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## AMP-Activated Protein Kinase Deficiency Enhances Myocardial Ischemia/Reperfusion Injury but Has Minimal Effect on the Antioxidant/Antinitrative Protection of Adiponectin

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### Abstract

**Background**—Diabetes increases the morbidity/mortality of ischemic heart disease, but the underlying mechanisms are incompletely understood. Deficiency of both AMP-activated protein kinase (AMPK) and adiponectin occurs in diabetes, but whether AMPK is cardioprotective or a central mediator of adiponectin cardioprotection in vivo remains unknown.

**Methods and Results**—Male adult mice with cardiomyocyte-specific overexpression of a mutant AMPK $\alpha_2$  subunit (AMPK-DN) or wild-type (WT) littermates were subjected to in vivo myocardial ischemia/reperfusion (MI/R) and treated with vehicle or adiponectin. In comparison to WT, AMPK-DN mice subjected to MI/R endured greater cardiac injury (larger infarct size, more apoptosis, and poorer cardiac function) likely as a result of increased oxidative stress in these animals. Treatment of AMPK-DN mice with adiponectin failed to phosphorylate cardiac acetyl-CoA carboxylase as it did in WT mouse heart. However, a significant portion of the cardioprotection of adiponectin against

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Disclosures

None.

### CLINICAL PERSPECTIVE

The incidence of obesity and type 2 diabetes mellitus is increasing worldwide, and both of these conditions increase the risk of cardiovascular disease. Adiponectin is an adipocyte-specific protein that plays a critical role in glucose and lipid metabolism regulation. Prospective studies have demonstrated the association between decreased plasma adiponectin levels and increased cardiovascular morbidity in diabetic patients. Clarification of the mechanisms by which adiponectin acts advantageously may contribute to our understanding of diabetic pathophysiology and provide insight to a potential therapeutic modality. Considerable evidence exists that the metabolic regulatory, anti-inflammatory, and vascular protective effects of adiponectin are largely mediated by activation of AMP-activated protein kinase (AMPK). However, our present study demonstrated for the first time that the anti-ischemic and cardioprotective effect of adiponectin is largely AMPK independent. In recent years, AMPK has become a major target in the search for effective treatments for metabolic syndromes, including type II diabetes. However, our present experimental results strongly suggest that adiponectin might be a better, more effective therapeutic target in the treatment of ischemic heart injury in diabetic patients. For instance, administration of adiponectin may still be an effective therapy under conditions in which the AMPK signaling-dependent cardioprotective agents become ineffective. Moreover, our present experimental results demonstrated that administration of globular domain of adiponectin reduced synchronized nitric oxide/superoxide overproduction from NADPH oxidase and inducible nitric oxide synthase and effectively blocked peroxynitrite formation in ischemic/reperfused hearts. As such, interventions with aims of bolstering adiponectin production may hold great potential in reducing diabetic patient mortality in those with ischemic heart disease.

MI/R injury was retained in AMPK-DN mice. Furthermore, treatment of AMPK-DN mice with adiponectin reduced MI/R-induced cardiac oxidative and nitrative stress to the same degree as that seen in WT mice. Finally, treating AMPK-DN cardiomyocytes with adiponectin reduced simulated MI/R-induced oxidative/nitrative stress and decreased cell death ( $P<0.01$ ).

**Conclusions**—Collectively, our results demonstrated that AMPK deficiency significantly increases MI/R injury in vivo but has minimal effect on the antioxidative/antinitrative protection of adiponectin.

### Keywords

adipocytokine; apoptosis; diabetes mellitus; myocardial infarction; signal transduction

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Ischemic heart disease is the leading cause of death in patients with diabetes.<sup>1</sup> Hyperglycemia and hyperlipidemia not only cause vascular injury that results in myocardial ischemia (MI) but also have a direct adverse impact on ischemic cardiomyocytes causing larger infarct size<sup>2,3</sup> and more severe heart failure<sup>4</sup> after MI. Identifying the molecular basis that links diabetes with increased morbidity and mortality of ischemic heart disease is not only scientifically important but may also reveal new therapeutic targets against diabetic heart injury.

### Clinical Perspective p 844

AMP-activated protein kinase (AMPK) is a heterotrimeric protein kinase expressed in most mammalian tissues including cardiac muscle, where it plays an essential role in the regulation of cellular energy metabolism.<sup>5,6</sup> Although the concept of AMPK as a cardioprotective molecule during ischemia is well accepted, whether AMPK activation is beneficial or detrimental during reperfusion remains an actively debated topic, largely because of its putative effect on cardiometabolic regulation and adverse effects such as acidosis and calcium overload.<sup>7</sup> Several recent studies in isolated buffer-perfused hearts have demonstrated that AMPK deficiency renders the hearts more susceptible to MI/reperfusion (MI/R injury), as evidenced by increased myocardial cell death<sup>8</sup> and poorer cardiac function recovery.<sup>9,10</sup> However, many additional factors (including metabolic substrates, inflammatory mediators, and neuronal/humoral factors) are operative in vivo that can influence the action of AMPK. Whether AMPK activation is detrimental or beneficial in clinically relevant animal models of regional MI/R remains largely unknown.

Adiponectin is an adipocytokine secreted from adipose tissue.<sup>11</sup> Several clinical observations have demonstrated that plasma adiponectin concentrations are significantly reduced in patients with type II diabetes and are inversely correlated with the risk of MI, indicating that reduced adiponectin function may be a major contributor to increased ischemic heart disease morbidity in diabetic patients.<sup>12,13</sup> Moreover, Walsh and colleagues<sup>14</sup> and our group<sup>15</sup> have recently demonstrated that MI/R injury is markedly increased in adiponectin knockout mice and that exogenous supplementation of adiponectin is cardioprotective, suggesting that reduced adiponectin levels found in diabetic patients may also play a causative role in increased cardiomyocyte death and high mortality in diabetic patients after MI/R. However, discernment of the specific intracellular signaling mechanisms responsible for the cardiomyocyte protective effect of adiponectin remains elusive. Considerable evidence from many investigators has demonstrated that AMPK plays an essential role in adiponectin intracellular signaling.<sup>16-18</sup> However, it is well known that AMPK is dramatically activated by MI itself, and whether additional AMPK activation by adiponectin under this specific pathological condition is responsible for the powerful cardioprotection of adiponectin against MI/R injury remains unknown.

Therefore, the aims of the present study were (1) to investigate the impact of AMPK deficiency on the severity of MI/R injury in intact animals; (2) to determine the degree of AMPK involvement in the cardioprotective signaling of adiponectin; and (3) to identify possible mechanisms by which adiponectin may directly protect cardiomyocytes during MI/R independent of AMPK.

## Methods

All experiments were performed on adult male mice overexpressing a dominant negative  $\alpha_2$  subunit (D157A) of AMPK (AMPK-DN) or male littermate controls expressing wild type of  $\alpha_2$  subunit of AMPK (WT). Generation, breeding, phenotype characteristics, and genotyping of these mice have previously been described in detail.<sup>10</sup> The experiments were performed in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Thomas Jefferson University Committee on Animal Care.

### Experimental Protocols

Mice were anesthetized with 2% isoflurane (total exposure time <5 minutes), and MI was produced as described in our previous studies.<sup>15</sup> Twenty minutes after MI, animals were randomized to 1 of the following groups: WT sham MI (n=12), WT MI+vehicle (n=13), WT MI+globular domain of adiponectin (gAPN) (2  $\mu$ g/g IP; n=13),<sup>15</sup> AMPK-DN sham MI (n=12), AMPK-DN MI+vehicle (n=14), and AMPK-DN MI+gAPN (n=14). After 30 minutes of MI, the slipknot was released, and the myocardium was reperfused. After 3 hours (all assays except cardiac function and infarct size) or 24 hours (cardiac function and infarct size only) of reperfusion, the ligature around the coronary artery was retied, and 1 mL of 2% Evans blue dye was injected into the left ventricular cavity. The Evans blue-negative stained part (ischemic/reperfused tissue or area at risk [AAR]) was isolated, and all assays including immunohistological, biochemical, and Western blot were performed with the tissue from AAR.

