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Effects of insulin replacement on cardiac apoptotic and survival pathways in streptozotocin-induced diabetic rats

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Increased myocyte apoptosis in diabetic hearts has been previously reported. Therefore, the purpose of this study was to evaluate the effects of insulin on cardiac apoptotic, hypertrophic, and survival pathways in streptozotocin (STZ)-induced diabetic rats. Forty-eight male Wistar rats at 8 weeks of age were randomly divided into control group (Control), STZ-induced (65 mg/kg STZ i.v.) Type 1-like diabetic rats (DM), and DM rats with 4 IU insulin replacement (DI) for 4 and 8 weeks, respectively. The levels of protein involved in cardiac apoptotic, hypertrophic, and survival pathways were measured by Western blotting. Cardiac mitochondrial-dependent apoptotic pathways, such as Bad, cytosolic cytochrome *c*, activated caspase 9 and 3, and calcineurin-nuclear factor activation transcription 3 (NFAT3) hypertrophic pathway in DM were increased compared to Control and attenuated in DI group after 8 weeks whereas those were not found after 4 weeks. Cardiac anti-apoptotic Bcl2 and phosphorylated-Bad were significantly decreased in DM group but not in DI group after 8 weeks. Insulin-like growth factor-I receptor (IGFIR), phosphatidylinositol 3'-kinase (PI3K), and the protein kinase B (Akt) were significantly decreased in DM relative to Control and DI after 8 weeks whereas those were not found after 4 weeks. Insulin replacement not only prevents activation of the cardiac mitochondrial-dependent apoptotic pathway and calcineurin-related NFAT3 hypertrophic pathway in diabetes but it also enhances the cardiac insulin/IGFIR–PI3K–Akt survival pathway, all of which are attenuated with insulin therapeutic duration-dependent manners. The findings may provide possible diabetes-related apoptotic, hypertrophic, and survival pathways for potentially preventing cardiac abnormality in diabetes. Copyright © 2009 John Wiley & Sons, Ltd.

KEY WORDS — diabetes; apoptosis; heart; hypertrophy

INTRODUCTION

Patients with diabetes mellitus have increased risks for cardiovascular diseases such as coronary heart disease and heart failure.[1] Diabetes, a common co-morbidity in patients with heart failure, is associated with worse long-term outcomes.[2] The cellular apoptosis in terminally differentiated cardiomyocyte is a very critical pathological mechanism to causes heart failure and, on the other hand, the process of apoptotic interruptus may allow development of a novel strategy to reverse or attenuate heart failure.[3] Diabetic hearts from patients' autopsy were characterized by an 85-fold increase in cardiomyocyte apoptosis.[4] An aggregate 30% cardiomyocyte loss were found in diabetic rat hearts.[5] However, cardiac apoptotic, hypertrophic, and survival pathways in diabetes are still not totally understood.

Apoptosis, a physiological program of cellular death, in heart may contribute to many cardiac disorders.[6,7] Cardiac apoptosis was found in many chronic metabolic and cardiovascular diseases such as obesity,[8,9] diabetes, and hypertension,[7,10] and in various stressful conditions, such as long-term hypoxia,[8,11] or smoke.[12] The occurrence of

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apoptosis has been often reported to contribute to the loss of cardiomyocytes in myocardial diseases, and is recognized as a predictor of adverse outcomes in patients with cardiac diseases or heart failure.[3] The mitochondrial-dependent apoptotic pathway is activated within cells and is resulted from the release of a number of pro-apoptotic factors from the intermembrane space of mitochondria.[13,14] The mitochondria is the main site of action for the apoptosis-regulating protein family exemplified by Bcl-2 family, such as Bcl-2 and Bad (Bcl-2 antagonist of cell death).[13] Bcl-2, an anti-apoptotic protein prevents cytochrome *c* release whereas Bad, a pro-apoptotic protein enhances cytochrome *c* release from mitochondria.[13] When cytochrome *c* releases from mitochondria into cytosol, it is responsible for activating caspase-9, which further activates caspase-3 and executes the apoptotic program.[15] However, it is unclear whether mitochondrial-dependent apoptotic pathway mediates diabetes-related cardiac apoptosis.

Calcineurin has been reported as a critical mediator for cardiac hypertrophy and cardiomyocyte apoptosis.[16–18] Transgenic mice hearts, when the activated forms of calcineurin were over-expressed, gradually developed cardiac hypertrophy and heart failure.[17] It has been reported that the activation of Ca^{2+} induces the calcineurin-nuclear factor activation transcription 3 (NFAT3) pathway and in turn enhances cardiac hypertrophy.[17,19] In addition, calcineurin has been reported to activate pro-apoptotic protein Bad and cause apoptosis of cardiomyocytes.[20]

Insulin and Insulin-like growth factor-I (IGFI) signaling is reported to contribute to the modulation of survival responses in cardiac tissues.[7,21] Phosphatidylinositol-3' kinase (PI3K) and protein kinase B (Akt) are key signaling molecules in insulin and IGFI receptor (IGFIR).[22–24] Impaired insulin and/or IGFIR signalings may contribute, at least partially, to the development of diabetes and the pathology of cardiac apoptosis in diabetic animal and human.[24]

The current study was to understand whether mitochondrial-dependent apoptotic pathway, calineurin-Bad apoptotic pathway, calcineurin-NFAT3 hypertrophic pathway, and impaired insulin/IGFIR–PI3K–Akt survival pathway in streptozotocin (STZ)-induced diabetic rats are worse than non-diabetic and whether these pathways can be improved by insulin replacement. We hypothesized that first, diabetes may predispose to more activated cardiac mitochondrial-dependent apoptotic pathway, more activated calcineurin-Bad apoptotic pathway, more activated calcineurin-NFAT3 hypertrophic pathway, and impaired insulin/IGFI–PI3K–Akt survival pathway as well as second, insulin replacement may prevent changes in these pathways in diabetes.

