, by the use of transgenic (TG) mice with cardiac specific over-expression of dominant negative (DN) 14-3-3 protein.

A number of studies have reported that hyperglycemia or diabetes induces cardiac hypertrophy and fibrosis,[10,11] where the left ventricular (LV) tissue expressions of atrial natriuretic peptide (ANP),[12,13] transforming growth factor (TGF) β1[14] and collagen III[15] were significantly elevated. Activation of protein kinase C (PKC) has been implicated in the diabetes-induced cardiovascular complications.[16] PKCβ2 isoform was found to be preferentially activated in the heart and aorta of diabetic animals.[16—19] Cardiac specific over-expression of PKCβ2 resulted in cardiac hypertrophy and fibrosis.[20,21] Moreover, 14-3-3 protein has been shown to bind to many PKC isoforms and inhibit their enzymatic activities [22,23] by directly binding to the cysteine rich domain of PKC.[24] However, the role of PKCβ2 in diabetic cardiomyopathy is not studied well. In the present study, we examined the co-relationship between 14-3-3 protein and PKCβ2 in the diabetic myocardium. Our results show for the first time that inactivation of 14-3-3 protein in DN-14-3-3 mice exacerbates diabetes-induced cardiac hypertrophy and fibrosis, which were positively correlated with the augmented expression of PKCβ2 in the LV tissue of DN-14-3-3 mice, relative to the non-transgenic (NTG) C57BL/6 animals.

MATERIALS AND METHODS

Generation of DN-14-3-3 Mice DN-14-3-3 mice were generated as described previously.[8] In brief, the coding region of the human DN (R56A and R60A) 14-3-3η cDNA with a 5′-Myc-1 epitope tag was subcloned into a vector containing the α-myosin heavy chain promoter and an SV40 polyadenylation site.[25] Linearized DNA was injected into the pronuclei of one-cell C57BL/6 XSJL embryos at the Neuroscience Transgenic Facility at Washington University School of Medicine. Progeny were backcrossed into the C57BL/6 genetic background and were analyzed by polymerase chain reaction to detect transgene integration[26] using mouse-tail DNA as template. Age matched C57BL/6 JAX mice (obtained from Charles River Japan Inc, Kanagawa, Japan) were used as NTG controls.

Diabetes Induction Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ; Sigma, St

* To whom correspondence should be addressed. e-mail: watanabe@niigata-pharm.ac.jp

Table 1. Changes in Blood Sugar Level, Body and Heart Weights and HW/BW during the Course of Diabetes in Non-transgenic (NTG) and Transgenic DN 14-3-3 (TG) Mice

	NTG (days after STZ injection)				NTG-normal-56	TG (days after STZ injection)				TG-normal-56
	0	7	28	56		0	7	28	56	
Blood sugar level (mg/dl)	151±19	333±22**	465±21**	532±38**	145±23	153±21	318±19##	479±29##	541±33##	162±28
Body weight (g)	23.2±0.36	22.7±0.42	19.9±0.39*	18.6±0.81**	28.9±0.64	25.7±0.54	24.9±0.73	22.5±0.59#	20.1±0.91##	31.4±0.74
Heart weight (mg)	101±4.6	98±4.3	87±4.9*	84±5.4**	126±5.6	105±4.8	101±5.9	95±6.9#	92±7.4##	130±6.5
HW/BW (mg/g)	4.34±0.25	4.33±0.15	4.39±0.16	4.52±0.19*	4.37±0.21	4.09±0.24	4.07±0.31	4.22±0.27#	4.59±0.18##	4.13±0.26

Values are mean±S.E.M., * $p<0.05$, ** $p<0.01$ *vs.* 0-d and 56-d age matched normal (non-STZ-induced) mice of NTG group (NTG-normal-56), # $p<0.05$, ## $p<0.01$ *vs.* 0-d and 56-d age matched normal mice of TG group (TG-normal-56).

