3-1-2010

Cardiomyocyte-derived adiponectin is biologically active in protecting against myocardial ischemia-reperfusion injury.

Yajing Wang

Wayne Bond Lau

Erhe Gao

Ling Tao

Yuexing Yuan

See next page for additional authors

Authors

Yajing Wang, Wayne Bond Lau, Erhe Gao, Ling Tao, Yuexing Yuan, Rong Li, Xiaoliang Wang, Walter J. Koch, and Xin-Liang Ma

Articles in PresS. Am J Physiol Endocrinol Metab (December 22, 2009). doi:10.1152/ajpendo.00663.2009

Cardiomyocyte-Derived Adiponectin is Biologically Active in Protecting Against Myocardial Ischemia/Reperfusion Injury

Yajing Wang, MD, PhD[1], Wayne Bond Lau, MD[1], Erhe Gao, MD, PhD[2], Ling Tao, MD, PhD[3], Yuexin Yuan, PhD[1], Rong Li, MD, PhD[1], Xiaoliang Wang, MD, PhD, Walter Koch, PhD[2], Xin-Liang Ma, MD, PhD[1#]

Running Title: Cardiac Adiponectin and Myocardial Protection

Address proofs to:
[#]Xin L Ma, M.D., Ph.D.
Department of Emergency Medicine
1020 Sansom Street
Thompson Building, Room 239
Philadelphia, PA 19107

Tel: (215)955-4994
Fax: (215)923-6225
E-mail: Xin.Ma@Jefferson.edu

Total Word Count: 4,971

[1]Department of Emergency Medicine, [2]Center for Translational Medicine, Thomas Jefferson University, Philadelphia, PA 19107, [3]Department of Cardiology, Xijing Hospital, 15 West Changle Road, Xian, PR China.

Abstract

Adiponectin (APN) has traditionally been viewed as an adipocyte-specific endocrine molecule with cardioprotective effects. Recent studies suggest that APN is also expressed in cardiomyocytes. However, biological significances of this locally produced APN remain completely unknown. The aim of this study was to investigate the pathologic and pharmacologic significance of cardiac-derived APN in cardiomyocyte pathology. Adult cardiomyocytes from wild type littermates (WT) or gene deficient mice were pre-treated with vehicle (V) or rosiglitazone (RSG) for 6 h followed by simulated ischemia/reperfusion (SI/R, 3h/12h). Compared to WT cardiomyocytes, myocytes from APN knockout (APN-KO) mice sustained greater SI/R injury, evidenced by greater oxidative/nitrative stress, caspase-3 activity, and LDH release ($P<0.05$). Myocytes from adiponectin receptor 1 knock-down (AdipoR1-KD) or AdipoR1-KD/AdipoR2-KO mice had slightly increased SI/R injury, but the difference was not statistically significant. RSG significantly ($P<0.01$) increased APN mRNA and protein expression, upregulated AdipoR1/AdipoR2 expression, reduced SI/R-induced apoptosis, and decreased LDH release in WT cardiomyocytes. However, the anti-oxidative/anti-nitrative and cell protective effects of RSG were completely lost in APN-KO cardiomyocytes ($P>0.05$ vs. vehicle group), although a comparable degree of AdipoR1/AdipoR2 upregulation was observed. The upregulatory effect of RSG on APN mRNA and protein expression was significantly potentiated in AdipoR1-KD/AdipoR2-KO cardiomyocytes. However, the cellular protective effects of RSG were significantly blunted, although not completely lost, in these cells. These results demonstrated that cardiomyocyte APN is biologically active in protecting cells against SI/R injury. Moreover, this locally produced APN achieves its protective effect primarily through paracrine/autocrine activation of APN receptors.

Keywords: Myocardial ischemia; diabetes; cytokines**;** oxidative stress

Strong epidemiological evidence exists that type-2 diabetes not only causes coronary vascular injury thus increasing ischemic heart disease prevalence in these patients, but also renders cardiomyocytes more susceptible to ischemia/reperfusion insult, and increases cardiomyocyte death after the onset of myocardial ischemia(5; 15; 19). Clarifying the molecular link between type-2 diabetes and cardiovascular injury may therefore help identify novel effective therapeutic interventions that attenuate post-ischemic myocardial injury, reduce myocardial ischemic morbidity, and ultimately decrease diabetic mortality of cardiovascular etiology.

Adiponectin (APN) is a protein hormone circulating in plasma as multimeric complexes at relatively high concentration (2-10 μg/ml)(21). Besides its well-defined insulin sensitization and metabolic regulatory effects, recent experimental and clinical studies demonstrate that APN is a potent endogenous cardioprotective molecule(10). Numerous epidemiological studies have shown that reduced APN levels correlate with increased risk of cardiovascular disease in obesity and diabetes(9; 12; 16; 29); high plasma APN concentrations are associated with a lower risk of MI in men(23). In addition, recent clinical observations have demonstrated that post-MI plasma APN levels correlated positively with myocardial salvage index and ejection fraction recovery(24). Persistently low plasma APN concentrations after acute myocardial infarction are predictive of future adverse cardiac events(1). As such, reduced APN production has been recognized as a risk factor for cardiovascular disease, and enhancing APN production has been accepted as a potential therapeutic modality for ameliorating diabetic cardiovascular injury.

Although APN receptors, including APN receptor 1 (AdipoR1) and APN receptor 2 (AdipoR2), are present in most organs including adult cardiomyocytes, it was generally accepted until recently that APN is exclusively synthesized in adipocytes. However, new studies reveal

that the APN gene is expressed in other cell types, including hepatocytes, myotubes, skeletal muscle, and osteoblasts(17; 25). Three recent studies have demonstrated that APN is also expressed in adult cardiomyocytes, and its production is increased by activation of peroxisome proliferator-activated receptor-γ (PPARγ)(4; 11; 22). However, whether this cardiac-derived APN is biologically active, contributing to physiological and pathologic regulation of cardiomyocyte function, remains completely unknown presently. Addressing this critical question may provide clues to the largely unknown regulation of cardiomyocyte metabolism and function in the diabetic heart.

Therefore, the aims of the present study were to determine whether cardiomyocyte-derived APN is biologically active and cardioprotective, and if so, to further investigate transmembrane and intracellular signaling mechanisms responsible for the cardioprotective actions of locally produced APN.

Materials and Methods:

Animals: Adult male C57BL/6, homozygous adiponectin knockout (APN-KO), AdipoR2 homozygous knockout (AdipoR2-KO), and their littermate wild type control weighing 27-32 g were purchased from Jackson Labs, Inc (Bar Harbor, ME). All procedures were performed in accordance 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.

In vivo siRNA-mediated AdipoR1 knock-down (AdipoR1-KD): AdipoR1 knockout mice were not commercially available when this study was performed. We thus utilized siRNA gene silencing technique to knock-down AdipoR1 expression in mouse heart (AdipoR1-KD). In brief, three pre-designed AdipoR1-specific siRNA (catalog numbers: s91209, s91210, and s91208, Ambion Silencer) or a control nonspecific siRNA oligos (Silencer Select Negative Control #1 siRNA, Ambion) were diluted in 5% glucose and mixed with *in vivo*-jet PEI (Genesee Scientific, San Diego, CA). Adult wild type (WT) or AdipoR2-KO mice were anesthetized with 2% isoflurane, and the heart was exposed via left thoracotomy at the fifth intercostal space. 20 µl (0.8 µg/g) siRNA or negative control was delivered via three separate intramyocardial injections (32½G needle) to temporarily blanch the left ventricular free wall. Based on our pilot experiments showing that AdipoR1 expression reaches its nadir 48 h after siRNA injection, hearts were isolated 48 h following siRNA treatment for adult cardiomyocyte harvest and preparation as described below.