### Determination of Myocardial Apoptosis and Myocardial Infarct Size

Myocardial apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, caspase 3 activity, and DNA fragmentation as described in our previous study.<sup>19</sup> Myocardial infarct size was determined by Evans blue-2,3,5-triphenyl tetrazolium chloride (TTC) double staining methods.

### Determination of Cardiac Function

Cardiac function was determined by echocardiography as well as left ventricular catheterization methods 24 hours after reperfusion before the chest was reopened, as described in our previous studies.<sup>15,19</sup>

### Quantification of Superoxide Production

Myocardial superoxide content (in AAR) was quantified by lucigenin-enhanced luminescence, and the cellular origin of reactive oxygen species was determined by dihydroethidium staining (Molecular Probes, Carlsbad, Calif), as described previously.<sup>15</sup>

### Determination of Total Nitric Oxide and Nitrotyrosine Content in Cardiac Tissue

The tissue nitric oxide (NO) and its metabolic products (NO<sub>2</sub> and NO<sub>3</sub>) were determined by use of a chemiluminescence NO detector (Siever 280i NO Analyzer), and nitrotyrosine content in MI/R cardiac tissue was quantified by enzyme-lined immunosorbent assay as described previously.<sup>15,19</sup>

**Western Blot Analysis**—Proteins were separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and incubated with primary antibodies (monoclonal antibody against phosphorylated acetyl-CoA carboxylase [ACC], inducible NO synthase [iNOS] [Upstate, Chicago, Ill], or gp91<sup>phox</sup> [Transduction Laboratories, San Jose, Calif] and horseradish peroxidase-conjugated secondary antibody. The blot was developed with a Supersignal Chemiluminescence detection kit (Pierce, Rockford, Ill), and band was visualized with a Kodak Image Station 400 (Rochester, NY).

### Adult Mouse Cardiomyocyte Culture

Adult mouse cardiomyocytes were isolated from WT or AMPK-DN mice and subjected to 3 hours of simulated ischemia and 6 hours of reperfusion (SI/R) as described in our previous studies.<sup>15</sup> The effect of gAPN (2  $\mu$ g/mL) on SI/R-induced cell death (determined by LDH release), superoxide (determined by dihydroethidium staining) and NO production, and nitrotyrosine formation was determined as described above.

### Statistical Analysis

All values in the text and figures are presented as mean $\pm$ SEM of n independent experiments. ANOVA across all investigated groups was conducted first, and post hoc pairwise tests for certain group pairs with assessment of statistical significance were then performed after Bonferroni correction of the overall significance level. Western blot densities were analyzed with the Kruskal-Wallis test followed by the Dunn post hoc test. Probabilities of  $\leq 0.05$  were considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

## Results

### Cardiomyocyte-Specific Overexpression of a Dominant Negative Mutant of AMPK $\alpha_2$ Subunit Increased Susceptibility to MI/R Injury

MI followed by reperfusion resulted in significant cardiac injury including cardiomyocyte apoptosis, myocardial infarction, and cardiac contractile as well as diastolic dysfunction in WT littermates (Figure 1, Table). Mice with cardiomyocyte-specific overexpression of a dominant negative mutant of AMPK $\alpha_2$  catalytic subunit developed normally and had normal cardiac function under basal conditions (sham MI/R groups in the Table). However, when subjected to in vivo MI/R, AMPK-DN animals manifested significantly greater cardiac injury than their littermate controls. Specifically, MI/R-induced cardiomyocyte apoptosis was increased (Figure 1A), infarct size was enlarged (Figure 1B), and cardiac function was further depressed (Figure 1C and 1D, Table).

### AMPK Deficiency Enhanced MI/R-Induced Oxidative Stress but Had No Significant Effect on MI/R-Induced Nitritative Stress

Oxidative and nitritative stresses are primary factors responsible for MI/R injury. As summarized in Figure 2, MI/R-induced superoxide production was further enhanced in AMPK-DN hearts, as evidenced by intensified dihydroethidium staining (Figure 2A) and increased lucigenin-enhanced luminescence signals (Figure 2B). However, MI/R-induced NO overproduction was not significantly changed (Figure 2C) in AMPK-DN hearts, and no significant difference in nitrotyrosine formation was observed between WT and AMPK-DN hearts subjected to MI/R (Figure 2D) with a relatively small sample size included in the present study.

### Overexpressing a Dominant Negative Mutant of AMPK $\alpha_2$ Subunit Abolished MI/R-Induced ACC Phosphorylation and Completely Blocked gAPN-Stimulated ACC Phosphorylation

ACC is a major substrate of AMPK, and its phosphorylation plays an essential role in the metabolic regulation of AMPK. Moreover, previous studies have demonstrated that much of the cardiometabolic effect of adiponectin is conducted by the AMPK-ACC signaling axis.<sup>13,20</sup> Consistent with these notions, a >2-fold increase in ACC phosphorylation was observed in WT hearts after MI/R, and treatment with gAPN (1.2-fold;  $P<0.05$ ) further enhanced ACC phosphorylation modestly (Figure 3, left panel). However, MI/R-induced ACC phosphorylation was lost in AMPK-DN hearts, and, more importantly, treatment with gAPN failed to stimulate ACC phosphorylation in the transgenic hearts (Figure 3, right panel).

### Cardioprotective Effect of gAPN Is Reduced But Not Lost in AMPK-DN Mice

Other investigators have demonstrated that many of the biological functions of adiponectin are completely abolished when AMPK signaling is blocked either genetically or pharmacologically,<sup>16-18,21-23</sup> and data from the present study summarized above confirmed the essential role of AMPK in gAPN-induced ACC phosphorylation. We next determined whether AMPK serves as the central mediator for the cardioprotective effects of gAPN. To better compare the cardioprotective effects of gAPN between WT littermates and AMPK-DN mice, data were normalized against the mean value of the respective vehicle group. Consistent with our previous observations,<sup>15</sup> treatment of WT animals with gAPN significantly reduced infarct size (Figure 4A left panel), attenuated MI/R-induced apoptosis determined by TUNEL staining (Figure 4B left panel), DNA fragmentation (Figure 4C left panel), and caspase 3 activation (Figure 4D left panel), and improved cardiac functional recovery after reperfusion (Table). However, although the cardioprotective effects of gADN were modestly reduced in AMPK-DN hearts compared with WT hearts (Figure 4; note that the percent reduction of infarct size and apoptosis by gAPN treatment is reduced in AMPK-DN mice compared with WT), a significant portion of cardioprotection from gAPN was retained in AMPK-DN hearts. Specifically, treatment with gAPN significantly reduced infarct size (Figure 4A, right panel), decreased cardiomyocyte apoptosis (less TUNEL-positive labeling in Figure 4B; weaker DNA fragmentation in Figure 4C; and lower caspase 3 activity in Figure 4D), and improved cardiac functional recovery (Table). These novel data suggest that mechanisms exist that are responsible for the cardioprotection of gAPN through signaling pathways other than AMPK.

