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Dynamic Alteration of Adiponectin/Adiponectin Receptor Expression and Its Impact on Myocardial Ischemia/Reperfusion in Type1 Diabetic Mice

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Running title: Adiponectin and diabetic myocardial injury

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Abstract

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 T1DM progression, and involved underlying mechanisms. Diabetic state was induced in mice via multiple intraperitoneal injections of low-dose streptozotocin (STZ). The dynamic change of plasma APN concentration and cardiac APN receptor-1 and -2 (AdipoR1, 2) expression were assessed immediately after diabetes onset (0 week), and 1, 3, 5, and 7 weeks thereafter. Indicators of MI/R injury (infarct size, apoptosis, and LDH release) were determined at 0, 1, and 7weeks of DM duration. The effect of APN upon MI/R injury was determined in mice subjected to different diabetic durations. Plasma APN levels (total and HMW form) increased while cardiac AdipoR1 expression decreased early after T1DM onset. With T1DM progression, APN levels reduced, and cardiac AdipoR1 expression increased. MI/R injury exacerbated with T1DM progression in a time-dependent manner. Administration of globular APN (gAD) failed to attenuate MI/R injury in 1-week T1DM mice, while an AMP-activated protein kinase (AMPK) activator (AICAR) reduced MI/R injury. However, administration of gAD (and AICAR) reduced infarct size and cardiomyocyte apoptosis in 7-week T1DM mice. In conclusion, our results demonstrate a dynamic dysfunction of APN/AdipoR1 during T1DM progression. Reduced cardiac AdipoR1 expression and APN concentration may respectively be responsible for increased I/R injury susceptibility at early and late T1DM stages. 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.

Keywords

Diabetes; Ischemia/reperfusion; Adiponectin receptor; AMP-activated protein kinase

Introduction

Global type1 diabetes mellitus (T1DM) incidence increases 2% to 5% annually; in the United States, the prevalence of T1DM is approximately 1/300 by 18 years of age (16). Pancreatic inability to produce insulin is the root mechanism for T1DM, a lifelong disease. Though its onset is possible at any age, T1DM has propensity for pediatric and young adult populations, and portends poor prognosis concerning cardiovascular disease complications, the most prevalent cause of diabetic-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 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 and 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 type1 diabetic heart model (and the time-dependency of any observed alteration); 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 weeks) were used for the present study. The animals were housed in a temperature- and humidity controlled room with a 12:12-hour 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 week diabetes duration, 10 days after initial STZ injection), and after 1, 3, 5, and 7 weeks 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 minutes of MI, the slipknot was released, and the myocardium was reperfused for 3 hours or 24 hours (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 (2μg/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 retied, 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 were 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 were expressed in international units (U) 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 HMW APN Concentration

Serum total and HMW APN concentrations were determined via mouse total and HMW APN ELISA kit (R&D Systems, Minneapolis, MN; Biovendor Laboratories Ltd, 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, Inc, Seattle, WA), phosphorylated AMPK and total AMPK (Cell Signaling Technology, Danvers, MA). Nitrocellulose membranes were then incubated with HRP-conjugated antirabbit immunoglobulin G antibody (Santa Cruz Biotechnology, Inc) for 1 hour. 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 mean±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 high-molecular-weight (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 anti-apoptotic 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, and the latter reported to be the most active APN isoform in the STZ-induced T1DM mouse model (9). As shown in **Figures 1A and 1B**, plasma total APN levels markedly increased after 1-week T1DM duration, which gradually decreased through the remainder of the study (7 weeks). In a consistent fashion, augmented levels of the HMWAPN isoform were observed after 1-week T1DM duration, also gradually decreasing until the study's conclusion (7 weeks).

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- week T1DM duration, returning to control levels by 5 weeks. By 7 weeks, AdipoR1 expression increased beyond control mice levels **(Figure 2A).** No significant difference in cardiac AdipoR2 protein was observed between control and diabetic mice via Western blot analysis **(Figure 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 **(Figure 3A)** and LDH release **(Figure 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 **Figure 4**, MI/R decreased AdipoR1 expression **(Figure 4A)** at 0 , 1 and 7weeks of T1DM duration, respectively. However, no significant difference in cardiac AdipoR2 protein **(Figure 4B)** was observed after MI/R injury during T1DM progression.

APN supplementation has no effect upon infarct size after 1-week T1DM duration

To determine whether decreased AdipoR1 expression is responsible for increased MI/R injury after 1 week T1DM duration, we examined the effect of exogenous APN treatment upon MI/R injury in the setting of reduced AdipoR1 expression after 1-week T1DM duration. Male adult control or diabetic mice were subjected to MI/R as described above, and treated with gAD 10 minutes before reperfusion. Infarct size and LDH release were determined. As illustrated in **Figure 5**, exogenous gAD supplementation did not attenuate infarct size (**Figure 5A**) or LDH release (**Figure 5B**) exacerbated by MI/R in diabetic mice compared to control.

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 AMPK activator (AICAR) 10 minutes before reperfusion, and determined infarct size and LDH release. As shown in **Figure 5A**, AICAR administration significantly reduced both infarct size and LDH release compared to vehicle (**Figure 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 upon cardiomyocyte apoptosis after 1-week 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 **Figure 6,** the proportion of TUNEL-positive cells and caspase-3 activity significantly increased in diabetic mice after MI/R compared to sham, consistent with previous infarct size data. Exogenous gAD supplementation did not reduce cardiomyocyte apoptosis, evidenced by unchanged TUNEL-positive cardiomyocyte proportion (**Figure 6B)** and caspase-3 activity (**Figure 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 (**Figure 6B)** and caspase-3 activity (**Figure 6C)**.

APN supplementation attenuated infarct size after 7-week T1DM duration

Our present study demonstrated that APN expression levels decreased while AdipoR1 levels were augmented after 7-week T1DM duration. To determine whether the decreased APN concentration was responsible for increased observed MI/R injury after 7- week T1DM duration, we investigated the effect of exogenous APN supplementation upon MI/R injury at this time period. As shown in **Figure 7A**, exogenous gAD treatment markedly decreased infarct size and LDH release compared to vehicle (**Figure 7B).** AICAR administration yielded similar cardioprotection as gAD against MI/R injury (**Figure 7**).