Louis, MO, U.S.A.) at the dose of 150 mg/kg body weight (BW) to 8—10 weeks old male DN-14-3-3 and NTG mice. STZ was dissolved in 20 mM sodium citrate saline buffer (pH 4.5) and injected within 5 min of preparation. Age matched DN-14-3-3 and NTG mice were injected with 100 μl of citrate buffer and they were used as non-diabetic control. Mice were maintained with free access to water and chow throughout the period of study, and the animals were treated in accordance with the guidelines for animal experimentation of our institute.

BW and Blood Glucose Measurements BW and blood glucose levels of animals were measured at a time course of 0, 7, 28, and 56 d after STZ injection. Animals' blood glucose level was determined using Medi-safe chips (Terumo Inc, Tokyo, Japan).

Ratio of Heart Weight (HW) to BW (HW/BW) After anesthetizing mice with single intraperitoneal injection of pentobarbital (50 mg/kg), hearts were excised at 0, 7, 28, and 56 d after STZ injection ($n=5$—6 for each time period). HW and HW/BW were determined for each animal. Left ventricle was quickly dissected and cut into two parts. One part was immediately transferred into liquid nitrogen and then stored at −80 °C for protein analysis. The other part was stored in 10% formalin to prepare paraffin section for histopathology.

Cell Size Measurement Paraffin embedded LV tissue sections stained with hematoxylin–eosin, were used for measuring cell size. Short axis diameter of cardiac myocyte was measured for 10 myocytes selected per field (about 50 fields were selected per section) at 400-fold magnification by light microscopy. Each average value was obtained based on the data from 10 myocytes and was used as an independent sampling data.

Measurement of Fibrosis The area of myocardial fibrosis in LV tissue sections stained with Azan-Mallory was quantified by a color image analyzer (CIA-102, Olympus, Tokyo, Japan), using the difference in color (blue fibrotic area as opposed to red myocardium).[27]

Protein Analysis by Western Blotting Western immuno-blotting was performed with LV cytosolic lysate obtained from both TG and NTG mice. Protein lysate was prepared as described previously.[9] Total protein concentration in samples was measured by modified Lowry method. Proteins were separated by SDS-PAGE and electrophoretically transferred into nitrocellulose filters. Filters were blocked with blotto solution [1% BSA and 1% non-fat dry milk in Tris-buffered saline (20 mM Tris, pH 7.6, 137 mM NaCl) with 0.1 % Tween 20]. Antibodies against ANP, TGFβ1, collagen III, PKCβ2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology. All antibodies were used at a dilution of 1 : 1000. After incubation with primary antibody, bound antibody was visualized with horseradish peroxidase coupled secondary antibody (Santa Cruz), and chemiluminescence developing agents (ECL Plus, Amersham). The level of expression of ANP, TGFβ1, collagen III and PKCβ2 was normalized with GAPDH protein expression in the same sample. The level of expression of each protein in 0-d NTG mice was considered as one arbitrary unit.

Statistical Analysis Data are presented as mean±standard error mean (S.E.M.). Statistical analysis between the groups was performed by one-way analysis of variance followed by Tukey's method. Correlations between groups of values were evaluated calculating the best fit based on least-squares regression analysis, and the coefficient of correlation (r) was indicated. Differences were considered significant at $p<0.05$.

RESULTS

Blood Glucose Levels in Mice Changes in blood glucose levels during different time courses of diabetes are listed in Table 1. Blood glucose level in TG and NTG mice were significantly ($p<0.01$) elevated from day-7 after STZ injection. However, the average blood glucose level between TG and NTG mice did not differ after STZ injection (Table 1). Animals which had blood glucose level higher than 300 mg/dl after STZ injection were used for further experiments.

Changes in BW, HW and HW/BW Changes in BW, HW and HW/BW during different courses of diabetes are listed in Table 1. BW and HW of TG and NTG mice were decreased significantly 28 and 56 d after STZ injection, compared to the 0-d and the 56-d age-matched normal animals of the same group (Table 1). But, HW/BW was significantly increased after STZ injection in both TG (on days 28 and 56) and NTG (on day 56) mice relative to the 0-d and the 56-d age-matched normal animals of the same group (Table 1). HW/BW in DN-14-3-3 mice was slightly lesser at 0 and 7 d time period when compared to the same time period in NTG mice, but this was reversed at day-56 after STZ injection (Table 1).