### Antioxidative/Antinitrative Effects of gAPN in the MI/R Hearts Are Not Mediated by AMPK

Having demonstrated that gAPN protects hearts against reperfusion injury partially through an AMPK-independent system, we further explored the potential mechanisms that might be responsible for the AMPK-independent cardioprotection of gAPN. We reported recently that gAPN cardioprotection involves the reduction of oxidative/nitrative stress.<sup>15</sup> Two series of experiments were thus performed to delineate the relationship between AMPK signaling and oxidative/nitrative stress in MI/R hearts. In the first series of experiments, MI/R-induced myocardial oxidative stress and nitrative stress were determined. As illustrated in Figure 5, MI/R-induced superoxide/NO overproduction (Figure 5A to 5C) and nitrotyrosine formation (Figure 5D) were significantly inhibited when WT and AMPK-DN animals were treated with gAPN. There was no statistically significant difference in the antioxidant and antinitrative effect of gAPN (expressed as percent reduction over their respective vehicle groups) between AMPK-DN and WT animals.

In the second series of experiments, we further determined the molecular sources that might be responsible for the AMPK-independent antioxidant/antinitrative actions of gAPN. As illustrated in Figure 6A, MI/R-induced overexpression of gp91<sup>phox</sup> was significantly increased in AMPK-DN hearts compared with WT hearts (Figure 6A; lane 2 versus lane 5,  $\#P<0.05$ ). This result is consistent with data presented in Figure 2A and 2B showing that MI/R-induced



superoxide overproduction was further enhanced in AMPK-DN hearts. Treatment of WT as well as AMPK-DN mice with gAPN significantly inhibited gp91<sup>phox</sup> expression (Figure 6A; \*\* $P < 0.01$ ). Although the absolute level of gp91<sup>phox</sup> expression remains greater in gAPN-treated AMPK-DN hearts than in gAPN-treated WT hearts (Figure 6A; lane 3 versus lane 6, # $P < 0.05$ ), the percent inhibitory effect of gAPN over their respective vehicle groups is comparable (40.4% and 44.3% reduction, respectively;  $P > 0.05$ ) between these 2 groups. Together with the data presented in Figure 2, these results demonstrated that MI/R-induced oxidative stress was increased when AMPK signaling is blocked, but the antioxidant effect of gAPN was not mediated by AMPK activation.

Data presented in Figure 5C and 5D demonstrated that gAPN inhibited MI/R-induced NO overproduction and nitrotyrosine formation in an AMPK-independent fashion. To further determine the NO source inhibited by gAPN, the effect of gAPN on iNOS expression was determined. As illustrated in Figure 6B, AMPK deficiency had no significant effect on MI/R-induced iNOS expression. These data are consistent with that presented in Figure 2C showing that AMPK deficiency had no significant effect on NO overproduction. Moreover, treatment of AMPK-DN hearts with gAPN reduced MI/R-induced iNOS expression to a level comparable to that seen in WT hearts. Together with the data presented in Figure 2, these results demonstrated that AMPK deficiency had no significant effect on MI/R-induced nitrate stress and that gAPN inhibited nitrate stress in an AMPK-independent fashion.

**gAPN Directly Inhibited Oxidative/Nitrate Stress in Cardiomyocytes in an AMPK-Independent Fashion**—Our aforementioned *in vivo* experimental results demonstrated that the cardioprotective effects of gADN are partially AMPK independent and that the antioxidant/antinitrate effect of gAPN is not mediated by AMPK. Because adiponectin is a potent anti-inflammatory molecule, it is thus possible that gAPN may achieve its antioxidant/antinitrate effects via an AMPK-mediated inhibitory effect on leukocyte/endothelial interactions in our cardiomyocyte-specific transgenic mice. To directly address this issue, adult cardiomyocytes were isolated from WT or AMPK-DN mice and subjected to SI/R as described in our previous publications.<sup>15</sup> Ten minutes before SI, cardiomyocytes were treated with either vehicle or gAPN (2  $\mu\text{g}/\text{mL}$ ), and the effect of gAPN on SI/R-induced cell death (LDH release) and oxidative/nitrate stress was determined. Consistent with our *in vivo* results, SI/R-induced cell death was significantly increased in cardiomyocytes isolated from AMPK-DN mice compared with WT mice (Figure 7A). However, SI/R-induced oxidative/nitrate stress was comparable in cardiomyocytes isolated from WT and AMPK-DN hearts (Figure 7B, 7C, 7D). Treatment of cardiomyocytes isolated from WT as well as AMPK-DN mice with gAPN significantly reduced SI/R-induced cardiomyocyte death (Figure 7A), superoxide and NO overproduction (Figure 7B, 7C), and protein nitration (Figure 7D). These results demonstrated that gAPN is capable of inhibiting oxidative/nitrate stress occurring in MI/R cardiomyocytes independent of AMPK and significantly reduced cell death.

## Discussion

Several important observations have been made in the present study. First, we have demonstrated that cardiomyocyte-specific AMPK deficiency significantly enhanced postischemic myocardial apoptosis, increased infarct size, and worsened cardiac functional recovery in a clinically relevant animal model of *in vivo* regional MI/R. Moreover, we have demonstrated that NADPH oxidase expression and superoxide production are significantly increased in AMPK-DN hearts after MI/R. These results not only provided additional evidence supporting a study published early this year by Calvert et al<sup>24</sup> showing that AMPK deficiency increases infarct size but, more importantly, also provided novel cellular mechanisms by which AMPK deficiency promotes myocardial cell death. Second, we have provided the first direct evidence that gAPN protects MI/R heart *in vivo* partially through AMPK-independent

mechanisms. Finally, we have proved that the antioxidative and antinitrative effects of gAPN in MI/R hearts are not mediated by AMPK.

### AMPK and MI/R Injury

Although the protective role of AMPK during cardiac ischemia has been investigated extensively and multiple mechanisms by which AMPK activation protects ischemic heart have been identified,<sup>8,10,24,25</sup> the role of AMPK in cardiac reperfusion after ischemia is much more complex. It was generally believed that AMPK activation during reperfusion can cause high rates of fatty acid oxidation that may adversely affect cardiac functional recovery.<sup>7</sup> Whereas the evidence from 2 recent *in vitro* studies<sup>8,9</sup> using AMPK-deficient cardiac models appears to disprove the notion that AMPK activation is detrimental to functional recovery during reperfusion, a conclusion that AMPK activation is beneficial to the heart during reperfusion cannot yet be made on the basis of these *ex vivo* experiments because of many limitations associated with the buffer-perfused heart model. In the present study, we utilized cardiomyocyte-specific AMPK $\alpha_2$  mutant transgenic mice and subjected them to regional MI/R *in vivo*. Our results demonstrated that compared with WT mice, MI/R in AMPK-DN mice caused greater cardiac injury, including larger infarct size, more apoptosis, and poorer cardiac function recovery (Figure 1 and Table). These results are important in defining the role of AMPK in MI/R injury.

The study by Russell et al<sup>8</sup> suggested that AMPK may protect reperfusion injury by limiting apoptotic activity rather than through its metabolic actions. Indeed, activation of AMPK by 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside attenuates endoplasmic reticulum stress and reduces apoptotic cell death in cultured cardiomyocytes.<sup>26</sup> However, the manner in which AMPK reduces reperfusion-induced endoplasmic reticulum stress and apoptosis remains unclear. Because overproduction of reactive oxygen species is a central cause of reperfusion-induced endoplasmic reticulum stress and apoptosis, our present study demonstrating increased expression of gp91<sup>phox</sup> (the essential membrane component of NADPH oxidase that accounts for the large portion of superoxide production in the MI/R heart) and enhanced superoxide production in AMPK-DN hearts provides the first likely mechanism that might be responsible for increased cardiomyocyte apoptosis observed in these animals.