APN supplementation decreased cardiomyocyte apoptosis after 7-week 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 (**Figure 8)**. While caspase-3 activity dramatically increased after MI/R injury, gAD treatment significantly blocked it (**Figure 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 (**Figure 8D).**

Discussion

In the present study, we have evaluated the alteration of cardiac sensitivity to adiponectin. During the early stage of T1DM, we demonstrated that exogenous gAD supplementation is ineffective in reducing I/R injury. However, with T1DM progression, we found that exogenous gAD's cardioprotection is restored due to the increased AdipoR1 expression. Although cardiac sensitivity to exogenous adiponectin is increased, endogenous adiponectin levels are decreased, ultimately resulting in time-dependent exacerbated MI/R injury if untreated. Overall, 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.

Adiponectin is an adipokine secreted nearly exclusively by adipocytes, which forms multimers and circulates in the serumin trimeric, hexameric, or HMW forms (23). Plasma adiponectin levels are useful markers for insulin sensitivity, and are markedly downregulated in association with obesity-linked diseases such as coronary artery disease or T2DM. However, discrepant data exists regarding APN concentrations in T1DM patients, as both elevated and decreased APN concentrations have been reported (7, 8, 15,17). More importantly, the relationship between APN and cardiovascular complications in T1DM are also contradictory. Some studies have reported that T1DM patients harboring elevated adiponectin concentrations suffered increased risk of microvascular complications (4, 5), whereas others have shown the opposite (6,7). The apparent inconsistency of this data may be explained by comprehension of dynamic APN level changes during T1DM progression. Our present study demonstrated that total plasma APN levels markedly increased after 1-week T1DM duration, which gradually decreased through the study remainder (7 weeks). HMW adiponectin has been reported to be the most biologically active APN isoform, whose levels are most predictive of insulin resistance(9). We have demonstrated for the first time that HMWAPN isoform levels are augmented after 1-week T1DM duration, gradually attenuating throughout the 7-week study duration, consistent with the total APN concentration trend. Previous results from the

STZ-treated diabetic model have also demonstrated that both the HMWAPN isoform and total APN are decreased (13, 26), however failing 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 suggests 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, clinical contradictory results about the relationship between APN and cardiovascular complications may be partly attributed to dysfunction of receptors for APN, thereby blocking physiologic APN signaling. Although some studies have demonstrated 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 weeks after successful establishment of T1DM. Interestingly, we also observed a restoration or even increased AdipoR1 expression at a later stage (5 to 7 weeks) 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 >50% of diabetic patient mortality (2). Diabetics suffer from increased incidence and severity of MI, and are more prone to heart failure compared to non-diabetics 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 non-diabetic 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 administrated to the 1-week T1DM mice 10 minutes before reperfusion. However, APN administration failed to reduce infarct size and cardiomyoctye apoptosis. AMPK is a key molecule mediating the cardioprotective actions of adiponectin. Furthermore, AICAR, an adenosine analogue, which activates AMPK through direct binding, decreased MI/R injury in 1-week 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 stage. With diabetic progression, AdipoR1 expression was increased, but circulating APN decreased. Both APN and AICAR administration decreased the MI/R injury in 7-week T1DM mice. T1DM is a metabolic disorder associated with massive reduction in adipose mass (23). We observed 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 in the advanced T1DM stages. More importantly, our results demonstrated 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 indicated 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 non-cardiac tissues (i.e. skeletal muscle and adipose tissue), as the highly aerobic heart is unique, subjected to a delicate balance of pro-oxidant production and antioxidant defense, processes in which various APN system

components are believed to be key regulators. Further studies are required to comprehensively determine alteration of APN and its receptors during type 2 diabetes progression, and any subsequent impact upon APN's cardioprotective efficacy.

Taken together, our results demonstrated 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 stage, 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 stage, endogenous APN production significantly attenuated, again rendering cardiomyocyte 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.

References

1. **Bonnard C, Durand A, Vidal H, and Rieusset J**. Changes in adiponectin, its receptors and AMPK activity in tissues of diet-induced diabetic mice. *Diabetes & Metabolism* 34: 52-61, 2008.

2. **Chiariello M, and Indolfi C**. Silent myocardial ischemia in patients with diabetes mellitus. *Circulation* 93: 2089-2091, 1996.

3. **Chowdhry MF, Vohra HA, and Galinanes M**. Diabetes increases apoptosis and necrosis in both ischemic and nonischemic human myocardium: role of caspases and poly-adenosine diphosphate-ribose polymerase. *J Thorac Cardiovasc Surg* 134: 124-131, 131 e121-123, 2007.

4. **Eefting F, Rensing B, Wigman J, Pannekoek WJ, Liu WM, Cramer MJ, Lips DJ, and Doevendans PA**. Role of apoptosis in reperfusion injury. *Cardiovasc Res* 61: 414-426, 2004.

5. **Fan Q, Gao F, Zhang L, Christopher TA, Lopez BL, and Ma XL**. Nitrate tolerance aggravates postischemic myocardial apoptosis and impairs cardiac functional recovery after ischemia. *Apoptosis* 10: 1235-1242, 2005.

6. **Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, and Lodish HF**. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci U S A* 98: 2005-2010, 2001.

7. **Galler A, Gelbrich G, Kratzsch J, Noack N, Kapellen T, and Kiess W**. Elevated serum levels of adiponectin in children, adolescents and young adults with type 1 diabetes and the impact of age, gender, body mass index and metabolic control: a longitudinal study. *Eur J Endocrinol* 157: 481-489, 2007.

8. **Guo Z, Xia Z, Yuen VG, and McNeill JH**. Cardiac expression of adiponectin and its receptors in streptozotocin-induced diabetic rats. *Metabolism* 56: 1363-1371, 2007.