Cardiac Cell Size The average cardiac cell diameter was markedly increased in both TG and NTG mice on 28 and 56 d after STZ injection, relative to the 0-d and the 56-d age-matched normal animals of the same group (Figs. 1A—D, 2A). However, the average cardiac myocyte diameter was significantly ($p<0.05$) increased in TG mice 28 and 56 d

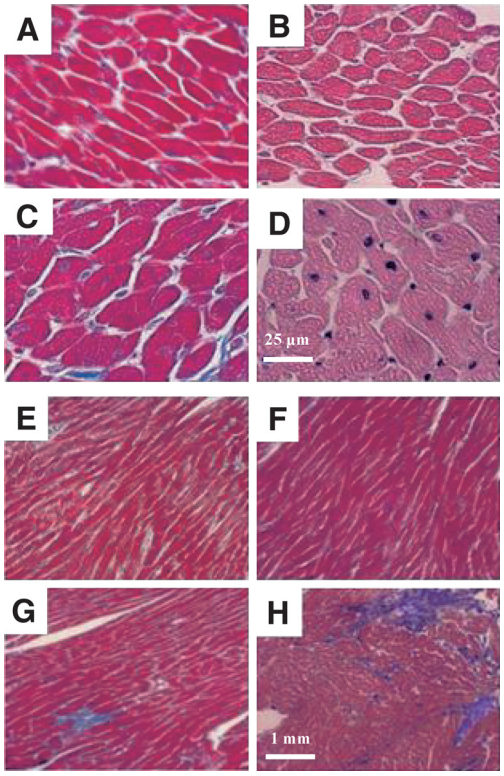

Fig. 1. Photomicrographs of Left Ventricular Tissue Sections Obtained from Transgenic DN-14-3-3 (TG) and Non-transgenic (NTG) Mice

Hematoxylin and eosin staining (A—D), demonstrating cardiac histology from (A)-NTG-0, (B)-TG-0, (C)-NTG-56 and (D)-TG-56 d after STZ injection. Cardiac cell diameter was significantly increased 56 d after STZ injection in both TG and NTG mice relative to their 0-d and 56-d age matched normal animals of the same group mice; however cardiac cell diameter was significantly increased in TG-56 (D), relative to NTG-56 (C), indicating exacerbation of cardiac hypertrophy in DN-14-3-3 mice. Azan-Mallory staining (E—H), for measuring cardiac fibrosis from (E)-NTG-0, (F)-TG-0, (G)-NTG-56 and (H)-TG-56 d after STZ injection. Blue areas in section (G) and (H) indicates fibrosis, which was significantly increased in (H)-TG-56 d mice, relative to (G)-NTG-56. (Magnification: A—D: ×400; E—H: ×100)

after STZ injection, compared to the same time period of NTG mice (Figs. 1A—D, 2A).

Cardiac Fibrosis The percentage of cardiac fibrosis was markedly increased 28 and 56 d after STZ injection in both TG and NTG mice, relative to the 0-d and the 56-d age-matched normal animals of the same group (Figs. 1E—H, 2B). However, the percentage of cardiac fibrosis was significantly ($p<0.05$) increased in TG mice 28 and 56 d after STZ injection, compared to the same time period in NTG group (Figs. 1E—H, 2B).