Preparation and culturing of adult mouse cardiomyocytes: Mice were anesthetized with 2% isoflurane. Hearts were removed and perfused at 37°C for ~3 min with a Ca^{2+}-free bicarbonate-based buffer. Enzymatic digestion was initiated by adding collagenase type B/D to the perfusion solution. After ~3 min of digestion, at which point the cardiac tissue became firm and swollen,

50 μM Ca^{2+} was added to the enzyme solution; ~7 min later, the left ventricle was removed, cut into several sections, and further digested in a shaker for 10 min at 37°C in the same enzyme solution. The supernatant containing the dispersed myocytes was filtered into a sterilized tube, and centrifuged at 800 x g for 1 min. The cell pellet was then resuspended in bicarbonate-based buffer containing 125 μM Ca^{2+}. After the myocytes were pelleted by gravity for ~10 min, the supernatant was aspirated, and the myocytes were resuspended in bicarbonate-based buffer containing 250 μM Ca^{2+}. Myocytes were plated at 0.5-1 × 10^4 cells.cm^{-2} in culture dishes pre-coated with mouse laminin.

Simulated ischemia/reperfusion (SI/R). After 1 h of culture in a 5% CO_2 incubator at 37° C, cardiomyocytes were randomized to receive either vehicle or rosiglitazone treatment (RSG, 10 μmol/L)(4). Six h after RSG or vehicle treatment, cells were either collected for assessment of APN and its receptor expression, or further subjected to simulated ischemia/reperfusion (SI/R) as originally described by Isner and colleagues(20) and modified in our recently published study(8). In brief, glucose-free culture medium was first gassed for 5 minutes with a hypoxic gas mixture (95%N_2/5%CO_2). Normal culture medium was quickly replaced with the hypoxia-hypoglycemic medium, and cardiomyocytes were placed in a Napco 8000WJ hypoxia (1%O_2/5%CO_2/94%N_2) incubator (Thermo Scientific, Waltham, MA). After 3 h of hypoxia-hypoglycemic culture, the hypoxia/hypoglycemic medium was replaced with normal culture medium. Cells were then incubated for an additional 12 h under normoxic conditions in a CO_2 incubator.

Measurements of mRNA levels in cultured cardiomyocytes: Total RNA was extracted from cultured cardiomyocytes with a Qiagen RNeasy kit (Qiagen, Valencia, CA). The expression levels of mRNA for APN, AdipoR1, and AdipoR2 were quantified by TaqMan one Step RT-PCR Master Mix reagent kit (Applied Biosystems, Foster City, CA) using FAM-labeled Taqman

probes (APN, AdipoR1, AdipoR2, Applied Biosystems) and 100 ng sample RNA to a final volume of 10 µl. Amplification reactions were performed in ABI 7900HT Sequence Detection System (Applied Biosystems) in the 384-well block format with the following cycle conditions: 50°C for 20 min for reverse transcription, 95°C denaturation and Ampli Taq Gold DNA polymerase activation for 10 min, followed by 40 cycles of 95°C denaturation for 15 s, and 60°C annealing and elongation for 60 s. Fluorescence spectra were recorded during the annealing phase of each cycle. On each plate, a standard curve is generated from 250 pg to 100 ng, and when the R^2 value was >0.99, the cycle threshold (Ct) values were accepted. The GAPDH housekeeping gene was used for normalization of target gene expression.

Analysis of APN secretion. Quantification of APN protein levels in cell culture supernatants was determined with mouse APN ELISA kit (Phoenix Pharmaceuticals, Inc., Belmont, CA) per manufacturer's instructions.

Western blot analysis: Cardiomyocyte lysates were separated by SDS/PAGE (12% acrylamide gel) and transferred to polyvinylidene difluoride membranes. Nonspecific binding sites were blocked by 5% (wt/vol) dried skimmed milk in TBS detergent (0.1% Tween 20). Membranes were then probed with specific primary antibodies followed by washing (3x for 15 min) with TBS detergent (0.1% Tween 20). The membranes were then incubated with either goat anti-mouse Ig or goat anti-rabbit Ig conjugated with horseradish peroxidase (1:10,000; Bio-Rad) for 1 h and washed (three times for 15 min) with TBS detergent (0.1% Tween 20). Immunoreactivity was visualized by using a Supersignal Chemiluminescence detection kit (Pierce, Rockford, Ill). Immunoblotting was visualized with a Kodak Image Station 400 (Rochester, NY), and the blot densities were analyzed with Kodak 1-dimensional software.

Measurement of caspase-3 activity: Apoptotic cell death was determined by caspase-3 activation, via a fluorometric kit (R&D System, Minneapolis, MN). Briefly, cardiomyocytes were harvested using caspase lysis buffers (50mM HEPES PH 7.4, 0.1% Chaps, 5mM DTT, 0.1 mM EDTA, 0.1% Triton-X100). 50 μg cell lysate samples were used to perform the fluorometric assay per manufacturer's instructions. The fluorescence emission of the 7-amino-4-trifluoromethyl-coumarin (AFC), released on proteolytic cleavage of the fluorogenic substrate DEVD-AFC by active caspase-3, was measured using Biotek FL600 microplate fluorescence reader (excitation wavelength, 400 nm; emission wavelength, 505 nm). Caspase-3 activity was expressed as nmol *AFC*/h/mg protein.

Lactate dehydrogenase (LDH) release assay: At the end of observation period, conditioned media was collected, and cells were lysed. LDH activity in conditioned media and cell lysates was determined spectrophotometrically (SpectraMax-Plus microplate spectrophotometer, Molecular Devices, Sunnyvale, CA). The percentage of LDH release was calculated as follows: (A-B)/(C-B) × 100, where A = LDH activity in conditioned media; B = LDH activity in culture media (without cells); C = LDH activity in cell lysates.

Quantification of cellular nitrotyrosine content: Cardiomyocyte nitrotyrosine content, an index of nitric oxide (NO) inactivation by superoxide and oxidative/nitrative stress, was determined by a nitrotyrosine ELISA kit (Cell Sciences, Canton, MA) per manufacturer's instructions.

Statistical Analysis: All values in the text and figures are presented as means ± SEM of *n* independent experiments. All data (except Western blot density) were subjected to ANOVA followed by Bonferroni correction for *post-hoc t* test. Western blot densities were analyzed with the Kruskal-Wallis test followed by Dunn's post-hoc test. Probabilities of 0.05 or less were considered to be statistically significant.

Results

Lack of APN, but not its receptors, significantly increased SI/R injury: Cardiomyocytes from adult male WT, APN-KO, AdipoR1-KD, and AdipoR2-KO mice were subjected to sham SI/R culture, or 3 h SI plus 12 h reperfusion. Cellular injury was determined by LDH release and caspase-3 activation. As illustrated in Figure 1, SI/R caused significant LDH release and caspase-3 activation in all groups studied (**P<0.01 vs. respective sham control). Lack of APN (APN-KO) significantly increased SI/R injury, as evidenced by higher percentage of LDH release (Figure 1A, second group, $^{\#}$P<0.05 vs. WT) and greater caspase-3 activity (Figure 1B, second group, $^{\#\#}$P<0.01 vs. WT). No significant difference in SI/R injury was observed between WT and APN receptor deficient cardiomyocytes (P>0.05), although LDH release and caspase-3 activation were slightly higher in cardiomyocytes from AdipoR1-KD/AdipoR2-KO mice. These results indicate that the basal level of APN produced by adult cardiomyocytes is biologically active in protecting cardiomyocytes against SI/R injury.