9. **Hara K, Horikoshi M, Yamauchi T, Yago H, Miyazaki O, Ebinuma H, Imai Y, Nagai R, and Kadowaki T**. Measurement of the high-molecular weight form of adiponectin in plasma is useful for the prediction of insulin resistance and metabolic syndrome. *Diabetes Care* 29: 1357-1362, 2006.

10. **Ingelsson E, Sundstrom J, Arnlov J, Zethelius B, and Lind L**. Insulin resistance and risk of congestive heart failure. *JAMA* 294: 334-341, 2005.

11. **Kadowaki T, and Yamauchi T**. Adiponectin and adiponectin receptors. *Endocr Rev* 26: 439-451, 2005.

12. **Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, and Tobe K**. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *The Journal of Clinical Investigation* 116: 1784-1792, 2006.

13. **Kusmic C, L'Abbate A, Sambuceti G, Drummond G, Barsanti C, Matteucci M, Cao J, Piccolomini F, Cheng J, and Abraham NG**. Improved myocardial perfusion in chronic diabetic mice by the up-regulation of pLKB1 and AMPK signaling. *J Cell Biochem* 109: 1033-1044, 2010.

14. **Li R, Xu M, Wang X, Wang Y, Lau WB, Yuan Y, Yi W, Wei X, Lopez BL, Christopher TA, Wang XM, and Ma XL**. Reduced vascular responsiveness to adiponectin in hyperlipidemic rats--mechanisms and significance. *J Mol Cell Cardiol* 49: 508-515, 2010.

15. **Lindstrom T, Frystyk J, Hedman CA, Flyvbjerg A, and Arnqvist HJ**. Elevated circulating adiponectin in type 1 diabetes is associated with long diabetes duration. *Clin Endocrinol (Oxf)* 65: 776-782, 2006.

16. **Maahs DM, West NA, Lawrence JM, and Mayer-Davis EJ**. Epidemiology of type 1 diabetes. *Endocrinol Metab Clin North Am* 39: 481-497, 2010.

17. **Morales A, Wasserfall C, Brusko T, Carter C, Schatz D, Silverstein J, Ellis T, and Atkinson M**. Adiponectin and leptin concentrations may aid in discriminating disease forms in children and adolescents with type 1 and type 2 diabetes. *Diabetes Care* 27: 2010-2014, 2004.

18. **Orlander PR, Goff DC, Morrissey M, Ramsey DJ, Wear ML, Labarthe DR, and Nichaman MZ**. The relation of diabetes to the severity of acute myocardial infarction and post-myocardial infarction survival in Mexican-Americans and non-Hispanic whites. The Corpus Christi Heart Project. *Diabetes* 43: 897-902, 1994.

19. **Pambianco G, Costacou T, Ellis D, Becker DJ, Klein R, and Orchard TJ**. The 30-year natural history of type 1 diabetes complications: the Pittsburgh Epidemiology of Diabetes Complications Study experience. *Diabetes* 55: 1463-1469, 2006.

20. **Paulson DJ**. The diabetic heart is more sensitive to ischemic injury. *Cardiovasc Res* 34: 104-112, 1997.

21. **Saito Y, Fujioka D, Kawabata K, Kobayashi T, Yano T, Nakamura T, Kodama Y, Takano H, Kitta Y, Obata JE, and Kugiyama K**. Statin reverses reduction of adiponectin receptor expression in infarcted heart and in TNF-alpha-treated cardiomyocytes in association with improved glucose uptake. *Am J Physiol Heart Circ Physiol* 293: H3490-3497, 2007.

22. **Shibata R, Sato K, Pimentel DR, Takemura Y, Kihara S, Ohashi K, Funahashi T, Ouchi N, and Walsh K**. Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms. *Nat Med* 11: 1096-1103, 2005.

23. **Takada J, Machado MA, Peres SB, Brito LC, Borges-Silva CN, Costa CEM, Fonseca-Alaniz MH, Andreotti S, and Lima FB**. Neonatal streptozotocin-induced diabetes mellitus: a model of insulin resistance associated with loss of adipose mass. *Metabolism* 56: 977-984, 2007.

24. **Tao L, Gao E, Bryan NS, Qu Y, Liu HR, Hu A, Christopher TA, Lopez BL, Yodoi J, Koch WJ, Feelisch M, and Ma XL**. Cardioprotective effects of thioredoxin in myocardial ischemia and reperfusion: role of S-nitrosation [corrected]. *Proc Natl Acad Sci U S A* 101: 11471-11476, 2004.

25. **Tao L, Gao E, Jiao X, Yuan Y, Li S, Christopher TA, Lopez BL, Koch W, Chan L, Goldstein BJ, and Ma XL**. Adiponectin cardioprotection after myocardial ischemia/reperfusion involves the reduction of oxidative/nitrative stress. *Circulation* 115: 1408-1416, 2007.

26. **Vu V, Kim W, Fang X, Liu YT, Xu A, and Sweeney G**. Coculture with primary visceral rat adipocytes from control but not streptozotocin-induced diabetic animals increases glucose uptake in rat skeletal muscle cells: role of adiponectin. *Endocrinology* 148: 4411-4419, 2007.

27. **Whitehead JP, Richards AA, Hickman IJ, Macdonald GA, and Prins JB**. Adiponectin--a key adipokine in the metabolic syndrome. *Diabetes Obes Metab* 8: 264-280, 2006.

28. **Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, and Kadowaki T**. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 8: 1288-1295, 2002.

29. **Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, and Kadowaki T**. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 7: 941-946, 2001.

Figure Legends:

Figure 1 Dynamic change in plasma levels of total and high-molecular-weight (HMW) APN isoforms during T1DM progression. Total (A) and HMW (B) plasma APN levels were determined by ELISA after various T1DM durations. N=6 to 8/group. **P*<0.05, ***P*<0.01 versus Control ,$^{\#}$*P*<0.05, $^{\#\#}$*P*<0.01 versus 1- week group.

Figure 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 to 5/group. **P*<0.05, ***P*<0.01 versus Control , $^{\#}$*P*<0.05, $^{\#\#}$*P*<0.01 versus 1- week group.

