Dynamic alteration of adiponectin/adiponectin receptor expression and its impact on myocardial ischemia/reperfusion in type 1 diabetic mice

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Ma Y, Liu Y, Liu S, Qu Y, Wang R, Xia C, Pei H, Lian K, Yin T, Lu X, Sun L, Yang L, Cao Y, Lau WB, Gao E, Wang H, Tao L. Dynamic alteration of adiponectin/adiponectin receptor expression and its impact on myocardial ischemia/reperfusion in type 1 diabetic mice. Am J Physiol Endocrinol Metab 301: E447–E455, 2011. First published May 17, 2011; doi:10.1152/ajpendo.00687.2010.—The present study determined the dynamic change of adiponectin (APN, a cardioprotective adipokine), its receptor expression, and their impact upon myocardial ischemia/reperfusion (MI/R) injury during type 1 diabetes mellitus (T1DM) progression, and involved underlying mechanisms. Diabetic state was induced in mice via multiple intraperitoneal injections of low-dose streptozotocin. The dynamic change of plasma APN concentration and cardiac APN receptor-1 and -2 (AdipoR1/2) expression were assessed immediately after diabetes onset (0 wk) and 1, 3, 5, and 7 wk thereafter. Indicators of MI/R injury (infarct size, apoptosis, and LDH release) were determined at 0, 1, and 7 wk of DM duration. The effect of APN on MI/R injury was determined in mice subjected to different diabetic durations. Plasma APN levels (total and HMW form) increased, whereas cardiac AdipoR1 expression decreased early after T1DM onset. With T1DM progression, APN levels were reduced and cardiac AdipoR1 expression increased. MI/R injury was exacerbated with T1DM progression in a time-dependent manner. Administration of globular APN (gAD) failed to attenuate MI/R injury in 1-wk T1DM mice, while an AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR) agonist reduced MI/R injury. However, administration of gAD and AICAR reduced infarct size and cardiomyocyte apoptosis in 7-wk T1DM mice. In conclusion, our results demonstrate a dynamic dysfunction of APN/AdipoR1 during T1DM progression. Reduced cardiac AdipoR1 expression and APN concentration may be responsible for increased MI/R injury susceptibility at early and late T1DM stages, respectively. Interventions bolstering AdipoR1 expression during early T1DM stages and APN supplementation during advanced T1DM stages may potentially reduce the myocardial ischemic injury in diabetic patients.

diabetes; AMP-activated protein kinase activator; apoptosis; adipokine

GLOBAL TYPE 1 DIABETES MELLITUS (T1DM) incidence increases 2–5% annually; in the United States, the prevalence of T1DM is ~1/300 by 18 years of age (16). Pancreatic inability to produce insulin is the root mechanism for T1DM, a lifelong disease. Although its onset is possible at any age, T1DM has a propensity for pediatric and young adult populations and portends poor prognosis concerning cardiovascular disease complications, the most prevalent cause of diabetes-associated morbidity and mortality (19).

T1DM patients and animal models manifest altered adipokine and metabolism profiles. Of primarily adipocyte origin, the protein adiponectin (APN) normally circulates at very high plasma concentrations (27). Attenuating inflammation and regulating glucose/lipid metabolism, APN additionally serves as an antiapoptotic adipokine (6, 28, 29). Increasing experimental evidence supports APN as a potential therapeutic molecule against cardiovascular disease, demonstrating a cardioprotective effect against myocardial ischemia-reperfusion (MI/R) injury (22, 25). APN exerts its effects primarily via two membrane receptors, APN receptor-1 and -2 (AdipoR1/2), mediating effects through AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR) (11).

Unlike obesity-linked diseases (such as coronary artery disease and type 2 diabetes), which manifest consistently reduced circulating APN levels, T1DM patients have been reported to harbor increased or decreased plasma APN concentrations (7, 8, 15, 17). Comprehensive information regarding dynamic fluctuations in APN levels during T1DM progression does not exist.

High-fat diet and obesity have been demonstrated to decrease APN concentration and AdipoR1/R2 expression levels, thereby reducing APN sensitivity. For instance, AdipoR1/2 expression was significantly decreased in high-fat-fed rats, resulting in reduced vascular responsiveness to APN treatment, leading to APN resistance (14). It is unknown whether APN resistance occurs in T1DM.

Therefore, the aims of the present study were 1) to determine whether APN concentration and APN receptor expression levels are altered in the well-established streptozotocin (STZ)-induced T1DM heart model (and the time-dependency of any observed alteration), and 2) to identify consequences of any observed dynamic change in APN/APN receptor levels in the cardioprotective effects of APN against MI/R injury.