A peroxisome proliferator-activated receptor-γ (PPARγ) agonist regulates cardiomyocyte APN and its receptors' expression: In order to better understand the biological function of locally produced APN, cardiomyocytes were treated with rosiglitazone (RSG), a PPARγ agonist known to stimulate APN expression and secretion in adipocytes. Based on our pilot time-course observation, APN mRNA/protein expression, APN protein secretion, AdipoR1, and AdipoR2 protein expression were determined 6 h after RSG treatment. As summarized in Figure 2, treatment of WT cardiomyocytes with RSG caused a 1.9-fold increase in APN mRNA expression, 2.7-fold increase in APN protein expression, and 1.6-fold increase in APN level in culture medium (P values for all <0.01). These results demonstrated that cardiomyocyte APN expression (at both mRNA and protein level) and secretion are regulated by the PPARγ system.

The adult cardiomyocytes used in this study has no adipocyte contamination as determined by Oil-red O staining (data not shown). To further enhance our confidence that PPARγ-stimulated APN production is of cardiomyocytes origin, H9C2 cells (rat cardiac cell line) were treated with vehicle or RSG for 6 hours. Comparable as that seen in adult cardiomyocytes, treatment of H9C2 cells also significantly increased APN mRNA expression (1.78±0.24 over vehicle group, $P<0.01$), APN protein expression (2.51±0.31 over vehicle group, $P<0.01$) and APN level in culture medium (1.71±0.22 over vehicle group, $P<0.01$).

To gain more insight into PPARγ regulation of cardiac APN systems, additional experiments were performed to determine the effect of RSG on APN, AdipoR1, and AdipoR2 expression in WT cardiomyocytes and gene-manipulated cardiomyocytes. As expected, APN expression was not detected in APN-KO cardiomyocytes; response to RSG was completely lost in these cells (Figure 3, second group). The AdipoR1-KD or AdipoR2-KO condition alone neither altered basal cardiomyocyte APN production, nor their response to RSG (Figure 3, third and fourth group). However, basal APN expression was significantly increased in AdipoR-KD/AdipoR2-KO cardiomyocytes ($P<0.05$), and the RSG response was significantly potentiated in these cells (Figure 3, last group. $P<0.01$).

RSG treatment significantly increased AdipoR1 expression in WT cardiomyocytes (Figure 4, first group), and lack of APN neither altered basal AdipoR1 expression, nor its response to RSG (Figure 4, second group). Our method of intra-myocardial siRNA delivery was highly successful. Basal AdipoR1 expression was markedly inhibited (>80%) when siRNA against AdipoR1 was injected, and upregulatory response to RSG was completely abolished in these cells (Figure 4, third group). Basal and RSG-stimulated AdipoR1 expression was unaltered in AdipoR2-KO cardiomyocytes (Figure 4, fourth group). Similar to AdipoR1, AdipoR2

expression was also upregulated by RSG. Lack of APN or AdipoR1 neither altered basal AdipoR2 expression, nor its upregulation by RSG (Figure 5).

Role of cardiomyocyte-derived APN in RSG cardioprotection and its receptor involvement: Having demonstrated that the lack of cardiomyocyte-derived basal APN production exaggerated SI/R injury (Figure 1), and that RSG upregulated the expression of APN and its receptors (Figures 2-5), we further determined whether cardiomyocyte-derived APN is also biologically active in mediating RSG's cardioprotective effects. Cardiomyocytes from WT, APN-KO, AdipoR1-KD, AdipoR2-KO, and AdipoR1-KD/AdipoR2-KO mice were treated with vehicle or RSG for 6 h as described above, followed by 3 h simulated ischemia and 12 h reperfusion. In WT cardiomyocytes, RSG pre-treatment significantly reduced SI/R injury, as evidenced by significantly reduced LDH release and attenuated caspase-3 activation (Figure 6, first group). In contrast, no significant cardioprotective effects of RSG were observed in cardiomyocytes from APN-KO mice (Figure 6, second group), indicating that *in vitro* cardioprotective effects of RSG are largely mediated by locally produced APN. Moreover, the cardioprotective effects of RSG was markedly reduced, but not completely lost, in cardiomyocytes isolated from AdipoR1-KD mice (Figure 6, third group). Although AdipoR2 is expressed in cardiomyocytes, complete knockout of adiponectin receptor 2 had less profound effect than adiponectin receptor 1 knock-down on RSG cardioprotection in this in vitro simulated ischemia/reperfusion model (Figure 6, fourth group). Finally, cardiomyocytes isolated from AdipoR1-KD/AdipoR2-KO mice responded to RSG similarly as AdipoR1-KD cardiomyocytes. These results demonstrated that increased cardiomyocyte APN production as a result of RSG treatment is largely responsible for the cardioprotective effects of RSG, and such effects are primarily mediated by AdipoR1 activation.

Role of APN in RSG anti-nitrative signaling and its receptor involvement: Our previous studies have demonstrated that *in vivo* systemic APN cardioprotective effects are largely mediated by its anti-oxidative/anti-nitrative effect(26). To further investigate the intracellular mechanisms responsible for cardiomyocyte-derived APN cardioprotection, the effect of manipulating APN or APN receptors on RSG anti-oxidative/anti-nitrative effect was assessed. In cardiomyocytes isolated from WT mice, SI/R caused a greater than 3-fold increase in cellular nitrotyrosine content, which was markedly reduced by RSG pre-treatment (Figure 7, first group). SI/R-induced cardiomyocyte nitrotyrosine overproduction was significantly further increased in APN-KO cardiomyocytes (Figure 7, second group). The anti-oxidant/anti-nitrative effect of RSG was lost in these cells, with a highly significant difference observed between RSG-treated WT versus RSG-treated APN-KO cardiomyocytes ($P<0.01$). Neither AdipoR1-KD nor AdipoR2-KO had significant effect on SI/R-induced nitrotyrosine overproduction (Figure 7, third to fifth group). However, the anti-oxidative/anti-nitrative effect of RSG was abrogated in AdipoR1-KD and AdipoR1-KD/AdipoR2-KO cardiomyocytes (Figure 7).

Discussion

APN is a protein hormone that modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism(2; 3). The primary sequence of the polypeptide contains a signal sequence (cleaved in the mature protein) and a non-conserved N-terminal domain, followed by 22 collagen repeats, and a C-terminal globular domain that has structural similarities to TNFα(13). Like all collagen domain-containing proteins, full length APN spontaneously forms a homotrimer basic unit. The homotrimer self-associates through conserved N-terminal cysteine residues to form disulfide-linked hexamers, which further assemble into high-molecular weight forms consisting of multiple oligomers of the basic trimer unit. A proteolytic cleavage product of APN containing the *globular* C-terminal domain (gAPN) has been postulated to exist *in vivo*(6). A recent study has shown that the cleavage of full length APN by leukocyte elastase (secreted by activated monocytes and/or neutrophils) could be responsible for gAPN generation(27).

For many years, APN was believed to be produced and secreted exclusively by adipocytes. As such, although APN has been well-accepted as a potent cardioprotective molecule, clinical and experimental studies have been completely focused on identifying the relationship between systemic APN and cardiovascular injury(1; 7; 12; 16; 23; 24). In 2005, Pineiro and colleagues reported that human and murine cardiomyocytes are capable of producing APN(22), an observation subsequently confirmed by other investigators(4; 11). However, a critical investigation concerning the biological activity and physiologic/pathologic relevance of cardiomyocyte-derived APN remained unexplored.