Figure 3 Dynamic change in MI/R injury during T1DM progression. (A) Myocardial infarct size assessed by Evans blue/TTC double staining. Evans blue stained areas (black) indicate non-ischemic/reperfused area; 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) stained areas (red staining) 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 to 8 hearts /group. **P*<0.05 versus 0- week group , $^{\#}$*P*<0.05, $^{\#\#}$*P*<0.01 versus 1- week group.

Figure 4 Dynamic change in APN receptor expression after M/IR injury during T1DM progression. Cardiac expression of AdipoR1 (A) and R2 (B), as determined by Western-blot analysis. N=4 to 5/group. **P*<0.05versus sham at 0- week group, $^{\#}$*P*<0.05versus sham at 1- week group, $^{\$}$*P*<0.05versus sham at 7- week group.

Figure 5 gAD supplementation failed to reduce MI/R injury after 1-week T1DM duration. (A) Myocardial infarct size assessed by Evans blue/TTC double staining. Evans blue stained areas (black) indicate non-ischemic/reperfused area;

2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) stained areas (red staining) 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 to 8 hearts /group. $^{**}P<0.01$ versus DM1+sham. $^{\#}P<0.05$ versus vehicle treated mice.

Figure 6 gAD supplementation failed to reduce cardiomyocyte apoptosis after 1-week 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 a percentage of the 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 to 8/group for TUNEL and caspase-3 assay. N=4 to 5/group for Western-blot analysis. $^{**}P<0.01$ versus DM1+sham mice. $^{\#}P<0.05$ versus vehicle treated mice.

Figure 7 gAD supplementation reduced MI/R injury after 7-week T1DM duration. (A) Myocardial infarct size assessed by Evans blue/TTC double staining. Evans blue stained areas (black) indicate non-ischemic/reperfused area; 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) stained areas (red staining) 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 to 8 hearts/group. $^{**}P<0.01$ versus DM7+sham mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus vehicle treated mice.

Figure 8 gAD supplementation reduced cardiomyocyte apoptosis after 7-week 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 a percentage of the 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 to 8/group for TUNEL and caspase-3 assay. N=4 to 5/group for Western-blot analysis. $^{**}P<0.01$ versus DM7+sham mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus vehicle treated mice.

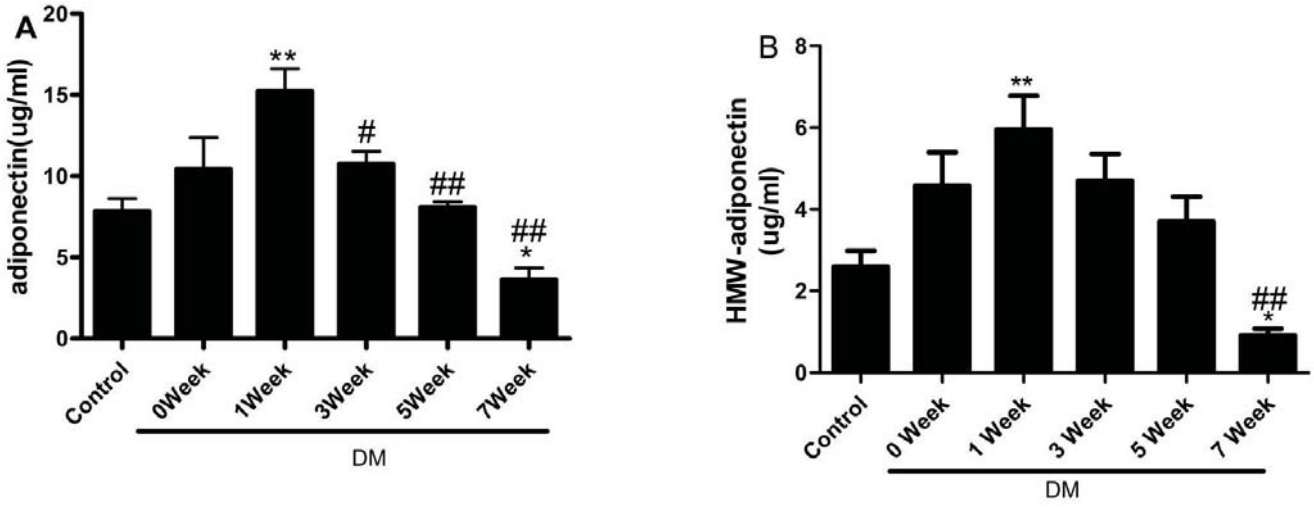
A
adiponectin(ug/ml)
20
15
10
5
0
**
#
##
##
*
Control
0Week
1Week
3Week
5Week
7Week
DM
B
HMW-adiponectin (ug/ml)
8
6
4
2
0
**
##
*
Control
0 Week
1 Week
3 Week
5 Week
7 Week
DM