MATERIALS AND METHODS

Experimental protocols. All experiments were performed in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Fourth Military Medical University Committee on Animal Care. Swiss mice (aged 6–8 wk) were used for the present study. The animals were housed in a temperature- and humidity controlled room with a 12:12-h light-dark cycle and were fed standard laboratory animal chow with free access to tap water. Diabetes was induced by intraperitoneal injection of 40
mg/kg STZ, diluted in citrate buffer (pH 4.5) for 5 consecutive days, and age-matched control mice were injected with an equal volume of citrate buffer. Diabetes onset was confirmed by hyperglycemia exceeding 300 mg/dl 10 days after initial STZ administration.

MI/R was performed immediately after diabetes onset (0 wk diabetes duration, 10 days after initial STZ injection) and after 1, 3, 5, and 7 wk of diabetes duration. Mice were anesthetized with 2% isoflurane, and myocardial infarction (MI) was produced by temporarily exteriorizing the heart via a left thoracic incision and placing a 6-0 silk suture slipknot around the left anterior descending coronary artery. After 30 min of MI, the slipknot was released, and the myocardium was reperfused for 3 or 24 h (for LDH release and infarct size assays). Ten minutes before reperfusion, mice were randomized to receive either vehicle (PBS, pH 7.5) or human recombinant gAd (2 µg/g) via intraperitoneal (ip) injection. Sham-operated control mice (sham MI/R) underwent the same surgical procedures except the suture placed under the left coronary artery was not tied. At the end of the reperfusion period, the ligature around the coronary artery was retracted, and 2% Evans Blue dye was injected into the left ventricular cavity. The heart was quickly excised, and the ischemic/reperfused cardiac tissue was isolated and processed per below protocols.

Measurement of myocardial infarct size. Twenty-four hours after reperfusion, mice were anesthetized and the hearts excised. Myocardial infarct size was determined by using Evans Blue/2,3,5-triphenyltetrazolium chloride (TTC) staining as previously described (24).

Assessment of myocardial injury. To quantitatively determine myocardial injury extent, blood samples were collected. Serum LDH release was measured per manufacturer’s protocol (NJJC, China). Values are expressed in international units per liter (5).

Determination of myocardial apoptosis. Myocardial apoptosis was determined via TUNEL staining and caspase-3 activity assay, inclusive of the entire ischemic/reperfused region commonly termed “area at risk” as described previously (24).

Quantitation of plasma total and high-molecular-weight APN concentration. Serum total and high-molecular-weight (HMW) APN concentrations were determined via mouse total and HMW APN ELISA kit (R&D Systems, Minneapolis, MN and Biovendor Laboratories, Czech Republic, respectively) per the manufacturer’s instructions.

Immunoblotting. Cardiac tissue homogenate proteins were separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and Western blotted with monoclonal antibody against AdipoR1 (Abcam, Cambridge, MA), AdipoR2 (LifeSpan Biosciences, Seattle, WA), and phosphorylated and total AMPK (Cell Signaling Technology, Danvers, MA). Nitrocellulose membranes were then incubated with HRP-conjugated antirabbit immunoglobulin G antibody (Santa Cruz Biotechnology) for 1 h. The blot was developed with an ECL-Plus chemiluminescence reagent kit and visualized with UVP Bio-Imaging Systems. Blot densities were analyzed with Vision Works LS Acquisition and Analysis Software.

Statistical analysis. All values in the text and figures are presented as means ± SD of n independent experiments. All data (except Western blot density) were subjected to ANOVA followed by Bonferroni correction for post hoc t-test. Western blot densities were analyzed with the Kruskal-Wallis test followed by Dunn post hoc test. P values <0.05 were considered statistically significant.

RESULTS

Dynamic changes in total and HMW plasma APN concentration during T1DM progression. Considerable evidence indicates that diabetic animals and patients are more sensitive to MI/R injury, with myocardial injury severity positively associated with duration of diabetic condition endured (3). Meanwhile, it has been demonstrated that APN acts as an antiapoptotic cytokine, exerting cardioprotection against MI/R injury (22, 25). Together, these data suggest the possibility of dynamically altering APN levels associated with T1DM progression. We assessed both the total and HMW isoforms of circulating APN, the latter reported to be the most active APN isoform in the STZ-induced T1DM mouse model (9). As shown in Fig. 1, A and B, plasma total APN levels markedly increased after 1-wk T1DM duration, which gradually decreased through the remainder of the study (7 wk). In a consistent fashion, augmented levels of the HMW APN isoform were observed after 1-wk T1DM duration, also gradually decreasing until the study’s conclusion (7 wk).

