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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 7 weeks 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

72 **Introduction**

73 Global type1 diabetes mellitus (T1DM) incidence increases 2% to 5% annually; in the
74 United States, the prevalence of T1DM is approximately 1/300 by 18 years of age
75 (16). Pancreatic inability to produce insulin is the root mechanism for T1DM, a
76 lifelong disease. Though its onset is possible at any age, T1DM has propensity for
77 pediatric and young adult populations, and portends poor prognosis concerning
78 cardiovascular disease complications, the most prevalent cause of diabetic-associated
79 morbidity and mortality (19).

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

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

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

100 Therefore, the aims of the present study were 1) to determine whether APN
101 concentration and APN receptor expression levels are altered in the well-established

102 streptozotocin (STZ)-induced type1 diabetic heart model (and the time-dependency of
103 any observed alteration); 2) to identify consequences of any observed dynamic change
104 in APN/APN receptor levels in the cardioprotective effects of APN against MI/R
105 injury.

106

107

108

109 **Materials and Methods**

110 **Experimental Protocols**

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

121 MI/R was performed immediately after diabetes onset (0 week diabetes duration,
122 10 days after initial STZ injection), and after 1, 3, 5, and 7 weeks diabetes duration.
123 Mice were anesthetized with 2% isoflurane, and myocardial infarction (MI) was
124 produced by temporarily exteriorizing the heart via a left thoracic incision and placing
125 a 6-0 silk suture slipknot around the left anterior descending coronary artery. After 30
126 minutes of MI, the slipknot was released, and the myocardium was reperfused for 3
127 hours or 24 hours (for LDH release and infarct size assays). Ten minutes before
128 reperfusion, mice were randomized to receive either vehicle (PBS, pH 7.5) or human
129 recombinant gAD (2 μ g/g) via intraperitoneal (IP) injection. Sham-operated control
130 mice (sham MI/R) underwent the same surgical procedures, except the suture placed
131 under the left coronary artery was not tied. At the end of the reperfusion period, the
132 ligature around the coronary artery was retied, and 2% Evans Blue dye was injected
133 into the left ventricular cavity. The heart was quickly excised, and the
134 ischemic/reperfused cardiac tissue was isolated and processed per below protocols.

135 **Measurement of Myocardial Infarct Size**

136 Twenty-four hours after reperfusion, mice were anesthetized, and the hearts were
137 excised. Myocardial infarct size was determined by using Evans

138 blue/2,3,5-triphenyltetrazolium chloride (TTC) staining as previously described (24) .

139 **Assessment of Myocardial Injury**

140 To quantitatively determine myocardial injury extent, blood samples were collected.

141 Serum LDH release was measured per manufacturer's protocol (NJJC, China). Values

142 were expressed in international units (U) per liter (5).

143 **Determination of Myocardial Apoptosis**

144 Myocardial apoptosis was determined via TUNEL staining and caspase-3 activity

145 assay, inclusive of the entire ischemic/reperfused region commonly termed

146 "area-at-risk" as described previously (24).

147 **Quantitation of Plasma Total and HMW APN Concentration**

148 Serum total and HMW APN concentrations were determined via mouse total and

149 HMW APN ELISA kit (R&D Systems, Minneapolis, MN; Biovondor Laboratories

150 Ltd, Czech Republic, respectively) per the manufacturer's instructions.

151 **Immunoblotting**

152 Cardiac tissue homogenate proteins were separated on SDS-PAGE gels, transferred

153 to nitrocellulose membranes, and Western blotted with monoclonal antibody against

154 AdipoR1 (Abcam, Cambridge, MA), AdipoR2 (LifeSpan Biosciences, Inc, Seattle,

155 WA), phosphorylated AMPK and total AMPK (Cell Signaling Technology, Danvers,

156 MA). Nitrocellulose membranes were then incubated with HRP-conjugated antirabbit

157 immunoglobulin G antibody (Santa Cruz Biotechnology, Inc) for 1 hour. The blot was

158 developed with an ECL-Plus chemiluminescence reagent kit and visualized with UVP

159 Bio-Imaging Systems. Blot densities were analyzed with Vision Works LS

160 Acquisition and Analysis Software.

161 **Statistical Analysis**

162 All values in the text and figures are presented as mean $\pm$ SD of n independent

163 experiments. All data (except Western blot density) were subjected to ANOVA

164 followed by Bonferroni correction for post hoc t test. Western blot densities were

165 analyzed with the Kruskal-Wallis test followed by Dunn post hoc test. P values <0.05

166 were considered statistically significant.