Figure 1

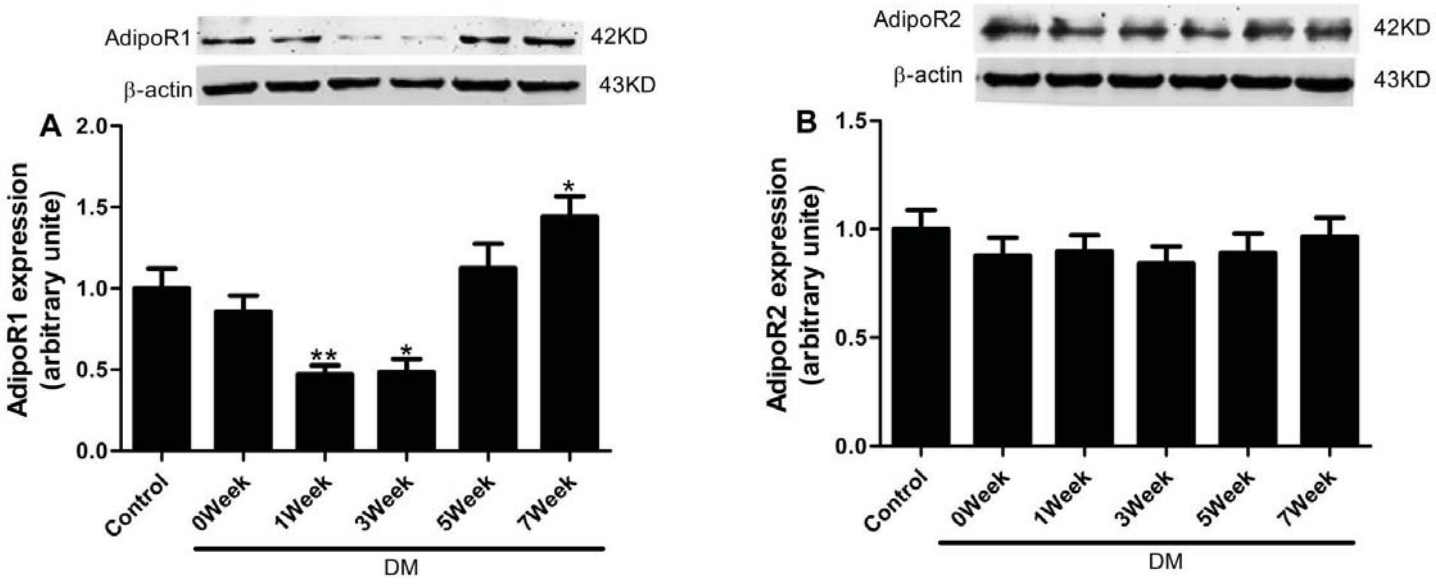
AdipoR1
42KD
β-actin
43KD
A
AdipoR1 expression (arbitrary unite)
2.0
1.5
1.0
0.5
0.0
**
*
*
Control
0Week
1Week
3Week
5Week
7Week
DM
AdipoR2
42KD
β-actin
43KD
B
AdipoR2 expression (arbitrary unite)
1.5
1.0
0.5
0.0
Control
0Week
1Week
3Week
5Week
7Week
DM

Figure 2

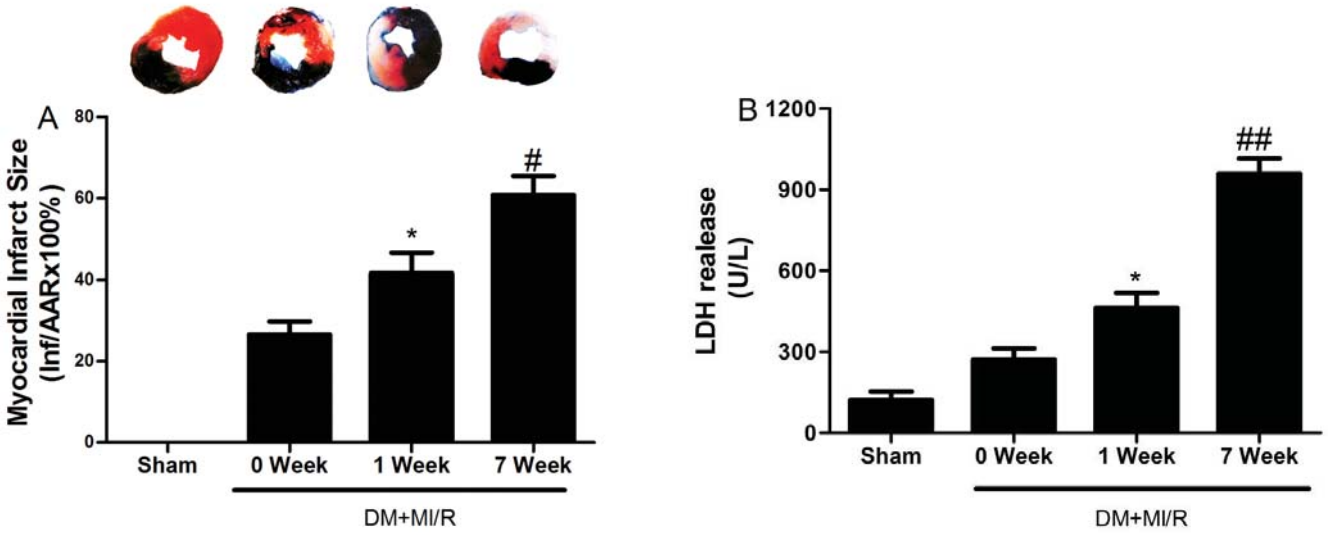
A
Myocardial Infarct Size
(Inf/AARx100%)
80
60
40
20
0
*
#
Sham
0 Week
1 Week
7 Week
DM+MI/R
B
LDH realease
(U/L)
1200
900
600
300
0
*
##
Sham
0 Week
1 Week
7 Week
DM+MI/R

