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AdipoRon, the First Orally Active Adiponectin Receptor Activator, Attenuates Post-Ischemic Myocardial Apoptosis through both AMPK-Mediated and AMPK-Independent Signalings

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Abstract

Adiponectin (APN) is a cardioprotective molecule. Its reduction in diabetes exacerbates myocardial ischemia/reperfusion (MI/R) injury. Although APN administration in animals attenuates MI/R injury, multiple factors limit its clinical application. The current study investigated whether AdipoRon, the first orally active molecule that binds APN receptors, may protect the heart against MI/R injury, and if so, to delineate the involved mechanisms. Wild type (WT), APN knockout (APNKO), and cardiomyocyte specific-AMPK dominant negative (AMPK-DN) mice were treated with vehicle or AdipoRon (50 mg/kg, 10 minutes prior to MI) and subjected to MI/R (30 minutes/3-24 hours). Compared to vehicle, oral administration of AdipoRon to WT mice significantly improved cardiac function, and attenuated post-ischemic cardiomyocyte apoptosis determined by DNA ladder formation, TUNEL staining, and caspase-3 activation (all, P value <0.01). MI/R-induced apoptotic cell death was significantly enhanced in mice deficient of either APN (APNKO) or AMPK (AMPK-DN). In APNKO mice, AdipoRon attenuated MI/R injury to the same degree observed in WT mice. In AMPK-DN mice, AdipoRon's anti-apoptotic action was partially inhibited, but not lost. Finally, AdipoRon significantly attenuated post-ischemic oxidative stress, as evidenced by reduced NADPH oxidase expression and superoxide production. Collectively, these results demonstrate for the first time that AdipoRon, an orally active APN receptor activator, effectively attenuated post-ischemic cardiac injury, supporting APN receptor agonists as a promising novel therapeutic approach treating cardiovascular complications caused by obesity-related disorders such as type 2 diabetes.

***Keywords*:** Reperfusion Injury; Diabetes, Apoptosis; Adipokines

Abbreviations

AAR	area at risk
AdipoR1	Adiponectin Receptor-1
AdipoR2	Adiponectin receptor-2
AMPK	5'-adenosine monophosphate-activated protein kinase
APN	Adiponectin
DHE	dihydroethidium
GPCR	G-protein coupled receptor
MI/R	Myocardial ischemia/reperfusion
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

Ischemic heart disease is the single-most important cause of death in developed countries. Accumulating evidence indicates that apoptosis, a gene-controlled programmed cell death pathway, contributes significantly to post-ischemic cardiomyocyte death, suggesting therapeutic interventions inhibiting apoptotic cell death may attenuate ischemic/reperfusion-induced heart injury(10).

Adiponectin (APN) is an adipocyte-derived cytokine. Most adipokines (e.g., TNFα) are pro-inflammatory and significantly increased in diabetic patients. The majority of currently published studies support APN as a potent cardiovascular protective molecule, and its levels are markedly reduced in type 2 diabetic patients(18; 26; 28). APN reduces oxidative/nitrative stress, protects cells from apoptosis, inhibits leukocyte-endothelial interaction, and decreases smooth muscle proliferation(9). Two APN receptors (AdipoR1 and AdipoR2) have been cloned(37). Belonging to a new family of membrane receptors (i.e., the progestin and AdipoQ receptor superfamily)(7; 16; 29) predicted to contain seven transmembrane domains, the APN receptors are topologically distinct from G-protein coupled receptors (GPCR). Although numerous studies, done by others and us, demonstrate exogenous recombinant APN supplementation significantly protects the heart from ischemia/reperfusion injury in experimental animals, clinical APN application is limited due to multiple factors such as the high cost of production.

An adiponectin receptor agonist, AdipoRon was recently discovered by Okada-Iwabu et al.(19) This synthetic small molecule is orally active, binds to and activates both AdipoR1 and AdipoR2, ameliorates insulin resistance and type 2 diabetes, and prolongs the shortened lifespan of db/db mice. These results in sum suggest AdipoRon may be a novel therapeutic

101 molecule that effectively treats type 2 diabetes. However, whether AdipoRon may possess
102 cardioprotective properties, attenuating post-ischemic cardiomyocyte death and improving
103 cardiac function, have not been previously investigated.

104 Therefore, the aims of the present study were 1) to determine whether oral administration
105 of AdipoRon may attenuate post-ischemic cardiomyocyte apoptosis and improve cardiac
106 function recovery, and 2) if so, to investigate the underlying molecular mechanisms.

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MATERIALS AND METHODS

Adult male WT mice and APN knockout ($APN^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). Cardiomyocyte-specific AMPK (5'-adenosine monophosphate-activated protein kinase)-α_2 subunit mutant transgenic mice (AMPK-DN) were kindly provided by Dr. R Tina (University of Washington). Generation, breeding, phenotype characteristics, and genotyping of AMPK-DN mice (>80% inhibition of cardiac AMPK activity) has previously been described in detail(36). 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.

Myocardial ischemia/reperfusion: Mice were anesthetized with 2% isoflurane. MI/R was induced 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. Ten minutes before coronary occlusion, animals were randomized to receive either vehicle or AdipoRon (50 mg/kg, Calbiochem, Cat # 509104) via a gavage tube. This dose was selected from previously published results demonstrating maximal blood concentration was achieved 30 minutes after a single oral dose of AdipoRon(19). After 30 minutes of MI, the slipknot was released. The myocardium was reperfused for either 3 hours (for all assays excluding cardiac functional measurement) or 24 hours (for cardiac functional assay). All assays were performed utilizing tissue from the ischemic/reperfused area, i.e. the area at risk (AAR) identified by Evans blue negative staining. Sham-operated control mice (Sham MI/R) underwent the same surgical procedure, except the suture placed under the left coronary artery was not tied. Cardiac function was determined by echocardiography and left ventricular catheterization methods 24 hours after reperfusion before thoracotomy, as described in our previous study(34).