### Role of AMPK in Adiponectin Cardioprotection Against MI/R

The biological activities of adiponectin have been investigated extensively in recent years. Four major functions, including metabolic regulation and anti-inflammatory, vasculoprotective, and cardioprotective effects, have been identified.<sup>27</sup> Substantial evidence has been accumulated supporting the conception that AMPK plays an essential role in adiponectin intracellular signaling. Specifically, pharmacological inhibition of AMPK activity or genetic inhibition of AMPK expression virtually abolishes the metabolic,<sup>16,18</sup> anti-inflammatory,<sup>28</sup> and vasculoprotective<sup>17,29</sup> effects of adiponectin. However, although the evidence for the cardioprotective effects of adiponectin in MI/R is strong, it is still unclear whether AMPK is the central mediator of this effect.<sup>20</sup> In the first study reporting the cardioprotective effect of adiponectin, Shibata et al<sup>14</sup> demonstrated that the importance of AMPK in cardiomyocyte injury varies greatly in different models. Specifically, adiponectin blocks hypoxia/reoxygenation-induced neonatal cardiomyocyte apoptosis exclusively through AMPK activation. In contrast, its inhibitory effect on lipopolysaccharide-induced tumor necrosis factor- $\alpha$  production is completely AMPK independent. Moreover, *in vivo* administration of NS398, a cyclooxygenase-2 inhibitor, abrogated the infarct-sparing action of exogenous adiponectin, suggesting that cyclooxygenase-2 plays a significant role in adiponectin cardioprotection after MI/R. However, 2 very recent *ex vivo* studies reported that AMPK is essential in the cardioprotective effects of adiponectin. The study by Shinmura et al<sup>30</sup> demonstrated that short-term caloric restriction protected the heart by increasing serum

adiponectin levels with subsequent AMPK activation. In a more recent study, Gonon et al<sup>31</sup> reported that adiponectin protected against MI/R injury via the AMPK/Akt/NO signaling axis. Although the data clearly demonstrated that administration of adiponectin in isolated perfused hearts resulted in AMPK phosphorylation, whether the protective effects were blocked when AMPK was inhibited was not determined, and a cause-effect relationship was not established. It thus remains unclear whether AMPK phosphorylation is a necessary step for adiponectin protection of the MI/R heart. Taken together, currently available data strongly suggest that the degree of AMPK involvement in adiponectin cardioprotection varies significantly under different experimental conditions, and an *in vivo* study utilizing AMPK gene-manipulated animals is essential to definitively discern the role of AMPK in adiponectin cardioprotection.

Our present study provided direct evidence that a large portion of gAPN cardioprotection is AMPK independent in intact animals. Moreover, our study demonstrated for the first time that gAPN inhibits MI/R-induced oxidative/nitrative stress in an AMPK-independent fashion and that these actions may account for the cardioprotection of gAPN observed in AMPK-DN animals. In a recent study, Ouedraogo<sup>32</sup> reported that treatment with gAPN suppressed glucose-induced superoxide overproduction in cultured endothelial cells and that this action cannot be blocked by pretreatment with AMPK inhibitors, indicating that gAPN reduces high glucose-induced superoxide overproduction in an AMPK-independent fashion. Because the same isoform of adiponectin (ie, globular domain of adiponectin) was used in these 2 studies (ie, Ouedraogo's study and our present study) and similar results were observed, it remains to be determined whether this AMPK-independent antioxidant action of adiponectin is isoform specific (ie, gAPN) or uniform to all isoforms of adiponectin.

It should be noted that AMPK $\alpha_2$  activity was markedly inhibited (78% reduction) but not completely lost and that AMPK $\alpha_1$  activity was unaltered in the transgenic animals used in our study.<sup>10</sup> Therefore, it is possible that residual AMPK $\alpha_2$  or AMPK $\alpha_1$  might be responsible for the antioxidant and antinitrative action of adiponectin, as was observed. Although additional studies utilizing AMPK $\alpha_{1/2}$  double knockout mice are necessary to definitely address this concern, currently available data argue against this possibility. The  $\alpha_2$  is the dominant form of AMPK in the heart, and the small amount of  $\alpha_1$  present in the  $\alpha_2$  mutant transgenic heart is maximally activated at baseline before ischemia.<sup>8,10</sup> Therefore, it is highly unlikely that AMPK $\alpha_1$  is responsible for the results of adiponectin treatment given 20 minutes after MI.<sup>10</sup> More importantly, our present study demonstrated that adiponectin-induced ACC phosphorylation is completely lost in AMPK-DN mice, indicating that the residual AMPK $\alpha_2$  and small amount of  $\alpha_1$  are insufficient to effectively transfer the signaling of adiponectin in the MI/R heart.

AMPK and adiponectin are 2 critical metabolic regulators, and their deficiency exists in obesity and diabetes.<sup>33-35</sup> Because of their well-recognized cardioprotective actions, it has been proposed that impaired adiponectin/AMPK signaling might be responsible for increased cardiovascular complications in patients with diabetes.<sup>36</sup> In recent years, AMPK has become a major target in the search for effective treatments for metabolic syndromes, including type II diabetes. However, our present experimental results strongly suggest that adiponectin might be a better, more effective therapeutic target in the treatment of ischemic heart injury in diabetic patients. For instance, administration of adiponectin may still be an effective therapy under conditions in which the AMPK signaling-dependent cardioprotective agents become ineffective. Indeed, a recent study by Lefer and colleagues<sup>24</sup> demonstrated that the cardioprotective effects of metformin, 1 of the most commonly prescribed antihyperglycemic agents for the treatment of type 2 diabetes, are completely lost in AMPK-DN animals. In addition, numerous studies have demonstrated that physiological or pharmacological concentrations of NO generated from endothelial NOS or NO donors exert significant cardioprotective effects against ischemia and MI/R injury,<sup>37</sup> and adiponectin has been shown



to stimulate NO production by endothelial NOS phosphorylation.<sup>21</sup> More importantly, it is well recognized that simultaneous overproduction of superoxide and NO not only causes the inactivation of a cytoprotective molecule (ie, NO) but also produces a highly cytotoxic molecule, peroxynitrite. Therefore, those therapeutic interventions that prevent concomitant, synchronized NO/superoxide overproduction effectively block peroxynitrite formation and offer great tissue protection. Our present experimental results demonstrated that AMPK activation may have an antioxidant effect via inhibiting NADPH oxidase expression but does not appear to have a significant role in MI/R-induced peroxynitrite formation. In contrast, administration of gAPN reduced synchronized NO/superoxide overproduction from NADPH oxidase and iNOS and effectively blocked peroxynitrite formation in MI/R hearts. As such, interventions with aims of bolstering adiponectin production may hold great potential in reducing diabetic patient mortality in those with ischemic heart disease.