MATERIALS AND METHODS

Animals and induction of diabetes

Forty-eight male Wistar rats at 8 weeks of age were obtained from National Laboratory Animal Center, Taiwan. Ambient temperature was maintained at 25°C and the animals were kept on an artificial 12-h light-dark cycle. The light period began at 7:00 A.M. Rats were provided with standard laboratory chow (Lab Diet 5001; PMI Nutrition International Inc., Brentwood, MO, USA) and water *ad libitum*. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals and all protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan.

All animals were allowed to adapt to the environment for 1 week after their arrival before the experiment started. All rats were divided into two groups, i.e., control group ($n = 16$) and STZ-injected group ($n = 32$). The rats in STZ-injected group were injected with STZ (65 mg/kg body weight in citrate buffer, pH 4.5) and the rats in control group were injected with an equal volume of vehicle via the lateral tail vein. The rats were considered to be diabetic if their fasting glucose levels maintain > 11.1 mM or > 200 mg/dl after 48 h after injection of STZ as detected by Accu Soft (Hoffmann-La Roche) test strips. Three days later, the 16 of 32 STZ-induced diabetic rats were treated subcutaneously with insulin 4 IU daily for 4 and 8 weeks at 6.00 P.M. There are six groups in current study, i.e., control group with citrate buffer (Control-4W), STZ-induced diabetic rats with citrate buffer (DM-4W), and STZ-induced diabetic rats with insulin replacement (DI-4W) for 4 weeks as well as control group with citrate buffer (Control-8W), STZ-induced diabetic rats with citrate buffer (DM-8W), and STZ-induced diabetic rats with insulin replacement (DI-8W) for 8 weeks.

Cardiac characteristics and heart weighting

The hearts of six rats in six groups (i.e., Control-4W, DM-4W, DI-4W, Control-8W, DM-8W, and DI-8W) were analyzed by heart weight index and Western blotting. The hearts of animals were excised and cleaned with PBS. The left ventricles were separated and weighed. The ratios of the whole heart weight (WHW) to body weight (BW), the ratios of the left ventricular weight (LVW) to BW, and the ratios of the LVW to the WHW were calculated.

Tissue extraction

Cardiac tissue extracts were obtained by homogenizing the left ventricle samples in a lysis buffer (20 mM Tris, 2 mM EDTA, 50 mM 2-mercaptoethanol, 10% glycerol, pH 7.4, proteinase inhibitor (Roche), phosphatase inhibitor cocktail (Sigma)) at a ratio of 100 mg tissue/1 ml buffer for 1 min. The homogenates were placed on ice for 10 min and then centrifuged twice at 12 000*g* for 40 min. The supernatant was collected and stored at −70°C for further experiments.

Separation of cytosolic and mitochondrial fractions

To detect cytosolic cytochrome *c*, cardiac tissue extracts were suspended in a buffer (Tris, EDTA, and proteinase inhibitor cocktail tablet (Roche)) for 1 min on ice, homogenized by Polytron, and centrifuged at 1200*g* for

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10 min. The supernatant was re-centrifuged at 10 000*g* for 15 min to collect the mitochondrion-enriched pellet and the supernatant as the cytosolic fraction. The pellet was resuspended in lysis buffer as the mitochondrial fraction.

Electrophoresis and western blot

Protein concentration of cardiac tissue extracts was determined by the Bradford method (Bio-Rad Protein Assay, Hercules, CA). Protein samples (50 μg/lane) were separated on a 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a constant voltage of 75 V. Electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, 0.45 μm pore size) with a transferring apparatus (Bio-Rad). PVDF membranes were incubated in 5% milk in TBS buffer. Primary antibodies including p-Bad, Bad, Bcl-2, calcineurin, PI3K, Akt, p-Akt (BD Transdution Laboratories), cytochrome *c* (Trevigen, Inc), caspase-9, caspase-3, NFAT3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and IGF-I receptor, and α-tubulin (NEO MARKERS) were diluted to 1:500 in antibody binding buffer overnight at 4°C. The immunoblots were washed three times in TBS buffer for 10 min and then immersed in the second antibody solution containing goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, or donkey anti goat IgG-HRP (Santa Cruz) for 1 h and diluted 500-fold in TBS buffer. The immunoblots were then washed in TBS buffer for 10 min three times. The immunoblotted proteins were visualized by using an enhanced chemiluminescence ECL Western blotting luminal Reagent (Santa Cruz, CA, USA) and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan). Densitometric analysis of immunoblots was performed by AlphaImager 2200 digital imaging system (Digital Imaging System, San Leandro, CA, USA).