167

168 **Results**

169 **Dynamic changes in total and high-molecular-weight (HMW) plasma APN** 170 **concentration during T1DM progression**

171 Considerable evidence indicates that diabetic animals and patients are more
172 sensitive to MI/R injury, with myocardial injury severity positively associated with
173 duration of diabetic condition endured (3). Meanwhile, it has been demonstrated that
174 APN acts as an anti-apoptotic cytokine, exerting cardioprotection against MI/R injury
175 (22, 25). Together, these data suggest the possibility of dynamically altering APN
176 levels associated with T1DM progression. We assessed both the total and HMW
177 isoforms of circulating APN, and the latter reported to be the most active APN
178 isoform in the STZ-induced T1DM mouse model (9). As shown in **Figures 1A and**
179 **1B**, plasma total APN levels markedly increased after 1-week T1DM duration, which
180 gradually decreased through the remainder of the study (7 weeks). In a consistent
181 fashion, augmented levels of the HMWAPN isoform were observed after 1-week
182 T1DM duration, also gradually decreasing until the study's conclusion (7 weeks).

183 **Dynamic change in cardiac APN receptor expression during T1DM progression**

184 Because APN's effects are mediated by its two membrane receptors AdipoR1 and
185 R2, we determined any parallel alteration in their levels. AdipoR1 expression
186 decreased dramatically after 1- and 3- week T1DM duration, returning to control
187 levels by 5 weeks. By 7 weeks, AdipoR1 expression increased beyond control mice
188 levels (**Figure 2A**). No significant difference in cardiac AdipoR2 protein was
189 observed between control and diabetic mice via Western blot analysis (**Figure 2B**).

190 **Dynamic change in MI/R injury during T1DM progression**

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

196 **Dynamic change in APN receptor expression after MI/R injury during T1DM** 197 **progression**

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

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

207 To determine whether decreased AdipoR1 expression is responsible for increased
208 MI/R injury after 1 week T1DM duration, we examined the effect of exogenous APN
209 treatment upon MI/R injury in the setting of reduced AdipoR1 expression after
210 1-week T1DM duration. Male adult control or diabetic mice were subjected to MI/R
211 as described above, and treated with gAD 10 minutes before reperfusion. Infarct size
212 and LDH release were determined. As illustrated in **Figure 5**, exogenous gAD
213 supplementation did not attenuate infarct size (**Figure 5A**) or LDH release (**Figure**
214 **5B**) exacerbated by MI/R in diabetic mice compared to control.

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

223 **APN supplementation has no effect upon cardiomyocyte apoptosis after 1-week**
224 **T1DM duration**

225 Apoptosis plays a critical role in cardiomyocyte loss, and is a major avenue of cell
226 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**).

257 **Discussion**

258 In the present study, we have evaluated the alteration of cardiac sensitivity to
259 adiponectin. During the early stage of T1DM, we demonstrated that exogenous gAD
260 supplementation is ineffective in reducing I/R injury. However, with T1DM
261 progression, we found that exogenous gAD's cardioprotection is restored due to the
262 increased AdipoR1 expression. Although cardiac sensitivity to exogenous adiponectin
263 is increased, endogenous adiponectin levels are decreased, ultimately resulting in
264 time-dependent exacerbated MI/R injury if untreated. Overall, we have demonstrated:
265 first, direct evidence of a dynamic change of APN and its receptors; and second, the
266 impact of such changes on gAD cardioprotection during different T1DM stages in the
267 setting of MI/R injury.