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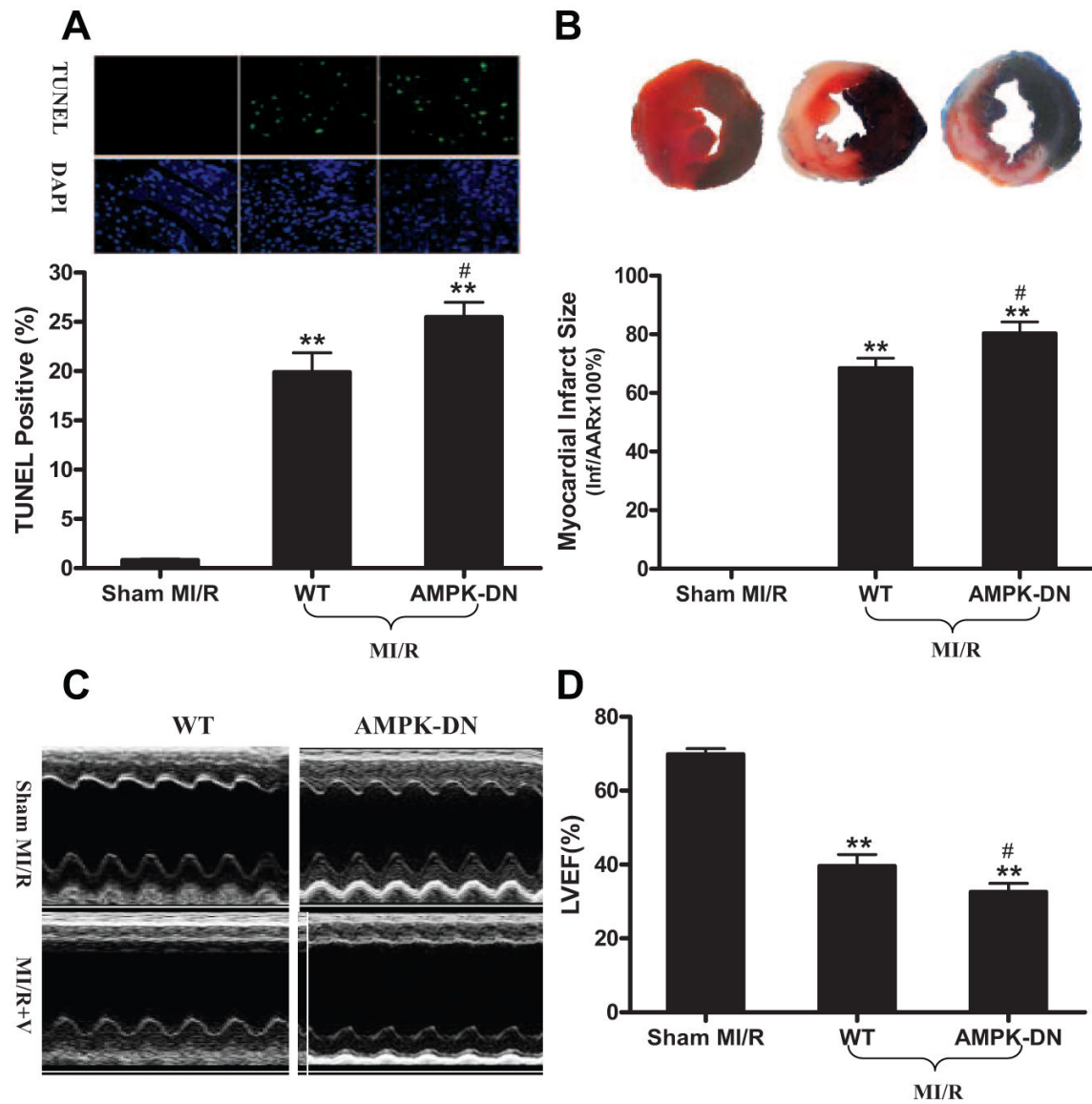
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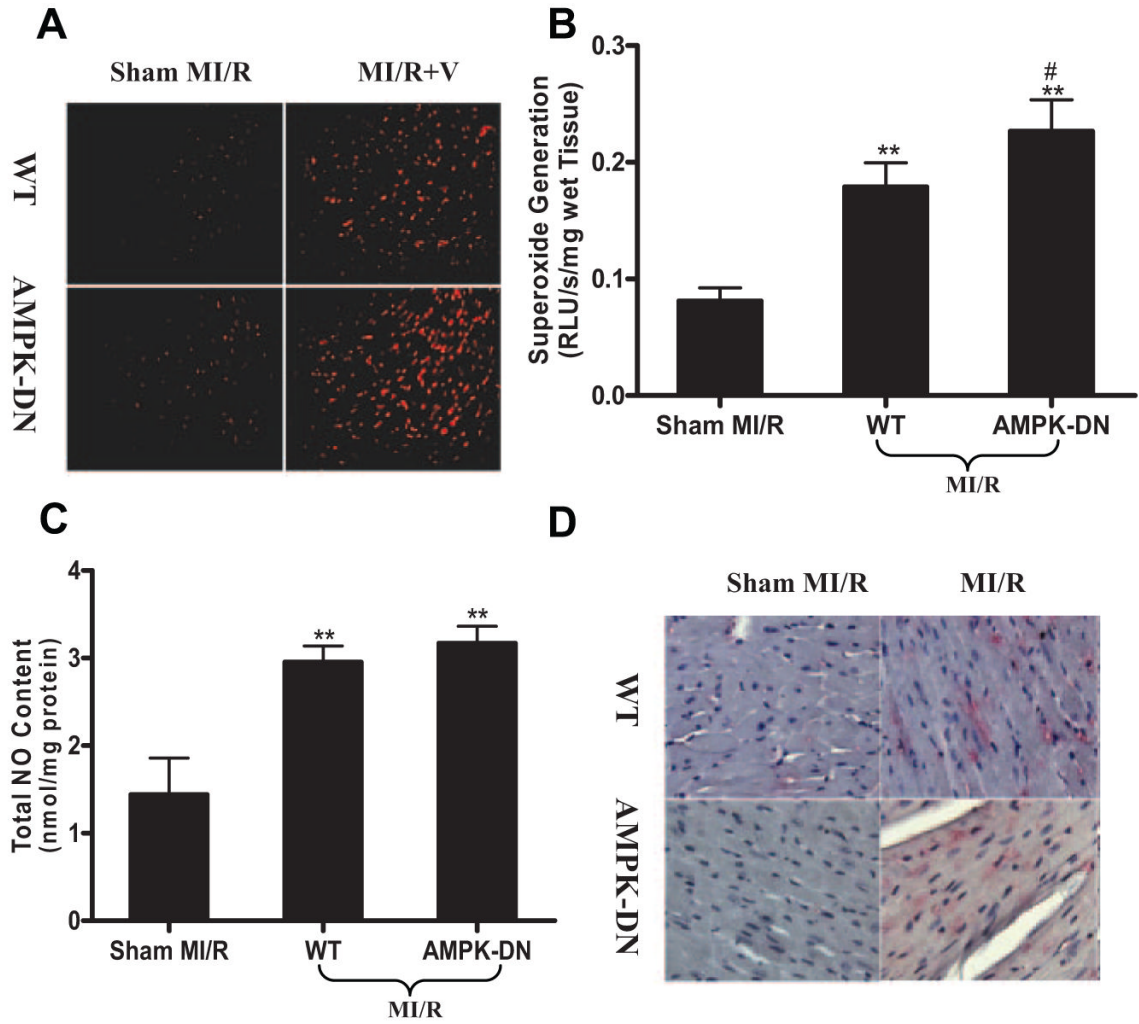
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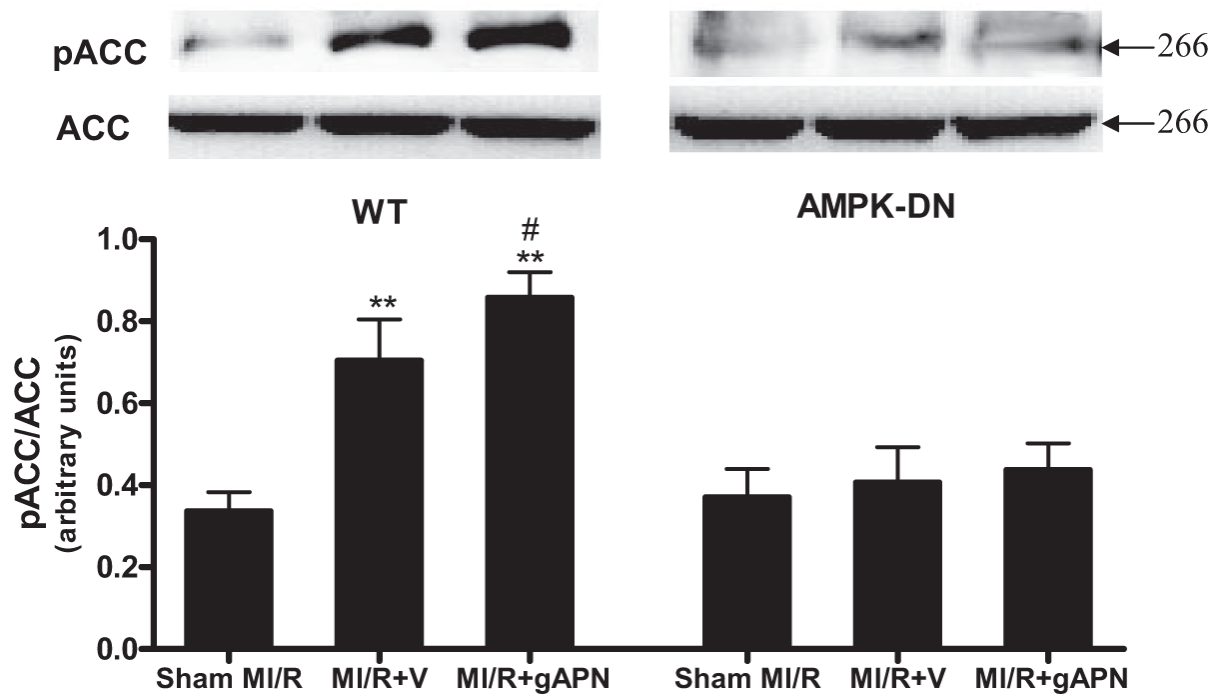
**Figure 1.**