Cardiac Hypertrophic and Fibrotic Marker Proteins' Expression The changes in levels of cardiac hypertrophic (ANP) and fibrotic (TGFβ1 and collagen III) marker proteins expressions during the course of diabetes are shown in Fig. 3. The levels of ANP, TGFβ1 and collagen III were markedly elevated in the left ventricle of TG (on days 28 and 56) and

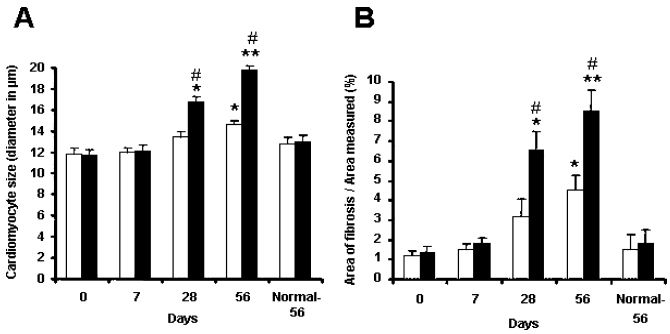

Fig. 2. DN-14-3-3 Exacerbates Cardiac Hypertrophy (A) and Fibrosis (B) in Experimental Diabetes

Myocyte size (microns) and area of fibrosis (shown as percent and normalized to area measured) were determined from histological sections of heart obtained from non-transgenic (open bar) and transgenic DN-14-3-3 (closed bar) mice at 0, 7, 28 and 56 d after STZ injection. Results are mean±S.E.M. from six animals. $*p<0.05$, $**p<0.01$ *vs*. 0-d and 56-d age-matched normal (non-STZ-induced) animals of the same group. $^{\#}p<0.05$ *vs*. the same day of non-transgenic group.

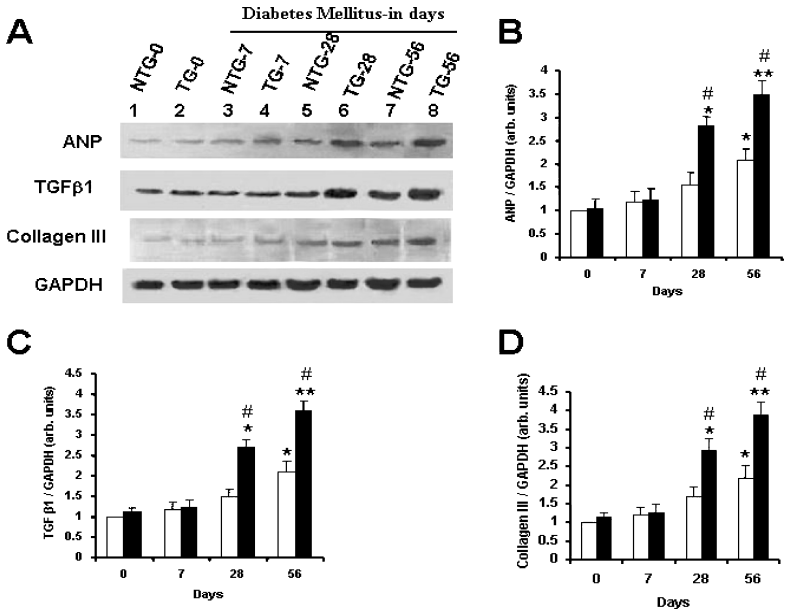

Fig. 3. DN-14-3-3-Induced Expression of Markers of Cardiac Hypertrophy and Fibrosis

(A) Western-Immunoblotting analysis of ANP, TGFβ1 and collagen III in protein lysate obtained from left ventricular tissue of transgenic DN-14-3-3 (TG) and non-transgenic (NTG) mice at 0, 7, 28 and 56 d after STZ injection. Equal amounts of total protein were loaded in each lane, and GAPDH protein expression was used as control. These immunoblots are representative of five separate experiments. From B—D: Quantification of ANP (B), TGFβ1 (C) and collagen III (D) protein expressions normalized with GAPDH, from NTG (open bar) and TG (closed bar) mice are plotted. Densitometric analysis of immuno-reactive bands was performed using Scion Image software, and data are represented as the mean signal intensity±S.E.M. from five experiments. $*p<0.05$, $**p<0.01$ *vs*. 0-d of the same group. $^{\#}p<0.05$ *vs*. the same day of NTG group.