Figure 3

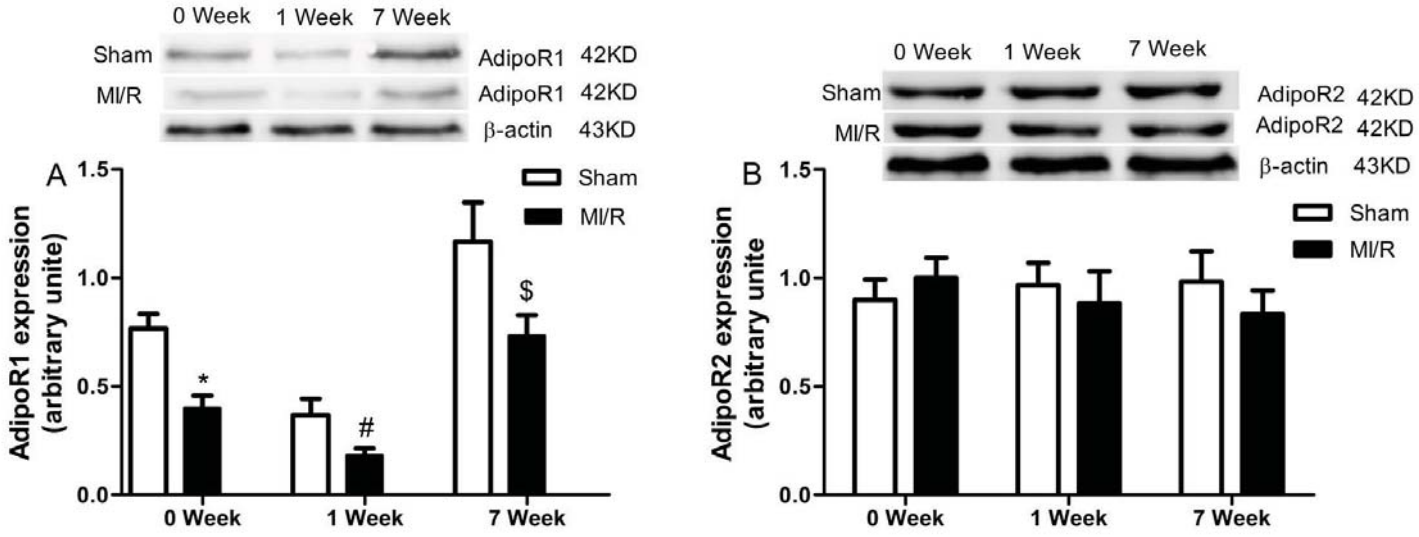
0 Week
1 Week
7 Week
Sham
MI/R
AdipoR1 42KD
AdipoR1 42KD
β-actin 43KD
A
AdipoR1 expression (arbitrary unite)
1.5
1.0
0.5
0.0
Sham
MI/R
*
#
$
0 Week
1 Week
7 Week
0 Week
1 Week
7 Week
Sham
MI/R
AdipoR2 42KD
AdipoR2 42KD
β-actin 43KD
B
AdipoR2 expression (arbitrary unite
1.5
1.0
0.5
0.0
Sham
MI/R
0 Week
1 Week
7 Week

Figure 4

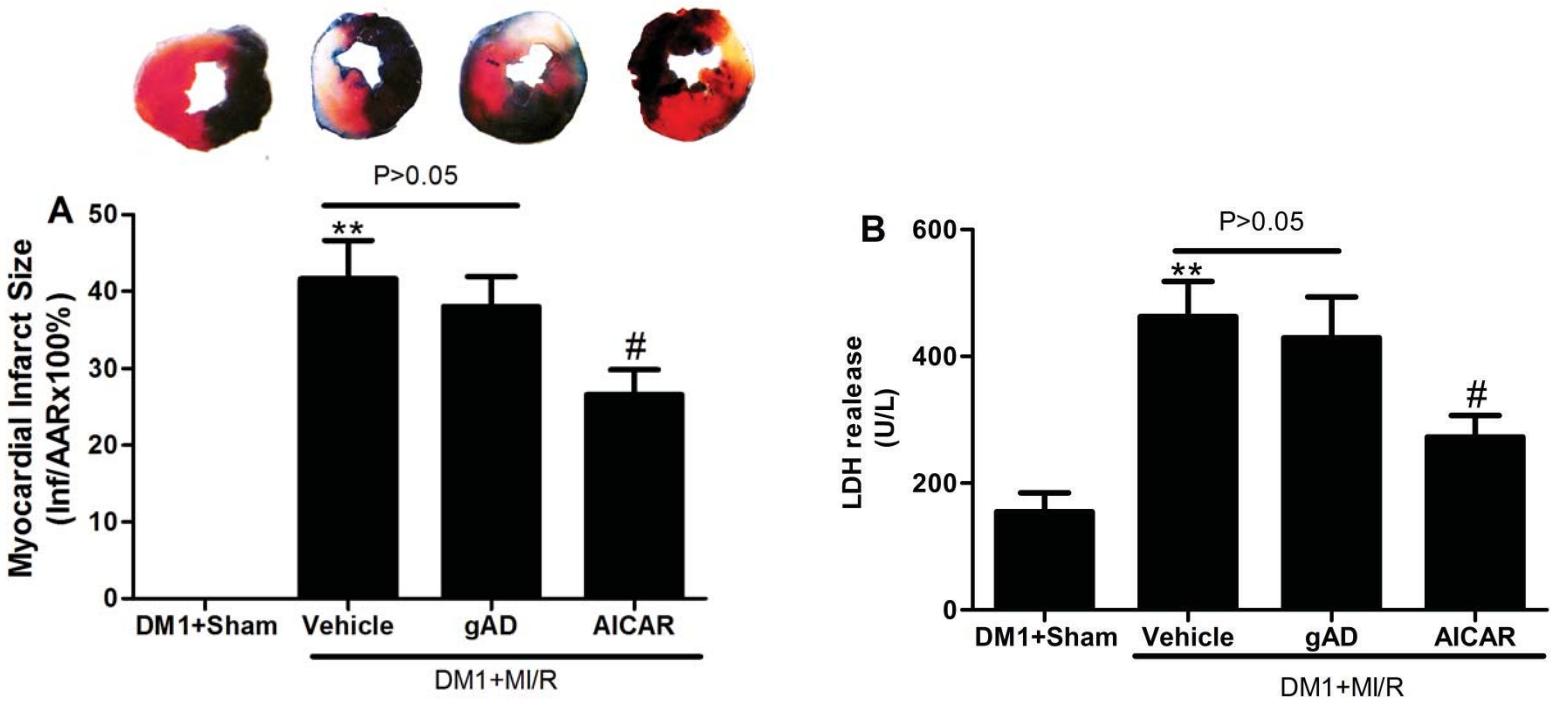
A
Myocardial Infarct Size
(Inf/AARx100%)
50
40
30
20
10
0
P>0.05
**
#
DM1+Sham
Vehicle
gAD
AICAR
DM1+MI/R
B
LDH realease
(U/L)
600
400
200
0
P>0.05
**
#
DM1+Sham
Vehicle
gAD
AICAR
DM1+MI/R