Statistical analysis

The data of whole heart weight index, glucose levels, and protein levels were compared among groups of animals in six groups (Control-4W, DM-4W, DI-4W, Control-8W, DM-8W, and DI-8W) using one-way analysis of variance (ANOVA) with pre-planned contrast comparison between 4 and 8 weeks. Control group serves as negative control group for DM groups and DM serves as non-therapeutic control group for therapeutic groups such as DI. In all cases, a difference at $p < 0.05$ was considered statistically significant.

RESULTS

Body weight and cardiac characteristics

BW, WHW, LVW, the whole heart weight corrected by body weight (WHW/BW), and the left ventricle weight corrected by body weight (LVW/BW) were similar among Control-4W, DM-4W, and DI-4W groups. BW was decreased but WHW/BW was increased in DM-8W, compared with those in Control-8W. WHW, LVW, and LVW/BW were similar among Control-8W, DM-8W, and DI-8W groups (Table 1). Daily blood glucose levels in DI-4W and DI-8W after insulin treatment for 6 h were significantly lower than those in DM-4W and DM-8W, respectively. However, blood glucose levels in DM-4W and DI-4W after insulin treatment for 48 h were significantly higher than those in Control-4W and blood glucose levels in DM-8W and DI-8W after insulin

Table 1. Heart weight index

4 Weeks	Control-4W	DM-4W	DI-4W
Body weight (BW), g	230 ± 12	220 ± 10	225 ± 9
Whole heart weight (WHW), g	0.76 ± 0.04	0.75 ± 0.06	0.79 ± 0.08
Left ventricular weight (LVW), g	0.51 ± 0.05	0.51 ± 0.04	0.50 ± 0.03
WHW/BW (10^2)	0.33 ± 0.03	0.34 ± 0.03	0.35 ± 0.02
LVW/BW	0.22 ± 0.02	0.23 ± 0.03	0.22 ± 0.03
Daily glucose, mg/dl (6 h)	131 ± 5	228 ± 21†	145 ± 48$^{\#\#}$
glucose, mg/dl (48 h)	145 ± 7	230 ± 31†	204 ± 28*
8 Weeks	**Control-8W**	**DM-8W**	**DI-8W**
Body weight (BW), g	290 ± 8	226 ± 7†	247 ± 9*,‡
Whole heart weight (WHW), g	0.76 ± 0.06	0.77 ± 0.06	0.79 ± 0.07
Left ventricular weight (LVW), g	0.56 ± 0.05	0.52 ± 0.05	0.54 ± 0.03
WHW/BW (10^2)	0.26 ± 0.04	0.34 ± 0.02*	0.32 ± 0.04
LVW/BW	0.19 ± 0.02	0.23 ± 0.02	0.22 ± 0.03
Daily glucose, mg/dl (6 h)	142 ± 10	228 ± 17†	146 ± 35$^{\#\#}$
Glucose, mg/dl (48 h)	160 ± 22	224 ± 15*	202 ± 14*

Values are means ± SEM ($n = 6$ in each group). Body weight and Heart weight index in Wistar rats with citrate buffer (Control), streptozotocin (STZ)-induced diabetic rats with citrate buffer (DM), and STZ-induced diabetic rats with insulin replacement (DI) for 4 and 8 weeks.
(6 h): Glucose level after insulin treatment for 6 h.
(48 h): Glucose level after insulin treatment for 48 h.
$^{*}p < 0.05$, significant differences versus control group.
$^{\dagger}p < 0.01$, significant differences versus control group.
$^{\ddagger}p < 0.05$, significant differences versus DM group.
$^{\#\#}p < 0.01$, significant differences versus DM group.

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treatment for 48 h were significantly higher than those in Control-8W (Table 1).

Upstream components of cardiac mitochondrial-dependent apoptotic pathways

To further understand the changes of the upstream components of mitochondrial-dependent apoptotic pathways in diabetes and diabetes treated with insulin, the protein levels of the Bcl-2 family (Bcl-2, p-Bad, and Bad) and cytosolic cytochrome *c* were measured in the excised hearts of six groups (Control-4W, DM-4W, DI-4W, Control-8W, DM-8W, and DI-8W) by Western blotting. The protein levels of Bad and cytosolic cytochrome *c* in DM-8W were significantly higher than those in Control-8W or DI-8W, but were similar among Control-4W, DM-4W, and DI-4W. The anti-apoptotic protein levels of Bcl2 and the ratio of p-Bad/Bad in DM-8W were significantly lower than those in DI-8W or Control-8W, but were similar among Control-4W, DM-4W, and DI-4W (Figure 1).