Assessment of cardiomyocyte apoptosis: Cardiomyocyte apoptosis was determined by DNA ladder formation, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, and caspase-3 activity as reported in our previous study(31). For the DNA fragmentation assay, total DNA was isolated with the Gentra Puregene Tissue DNA Isolation Kit (QIAgen, Valencia, CA) per manufacturer's instructions. 10 μg of DNA was loaded into 1.8% agarose gel containing 0.5 μg ml^{-1} ethidium bromide. DNA electrophoresis commenced at 60 V for 1-2 hours. DNA ladder formation, a "hallmark" of tissue apoptosis, was visualized under ultraviolet light, and photographed for permanent documentation.

TUNEL staining was performed via In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Manheim, Germany) per manufacturer's protocol. In brief, cardiomyocytes from at least four random slides per block were evaluated immunohistochemically to determine the number and percentage of cells exhibiting apoptotic-positive staining. The slides were covered with the mounting medium containing DAPI for total nuclei detection. By 20 X objective, the entire ischemic/reperfused area was digitally photographed with a QICAM-Fast Digital Camera mounted atop an Olympus BX51 Fluorescence Microscope. Total nuclei (blue) and the TUNEL positive nuclei (green) were counted by IP Lab Imaging Analysis Software (Version 3.5, Scanalytics, Fairfax, VA) with a custom-made script (by Mr. Ken Anderson, Bio Vision Technologies, North Exton, PA). The index of apoptosis (number of TUNEL positive nuclei/total number of nuclei x 100) was automatically calculated and exported to Microsoft Excel for further analysis. Results from different fields taken from the same animal were averaged and counted as 1 sample. The caspase-3 activity was determined by utilizing the fluorogenic substrate DEVD-7-amino-4-trifluoromethyl-coumarin (AFC).

Briefly, cells or mouse heart tissue were lysed by 1X caspase-3 lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 5 mM DTT, 0.1 mM EDTA, 0.1% Triton X-100), and total protein concentration was determined by the Bradford method (Bio-Rad). To each well of a 96-well plate, supernatant containing 50 μg/50μl of protein was loaded and incubated with 3.645 μg of Ac-DEVD-AFC (Biomol, P-409) in 50μl 2X Assay buffer (100 mM HEPES, 200mM NaCl, 0.2% CHAPS, 2mM EDTA,10% glycerol, 10mM DTT, pH7.4) at 37°C for 1.5 hours. AFC was cleaved from Ac-DEVD-AFC by activated caspase-3, and the free AFC was quantified with Spectra Max M5 fluorescence microplate reader (excitation wavelength, 400 nm; emission wavelength, 508 nm, Molecular Devices, Sunnyvale, CA) by AFC standard curve (Biomol, KI-108). Caspase-3 activity was expressed as nanomoles of AFC formation per hour per milligram of protein.

Quantification of superoxide production: Myocardial superoxide content (in the area-at-risk) was determined by lucigenin-enhanced luminescence as previously described(34). Approximately 30 mg protein of ischemic left ventricular region was separated, immediately minced, and incubated in 5ml of oxygen-equilibrated Krebs-Henseleit solution containing 10 mM HEPES-NaOH (pH 7.4) for 20 minutes at room temperature. The samples were placed into glass tubes containing 10 μM lucigenin in a final volume of 1 ml Krebs-Henseleit solution. Superoxide production was expressed as relative light units (RLU) per second per mg heart weight (RLU/s/mg wet tissue). The cellular origin of reactive oxygen species was determined by dihydroethidium (DHE) staining per manufacturer's protocol (Molecular Probes, Carlsbad, CA).

Western blot analysis: Proteins were separated on SDS-PAGE gels, transferred to

nitrocellulose membranes, and incubated with primary antibodies against Acetyl-CoA carboxylase (ACC) phosphorylated ACC (pACC), $gp91^{phox}$ or GAPDH (Cell Signaling Technology, Danvers, MA) followed by HRP-conjugated secondary antibody. The blot was developed with a Supersignal Chemiluminescence detection kit (Pierce, Rockford, IL). The band was visualized by a Kodak Image Station 4000R Pro (Rochester, NY).

Determination of cardiac function: Cardiac function was determined by echocardiography and left ventricular (LV) catheterization methods 24 hours after reperfusion, prior to chest reopening, as described previously(31; 32).

Statistical Analysis: All data in the text and figures are presented as means±S.E. of n independent experiments. Hemodynamic data were analyzed by two-way ANOVA. All other data were analyzed by one-way ANOVA followed by the Bonferroni correction for post hoc *t* tests (GraphPad Prism, San Diego, CA). Probabilities of 0.05 or less with Bonferroni correction were considered statistically significant.

RESULTS

AdipoRon treatment significantly improved cardiac functional recovery after reperfusion: Myocardial ischemia/reperfusion causes severe cardiac functional impairment 24 hours after reperfusion (Figure 1 and Figure 2, WT). Treatment with AdipoRon significantly improved cardiac functional recovery as evidenced by improved maximal positive and negative dP/dt (Figure 1) and increased left ventricular ejection fraction (EF%, Figure 2 WT). MABP was slightly decreased in the MI/R group 24 hours after reperfusion in comparison to the sham MI group. However, the difference was not statistically different. There was no difference in heart rate among the three groups studied (data not shown).