Dynamic change in cardiac APN receptor expression during T1DM progression. Because APN’s effects are mediated by its two membrane receptors AdipoR1 and R2, we determined any parallel alteration in their levels. AdipoR1 expression decreased dramatically after 1- and 3-wk T1DM duration, returning to control levels by 5 wk. By 7 wk, AdipoR1 expression increased beyond control mice levels (Fig. 2A). No significant difference in cardiac AdipoR2 protein was observed between control and diabetic mice via Western blot analysis (Fig. 2B).

Dynamic change in MI/R injury during T1DM progression. To investigate whether observed dynamic APN/APN receptor expression has association with MI/R injury, we determined
LDH release and infarct size after inducing myocardial ischemia. MI/R injury was augmented in a time-dependent fashion, evidenced by enhanced infarct size (Fig. 3A) and LDH release (Fig. 3B) as duration of T1DM increased.

Dynamic change in APN receptor expression after MI/R injury during T1DM progression. Previous studies have demonstrated that I/R injury decreases AdipoR1 expression (21), indicating the involvement of APN receptor with myocardial ischemic injury. Therefore, we investigated the dynamic change of APN receptor expression after MI/R injury during T1DM Progression. As illustrated in Fig. 4, MI/R decreased AdipoR1 expression (Fig. 4A) at 0-, 1-, and 7-wk T1DM duration, respectively. However, no significant difference in cardiac AdipoR2 protein (Fig. 4B) was observed after MI/R injury during T1DM progression.

APN supplementation has no effect upon infarct size after 1-wk T1DM duration. To determine whether decreased AdipoR1 expression is responsible for increased MI/R injury after 1-wk T1DM duration, we examined the effect of exogenous APN treatment on MI/R injury in the setting of reduced AdipoR1 expression after 1-wk T1DM duration. Male adult control or diabetic mice were subjected to MI/R as described above and treated with gAD 10 min before reperfusion. Infarct size and LDH release were determined. As illustrated in Fig. 5, exogenous gAD supplementation did not attenuate infarct size (Fig. 5A) or LDH release (Fig. 5B) exacerbated by MI/R in diabetic mice compared with controls.

AMPK is a downstream signaling molecule known to be partially responsible for APN/AdipoR1 cardioprotection. To further identify the mechanism responsible for impaired APN cardioprotection, we administrated an AMPK activator (AICAR) 10 min before reperfusion and determined infarct size and LDH release. As shown in Fig. 5A, AICAR administration significantly reduced both infarct size and LDH release compared with vehicle (Fig. 5B). These results suggest that reduced AdipoR1 expression may be responsible for impaired APN-mediated cardioprotection in the early stage of T1DM.

APN supplementation has no effect on cardiomyocyte apoptosis after 1-wk T1DM duration. Apoptosis plays a critical role in cardiomyocyte loss and is a major avenue of cell death (4). To investigate the apoptotic extent in the area-at-risk (AAR) region, we assessed cellular TUNEL positivity and caspase-3 activity. As shown in Fig. 6A, the proportion of TUNEL-positive cells was not significantly different between control and diabetic groups.

LDH release and infarct size after inducing myocardial ischemia. MI/R injury was augmented in a time-dependent fashion, evidenced by enhanced infarct size (Fig. 3A) and LDH release (Fig. 3B) as duration of T1DM increased.

Dynamic change in cardiac APN receptor expression during T1DM progression. Cardiac expression of AdipoR1 (A) and -R2 (B), as determined by Western blot analysis; n = 4–5/group. *P < 0.05, **P < 0.01 vs. Control.

Fig. 2. Dynamic change in cardiac APN receptor expression during T1DM progression. Cardiac expression of AdipoR1 (A) and -R2 (B), as determined by Western blot analysis; n = 4–5/group. *P < 0.05, **P < 0.01 vs. Control.