Figure 5

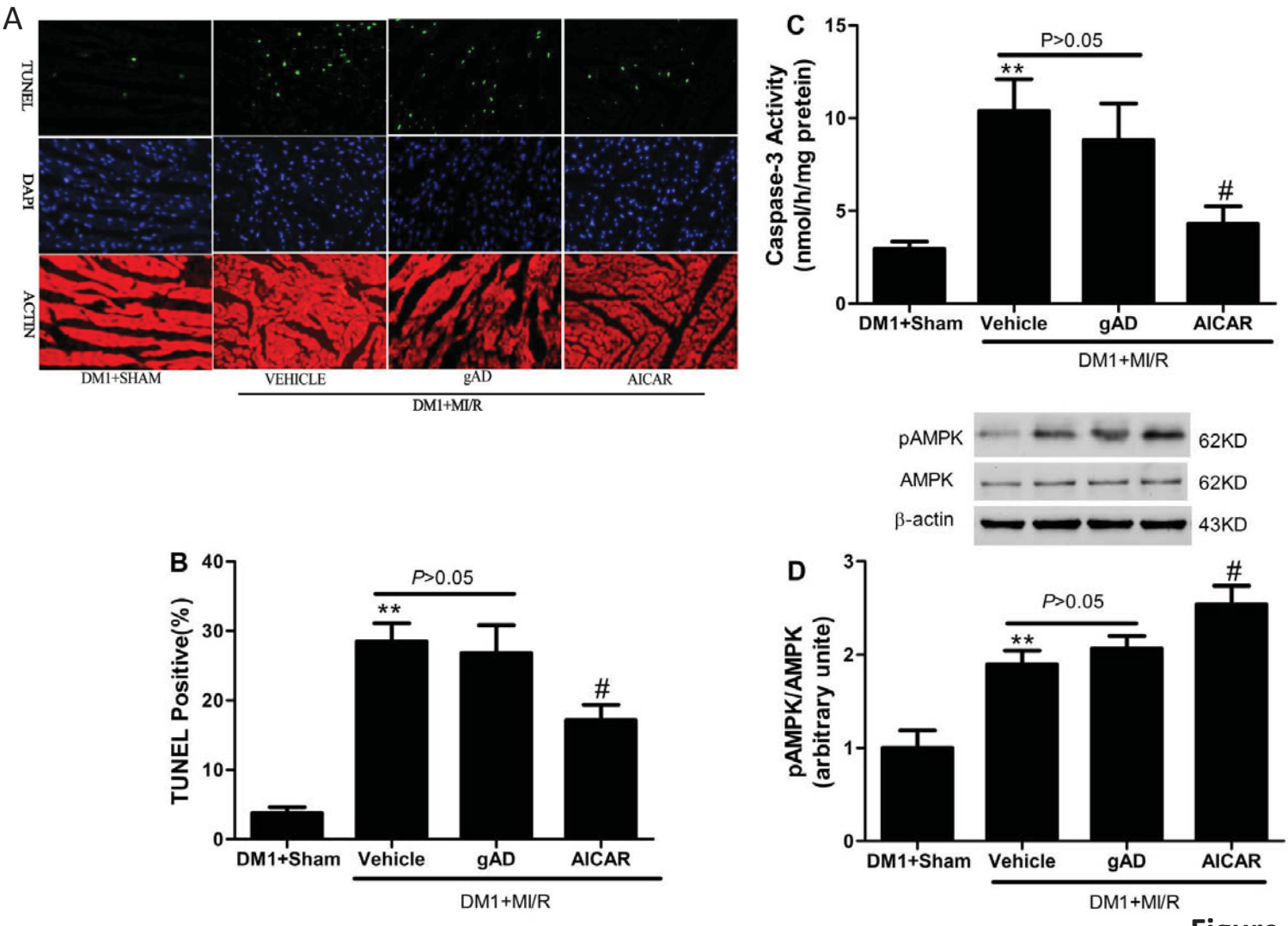
A
TUNEL
DAPI
ACTIN
DM1+SHAM
VEHICLE
gAD
AICAR
DM1+MI/R
C
Caspase-3 Activity (nmol/h/mg pretein)
P>0.05
**
#
DM1+Sham
Vehicle
gAD
AICAR
DM1+MI/R
pAMPK
AMPK
β-actin
62KD
62KD
43KD
B
TUNEL Positive(%)
P>0.05
**
#
DM1+Sham
Vehicle
gAD
AICAR
DM1+MI/R
D
pAMPK/AMPK (arbitrary unite)
P>0.05
**
#
DM1+Sham
Vehicle
gAD
AICAR
DM1+MI/R

Figure 6

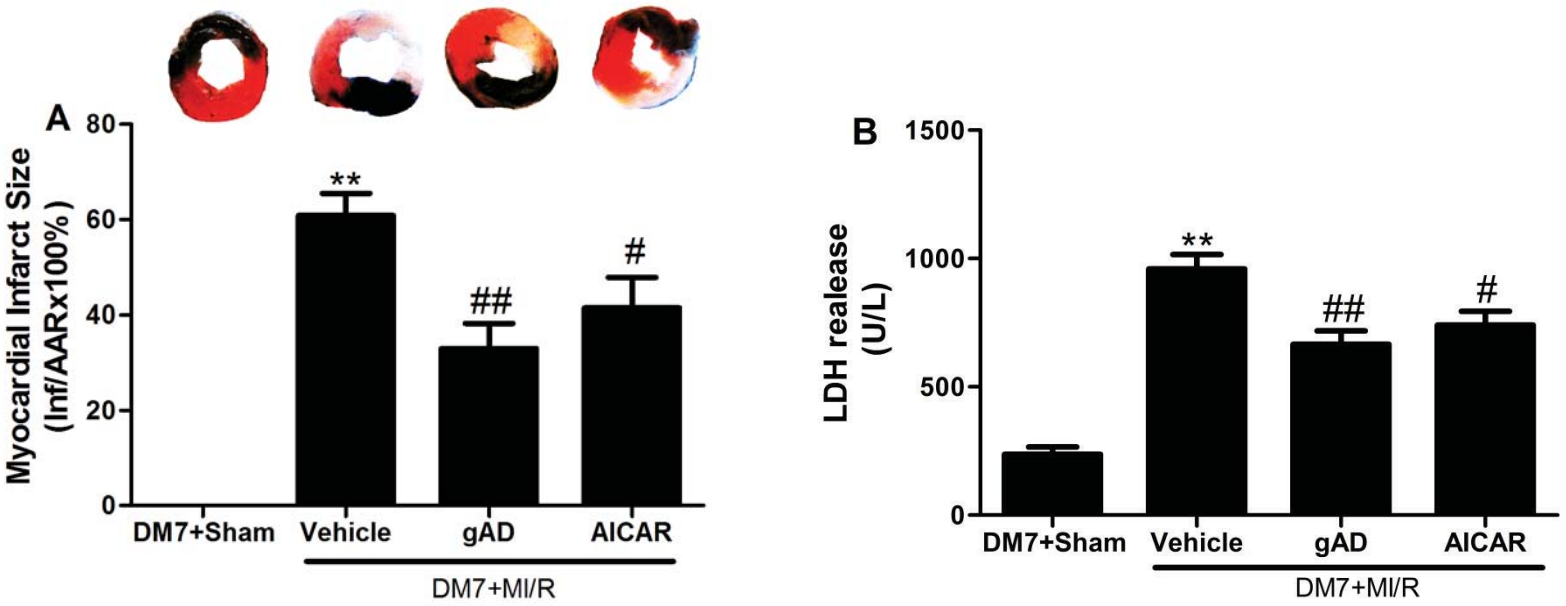
A
Myocardial Infarct Size
(Inf/AARx100%)
80
60
40
20
0
**
##
#
DM7+Sham
Vehicle
gAD
AICAR
DM7+MI/R
B
LDH realease
(U/L)
1500
1000
500
0
**
##
#
DM7+Sham
Vehicle
gAD
AICAR
DM7+MI/R