AdipoRon significantly inhibited post-MI apoptosis: To determine the cellular mechanism responsible for cardioprotective effect of AdipoRon, we first determined the effect of AdipoRon treatment on cardiomyocyte apoptotic death by DNA ladder formation, a hall marker of apoptotic cell death. In myocardial tissue from sham MI hearts, no DNA ladder was detected (Figure 3A WT, lanes 1). In contrast, the formation of DNA nucleosome ladders was clearly detected in myocardial tissues obtained from MI/R hearts receiving only vehicle (Figure 3A WT, lanes 2). Most importantly, hearts treated with AdipoRon exhibited markedly decreased DNA fragmentation (Figure 3A WT, lane 3).

To determine the effect of AdipoRon on apoptosis in a quantitative manner, caspase-3 activation and TUNEL staining were performed. AdipoRon treatment markedly reduced ischemia/reperfusion-induced caspase-3 activation (Figure 3B WT). In sham MI hearts, an extremely low level of TUNEL positive cells (Figure 4 WT) was observed. In contrast, tissues from ischemic-reperfused hearts receiving only vehicle manifested prevalent

TUNEL-positive nuclei (Figure 4 WT). AdipoRon treatment reduced the number of TUNEL-positive cells (Figure 4 WT).

Enhanced cardiomyocyte apoptosis in APN deficient mice is rescued by AdipoRon administration: To determine whether AdipoRon is effective in rescuing the heart from enhanced MI/R injury in APN deficient animals, the effect of AdipoRon on cardiac dysfunction and cardiomyocyte apoptosis was determined in $APN^{-/-}$ mice. AdipoRon significantly improved cardiac function (Figure 1, Figure 2, $APN^{-/-}$) and reduced post-MI cardiomyocyte apoptosis, as evidenced by attenuated ladder formation (Figure 3A, $APN^{-/-}$), reduced caspase-3 activity (Figure 3B, $APN^{-/-}$), and decreased TUNEL positive cells (Figure 4, $APN^{-/-}$).

Anti-apoptotic effect of AdipoRon is attenuated but not lost in AMPK-DN mice: Compared to WT, cardiac dysfunction and apoptotic cell death caused by MI/R was increased in the AMPK-DN heart. We have reported previously the cardioprotective effects of APN are only partially mediated by AMPK activation(34). In similar fashion, the beneficial effects of AdipoRon upon cardiac dysfunction are blunted (Figure 1 and Figure 2, AMPK-DN vs. WT) but not lost in AMPK-DN mice. Similarly, the anti-apoptotic effect of AdipoRon is partially blocked but not completely lost in AMPK-DN mice. Specifically, administration of AdipoRon reduced caspase-3 activation by 39% (compared to 73% reduction in WT mice, Figure 3) and TUNEL staining by 33% (comparing to 50% reduction in WT mice, Figure 4) in AMPK-DN mice. In the AMPK-DN heart, there was complete blockade of AdipoRon-induced phosphorylation of ACC (pACC, Figure 5A). Therefore, the remaining portion of anti-apoptotic effect of AdipoRon in AMPK-DN mice can be attributed to AMPK

independent signaling mechanisms.

AdipoRon significantly reduced NADPH oxidase expression and inhibited superoxide production in ischemic/reperfused heart: Our previous study demonstrated the anti-oxidant effect of APN is not mediated by AMPK(34). To determine whether AdipoRon may have any anti-oxidant effect (potentially contributive to the remaining anti-apoptotic effect of AdipoRon observed in AMPK-DN mice), the effect of AdipoRon upon post-ischemic superoxide production and $gp91^{phox}$ (the primary sub-form of NADPH oxidase expressed in adult cardiomyocytes) expression were determined in AMPK-DN mice. As summarized in Figure 5, AdipoRon administration significantly reduced superoxide production assessed by DHE staining (B) and lucigenin-enhanced luminescence assay (C). Moreover, AdipoRon treatment significantly attenuated ischemia/reperfusion induced $gp91^{phox}$ overexpression (D).

Discussion

Early reperfusion after coronary occlusion remains the most effective means of limiting ischemic myocardial injury. However, evidence from animal studies, as well as clinical observations, demonstrates reperfusion itself may cause additional cell death, defined as "reperfusion injury"(6). Strong epidemiological evidence suggests type-2 diabetes not only causes coronary vascular injury thereby increasing ischemic heart disease prevalence, but also exacerbates cardiac injury after ischemia/reperfusion insult in these patients(4; 13; 17). Adiponectin (APN) is a protein hormone primarily produced by adipocytes (20). In contrast to the majority of adipokines (such as TNFα), which are pro-inflammatory, and significantly increased in diabetic patients, APN is markedly reduced in diabetic patients, and is potently protective of the vasculature(18; 26; 28). Plasma APN levels significantly decrease after tissue injury, such as acute lung injury caused by ovalbumin challenge(27). Numerous epidemiological studies reveal the association between hypoadiponectinemia and increased cardiovascular disease risk in obesity and diabetes(8; 12; 15; 38). Additionally, recent clinical observations demonstrate post-MI plasma APN levels correlate positively with myocardial salvage index and ejection fraction recovery(23). Persistent plasma hypoadiponectinemia post myocardial infarction is predictive of future adverse cardiac events(2). Moreover, recent experimental studies demonstrate that myocardial reperfusion injury is significantly enhanced in APNKO mice. Replenishment of recombinant APN in APNKO mice was cardioprotective and fully rescued phenotypic alteration(20-22; 24). Importantly, multiple investigations [including the seminal study by Walsh and colleagues(25), a large animal model study by Kondo and colleagues (14), and our recent study (31)] have documented APN administration

in WT mice and pigs significantly reduces infarct size and improves cardiac function. Despite clear experimental evidence that supplementation of recombinant human APN exerts significant anti-diabetic and cardioprotective actions, APN's complex quaternary structure and rapid turnover are major disadvantages to producing and administering APN in the requisite amounts for appropriate clinical care. Thus, the field has been awaiting the advent of low molecular weight APN receptor agonists capable of overcoming such hindrances.