Fig. 3. Dynamic change in myocardial ischemia/reperfusion (MI/R) injury during T1DM progression. A: myocardial infarct size assessed by Evans blue/TTC (2,3,5-triphenyl-2H-tetrazolium chloride) double staining. Evans blue-stained areas (black) indicate nonischemic/reperfused area; TTC-stained areas (red) indicate ischemic but viable tissue; Evans blue/TTC staining negative areas indicate infarcted myocardium. Infarct size quantification expressed as the ratio of infarct area (Inf) to total ischemic/reperfused area (area-at-risk, AAR). B: Plasma LDH release determined by ELISA; n = 6–8 hearts/group. *P < 0.05 vs. 0-wk group; #P < 0.05, ##P < 0.01 vs. 1-wk group.
positive cells and caspase-3 activity significantly increased in diabetic mice after MI/R compared with sham, consistent with previous infarct size data. Exogenous gAD supplementation did not reduce cardiomyocyte apoptosis, evidenced by unchanged TUNEL-positive cardiomyocyte proportion (Fig. 6B) and caspase-3 activity (Fig. 6C). In addition to serving as a downstream APN signaling molecule, AMPK is known to protect against I/R injury (22). AICAR treatment markedly attenuated the I/R-induced TUNEL-positive cardiomyocyte proportion (Fig. 6B) and caspase-3 activity (Fig. 6C).

APN supplementation decreased cardiomyocyte apoptosis after 7-wk T1DM duration. Apoptotic extent was determined by TUNEL staining and caspase-3 activity. Representative photographs revealed a higher TUNEL-positive cardiomyocyte proportion in diabetic mouse myocardium after MI/R injury, which was significantly reduced by gAD treatment (Fig. 8). Although caspase-3 activity dramatically increased after MI/R injury, gAD treatment significantly blocked it (Fig. 8C). AICAR exhibited similar potency to gAD in reducing cardiomyocyte apoptosis. Reduction in cardiomyocyte apoptosis by gAD and AICAR treatment was associated with increased levels of phosphorylated AMPK (pAMPK) in diabetic mice (Fig. 8D).

**DISCUSSION**

In the present study, we have evaluated the alteration of cardiac sensitivity to adiponectin. We have demonstrated that, during the early stage of T1DM, exogenous gAD supplementation is ineffective in reducing I/R injury. However, with T1DM progression, we have found that exogenous gAD’s cardioprotection is restored due to the increased AdipoR1 expression. Although cardiac sensitivity to exogenous adi-
Adiponectin is an adipokine, secreted nearly exclusively by adipocytes, which forms multimers and circulates in the serum.

Fig. 6. gAD supplementation failed to reduce cardiomyocyte apoptosis after 1-wk T1DM duration. A: myocardial apoptosis determined by TUNEL staining. TUNEL staining (green) indicates apoptotic nuclei; DAPI counterstaining (blue) indicates total nuclei. B: quantification of apoptotic nuclei. TUNEL-positive nuclei are expressed as % total number of nuclei, automatically counted and calculated by Image-Pro Plus software. C: myocardial apoptosis determined by caspase-3 activity assay. D: pAMPK/AMPK expression determined by Western blot analysis; n = 4–5/group for TUNEL and caspase-3 assay, n = 6–8/group for Western blot analysis. **P < 0.01 vs. DM1+sham mice; #P < 0.05 vs. vehicle-treated mice.

Adiponectin is increased, endogenous adiponectin levels are decreased, ultimately resulting in time-dependent exacerbated MI/R injury if untreated. Over all, we have demonstrated first, direct evidence of a dynamic change of APN and its receptors and second, the impact of such changes on gAD cardioprotection during different T1DM stages in the setting of MI/R injury.
We have demonstrated for the first time that HMW adiponectin, whose levels are most predictive of insulin resistance (9), are decreased (13, 26), failing, however, to unveil the dynamic alteration in the progression of diabetes. We have provided direct evidence that APN concentration varies according to different stages of T1DM duration. Our data suggest that the dynamic change of APN concentration may be responsible for the contradictory clinical APN concentration data from T1DM patients.

APN exerts its effects primarily via two membrane receptors, AdipoR1 and AdipoR2, and mostly via stimulation of AMPK and PPAR (11). Therefore, contradictory clinical results about the relationship between APN and cardiovascular complications may be partly attributed to dysfunction of receptors for APN, thereby blocking physiological APN signaling. Although some studies have demonstrated that AdipoR expression level is decreased in the skeletal muscle of diabetic mice (1), few studies elucidate the dynamic change of cardiac AdipoR expression, which is essential in understanding the role of the APN/AdipoR system in diabetic complication progression. In this study, we found that AdipoR1 expression was significantly reduced 1 and 3 wk after successful establishment of T1DM. Interestingly, we also observed a restoration or even increased AdipoR1 expression at a later stage (5–7 wk) after establishment of T1DM. No significant difference in cardiac AdipoR2 was observed between control and diabetic mice.