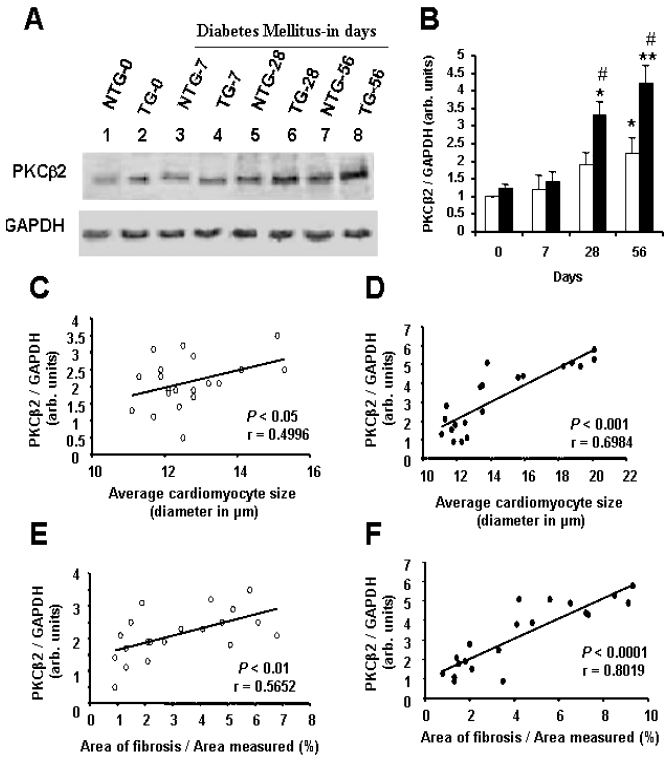

Fig. 4. DN-14-3-3-Induced Expression of PKCβ2 in Diabetic Myocardium

(A) Western immuno-blotting images of PKCβ2 and GAPDH protein expressions in non-transgenic (NTG) and transgenic DN-14-3-3 (TG) mice heart at 0, 7, 28 and 56 d after STZ injection. (B) Quantification of PKCβ2 protein expressions from NTG (open bar) and TG (closed bar) mice are plotted. Densitometric analysis of immuno-reactive bands was performed using Scion Image software, and data are represented as the mean signal intensity±S.E.M. from five experiments. (C—F) Correlation analysis between the average cardiomyocyte size and PKCβ2 expression (C, D) and between the % of cardiac fibrosis and PKCβ2 expression (E, F) in the diabetic myocardium of NTG (open circle) and TG (closed circle) mice. $*p<0.05$, $**p<0.01$ *vs.* 0-d of the same group. $^{\#}p<0.05$ *vs.* the same day of NTG group.

NTG mice (on day 56) after STZ injection, compared to the 0-d level of the same group (Figs. 3A—D). However, the levels of ANP, TGFβ1 and collagen III were significantly ($p<0.05$) higher in TG mice 28 and 56 d after STZ injection, relative to the same time period in NTG group (Figs. 3A—D). A slight but insignificant increase in the levels of ANP, TGFβ1 and collagen III were observed 7 d after STZ injection in both TG and NTG mice.

Cardiac Expression of PKCβ2 The basal level of PKCβ2 was slightly higher in TG mice compared to NTG mice. Marked increase in the level of PKCβ2 was found after STZ injection in the left ventricle of both TG (on days 28 and 56) and NTG mice (on day 56) (Figs. 4A, B). However, significant ($p<0.05$) increase in the level of PKCβ2 was found in the left ventricle of TG mice 28 and 56 d after STZ injection, compared to the same time period in NTG mice (Figs. 4A, B). Figures 4C and D show that the correlation between the level of expression of PKCβ2 and the average cardiac cell diameter is significant. [For NTG mice: $r=0.4996$, $p<0.05$ (Fig. 4C); For TG mice: $r=0.6984$, $p<0.001$ (Fig. 4D)]. As shown in Figs. 4E and F, the correlation between the level of expression of PKCβ2 and the % of cardiac fibrosis is significant [For NTG mice: $r=0.5652$, $p<0.01$ (Fig. 4E); For TG mice: $r=0.8019$, $p<0.0001$ (Fig. 4F)]. Our results show that the correlation coefficient (r) values were higher in DN-14-3-3 mice, relative to NTG mice.