In an effort to identify small synthetic molecules capable of activating the APN receptors (AdipoR1 and AdipoR2), Kadowaki and colleagues recently screened a compound library, and identified several molecules activating APN receptors, but focused their in-depth analysis upon one, "AdipoRon."(19) AdipoRon binds, at a low micromolar concentration, to both AdipoR1 and AdipoR2. Like APN, it activates AMPK in cultured mammalian cells, an enzyme involved in many metabolic processes including insulin release, lipid synthesis inhibition, and glucose uptake stimulation. It also activates the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), which boosts mitochondrial proliferation and energy metabolism. Like APN, AdipoRon improved glucose metabolism, lipid metabolism, and insulin sensitivity in both cultured cells and mice, via APN receptor-dependent mechanisms. When *db/db* mice fed a high-fat diet were treated with AdipoRon, their metabolic improvements extended their life span. Furthermore, AdipoRon administration to chow-fed wild-type mice augmented exercise endurance capacity. Their study convincingly demonstrated the viable strategy of targeting adiponectin receptors with low molecular weight agonists.

294 Strong clinical and experimental evidence supports the contribution of
295 hypoadiponectinemia to enhanced cardiovascular injury in the diabetic population. We and
296 others have previously demonstrated MI/R injury is significantly exacerbated in APN
297 deficient mice, a phenotype fully rescued by recombinant APN replenishment. In the current
298 study, AdipoRon administration rescued the pathological cardiac phenotype in APN deficient
299 mice, similar to our previous report concerning APN administration(31). Moreover, our
300 current study provides direct evidence that oral AdipoRon administration in WT mice also
301 significantly improved post-ischemic cardiac function, as evidenced by increased $\pm dP/dt_{max}$
302 and enhanced left ventricular ejection fraction. These results demonstrate AdipoRon, an
303 orally active small molecule previously shown to mimic the metabolic benefits of
304 recombinant APN, is biologically active in protecting heart from ischemia/reperfusion injury.
305 Apoptotic cell death is the primary cell death pathway following MI/R and significantly
306 contributes to post-ischemic cardiac dysfunction(3; 5). DNA ladder formation is highly
307 specific for apoptotic cell death, but lacks sensitivity, and is difficult to quantify. In contrast,
308 TUNEL staining of nuclei is extremely sensitive, but is less specific for apoptosis as some
309 necrotic cells may stain TUNEL positive. Caspase-3 activation is the final common pathway
310 leading to caspase-8 and caspase-9 induced apoptotic cell death. These three methods were
311 used in combination to improve the accuracy and reliability of our results. AdipoRon reduced
312 DNA ladder formation (Figure 3A), inhibited caspase-3 activation (Figure 3B), and decreased
313 TUNEL staining (Figure 4), indicating AdipoRon possesses clear anti-apoptotic property in
314 ischemic/reperfused cardiomyocytes.
315 AMPK was once considered the most important downstream molecule mediating APN

316 biological function. The effect of cardiac-specific AMPK inhibition upon the anti-apoptotic
317 effects of AdipoRon was determined. The beneficial effects of AdipoRon upon cardiac
318 dysfunction (Figures 1,2) and apoptotis (Figures 3,4) after MI/R were clearly blunted in
319 AMPK-DN mice. These results indicate AMPK activation contributes to the cardioprotective
320 effect of AdipoRon. However, our results also clearly demonstrate the cardioprotective effect
321 of AdipoRon is not completely lost in AMPK-DN mice. Specifically, administration of
322 AdipoRon in AMPK-DN increased $\pm dP/dt_{max}$ (1.35- and 1.34-fold), enhanced left ventricular
323 ejection fraction (1.27-fold), reduced caspase-3 activation (39%), and decreased TUNEL
324 staining (33%). That AdipoRon retained a significant portion of anti-apoptotic effect in
325 AMPK-DN mice suggests the existence and contribution of mechanisms independent of
326 AMPK signaling to AdipoRon-mediated anti-apoptotic function in the ischemic/reperfused
327 heart. This result is consistent with our previous study showing that the cardioprotective
328 effect of adiponectin is partially mediated by its AMPK-independent anti-nitrative action(35).
329 Our current study provides supporting evidence the remaining anti-apoptotic action of
330 AdipoRon in AMPK-DN mice is mediated by anti-oxidative effect. This notion is supported
331 by following three lines of evidence. First, oxidative stress plays critical causative roles in
332 post-ischemic myocardial apoptosis and cardiac dysfunction(1; 30; 33); Second, the
333 anti-oxidative effect of APN is AMPK-independent; and third, AdipoRon inhibits NADPH
334 oxidase overexpression and superoxide overproduction in the ischemic/reperfused heart.
335 In summary, our study demonstrated that the oral APN receptor agonist AdipoRon is
336 effective in attenuating post-ischemic cardiac injury, indicating APN receptor agonists are a

promising novel therapeutic approach for treating cardiovascular complications caused by obesity-related disorders such as type 2 diabetes(11).

Disclosures

None

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462 **Figure Legends**

463 **Figure 1**. Oral AdipoRon administration significantly improved dP/dt_{max} and $-dP/dt_{max}$ in
464 wild type (WT), Adiponectin knockout ($APN^{-/-}$) and cardiomyocyte-specific AMPK-α_2
465 subunit mutant transgenic mice (AMPK-DN). N=14-16 animals/group. *P<0.05, **P<0.01 vs.
466 MI/R+vehicle. P values between MI/R vs. sham are all less than 0.01 (not labeled, the same
467 for all figures).