Downstream components of cardiac mitochondrial-dependent apoptotic pathways

To identify the changes of the downstream components of cardiac mitochondrial-dependent apoptotic pathways in

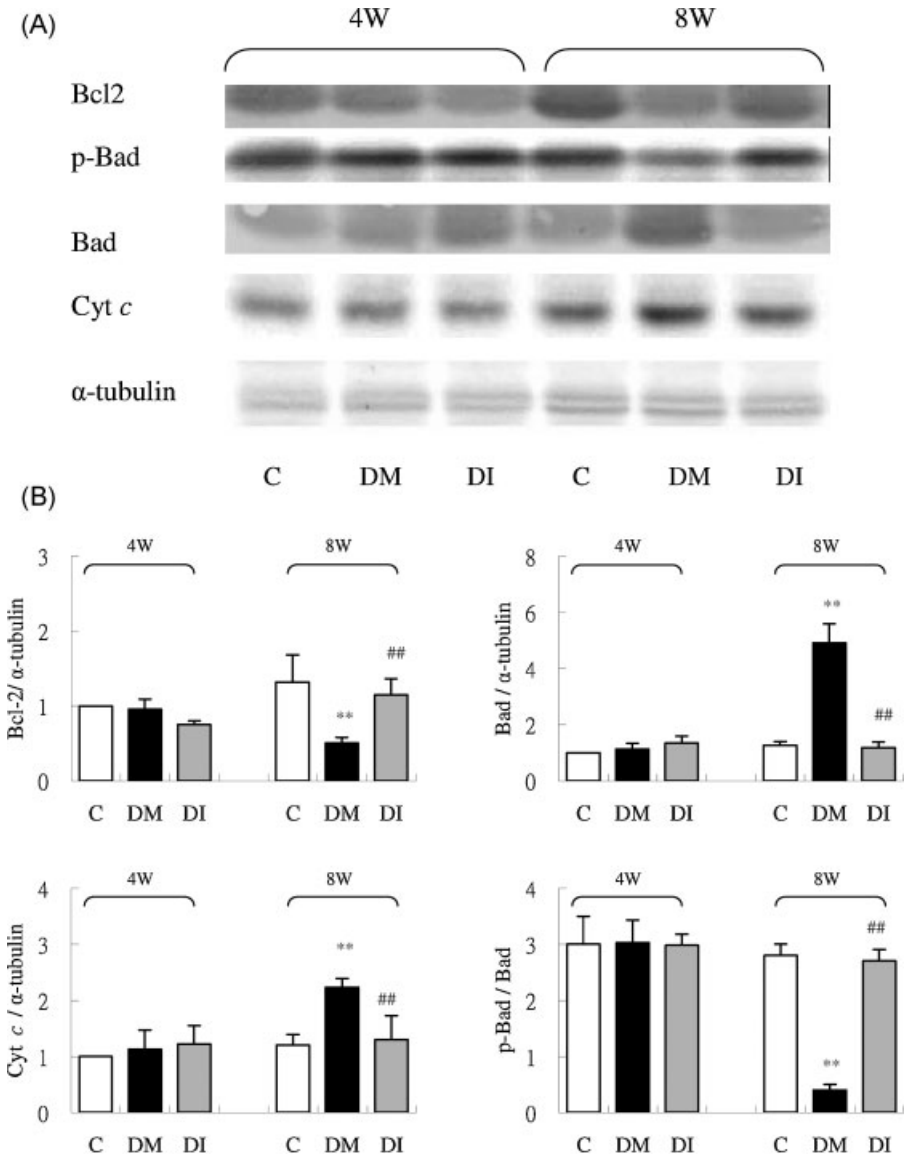

Figure 1. (A) The representative protein levels of Bcl2, phosphorylated Bad (p-Bad), Bad, and cytosolic cytochrome *c* extracted from the left ventricles of excised hearts in Wistar rats with citrate buffer (Control, C), streptozotocin (STZ)-induced diabetic rats with citrate buffer (DM), and STZ-induced diabetic rats with insulin replacement (DI) for 4 and 8 weeks were measured by Western blotting analysis. (B) Bars represent the relative protein quantification of Bcl2, Bad, cytosolic cytochrome *c*, and p-Bad/Bad normalized to α-tubulin, and indicate mean values ± SD ($n = 6$ in each group). $^{*}p < 0.05$, $^{**}p < 0.01$, significant differences from control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant differences from STZ group

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diabetes and diabetes treated with insulin, the protein levels of activated caspase 9 and 3 were measured in the excised hearts of six groups (Control-4W, DM-4W, DI-4W, Control-8W, DM-8W, and DI-8W) by Western blotting. The activated forms of caspase 9 and 3 protein levels were significantly higher in DM-8W than those in Control-8W or DI-8W, but were similar among Control-4W, DM-4W, and DI-4W (Figure 2).

Calcineurin-NFAT3 hypertrophic pathway

To identify the activities of calcineurin-NFAT3 hypertrophic pathway in diabetes and diabetes treated with insulin, the protein levels of calcineurin and NFAT3 were measured in the excised hearts of six groups (Control-4W, DM-4W, DI-4W, Control-8W, DM-8W, and DI-8W) by Western blotting. The protein levels of calcineurin were significantly higher in DM-4W than those in Control-4W or DI-4W and were also significantly higher in DM-8W than those in Control-8W or DI-8W. The protein levels of NFAT3 were significantly higher in DM-8W than those in Control-8W or DI-8W, but were similar among Control-4W, DM-4W, and DI-4W (Figure 3).