Using two different approaches in our study, we have obtained clear evidence that cardiomyocyte-derived APN is both biologically active and pathologically relevant. First, we

297 have demonstrated that simulated ischemia/reperfusion injury is significantly potentiated in
298 cardiomyocytes lacking APN, indicating that APN is constantly produced by adult
299 cardiomyocytes, and that this basal level of locally produced APN is indeed biologically active
300 in its protection of cardiomyocytes from ischemia/reperfusion injury. Second, we have
301 demonstrated that cardiomyocyte APN production is upregulated by the PPARγ system, and
302 more importantly, the cardioprotective effect of RSG was completely lost in cardiomyocytes
303 lacking APN. To our knowledge, this is the first direct evidence demonstrating that
304 cardiomyocyte-derived APN is the most important molecule mediating the cardioprotective
305 actions of RSG- clearly making APN of local origin a key player in pharmacologic therapeutic
306 intervention success.

307 Currently, it is generally accepted that APN regulates cellular function by
308 binding/activating its specific receptors. Two types of APN receptor have been cloned(28).
309 Whereas AdipoR1 is abundantly expressed in muscular cells, AdipoR2 is predominantly
310 expressed in the liver. They belong to a new family of membrane receptors predicted to contain
311 seven transmembrane domains, but are structurally and topologically distinct from G-protein
312 coupled receptors. APN binds to the C-terminal extracellular domain of AdipoR, whereas the N-
313 terminal cytoplasmic domain interacts with an adaptor protein, APPL1(18). In addition to these
314 two receptors, T-cadherin has been proposed to be a receptor for hexameric and high molecular
315 weight forms of APN(14). However, the biologic function of APN/T-cadherin binding remains
316 unclear, because T-cadherin lacks an intracellular domain.

317 The current study raised several interesting observations concerning APN receptors and
318 their involvement in locally produced APN-mediated cardioprotection. First, the absence of APN
319 did not alter basal expression of the APN receptor, and it did not affect RSG-induced receptor

upregulation. However, although the absence of one of the two APN receptor types did not affect APN expression, deficiency of both APN receptor types significantly upregulated APN basal expression, and potentiated RSG response. The pathological significance of this interesting phenomenon warrants more direct investigation. Second, APN deficiency had more significant impact on cardiomyocyte response to ischemia/reperfusion than its receptors had. Specifically, under basal condition, cardiomyocytes lacking APN, but not APN receptors, had significantly higher LDH release and caspase-3 activation after SI/R (Figure 1). Under RSG stimulatory condition, the RSG cardioprotective effect was completely lost in cardiomyocytes lacking APN, but not in cardiomyocytes lacking APN receptors. Precise mechanisms responsible for this discrepancy can not be addressed in the current study. However, several possibilities exist. In the current study, AdipoR1 siRNA caused approximately 80% reduction of AdipoR1 expression. The remaining AdipoR1 might be responsible for partial protection in AdipoR-KD cardiomyocytes. Alternatively, other APN receptors, such as T-cadherin, might be present in adult cardiomyocytes and be able to translate APN cardioprotective signaling in AdipoR1-KD/AdipoR2-KO cardiomyocytes. Finally, cardiomyocyte-derived APN may participate in cardioprotective signaling via intracellular protein-protein interaction. Additional experiments directly investigating these possibilities are currently under investigation.

Third, although AdipoR1 and AdipoR2 are known to be constitutively expressed in adult cardiomyocytes, each receptor's relative contribution to APN cardioprotective signaling remains unclear. As illustrated in Figures 6 and 7, manipulation of AdipoR1 had greater impact on RSG's cardioprotective and anti-oxidative/anti-nitrative actions than alteration of AdipoR2, despite the complete knockout of AdipoR2, whereas AdipoR1 was only partially (knock-down) lost in cardiomyocytes studied. At least two possibilities may explain this discrepancy. First, it is

possible that the cardioprotective effects of locally produced APN are primarily mediated by AdipoR1, since cardiomyocytes are AdipoR1 dominant. Second, cardiomyocyte-derived APN may contain more low-molecular weight APN, which has high affinity to AdipoR1. Additional experiments are currently undertaken to directly address these possibilities.

In summary, our studies demonstrated for the first time that locally-produced APN by cardiomyocytes is biologically active, pathologically significant, and pharmacologically important. This locally-produced APN protects cardiomyocyte against ischemia/reperfusion injury primarily via paracrine/autocrine activation of AdipoR1. Furthermore, similar to circulatory APN, locally-produced APN protects against cardiomyocyte injury through reduction of ischemia/reperfusion-induced oxidative/nitrative stress.

354 References
355

356 1. **Behrends M, Schulz R, Post H, Alexandrov A, Belosjorow S, Michel MC and Heusch**
357 **G**. Inconsistent relation of MAPK activation to infarct size reduction by ischemic
358 preconditioning in pigs. *Am J Physiol* 279: 1111-1119, 2000.

359 2. **Berg AH, Combs TP and Scherer PE**. ACRP30/adiponectin: an adipokine regulating
360 glucose and lipid metabolism. *Trends Endocrinol Metab* 13: 84-89, 2002.

361 3. **Chandran M, Phillips SA, Ciaraldi T and Henry RR**. Adiponectin: more than just
362 another fat cell hormone? *Diabetes Care* 26: 2442-2450, 2003.

363 4. **Ding G, Qin Q, He N, Francis-David SC, Hou J, Liu J, Ricks E and Yang Q**.
364 Adiponectin and its receptors are expressed in adult ventricular cardiomyocytes and
365 upregulated by activation of peroxisome proliferator-activated receptor gamma. *J Mol Cell*
366 *Cardiol* 43: 73-84, 2007.

367 5. **Forrat R, Sebbag L, Wiernsperger N, Guidollet J, Renaud S and De Lorgeril M**.
368 Acute myocardial infarction in dogs with experimental diabetes. *Cardiovasc Res* 27: 1908-
369 1912, 1993.

370 6. **Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE**
371 **and Lodish HF**. Proteolytic cleavage product of 30-kDa adipocyte complement-related
372 protein increases fatty acid oxidation in muscle and causes weight loss in mice. *PNAS* 98:
373 2005-2010, 2001.

7. **Frystyk J, Berne C, Berglund L, Jensevik K, Flyvbjerg A and Zethelius B**. Serum adiponectin is a predictor of coronary heart disease: a population-based 10-year follow-up study in elderly men. *J Clin Endocrinol Metab* 92: 571-576, 2007.

8. **Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA and Ma XL**. Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: the roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. *Circulation* 105: 1497-1502, 2002.

9. **Goldstein BJ and Scalia R**. Adipokines and vascular disease in diabetes. *Curr Diab Rep* 7: 25-33, 2007.

10. **Goldstein BJ, Scalia RG and Ma XL**. Protective vascular and myocardial effects of adiponectin. *Nat Clin Pract Cardiovasc Med* 6: 27-35, 2009.

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

12. **Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T and Matsuzawa Y**. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol* 20: 1595-1599, 2000.

13. **Hug C and Lodish HF**. The role of the adipocyte hormone adiponectin in cardiovascular disease. *Curr Opin Pharmacol* 5: 129-134, 2005.

14. **Hug C, Wang J, Ahmad NS, Bogan JS, Tsao TS and Lodish HF**. T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. *Proc Natl Acad Sci U S A* 101: 10308-10313, 2004.