468 **Figure 2**. Oral AdipoRon administration significantly enhanced left ventricular ejection
469 fraction (EF%) determined by echocardiography in WT, $APN^{-/-}$ and AMPK-DN. Note
470 attenuated response to AdipoRon treatment in AMPK-DN mice compared to WT mice.
471 However, a significant portion of cardioprotection is retained in AMPK-DN mice. N=14-16
472 animals/group. *P<0.05, **P<0.01 vs. MI/R+vehicle.

473 **Figure 3**. Oral AdipoRon administration significantly attenuated post-ischemic cardiac
474 apoptosis determined by DNA ladder formation (A) and caspase-3 activation (B) in WT and
475 $APN^{-/-}$ mice. AdipoRon also significantly inhibited DNA ladder formation and reduced
476 caspase-3 activity in AMPK-DN mice, albeit to lesser extent seen in WT mice. N=6-8
477 animals/group. *P<0.05, **P<0.01 vs. MI/R+vehicle.

478 **Figure 4.** Oral AdipoRon administration significantly attenuated post-ischemic cardiac
479 apoptosis determined by TUNEL staining in WT and $APN^{-/-}$ mice. AdipoRon also
480 significantly inhibited TUNEL staining in AMPK-DN mice, albeit to lesser extent seen in WT
481 mice. N=6-8 animals/group. *P<0.05, **P<0.01 vs. MI/R+vehicle.

482 **Figure 5**. AdipoRon activated AMPK signaling (determined by ACC phosphorylation) in WT,

483 but not in AMPK-DN, animals (A). AdipoRon significantly inhibited ischemia/reperfusion
484 induced superoxide production, determined by DHE staining (B), lucigenin-enhanced
485 luminescence assay (C), and $gp91^{phox}$ overexpression (D) in AMPK-DN mice. N=6-8
486 animals/group for DHE staining and Western analysis; N=9-11 animals/group for
487 lucigenin-enhanced luminescence assay. *P<0.05, **P<0.01 vs. MI/R+vehicle.

488

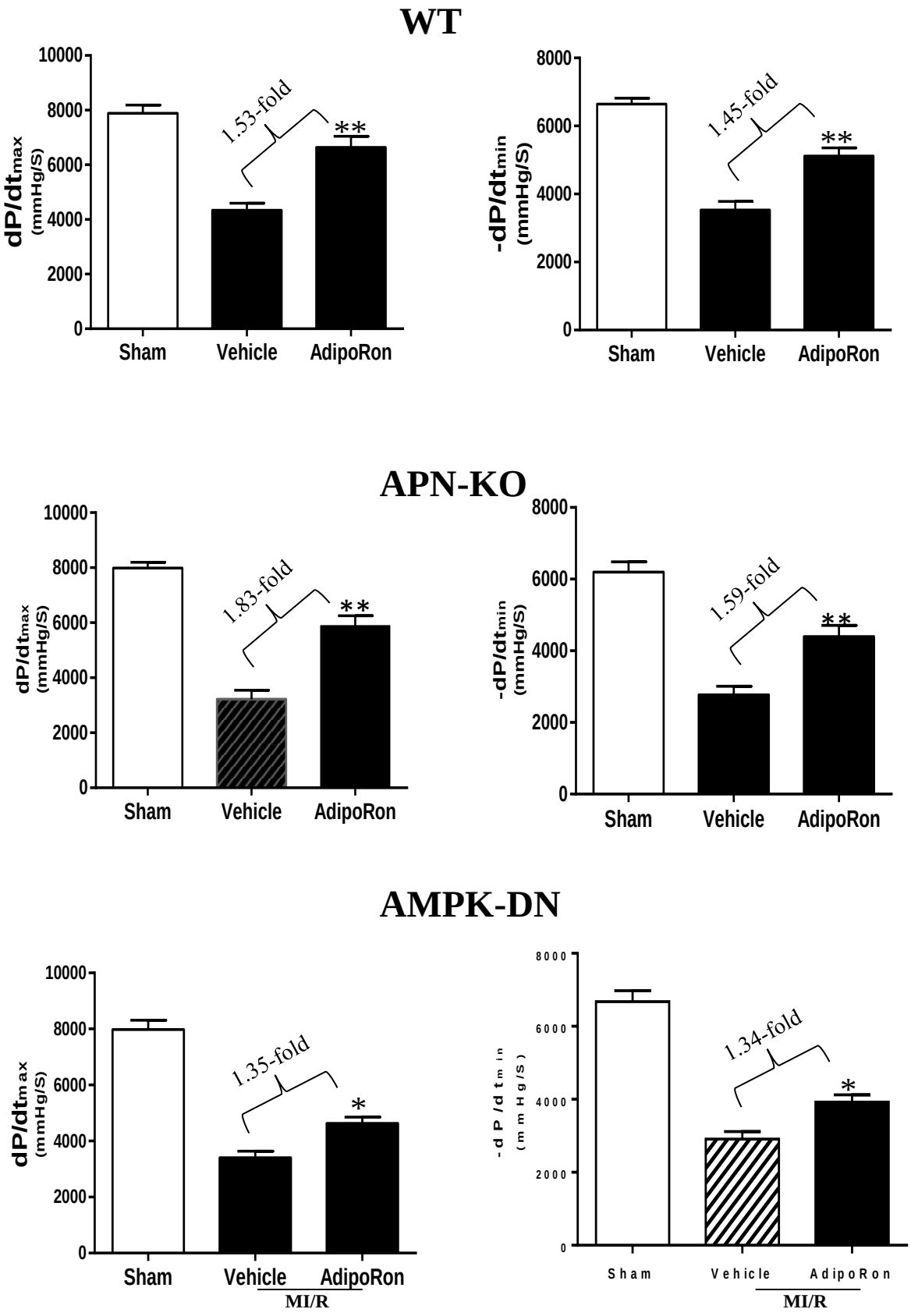
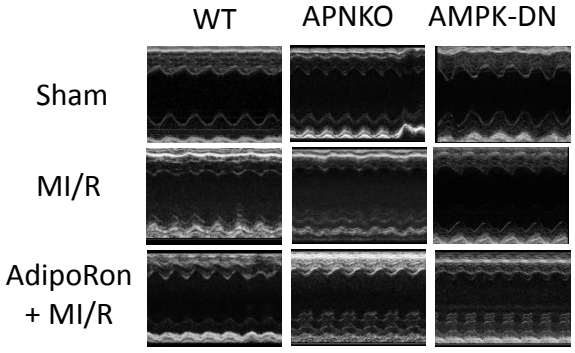
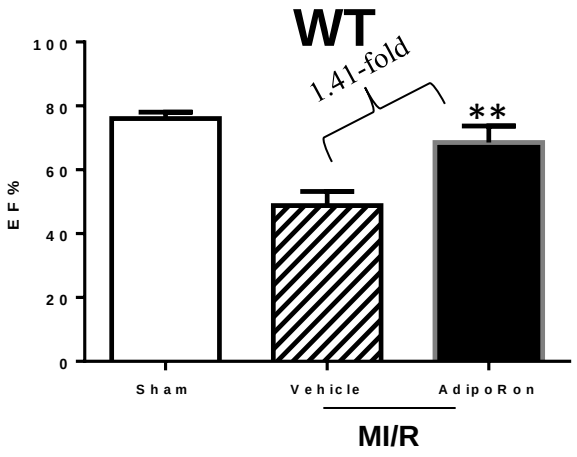
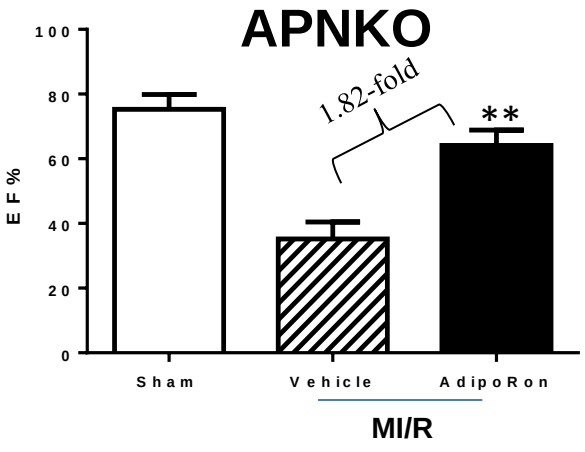