IGFIR, PI3K, and p-Akt survival pathway

To identify insulin-related PI3K–Akt survival pathway and IGFIR-related PI3K–Akt survival pathway in diabetes and diabetes treated with insulin, the protein levels of IGFIR, PI3K, Akt, and p-Akt were measured in the excised hearts of six groups (Control-4W, DM-4W, DI-4W, Control-8W, DM-8W, and DI-8W) by Western blotting. The protein levels of IGFIR, PI3K, and p-Akt and the ratio of p-Akt to Akt were similar among Control-4W, DM-4W, and DI-4W. The protein levels of IGFIR, PI3K, and p-Akt and the ratio of p-Akt to Akt were significantly decreased in DM-8W relative to Control-8W. These levels of IGFIR, PI3K, p-Akt, and p-Akt/Akt in DI-8W were significantly increased relative to DM-8W (Figure 4).

DISCUSSION

Our main findings can be summarized as follows: (1) Body weight was decreased in STZ-induced diabetes after 8 weeks but the ratio of WHW to BW was increased, compared with Control group. Changes in heart weight index in STZ-induced diabetes and diabetes with insulin replacement for 4 weeks were not found. (2) The key components of cardiac mitochondrial-dependent apoptotic pathways, such as Bad, cytosolic cytochrome *c*, activated caspase 9, and activated caspase 3 in STZ-induced diabetes were significantly increased compared with non-diabetes and these hyperglycemia-induced mitochondrial-dependent apoptotic pathways were significantly attenuated after insulin replacement for 8 weeks, not 4 weeks. (3) Calcineurin-NFAT3 hypertrophic pathways in STZ-induced diabetes were significantly increased compared with non-diabetes and these hyperglycemia-induced calcineurin-NFAT3 hyper-

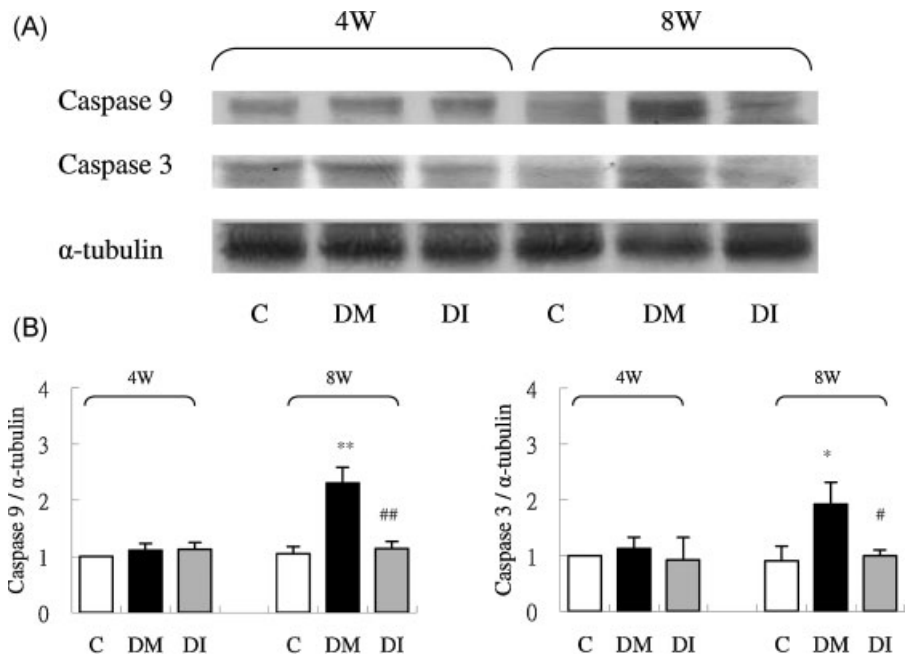

Figure 2. (A) The representative protein levels of activated caspase-9 and activated caspase-3 extracted from the left ventricles of excised hearts in Wistar rats with citrate buffer (Control, C), streptozotocin (STZ)-induced diabetic rats with citrate buffer (DM), and STZ-induced diabetic rats with insulin replacement (DI) for 4 and 8 weeks were measured by Western blotting analysis. (B) Bars represent the relative protein quantification of activated caspase-9 and activated caspase-3 normalized to α-tubulin, and indicate mean values $\pm$ SD ($n = 6$ in each group). $^{*}p < 0.05$, $^{**}p < 0.01$, significant differences from control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant differences from STZ group