15. **Jagasia D and McNulty PH**. Diabetes mellitus and heart failure. *Congest Heart Fail* 9: 133-139, 2003.

16. **Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, Arita Y, Okamoto Y, Shimomura I, Hiraoka H, Nakamura T, Funahashi T and Matsuzawa Y**. Association of hypoadiponectinemia with coronary artery disease in men. *Arterioscler Thromb Vasc Biol* 23: 85-89, 2003.

17. **Liu Y, Chewchuk S, Lavigne C, Brule S, Pilon G, Houde V, Xu A, Marette A and Sweeney G**. Functional significance of skeletal muscle adiponectin production, changes in animal models of obesity and diabetes, and regulation by rosiglitazone treatment. *Am J Physiol Endocrinol Metab* 297: E657-E664, 2009.

18. **Mao X, Kikani CK, Riojas RA, Langlais P, Wang L, Ramos FJ, Fang Q, Christ-Roberts CY, Hong JY, Kim RY, Liu F and Dong LQ**. APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. *Nat Cell Biol* 8: 518-523, 2006.

19. **Marfella R, D'Amico M, Di Filippo C, Piegari E, Nappo F, Esposito K, Berrino L, Rossi F and Giugliano D**. Myocardial infarction in diabetic rats: role of hyperglycaemia on infarct size and early expression of hypoxia-inducible factor 1. *Diabetologia* 45: 1172-1181, 2002.

20. **Namiki A, Brogi E, Kearney M, Kim EA, Wu T, Couffinhal T, Varticovski L and Isner JM**. Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. *J Biol Chem* 270: 31189-31195, 1995.

21. **Ouchi N, Shibata R and Walsh K**. Cardioprotection by adiponectin. *Trends Cardiovasc Med* 16: 141-146, 2006.

22. **Pineiro R, Iglesias MJ, Gallego R, Raghay K, Eiras S, Rubio J, Dieguez C, Gualillo O, Gonzalez-Juanatey JR and Lago F**. Adiponectin is synthesized and secreted by human and murine cardiomyocytes. *FEBS Lett* 579: 5163-5169, 2005.

23. **Pischon T, Girman CJ, Hotamisligil GS, Rifai N, Hu FB and Rimm EB**. Plasma adiponectin levels and risk of myocardial infarction in men. *JAMA* 291: 1730-1737, 2004.

24. **Shibata R, Numaguchi Y, Matsushita K, Sone T, Kubota R, Ohashi T, Ishii M, Kihara S, Walsh K, Ouchi N and Murohara T**. Usefulness of adiponectin to predict myocardial salvage following successful reperfusion in patients with acute myocardial infarction. *Am J Cardiol* 101: 1712-1715, 2008.

25. **Shinoda Y, Yamaguchi M, Ogata N, Akune T, Kubota N, Yamauchi T, Terauchi Y, Kadowaki T, Takeuchi Y, Fukumoto S, Ikeda T, Hoshi K, Chung UI, Nakamura K and Kawaguchi H**. Regulation of bone formation by adiponectin through autocrine/paracrine and endocrine pathways. *J Cell Biochem* 99: 196-208, 2006.

26. **Tao L, Gao E, Jiao X, Yuan Y, Li S, Christopher TA, Lopez BL, Koch W, Chan L, Goldstein BJ and Ma XL**. Adiponectin cardioprotection after myocardial

ischemia/reperfusion involves the reduction of oxidative/nitrative stress. *Circulation* 115: 1408-1416, 2007.

27. **Waki H, Yamauchi T, Kamon J, Kita S, Ito Y, Hada Y, Uchida S, Tsuchida A, Takekawa S and Kadowaki T**. Generation of globular fragment of adiponectin by leukocyte elastase secreted by monocytic cell line THP-1. *Endocrinology* 146: 790-796, 2005.

28. **Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R and Kadowaki T**. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 423: 762-769, 2003.

29. **Zhu M, Miura J, Lu LX, Bernier M, DeCabo R, Lane MA, Roth GS and Ingram DK**. Circulating adiponectin levels increase in rats on caloric restriction: the potential for insulin sensitization. *Exp Gerontol* 39: 1049-1059, 2004.

Funding Sources

This research was supported by the following grants: NIH 2R01HL-63828, American Diabetes Association Research Award 7-08-RA-98, American Heart Association Grant-in-Aid 0855554D (to X.L.M.), and the Emergency Medicine Foundation Career Development Grant (to W.B.L.).

Conflict of interest: none declared

462 **Figure Legend**

463

464 Figure 1. Simulated ischemia/reperfusion injury assessed by LDH release (A) and caspase-3
465 activation (B). Assays were performed at the end of 3 h of SI and 12 h of reperfusion
466 (SI/R) or 15 h of normal culture (Sham). **P<0.01 vs. sham SI/R; $^{\#}$P<0.05, $^{\#\#}$P<0.01
467 vs. WT cardiomyocytes with SI/R. N=14-16 wells/group with cardiomyocytes
468 isolated from 6 to 8 mice.

469

470 Figure 2. Effect of rosiglitazone (RSG) on adiponectin (APN) mRNA expression (A), APN
471 protein expression (B) and APN secretion (C) in C57BL/6 cardiomyocytes. Assays
472 were performed 6 h after vehicle or RSG treatment. **P<0.01 vs. vehicle; N=14-16
473 wells/group with cardiomyocytes isolated from 6 to 8 mice.

474

475 Figure 3. Effect of RSG on APN protein expression in cardiomyocytes isolated from WT or
476 gene manipulated mice. Assays were performed 6 h after vehicle or RSG treatment.
477 *P<0.05, **P<0.01 vs. vehicle in the same group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. WT
478 cardiomyocytes with the same treatment. N=14-16 wells/group with cardiomyocytes
479 isolated from 6 to 8 mice.

480

481 Figure 4. Effect of RSG on adiponectin receptor 1 (AdipoR1) expression in cardiomyocytes
482 isolated from WT or gene manipulated mice. Assays were performed 6 h after vehicle
483 or RSG treatment. *P<0.05 vs. vehicle in the same group; N=14-16 wells/group with
484 cardiomyocytes isolated from 6 to 8 mice.

Figure 5. Effect of RSG on adiponectin receptor 2 (AdipoR2) expression in cardiomyocytes isolated from WT or gene manipulated mice. Assays were performed 6 h after vehicle or RSG treatment. *P<0.05 vs. vehicle in the same group; N=14-16 wells/group with cardiomyocytes isolated from 6 to 8 mice.

Figure 6. Effect of APN, AdipoR1 and AdipoR2 deficiency on RSG cardioprotection against SI/R injury. Cardiomyocytes were pretreated with vehicle or RSG for 6 h followed by 3 h of SI and 12 h of reperfusion. All data were normalized against mean value of their own vehicle group. *P<0.05, **P<0.01 vs. vehicle in the same group; $^{\#\#}$P<0.01 vs. WT cardiomyocytes with the same treatment. N=14-16 wells/group with cardiomyocytes isolated from 6 to 8 mice.

Figure 7. Effect of APN, AdipoR1, and AdipoR2 deficiency on RSG mediated anti-oxidative/anti-nitrative effect in SI/R cardiomyocytes. Cardiomyocytes were pretreated with vehicle or RSG for 6 h followed by 3 h of SI and 12 h of reperfusion. All data were normalized against mean value of their own vehicle group. *P<0.05, **P<0.01 vs. vehicle in the same group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. WT cardiomyocytes with the same treatment. N=14-16 wells/group with cardiomyocytes isolated from 6 to 8 mice.