WT
dP/dtmax (mmHg/S)
10000
8000
6000
4000
2000
0
1.53-fold
**
Sham
Vehicle
AdipoRon
-dP/dtmin (mmHg/S)
8000
6000
4000
2000
0
1.45-fold
**
Sham
Vehicle
AdipoRon
APN-KO
dP/dtmax (mmHg/S)
10000
8000
6000
4000
2000
0
1.83-fold
**
Sham
Vehicle
AdipoRon
-dP/dtmin (mmHg/S)
8000
6000
4000
2000
0
1.59-fold
**
Sham
Vehicle
AdipoRon
AMPK-DN
dP/dtmax (mmHg/S)
10000
8000
6000
4000
2000
0
1.35-fold
*
Sham
Vehicle
AdipoRon
MI/R
-dP/dtmin (mmHg/S)
8000
6000
4000
2000
0
1.34-fold
*
Sham
Vehicle
AdipoRon
MI/R

Figure 1


WT
APNKO
AMPK-DN
Sham
MI/R
AdipoRon
+ MI/R


WT
1.41-fold
**
EF%
100
80
60
40
20
0
Sham
Vehicle
AdipoRon
MI/R


APNKO
1.82-fold
**
EF%
100
80
60
40
20
0
Sham
Vehicle
AdipoRon
MI/R

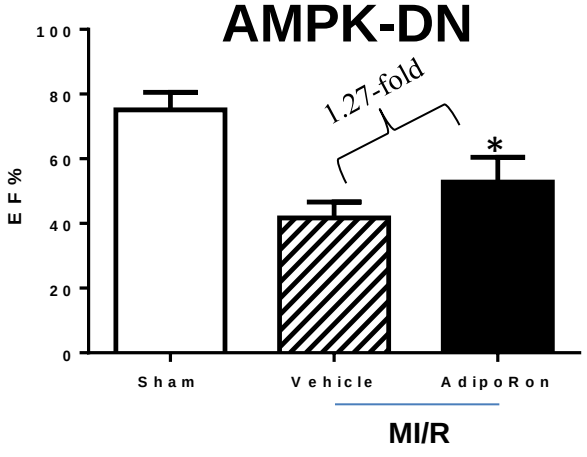

AMPK-DN
1.27-fold
*
EF%
100
80
60
40
20
0
Sham
Vehicle
AdipoRon
MI/R

Figure 2

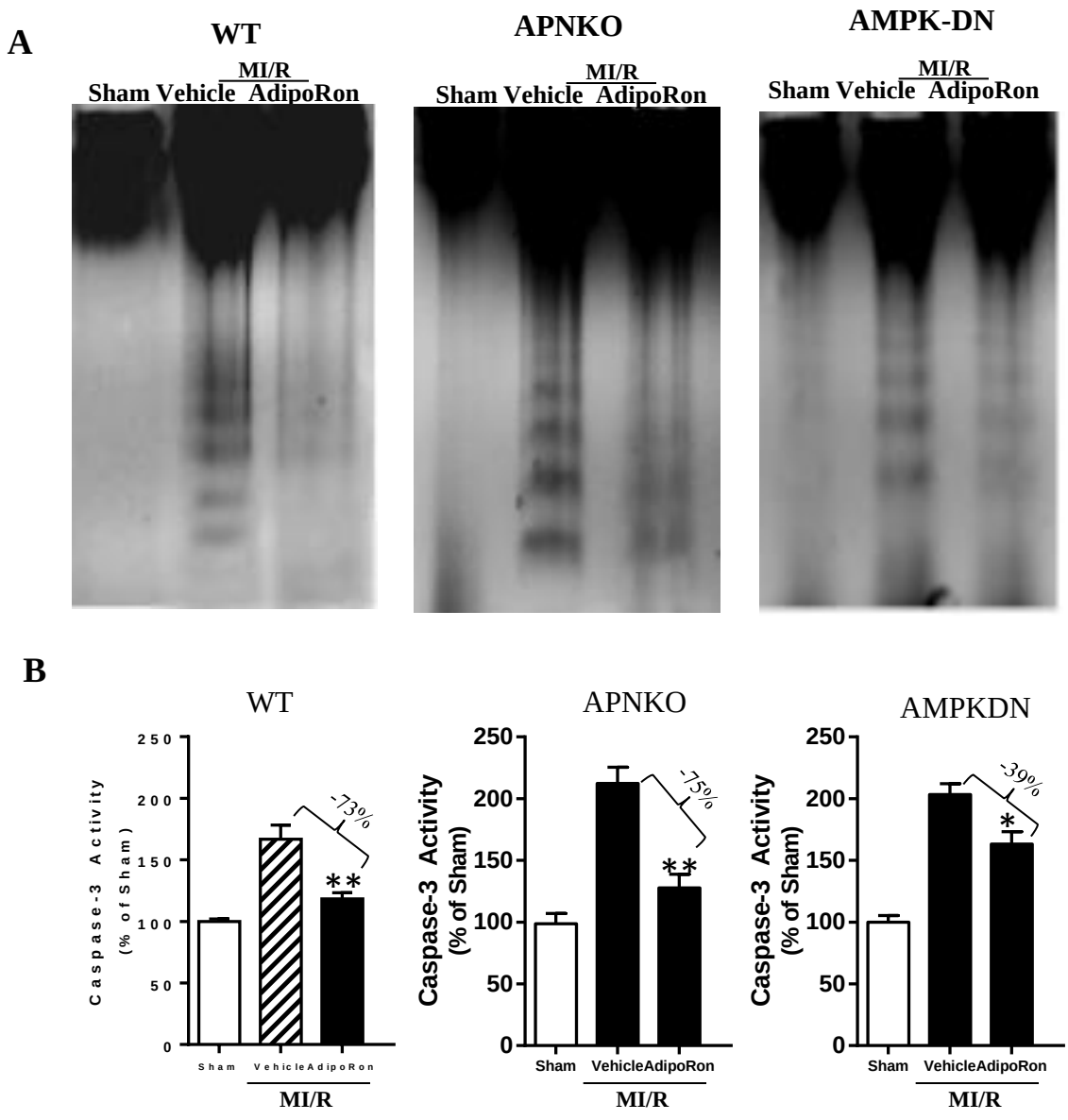

A
WT
APNKO
AMPK-DN
MI/R
Sham Vehicle AdipoRon
B
WT
APNKO
AMPKDN
Caspase-3 Activity (% of Sham)
250
200
150
100
50
0
-73%
-75%
-39%
**
*
Sham
Vehicle
AdipoRon
MI/R