A, Cardiomyocyte apoptosis determined by TUNEL (n=5 to 6 hearts per group). B, Myocardial infarction determined by Evans blue/TTC double staining (n=12 to 14 hearts per group). C/D, Cardiac function determined by echocardiography (n=12 to 14 animals per group). \*\* $P < 0.01$  vs sham MI/R; # $P < 0.05$  vs WT with MI/R. V indicates vehicle; LVEF, left ventricular ejection fraction.

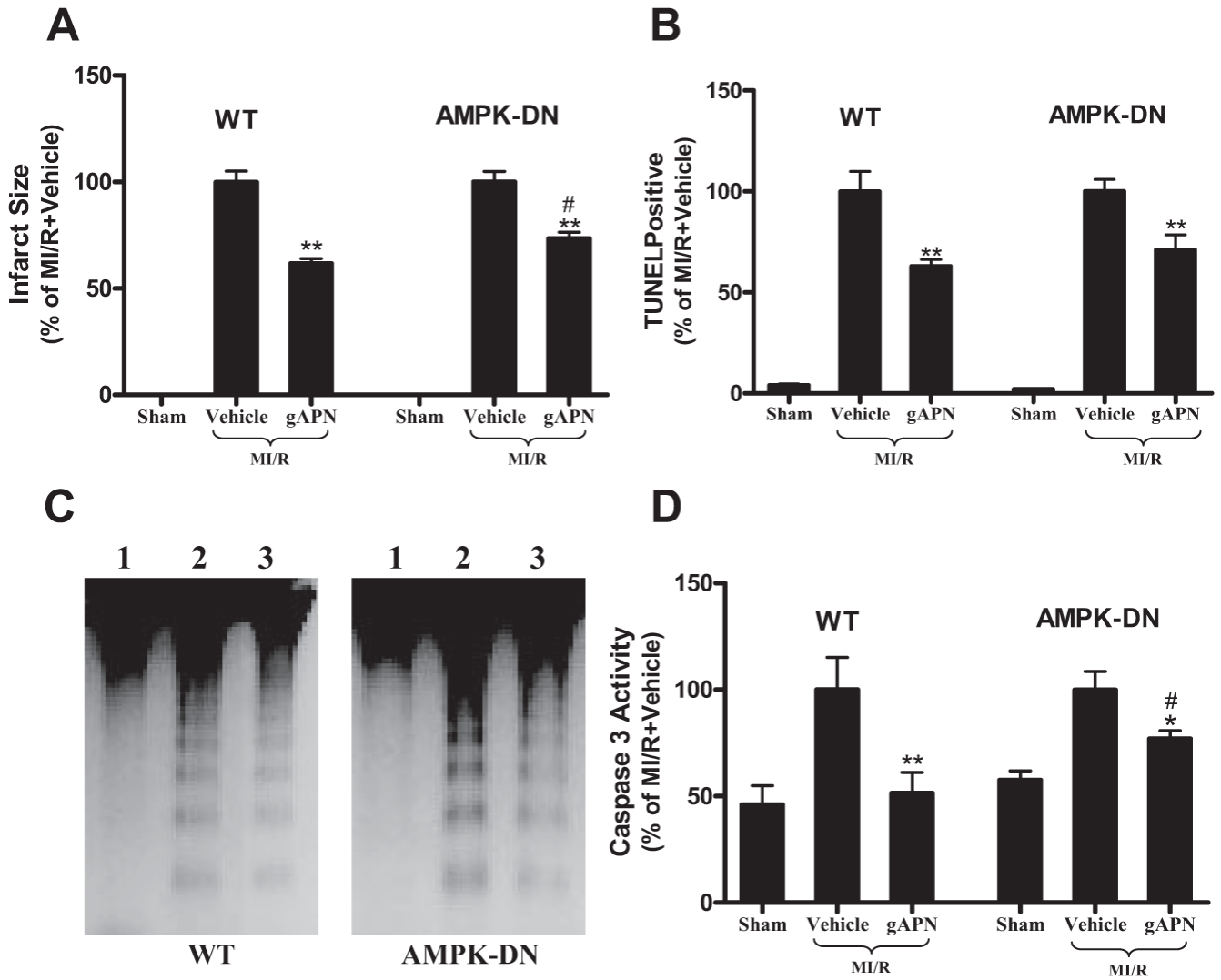


**Figure 2.** AMPK deficiency increases superoxide production in MI/R cardiac tissue without altering NO and peroxynitrite formation. A, In situ superoxide detection performed with dihydroethidium (n=4 to 5 hearts per group) staining. B, Superoxide content quantified with lucigenin-enhanced luminescence (n=9 to 12 hearts per group). RLU indicates relative light units. C, Total NO content determined by chemiluminescence NO detector (n=9 to 12 hearts per group). D, Immunohistological sections stained with an antibody against nitrotyrosine to determine the nitrotyrosine formation in the MI/R heart, a footprint of in vivo peroxynitrite formation and an index for nitrative stress (n=5 to 6 hearts per group). \*\**P*<0.01 vs sham MI/R; #*P*<0.05 vs WT with MI/R.