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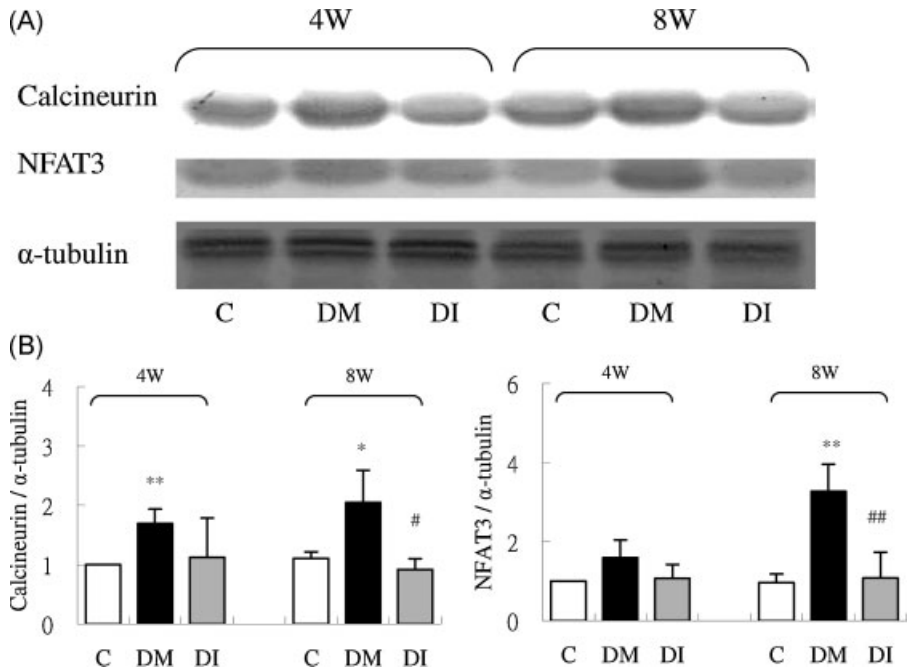

Figure 3. (A) The representative protein levels of calcineurin and nuclear factor activation transcription 3 (NFAT3) extracted from the left ventricles of excised hearts in Wistar rats with citrate buffer (Control, C), streptozotocin (STZ)-induced diabetic rats with citrate buffer (DM), and STZ-induced diabetic rats with insulin replacement (DI) for 4 and 8 weeks were measured by Western blotting analysis. (B) Bars represent the relative protein quantification of calcineurin and NFAT3 normalized to α-tubulin, and indicate mean values $\pm$ SD ($n = 6$ in each group). $^{*}p < 0.05$, $^{**}p < 0.01$, significant differences from control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, significant differences from STZ group

trophic pathways were significantly attenuated after insulin replacement for 8 weeks. (4) Cardiac IGFIR, PI3K, and Akt were significantly decreased in diabetes compared to non-diabetes and these hyperglycemia-induced impaired insulin/IGFIR–PI3K–Akt pathways were improved after insulin replacement for 8 weeks, not for 4 weeks. After integrating our current findings into previously proposed theories, our hypothesis proposed that insulin replacement not only prevented hyperglycemia-induced cardiac mitochondrial-dependent apoptotic pathway and calcineurin-NFAT3-related hypertrophic pathway but also enhanced cardiac insulin/IGFIR–PI3K–Akt survival pathway (Figure 5).

The STZ-induced diabetes (hyperglycemic) rats present many of the same pathophysiologic deficits as noted in diabetic humans, such as hypoinsulinemia, hyperglycemia, atherosclerosis, cardiac hypertrophy, cardiomyopathy, cardiovascular dysfunction, and heart failure.[25–28] Diabetes predisposes to develop a multi-system disorder and had shown to be impacted by multiple factors, such as endocrine dysfunctions, metabolic disorders, oxidative stresses, inflammatory processes, or immune systems.[29,30] Therefore, in the current experimental design, we have to add a cautious note that any deleterious effect of STZ-induced diabetes or any preventive effect of insulin replacement on cardiac changes cannot be isolated to one specific factor or any specific system, but may be affected directly or indirectly by various factors, such as hypoinsulinemia, hyperglycemia, endocrine dysfunctions, oxidative stress, inflammatory status, autoimmune, or unclear interacting factors.

Diabetes is associated with a high incidence of cardiovascular disease which is the major cause of morbidity and mortality.[30] The presence of diabetes is a powerful risk factor for the development of cardiac hypertrophy, cavity dilation, and heart failure in humans.[4,31] Left ventricular dysfunction was significantly impaired in diabetic animals and humans.[4,32] Individuals with diabetes have an increased risk of developing heart failure although a specific diabetic cardiomyopathy, secondary to a microangiopathy, may also exist.[33] In addition, diabetes is an independent risk factor for chronic heart failure, probably in part due to disturbances in myocardial metabolism.[34] Characteristic features of chronic heart failure are progressive deterioration of the left ventricular function and the loss of cardiomyocytes via apoptosis or necrosis.[35] An 85-fold increase in cardiomyocyte apoptosis was found in diabetic human heart[4] and an aggregate 30% myocyte loss was found in diabetic rat heart.[5]

The mitochondrial-dependent apoptotic pathway is mediated by Bcl2 family, such as Bad and Bcl2.[36] Shifting the balance of Bcl2 family members toward pro-apoptotic effects will enhance cytochrome *c* release and will activate caspase-9 which further activates caspase-3 and executes the apoptotic program.[15] Prolonged exposure of the isolated neonatal cardiomyocyte cells to medium containing insulin and high glucose led to increased susceptibility to apoptosis with an increased Bax/Bcl-2 ratio.[37] Previous study shows increased apoptosis via increases in caspase-9 and caspase-3 activities in STZ-induced diabetic rat heart.[38] After infarction, the numbers of apoptotic cells and cardiac