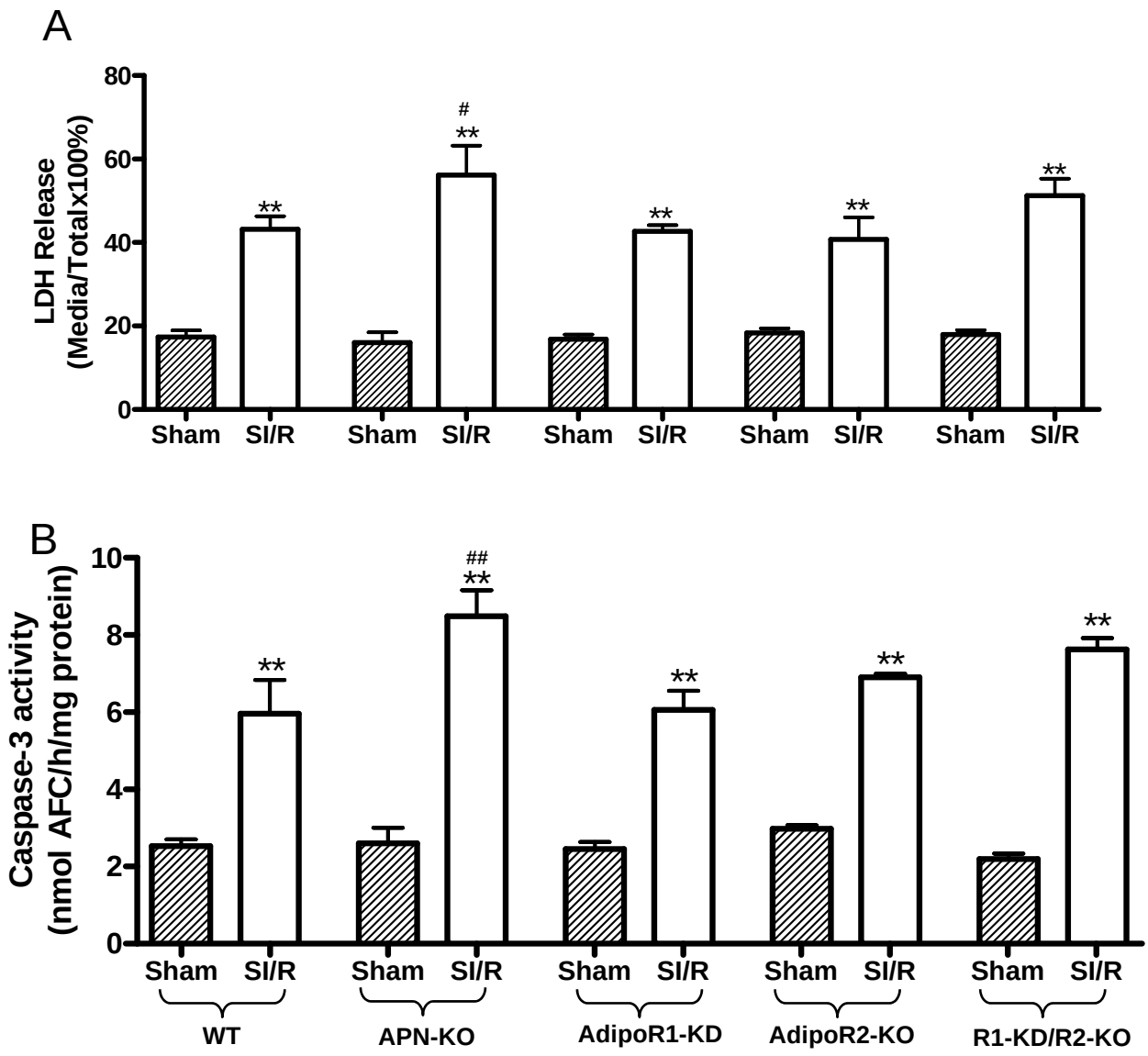
A
LDH Release
(Media/Total x100%)
80
60
40
20
0
#
**
**
**
**
**
**
Sham
SI/R
Sham
SI/R
Sham
SI/R
Sham
SI/R
Sham
SI/R
B
Caspase-3 activity
(nmol AFC/h/mg protein)
10
8
6
4
2
0
##
**
**
**
**
**
**
Sham
SI/R
Sham
SI/R
Sham
SI/R
Sham
SI/R
Sham
SI/R
WT
APN-KO
AdipoR1-KD
AdipoR2-KO
R1-KD/R2-KO

Figure 1

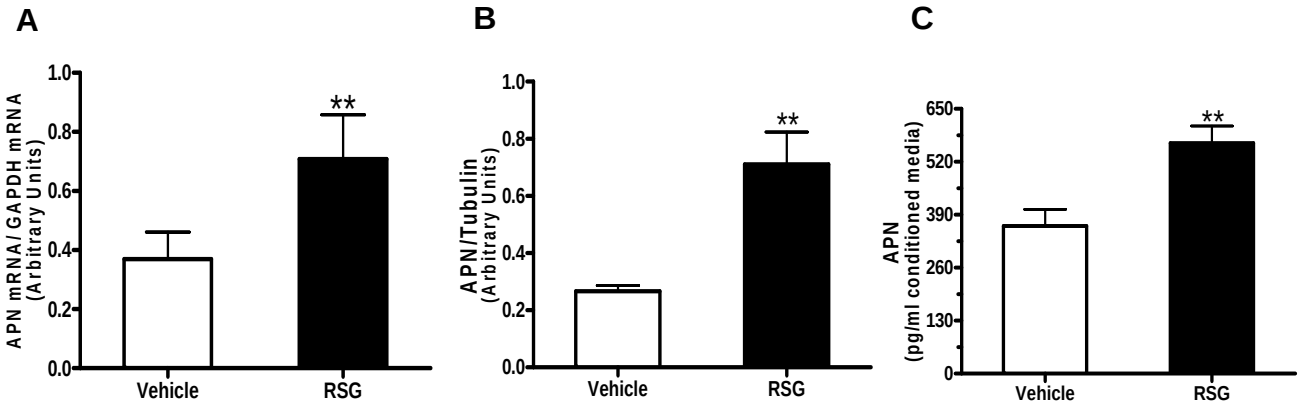
A
APN mRNA/GAPDH mRNA (Arbitrary Units)
1.0
0.8
0.6
0.4
0.2
0.0
**
Vehicle
RSG
B
APN/Tubulin (Arbitrary Units)
1.0
0.8
0.6
0.4
0.2
0.0
**
Vehicle
RSG
C
APN (pg/ml conditioned media)
650
520
390
260
130
0
**
Vehicle
RSG