Figure 7

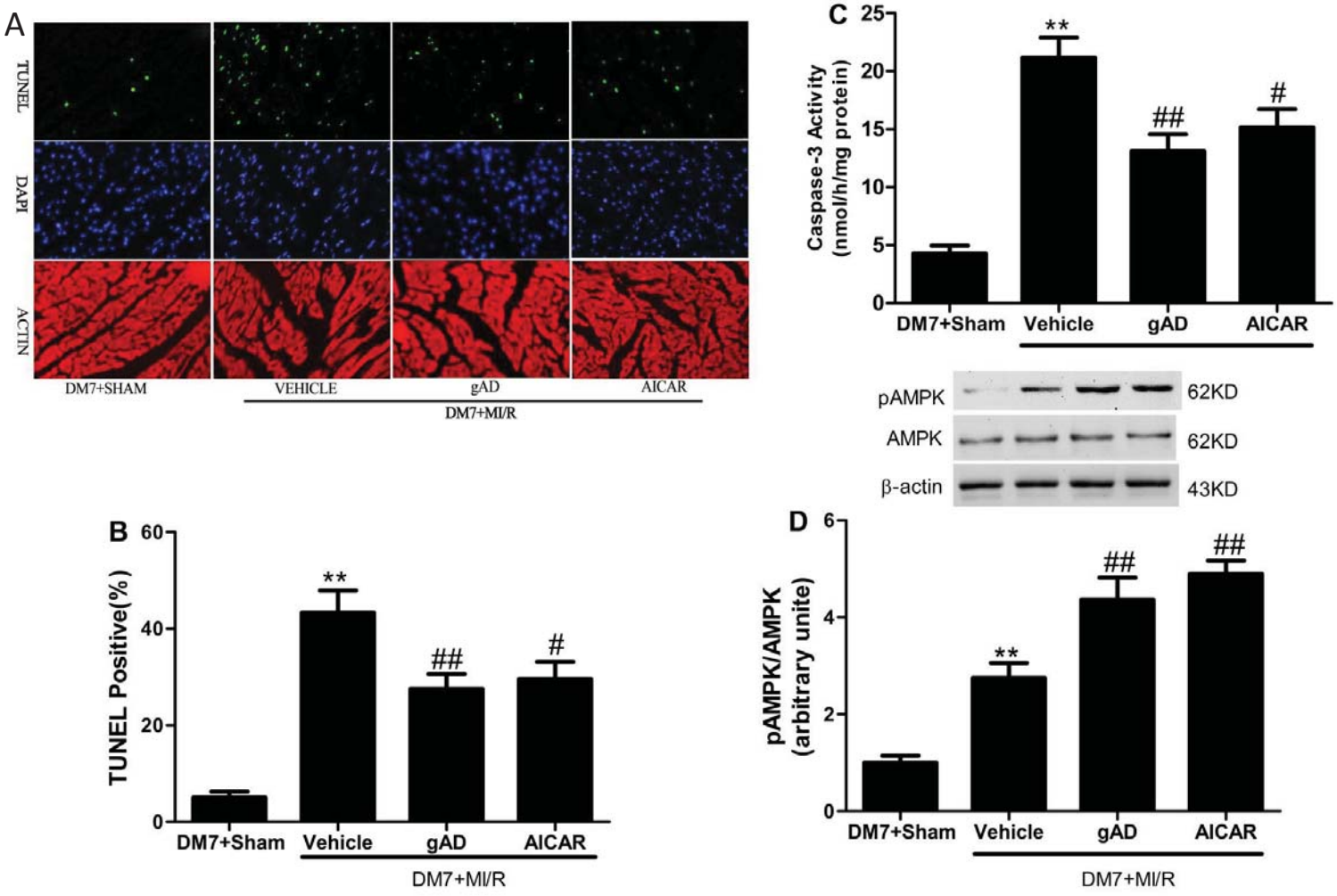
A
TUNEL
DAPI
ACTIN
DM7+SHAM
VEHICLE
gAD
AICAR
DM7+MI/R
B
TUNEL Positive(%)
60
40
20
0
**
##
#
DM7+Sham
Vehicle
gAD
AICAR
DM7+MI/R
C
Caspase-3 Activity (nmol/h/mg protein)
25
20
15
10
5
0
**
##
#
DM7+Sham
Vehicle
gAD
AICAR
pAMPK
AMPK
β-actin
62KD
62KD
43KD
D
pAMPK/AMPK (arbitrary unite)
6
4
2
0
**
##
##
DM7+Sham
Vehicle
gAD
AICAR
DM7+MI/R

Figure 8