DISCUSSION

The major findings of the present study are: 1) cardiac specific overexpression of DN-14-3-3 protein exacerbates diabetes-induced cardiac hypertrophy and fibrosis, and 2) the exacerbation of cardiac hypertrophy and fibrosis in DN-14-3-3 mice is positively correlated with the increased protein expression of PKCβ2 in the LV tissue. 14-3-3 proteins are dimeric, phosphoserine binding molecules that regulate signal transduction, apoptotic and checkpoint control pathways.[3,4] However, the biological functions of mammalian 14-3-3 protein are limited. In the present study, we tested whether 14-3-3 protein regulates cardiac hypertrophy and fibrosis in STZ-induced diabetic mice. DN-14-3-3 mice appeared normal at birth, had normal cardiac function and morphology, and lived normal lifespan in the absence of experimental intervention.[8] Moreover, in DN-14-3-3 TG mice, DN-14-3-3 protein represented 50% of total 14-3-3 protein.[8]

Diabetes mellitus is associated with cardiac hypertrophy and fibrosis.[10,11] In our study, the decrease of BW, a hallmark of diabetes mellitus, was in proportionate with the decrease of HW after STZ injection in both NTG and TG group mice. However, HW/BW was found increased in diabetic animals of both groups, as indicated in Table 1. The occurrence of cardiac hypertrophy in our model was confirmed by the increased myocardial cell size and LV expression of ANP in both DN-14-3-3 and NTG mice; where the cardiac cell size and ANP expression were significantly increased in DN-14-3-3 mice 28 and 56 d after STZ injection, relative to the same time period in NTG mice. Indeed, ANP is a cardiac homeostatic molecule,[28] preferentially expressed in the atrium and found at low levels in adult ventricular myocytes.[29] ANP expression was shown to be elevated in pathological cardiac hypertrophy[30] including diabetes-induced cardiac hypertrophy,[12,13] and the total level of circulating ANP is strongly correlated with the degree of ventricular dysfunction and eventually mortality.[31] Our study results indicate that cardiac hypertrophy induced by experimental diabetes is exacerbated in DN-14-3-3 mice.

TGFβ1 and collagen III is shown to be highly expressed during cardiac hypertrophy and fibrosis.[32,33] Our results are in accordance with the previous findings,[14,15,34] where the levels of TGFβ1 and collagen III have been shown to be increased in diabetes-induced cardiac hypertrophy and fibrosis. In this study, the percentage of cardiac myocyte fibrosis as well as the expression of cytokine TGFβ1 and extra cellular fiber collagen III were significantly elevated in the left ventricle of TG mice relative to NTG mice 28 and 56 d after STZ injection; which indicates that experimental diabetes-induced cardiac fibrosis is intensified in DN-14-3-3 mice.

PKC plays an important role in intracellular signaling for modulating cardiac myocyte development, inotropic functions, and cellular growth.[35,36] Activation of PKC has been implicated in the diabetes-induced cardiovascular complications.[16] Glucose-induced activation of PKC augmented the production of extracellular matrix macromolecules that accumulate during atherosclerotic lesion formation.[16] PKC family consists of at least 11 different isoforms, out of which, PKCβ2, which is a Ca^{2+} dependent one, was found to be preferentially activated in the diabetic myocardium.[16—19] Recently, Guo *et al.*[19] have shown that PKCβ2 is significantly