Figure 3

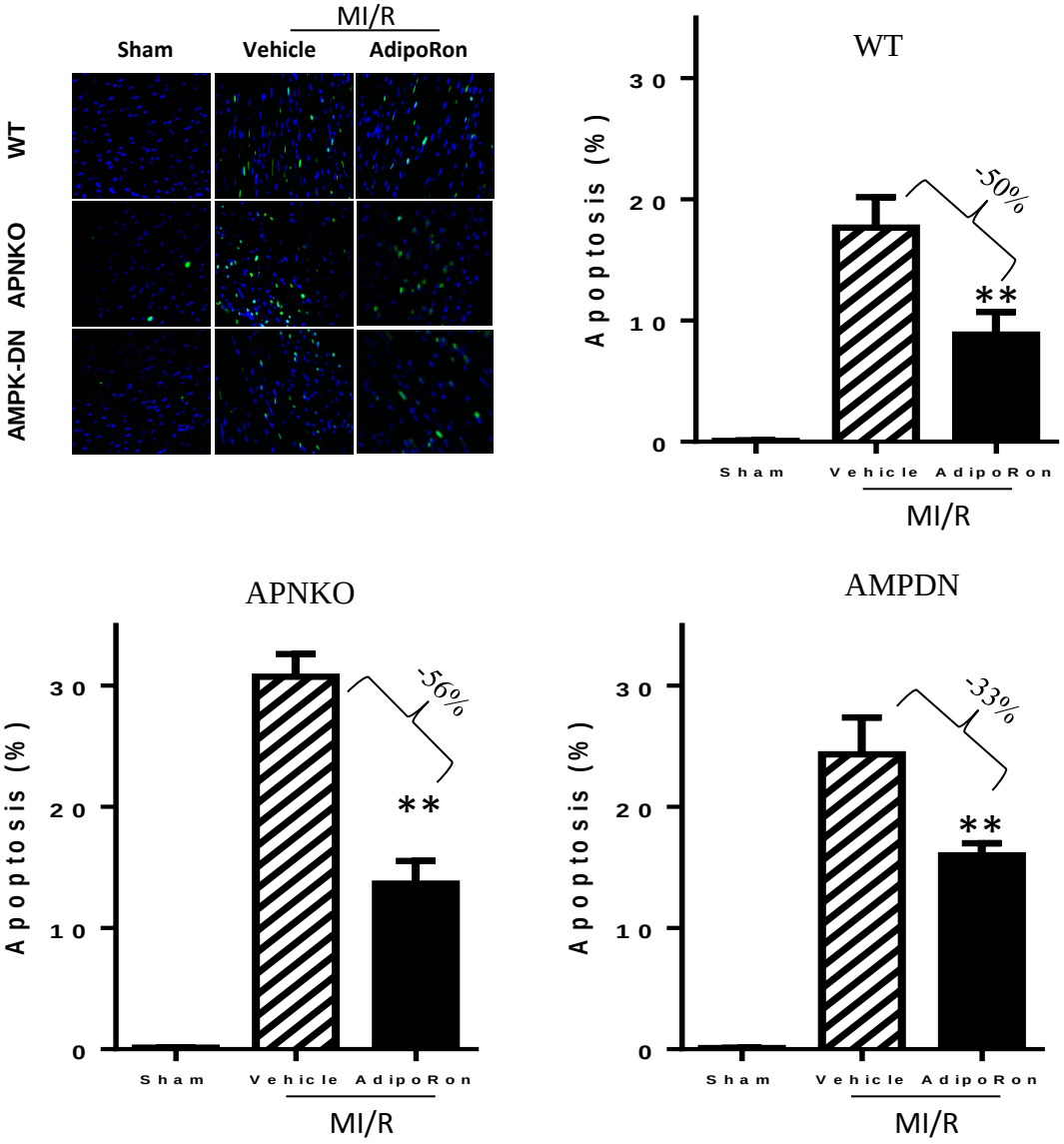
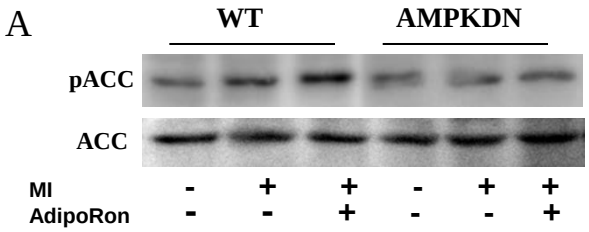
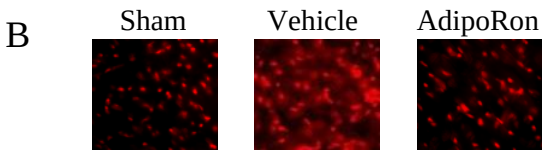
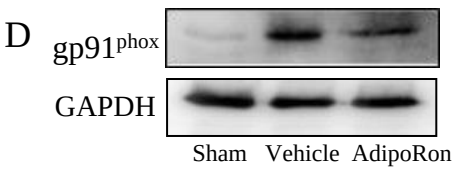
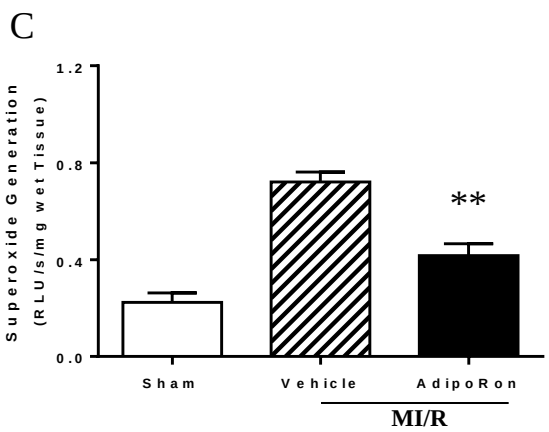

MI/R
Sham
Vehicle
AdipoRon
WT
APNKO
AMPK-DN
WT
Apoptosis (%)
30
20
10
0
-50%
**
Sham
Vehicle
AdipoRon
MI/R
APNKO
-56%
AMPDN
-33%

Figure 4


A
WT
AMPKDN
pACC
ACC
MI
AdipoRon


B
Sham
Vehicle
AdipoRon


D
gp91phox
GAPDH
Sham
Vehicle
AdipoRon


C
Superoxide Generation
(RLU/s/mg wet Tissue)
1.2
0.8
0.4
0.0
**
Sham
Vehicle
AdipoRon
MI/R

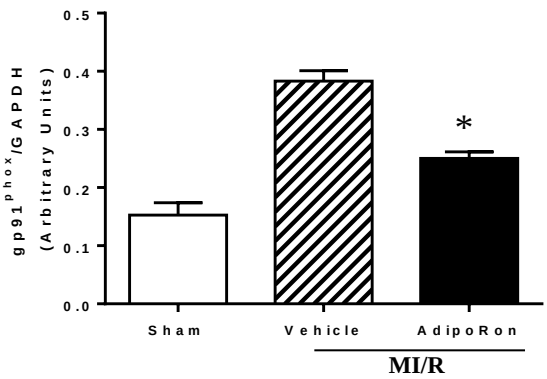

gp91phox/GAPDH
(Arbitrary Units)
0.5
0.4
0.3
0.2
0.1
0.0
*
Sham
Vehicle
AdipoRon
MI/R

Figure 5