Figure 2

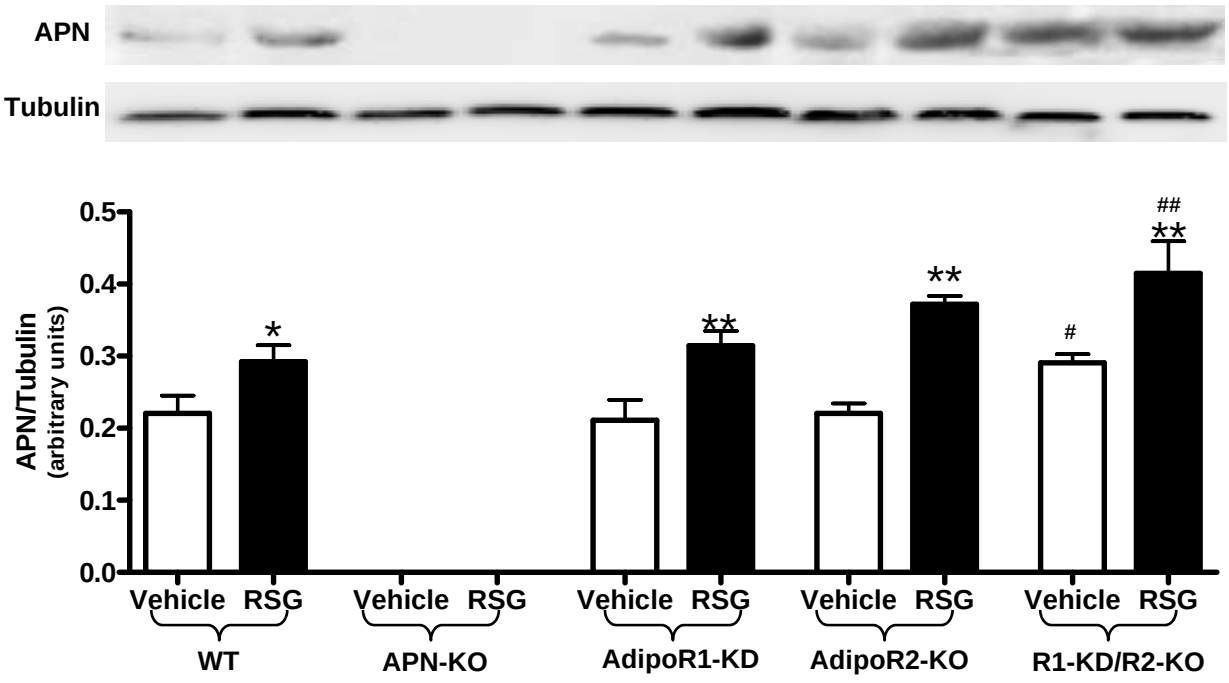
APN
Tubulin
APN/Tubulin
(arbitrary units)
0.5
0.4
0.3
0.2
0.1
0.0
*
**
**
#
##
**
Vehicle
RSG
WT
APN-KO
AdipoR1-KD
AdipoR2-KO
R1-KD/R2-KO

Figure 3

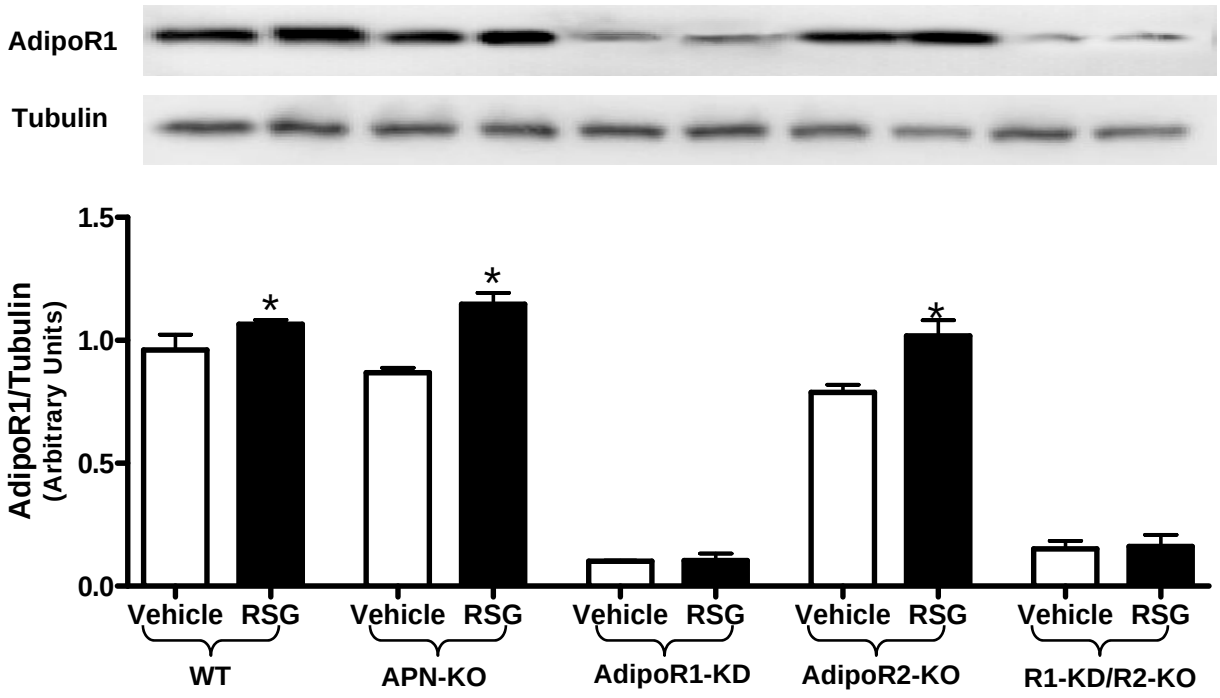
AdipoR1
Tubulin
AdipoR1/Tubulin
(Arbitrary Units)
1.5
1.0
0.5
0.0
*
*
*
Vehicle
RSG
WT
APN-KO
AdipoR1-KD
AdipoR2-KO
R1-KD/R2-KO

Figure 4

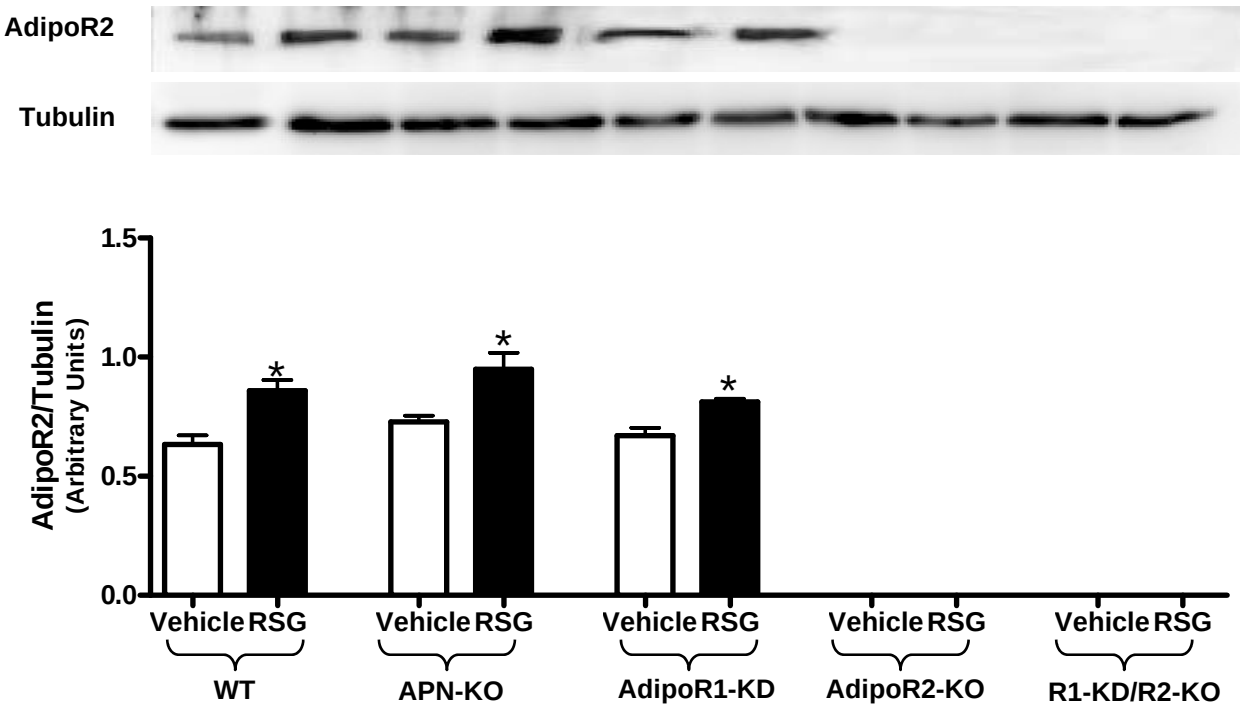
AdipoR2
Tubulin
AdipoR2/Tubulin
(Arbitrary Units)
1.5
1.0
0.5
0.0
*
*
*
Vehicle
RSG
WT
APN-KO
AdipoR1-KD
AdipoR2-KO
R1-KD/R2-KO