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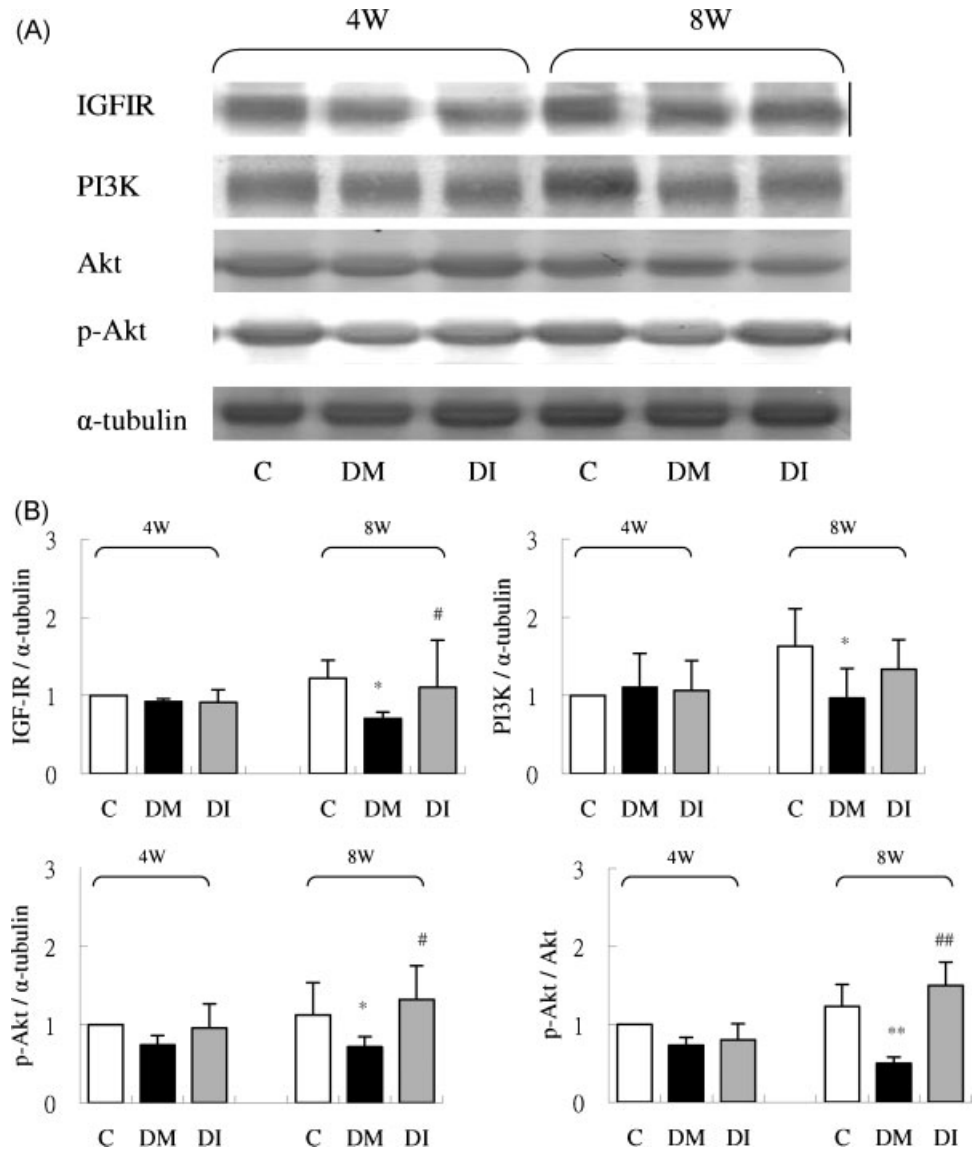

Figure 4. (A) The representative protein levels of insulin-like growth factor-I receptor (IGFIR), phosphatidylinositol 3'-kinase (PI3K), Akt, and phosphorylated Akt (p-Akt) extracted from the left ventricles of excised hearts in Wistar rats with citrate buffer (Control, C), streptozotocin (STZ)-induced diabetic rats with citrate buffer (DM), and STZ-induced diabetic rats with insulin replacement (DI) for 4 and 8 weeks were measured by Western blotting analysis. (B) Bars represent the relative protein quantification of IGFIR, PI3K, p-Akt, and p-Akt/Akt normalized to α-tubulin, and indicate mean values $\pm$ SD ($n = 6$ in each group). $^{*}p < 0.05$, significant differences from control group. $^{\#}p < 0.05$, significant differences from STZ group

caspase 3 were higher in the diabetic rats as compared to non-diabetic rats in the border zone of infarction and in non-infarcted area.[39] In the current study, after 8 weeks but not 4 weeks, the mitochondrial-dependent apoptotic pathways were significantly activated in cardiac tissues in diabetic rats and the hyperglycemia-induced mitochondrial-dependent apoptotic pathways were suppressed with the dosage of 4 IU insulin from a series of evidence, such as cardiac Bad, Bcl2, cytosolic cytochrome *c*, activated caspase-9, and activated caspase-3 levels. Therefore, our findings suggest that longer duration of diabetes did cause more mitochondrial-dependent apoptosis as well as insulin replacement significantly attenuated diabetes-induced cardiac mitochondrial-dependent apoptosis.