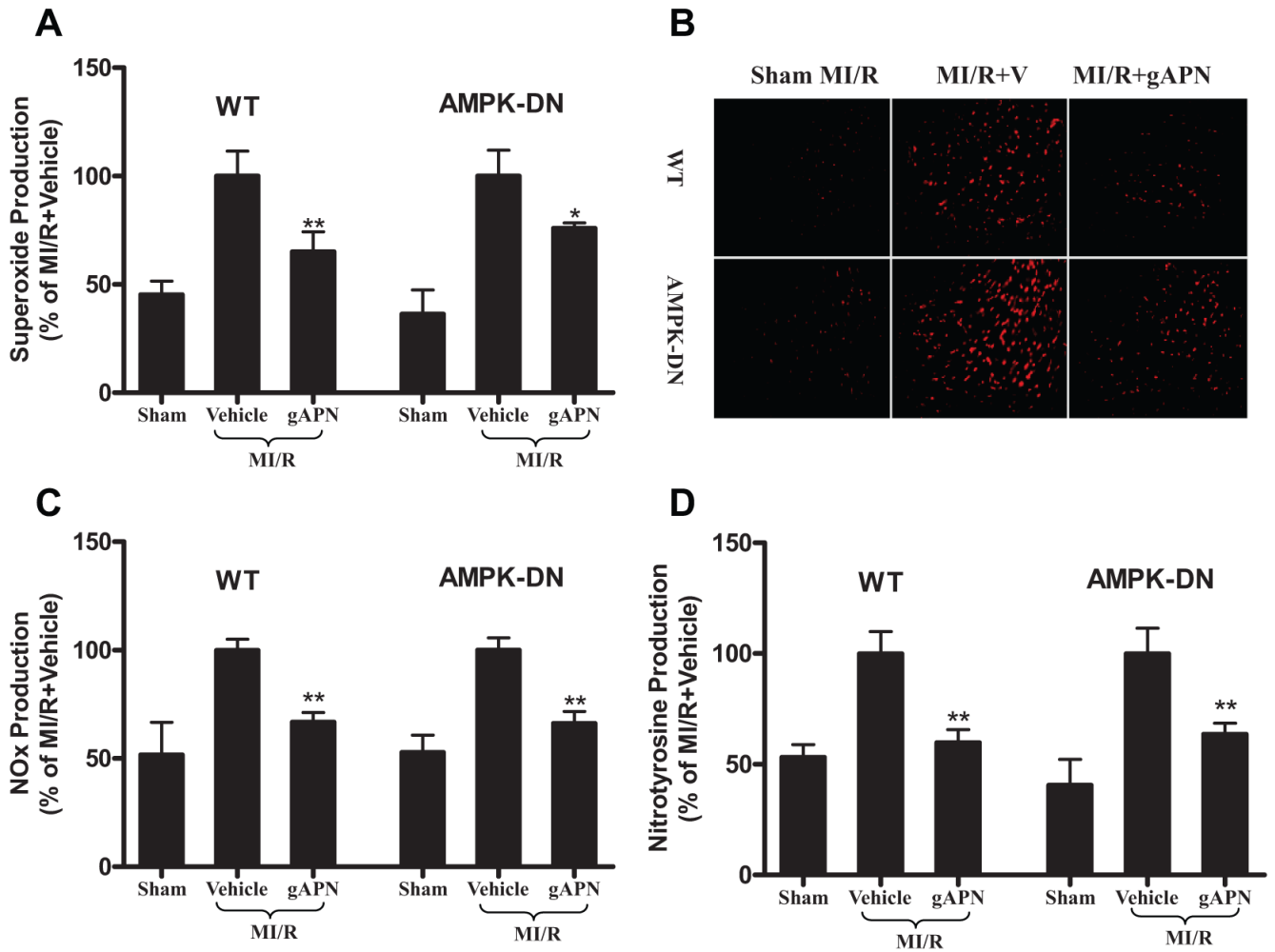




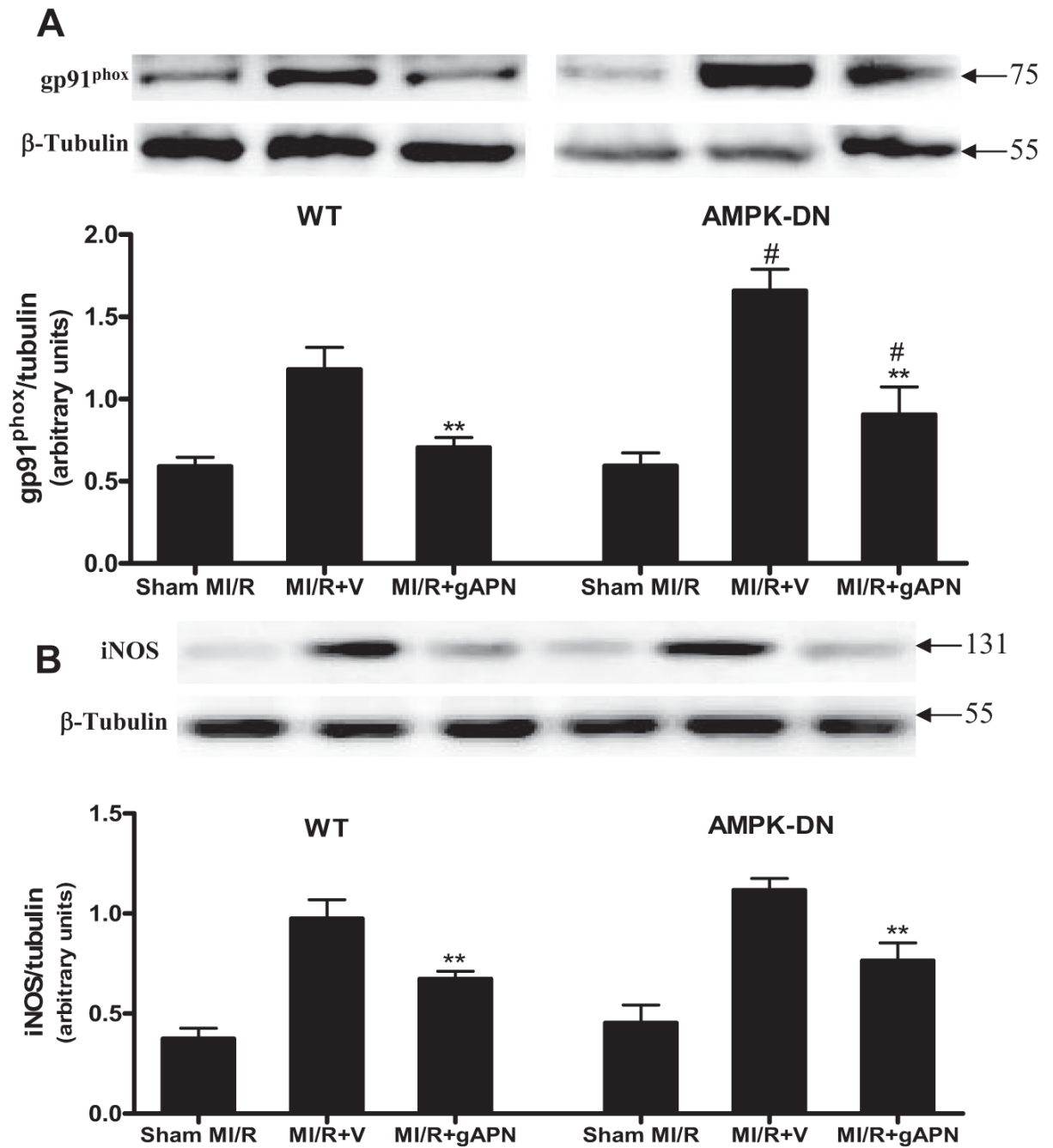
**Figure 3.** Effect of MI/R and adiponectin treatment on ACC phosphorylation in WT mice (left) and AMPK-DN mice (right). Inserts, Representative Western blots. Bar graph, Density analysis results from 5 to 8 hearts per group. \*\* $P < 0.01$  vs sham MI/R; # $P < 0.05$  vs MI/R+V. V indicates vehicle.