Coronary artery disease is a major complication of diabetes mellitus, responsible for more than 50% of diabetic patient mortality (2). Diabetics suffer from increased incidence and severity of MI, and are more prone to heart failure than nondiabetics post-MI (10, 18). In our present study, we demonstrated T1DM-duration time-dependent exacerbation of MI/R injury (evidenced by cardiac infarct size and LDH release), consistent with previous reports (20). APN is a natural cardioprotective molecule against I/R injury. Ischemic injury has been shown to downregulate APN concentration and AdipoR1 expression in nondiabetic mice (21). Our present study revealed decreased levels of AdipoR1 expression after MI/R in both early and advanced T1DM stages. In the early stage, AdipoR1 expression was decreased, but circulating APN levels were increased. We speculated that increased APN expression may represent a compensatory cardioprotective mechanism elicited by significantly decreased AdipoR1 expression, compensating for potential downregulation of the adiponectin signaling system. However, this compensatory APN upregulation appears unable to achieve complete efficacy, as evidenced by an increased MI/R injury. To verify the hypothesis, we conducted the following experiment. Exogenous APN was administered to the 1-wk T1DM mice 10 min before reperfusion. However, APN administration failed to reduce infarct size and cardiomyocyte apoptosis. AMPK is a key molecule mediating the cardioprotective actions of adiponectin. Furthermore, AICAR, an adenosine analog, which activates AMPK through direct binding, decreased MI/R injury in 1-wk T1DM mice. Our results indicate that the decreased AdipoR1 expression may be responsible for the loss of APN’s cardioprotective effect in the early T1DM stages. With diabetic progression, AdipoR1 expression was increased, but circulating APN decreased. Both APN and AICAR administration decreased the MI/R injury in 7-wk T1DM mice. T1DM is a metabolic disorder associated with
massive reduction in adipose mass (23). We observed a confirmatory decrease of visceral animal fat with T1DM progression (data not provided). Adiponectin is an adipokine secreted exclusively by adipocytes (12). Therefore, adiponectin concentration decrease may stem from attenuated adiposity, with AdipoR1 upregulation serving a compensatory function. Our results indicate that the decreased circulating APN is the key factor exacerbating MI/R injury.

Fig. 8. gAD supplementation reduced cardiomyocyte apoptosis after 7-wk T1DM duration. A: myocardial apoptosis determined by TUNEL staining. TUNEL staining (green) indicates apoptotic nuclei; DAPI counterstaining (blue) indicates total nuclei. B: quantification of apoptotic nuclei. TUNEL-positive nuclei are expressed as % total number of nuclei, automatically counted and calculated by Image-Pro Plus software. C: myocardial apoptosis determined by caspase-3 activity assay. D: pAMPK/AMPK expression determined by Western-blot analysis; n = 6–8/group for TUNEL and caspase-3 assay, n = 4–5/group for Western blot analysis. **P < 0.01 vs. DM7 + sham mice; #P < 0.05, ##P < 0.01 vs. vehicle-treated mice.
in the advanced T1DM stages. More importantly, our results demonstrate that decreased endogenous APN production in late T1DM stages renders cardiomyocytes more susceptible to I/R injury than AdipoR1 downregulation in the early T1DM stages.

It should be noted that the current findings of dynamic expression alteration in APN and its receptors in a T1DM mouse model may not readily be applied to type 2 diabetic models, which possess a distinctly different genetic profile and metabolic properties. Similarly, we cannot apply the pattern of APN expression change in cardiac tissue to noncardiac tissues (i.e., skeletal muscle and adipose tissue), as the highly aerobic heart is unique, subjected to a delicate balance of prooxidant production and antioxidant defense, processes in which various APN system components are believed to be key regulators. Further studies are required to comprehensively determine the alteration of APN and its receptors during type 2 diabetes progression and any subsequent impact on APN’s cardioprotective efficacy.

Taken together, our results demonstrate for the first time that there is systemic APN dysfunction in T1DM potentially contributive to cardiovascular injury via different mechanisms during different T1DM stages. In the early T1DM stages both endogenous and exogenous APN failed to provide cardioprotection against I/R injury, likely due to significantly reduced cardiac AdipoR1 expression. In contrast, although cardiac AdipoR1 expression gradually returned to normal during the later T1DM stages endogenous APN production significantly attenuated, again rendering cardiomyocytes more susceptible to I/R injury. Accordingly, divergent strategies must be developed to restore APN cardioprotection during different stages of T1DM. Specifically, therapeutic strategies capable of bolstering AdipoR1 expression might be more cardioprotective during the early T1DM stages, whereas exogenous APN administration may be more efficacious during late T1DM stages to mitigate MI/R injury.

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