Calcineurin and its downstream transcriptional effector NFAT3 have been reported as a critical mediator for cardiac hypertrophy and cardiac myocyte apoptosis.[16,17] In addition, calcineurin will activate pro-apoptotic protein Bad and induce cytochrome *c* release from mitochondria to cause cardiomyocyte apoptosis.[20] Cardiac hypertrophy was previously observed in STZ-induced diabetic rats.[40] The current study represents the first to report that calcineurin-NFAT3 hypertrophic pathway and calcineurin-Bad related apoptotic pathway were activated in STZ-induced diabetes

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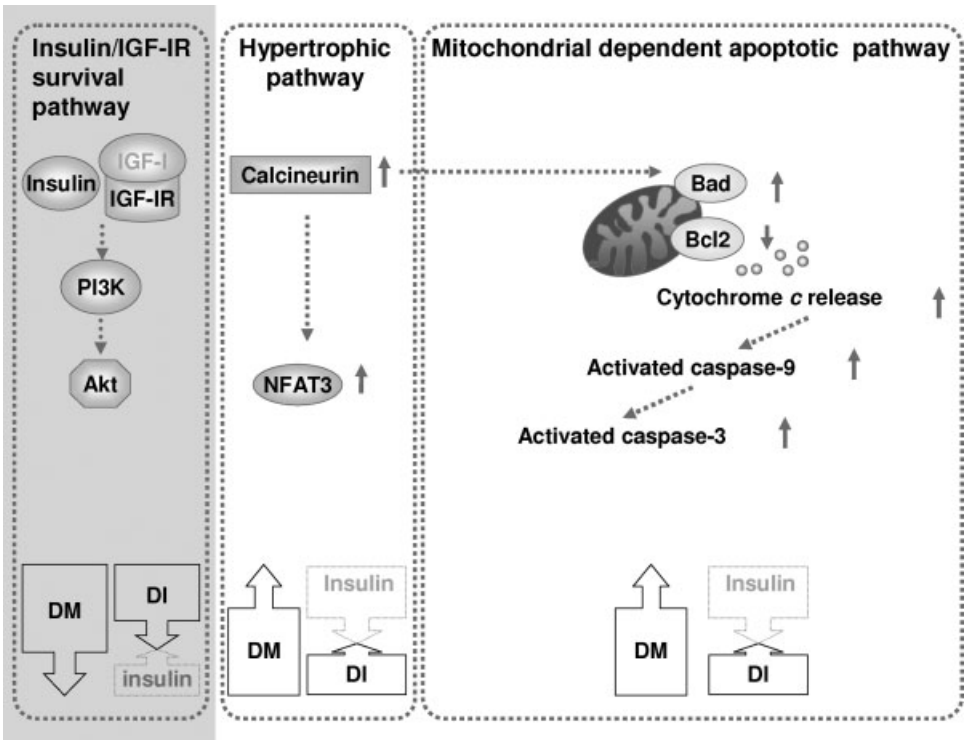

Figure 5. Our proposed hypothesis that cardiac mitochondrial-dependent apoptotic pathway may be more activated in diabetes due to decreased anti-apoptotic Bcl2, increased pro-apoptotic Bad, increased cytochrome c release, increased activated caspase-9, and increased caspase-3. These hyperglycemia-induced mitochondrial-dependent apoptotic pathways were significantly attenuated after insulin replacement (right column). Up arrows and down arrows on the right side represent increases and decreases, respectively. Calcineurin-NFAT3 hypertrophic pathways in diabetes may be more activated in diabetes and these hyperglycemia-induced calcineurin-NFAT3 hypertrophic pathways were significantly attenuated after insulin replacement (middle column). Insulin-like growth factor-I receptor (IGFIR), phosphatidylinositol 3'-kinase (PI3K), and the protein kinase B (Akt) may be more activated in diabetes and these hyperglycemia-induced impaired insulin/IGFIR related PI3K-Akt pathways were improved after insulin replacement (left column)

and insulin replacement significantly reversed the deleterious effects of diabetes.

Insulin/IGFIR and their downstream PI3K and Akt signaling pathways were reported to contribute to the modulation of survival responses in cardiac tissue.[22,23] High glucose in isolated neonatal cardiomyocytes was reported to diminish insulin signaling and reduce phospho-Akt levels.[37] One previous study suggests that exogenous IGF-I treatment may ameliorate contractile disturbances in cardiomyocytes from diabetic animals and could provide therapeutic potential in the treatment of diabetic cardiomyopathy.[41] The current study represents the first to report that impaired insulin/IGFIR–PI3K–Akt survival pathways were found in diabetic hearts and these impairments were significantly improved after insulin replacement.

Hypothesized clinical application

Since cardiac tissues are difficult to be obtained from diabetic patients, the findings of the current STZ-induced diabetic animal experiment should provide an important mechanism for explaining the diabetes-related cardiac diseases. If diabetes-related cardiac apoptosis and diabetes-related cardiac hypertrophy also occur in human, diabetic patients should be highly aware of the progressive development in cardiac abnormality and should actively promote cardiac health. The apoptotic, hypertrophic, and survival pathways might provide one possible mechanism to interrupt the development of heart failure and pathological cardiac hypertrophy. Of course, further clinical and experimental studies are required to clarify the apoptotic, hypertrophic, or survival mechanisms in human diabetic hearts.

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