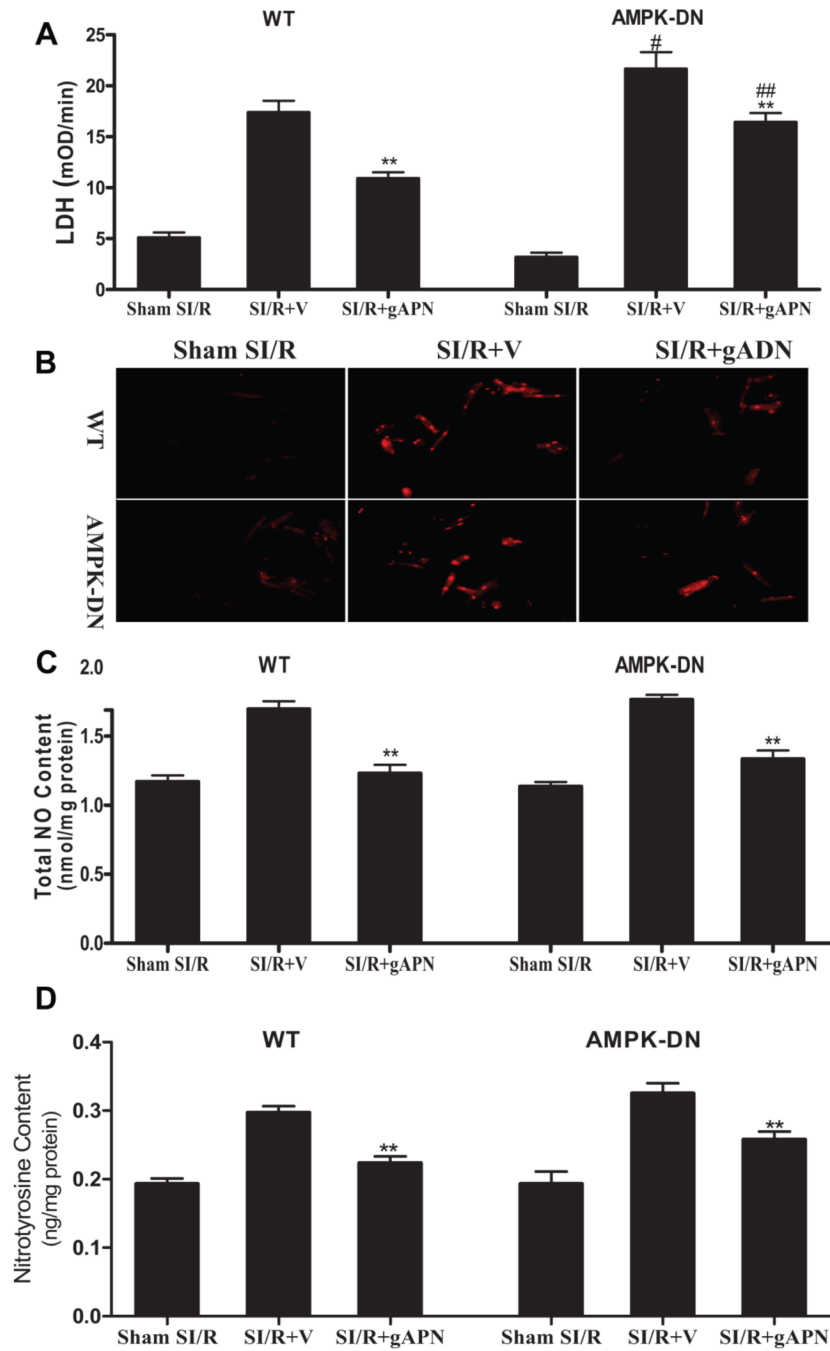
**Figure 4.** Cardioprotective effects of adiponectin are blunted but not lost in AMPK-DN mice. Mean values obtained from vehicle-treated MI/R animals were treated as 100%, and individual values from each animal were normalized against the mean values. Myocardial infarct size was determined by Evans blue/TTC double staining (A, n=12 to 14 hearts per group). Myocardial apoptosis was determined by TUNEL labeling (B, n=5 to 6 hearts per group), DNA ladder formation (C, n=4 to 6 hearts per group; lane 1=sham MI/R, lane 2=MI/R+vehicle, lane 3=MI/R+gAPN), and caspase 3 activation (D, n=10 to 12 hearts per group). \* $P < 0.05$ , \*\* $P < 0.01$  vs MI/R+vehicle; # $P < 0.05$  vs WT mice treated with adiponectin.



**Figure 5.** Treatment with adiponectin reduced superoxide production determined by lucigenin-enhanced luminescence (A, n=9 to 12 hearts per group) and dihydroethidium staining (B, n=4 to 5 hearts per group), reduced nitric oxide (NOx) production (C, n=9 to 12 hearts per group), and decreased peroxynitrite formation (D, n=10 to 12 hearts per group) in WT as well as in AMPK-DN mice. \* $P < 0.05$ , \*\* $P < 0.01$  vs MI/R+vehicle. V indicates vehicle.



**Figure 6.** Treatment with adiponectin inhibited gp91<sup>phox</sup> (A) and iNOS expression (B) in MI/R hearts. Inserts, Representative Western blots. Bar graph, Density analysis results from 5 to 8 hearts per group. \*\* $P < 0.01$  vs MI/R+V; # $P < 0.05$  vs WT mice. V indicates vehicle.



**Figure 7.** Adiponectin reduced cell death (A,  $n=12$  to 14 dishes isolated from at least 4 hearts per group, the same for all other assays) and inhibited production of superoxide (B), nitric oxide (C), and peroxynitrite (D) in cardiomyocytes isolated from WT as well as from AMPK-DN mice. \*\* $P<0.01$  vs SI/R+V; # $P<0.05$  vs WT cardiomyocytes subjected to SI/R treated with vehicle; # $P<0.05$ , ## $P<0.01$  vs cardiomyocytes isolated from WT. V indicates vehicle.



Table  
Effect of gADN Treatment on Hemodynamic Changes in WT and AMPK-DN Mice Subjected to MI/R

	WT			AMPK-DN		
	Sham MI/R	MI/R+Vehicle	MI/R+gADN	Sham+MI/R	MI/R+Vehicle	MI/R+gADN
Heart rate, bpm	369±14	378±22	413±28	404±34	446±16	432±21
LVEDP, mm Hg	4.5±0.6	17.1±2.1 <sup>†</sup>	6.2±0.5 <sup>*‡</sup>	5.0±0.4	23.4±1.9 <sup>†</sup>	11.9±1.5 <sup>†‡</sup>
dP/dt <sub>max</sub> , mm Hg/s	7429±451	4055±724 <sup>†</sup>	5839±288 <sup>*‡</sup>	7986±208	3458±448 <sup>†</sup>	6209±359 <sup>*‡</sup>
-dP/dt <sub>max</sub> , mm Hg/s	5877±430	3102±557 <sup>†</sup>	4119±367 <sup>*‡</sup>	5199±275	2296±320 <sup>†</sup>	3900±316 <sup>*‡</sup>

Treatment with gADN 10 minutes before reperfusion had no effect of heart rate but significantly reduced left ventricular end-diastolic pressure (LVEDP) and increased maximal positive and negative values of the instantaneous first derivative of left ventricular pressure (dP/dt<sub>max</sub> and -dP/dt<sub>max</sub>) in WT as well as AMPK-DN mice subjected to 30 minutes of MI and 24 hours of reperfusion.

Hemodynamic measurements were made by using a 1.2F micromanometer advanced to the left ventricular cavity through the right common carotid artery. The left ventricular pressure was recorded, and heart rate, LVEDP, and dP/dt<sub>max</sub> and -dP/dt<sub>max</sub> were obtained with the use of computer algorithms and an interactive videographics program.

\*  $P < 0.05$

<sup>†</sup>  $P < 0.01$  vs sham MI/R

<sup>‡</sup>  $P < 0.05$  vs MI/R vehicle.