268 Adiponectin is an adipokine secreted nearly exclusively by adipocytes, which
269 forms multimers and circulates in the serum in trimeric, hexameric, or HMW forms
270 (23). Plasma adiponectin levels are useful markers for insulin sensitivity, and are
271 markedly downregulated in association with obesity-linked diseases such as coronary
272 artery disease or T2DM. However, discrepant data exists regarding APN
273 concentrations in T1DM patients, as both elevated and decreased APN concentrations
274 have been reported (7, 8, 15,17). More importantly, the relationship between APN and
275 cardiovascular complications in T1DM are also contradictory. Some studies have
276 reported that T1DM patients harboring elevated adiponectin concentrations suffered
277 increased risk of microvascular complications (4, 5), whereas others have shown the
278 opposite (6,7). The apparent inconsistency of this data may be explained by
279 comprehension of dynamic APN level changes during T1DM progression. Our
280 present study demonstrated that total plasma APN levels markedly increased after
281 1-week T1DM duration, which gradually decreased through the study remainder (7
282 weeks). HMW adiponectin has been reported to be the most biologically active APN
283 isoform, whose levels are most predictive of insulin resistance(9). We have
284 demonstrated for the first time that HMWAPN isoform levels are augmented after
285 1-week T1DM duration, gradually attenuating throughout the 7-week study duration,
286 consistent with the total APN concentration trend. Previous results from the

287 STZ-treated diabetic model have also demonstrated that both the HMWAPN isoform
288 and total APN are decreased (13, 26), however failing to unveil the dynamic alteration
289 in the progression of diabetes. We have provided direct evidence that APN
290 concentration varies according to different stages of T1DM duration. Our data
291 suggests that the dynamic change of APN concentration may be responsible for the
292 contradictory clinical APN concentration data from T1DM patients.

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

306 Coronary artery disease is a major complication of diabetes mellitus, responsible
307 for >50% of diabetic patient mortality (2). Diabetics suffer from increased incidence
308 and severity of MI, and are more prone to heart failure compared to non-diabetics
309 post-MI (10,18). In our present study, we demonstrated T1DM-duration
310 time-dependent exacerbation of MI/R injury (evidenced by cardiac infarct size and
311 LDH release), consistent with previous reports(20). APN is a natural cardioprotective
312 molecule against I/R injury. Ischemic injury has been shown to downregulate APN
313 concentration and AdipoR1 expression in non-diabetic mice (21). Our present study
314 revealed decreased levels of AdipoR1 expression after MI/R in both early and
315 advanced T1DM stages. In the early stage, AdipoR1 expression was decreased, but
316 circulating APN levels were increased. We speculated that increased APN expression

317 may represent a compensatory cardioprotective mechanism elicited by significantly
318 decreased AdipoR1 expression, compensating for potential downregulation of the
319 adiponectin signaling system. However, this compensatory APN upregulation appears
320 unable to achieve complete efficacy, as evidenced by an increased MI/R injury. To
321 verify the hypothesis, we conducted the following experiment. Exogenous APN was
322 administrated to the 1-week T1DM mice 10 minutes before reperfusion. However,
323 APN administration failed to reduce infarct size and cardiomyocyte apoptosis. AMPK
324 is a key molecule mediating the cardioprotective actions of adiponectin. Furthermore,
325 AICAR, an adenosine analogue, which activates AMPK through direct binding,
326 decreased MI/R injury in 1-week T1DM mice. Our results indicate that the decreased
327 AdipoR1 expression may be responsible for the loss of APN's cardioprotective effect
328 in the early T1DM stage. With diabetic progression, AdipoR1 expression was
329 increased, but circulating APN decreased. Both APN and AICAR administration
330 decreased the MI/R injury in 7-week T1DM mice. T1DM is a metabolic disorder
331 associated with massive reduction in adipose mass (23). We observed confirmatory
332 decrease of visceral animal fat with T1DM progression (data not provided).
333 Adiponectin is an adipokine secreted exclusively by adipocytes (12). Therefore,
334 adiponectin concentration decrease may stem from attenuated adiposity, with
335 AdipoR1 upregulation serving a compensatory function. Our results indicate that the
336 decreased circulating APN is the key factor exacerbating MI/R injury in the advanced
337 T1DM stages. More importantly, our results demonstrated that decreased endogenous
338 APN production in late T1DM stages renders cardiomyocytes more susceptible to I/R
339 injury than AdipoR1 downregulation in the early T1DM stages.