Figure 5

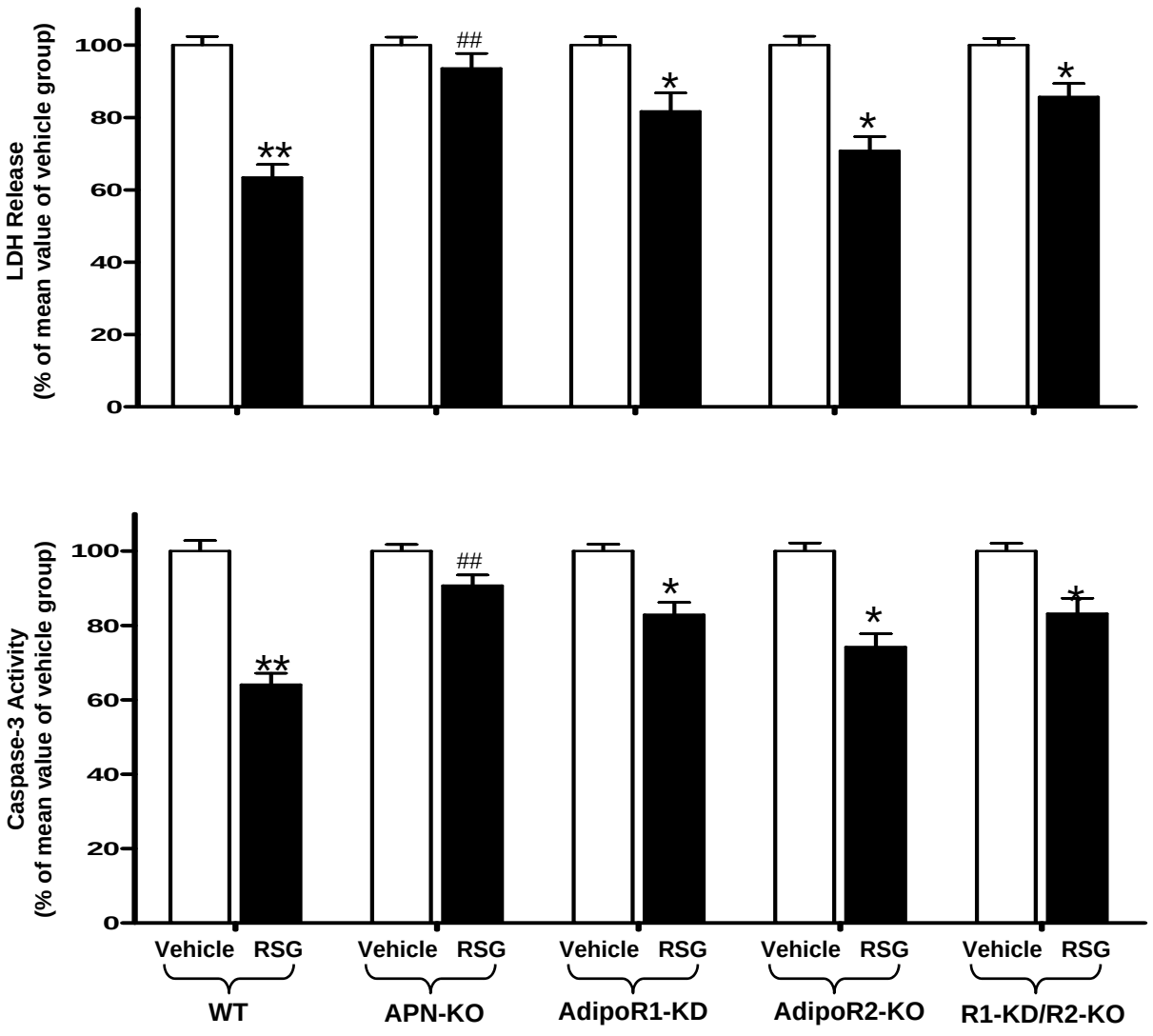
LDH Release
(% of mean value of vehicle group)
100
80
60
40
20
0
**
##
*
*
*
Caspase-3 Activity
(% of mean value of vehicle group)
100
80
60
40
20
0
**
##
*
*
*
Vehicle RSG
Vehicle RSG
Vehicle RSG
Vehicle RSG
Vehicle RSG
WT
APN-KO
AdipoR1-KD
AdipoR2-KO
R1-KD/R2-KO

Figure 6

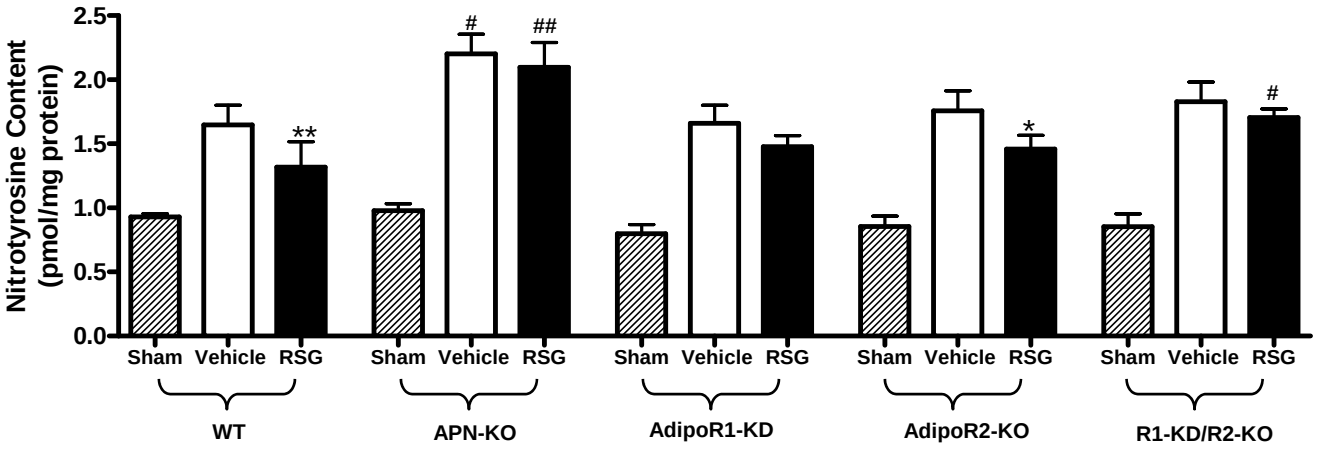
Nitrotyrosine Content
(pmol/mg protein)
2.5
2.0
1.5
1.0
0.5
0.0
**
#
##
*
#
Sham
Vehicle
RSG
WT
APN-KO
AdipoR1-KD
AdipoR2-KO
R1-KD/R2-KO

Figure 7