upregulated in the diabetic heart at both the transcriptional and translational levels. It is noteworthy that targeted overexpression of PKCβ2 in mouse myocardium resulted in LV hypertrophy and fibrosis.[20,21] Earlier reports indicate that 14-3-3 protein interacts with PKC,[22—24] where, Aitken *et al*.[23] have shown by *in vitro* studies that 14-3-3 protein isolated from sheep brain inhibits PKCβ2 in addition to other PKC isozymes. Also, overexpression of 14-3-3 protein inhibited the translocation of PKC.[37] Even though PKC have been implicated in the diabetic myocardium, its regulatory mechanism is not understood completely. In the present study, we examined the *in vivo* co-relationship between 14-3-3 protein and PKCβ2 in the diabetic myocardium. Our results show that PKCβ2 is significantly increased 28 and 56 d after STZ injection in the LV cytosolic lysate of DN-14-3-3 mice compared to the same time period in NTG mice (Figs. 4A, B). Also, the basal level of PKCβ2 is slightly higher in TG mice, compare to NTG mice (Figs. 4A, B). Moreover, the correlation coefficient (r) values obtained between the expression of PKCβ2 with the average cardiac cell diameter (Figs. 4C, D), and with the % of cardiac fibrosis (Figs. 4E, F) were higher in DN-14-3-3 mice than in NTG mice. These results indicate that the increased level of PKCβ2 in DN-14-3-3 mice is directly related to the exacerbation of diabetes-induced cardiac hypertrophy and fibrosis.

The role of 14-3-3 protein in the prevention of diabetes-induced cardiomyopathy can be explained as follows: 14-3-3 binds with PKC and maintains the activity of PKC at minimum level.[22—24] Hyperglycemia or diabetes activates diacylglycerol (DAG), which in turn activates PKC,[17,18] where, the level of DAG in the diabetic heart is positively correlated with the level of glucose in blood.[17] Robinson *et al*.[24] have shown by *in vitro* studies that DAG (40 μM) and phorbol ester (200 nM) in the presence of phosphatidyl serine, overcomes about 60% of the 14-3-3 mediated inhibition of PKC. This may be due to DAG or phorbol ester produces a conformation of PKC which is less susceptible to inhibition by 14-3-3, and more likely that the interaction site of 14-3-3 on PKC is at or near the DAG/phorbol ester binding site, *i.e.* the cysteine-rich domain.[24] In our study, the blood glucose level did not differ between DN-14-3-3 and NTG mice after STZ injection (Table 1), but the LV tissue expression of PKCβ2 was significantly elevated in DN-14-3-3 mice, compared to NTG mice. These results clearly demonstrate that the functional inactivation of 14-3-3 protein in DN-14-3-3 mice augmented the protein expression of PKCβ2 in the diabetic myocardium, and thus elevated PKCβ2 probably contributes for the exacerbation of diabetes-induced cardiac hypertrophy and fibrosis.

Mitogen activated protein kinase (MAPK) pathways play an important role in mediating intracellular signaling for cardiac hypertrophy.[38] Previously, we have shown that the activation of p38 MAPK is significantly increased 28 and 56 d after STZ injection in DN-14-3-3 mice.[9] It is noteworthy that expression of PKCβ2 coincides with the expression of p38 MAPK in our model; hence it is conceivable that they may have a common axis. Since earlier reports suggest that glucose activates p38 MAPK *via* PKC,[39,40] we speculate that the enhanced expression of PKCβ2 in our model of diabetic cardiomyopathy may be an upstream event to p38 MAPK activation; however, further studies are required. Moreover, it is important to investigate the role of PKCβ2 and its relationship with 14-3-3 protein in the diabetic myocardium by pharmacological intervention of PKCβ2 and over-expression of 14-3-3 protein *in situ* to comprehensively elucidate the association between them in diabetes-induced cardiac hypertrophy and fibrosis.

In conclusion, our study results show that the functional inactivation of 14-3-3 protein in DN-14-3-3 mice exacerbates diabetes-induced cardiac hypertrophy and fibrosis. The exacerbation of cardiac hypertrophy and fibrosis were significantly and positively correlated with the enhanced expression of PKCβ2 in DN-14-3-3 mice. To the best of our knowledge, this is the first report to indicate that 14-3-3 protein negatively regulates cardiac hypertrophy and fibrosis, possibly through controlling the expression of PKCβ2 in the diabetic myocardium.

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