340 It should be indicated that the current findings of dynamic expression alteration in
341 APN and its receptors in a T1DM mouse model may not readily be applied to type
342 2 diabetic models, which possess a distinctly different genetic profile and metabolic
343 properties. Similarly, we cannot apply the pattern of APN expression change in
344 cardiac tissue to non-cardiac tissues (i.e. skeletal muscle and adipose tissue), as the
345 highly aerobic heart is unique, subjected to a delicate balance of pro-oxidant
346 production and antioxidant defense, processes in which various APN system

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

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

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453 **Figure Legends:**

454 **Figure 1 Dynamic change in plasma levels of total and high-molecular-weight**
455 **(HMW) APN isoforms during T1DM progression.** Total (A) and HMW (B) plasma
456 APN levels were determined by ELISA after various T1DM durations. N=6 to
457 8/group. * $P<0.05$, ** $P<0.01$ versus Control, # $P<0.05$, ## $P<0.01$ versus 1- week group.

458
459 **Figure 2 Dynamic change in cardiac APN receptor expression during T1DM**
460 **progression.** Cardiac expression of AdipoR1 (A) and R2 (B), as determined by
461 Western-blot analysis. N=4 to 5/group. * $P<0.05$, ** $P<0.01$ versus Control, # $P<0.05$,
462 ## $P<0.01$ versus 1- week group.

463
464 **Figure 3 Dynamic change in MI/R injury during T1DM progression.** (A)
465 Myocardial infarct size assessed by Evans blue/TTC double staining. Evans blue
466 stained areas (black) indicate non-ischemic/reperfused area;
467 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) stained areas (red staining) indicate
468 ischemic but viable tissue; Evans blue/TTC staining negative areas indicate infarcted
469 myocardium. Infarct size quantification expressed as the ratio of infarct area (Inf) to
470 total ischemic/reperfused area (area-at-risk, AAR). (B) Plasma LDH release
471 determined by ELISA. N=6 to 8 hearts /group. * $P<0.05$ versus 0- week group ,
472 # $P<0.05$, ## $P<0.01$ versus 1- week group.

473
474 **Figure 4 Dynamic change in APN receptor expression after M/IR injury during**
475 **T1DM progression.** Cardiac expression of AdipoR1 (A) and R2 (B), as determined
476 by Western-blot analysis. N=4 to 5/group. * $P<0.05$ versus sham at 0- week group,
477 # $P<0.05$ versus sham at 1- week group, \$ $P<0.05$ versus sham at 7- week group.

478
479 **Figure 5 gAD supplementation failed to reduce MI/R injury after 1-week T1DM**
480 **duration.** (A) Myocardial infarct size assessed by Evans blue/TTC double staining.
481 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

512 staining (green) indicates apoptotic nuclei; DAPI counterstaining (blue) indicates total
513 nuclei. (B) Quantification of apoptotic nuclei. TUNEL-positive nuclei are expressed
514 as a percentage of the total number of nuclei, automatically counted and calculated by
515 Image-Pro Plus software. (C) Myocardial apoptosis determined by caspase-3 activity
516 assay. (D) pAMPK/AMPK expression determined by Western-blot analysis. N=6 to
517 8/group for TUNEL and caspase-3 assay. N=4 to 5/group for Western-blot analysis.
518 ** $P < 0.01$ versus DM7+sham mice. [#] $P < 0.05$, ^{##} $P < 0.01$ versus vehicle treated mice.

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Figure 1

Figure 2

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Figure 4

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Figure 8