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Adiponectin Inhibits Oxidative/Nitrative Stress during Myocardial Ischemia and Reperfusion
via PKA Signaling

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Running Head: PKA in APN's cardioprotective signaling

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38 **Abstract**

39 The cardioprotective effects of adiponectin (APN) against myocardial ischemia/reperfusion
40 (MI/R) injury are well known. However, comprehension of the mechanisms mediating
41 intracellular APN signaling remains incomplete. We recently demonstrate the
42 antioxidant/antinitrative effects of APN are not dependent upon AMPK. Protein Kinase A
43 (PKA) has been previously shown to be activated by APN, with uncertain relevance to APN
44 cardiac protection. The current study determined whether the anti-oxidative/anti-nitrative
45 effect of APN is mediated by PKA. Administration of APN (2 µg/g) 10 minutes before
46 reperfusion significantly enhanced cardiac PKA activity, reduced oxidative stress and
47 decreased infarct size. Knockdown of cardiac PKA expression (PKA-KD) by intramyocardial
48 injection of PKA-siRNAs (>70% suppression) significantly inhibited APN cardioprotection
49 determined by cardiac apoptosis, infarct size, and cardiac function. Moreover, PKA-KD
50 virtually abolished the suppressive effect of APN on MI/R induced NADPH oxidase
51 overexpression and superoxide overproduction, and partially inhibited the effect of APN on
52 nitrative protein modification in MI/R heart. Mechanistically, APN significantly inhibited
53 MI/R-induced IKK/IκB phosphorylation and NFκB activation, which were blocked in
54 PKA-KD heart. Finally, the PKA-mediated antioxidant/antinitrative and cardioprotective
55 effects of APN are intact in AMPK deficiency mice, suggesting that there is no cross-talk
56 between AMPK and PKA signaling in APN cardioprotection. Collectively, we demonstrate
57 for the first time that APN inhibits oxidative/nitrative stress during MI/R via PKA-dependent
58 NFκB inhibition.

59

60 **Keywords:** Protein Kinase A; Adiponectin; Reperfusion Injury; Oxidative Stress;

61 The adipokine adiponectin (APN), of primarily adipose origin, regulates glucose and
62 lipid metabolism(23). Prospective clinical observations and animal studies have demonstrated
63 that reduced adiponectin function may be a major contributor to increased ischemic heart
64 disease morbidity in diabetic patients(21). Administration of the adiponectin attenuates
65 myocardial ischemia and reperfusion (MI/R) injury in mice(20; 24). However, the detailed
66 molecular mechanisms responsible for the cardioprotective effect of adiponectin remain
67 elusive.

68 It is well accepted that excess superoxide-mediated oxidative stress plays an important
69 role in MI/R injury. Superoxide reacts with nitric oxide (NO) to form the highly cytotoxic
70 molecule peroxynitrite (ONOO⁻), which induces nitrative stress and exacerbates MI/R injury.
71 We demonstrated that APN deficient mice exhibited much more severe I/R injury from
72 overproduction of superoxide, peroxynitrite, and resultant nitrative stress, reversible by
73 gAPN administration (22). Therefore, APN is cardioprotective against ischemia/reperfusion
74 injury by inhibiting oxidative/nitrative stress. We have recently demonstrated that the
75 anti-oxidative/anti-nitrative property of APN is not mediated by AMP activated protein kinase
76 (AMPK), the most recognized APN intracellular signaling molecule (24). A comprehensive
77 understanding of intracellular APN anti-oxidative signaling mechanisms remains lacking.

78 Protein kinase A (PKA) is an important mediator of signal transduction downstream of
79 G-protein-coupled receptors, and plays a key role in the regulation of metabolism and
80 triglyceride storage. In endothelial cells, APN inhibits palmitate-induced apoptosis by
81 suppressing reactive oxygen species (ROS) generation via PKA pathways (27). However,
82 whether PKA signaling mediates APN's anti-oxidative/anti-nitrative effect during MI/R
83 injury has never been previously investigated.

84 Therefore, the aims of the present study were to investigate whether PKA mediates
85 APN's anti-oxidative/anti-nitrative and cardioprotective effects against MI/R injury; and if so

86 to identify the possible responsible intracellular signaling mechanisms.

87

88 **Materials and Methods**

89 Adult male WT mice, adult male AMPK-DN mice or their male littermate controls
90 were utilized in this study. Generation, breeding, phenotype characteristics, and genotyping
91 of AMPK-DN mice (>80% inhibition of cardiac AMPK activity) have been previously
92 described in detail (28). The experiments were performed in adherence with the National
93 Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the
94 Thomas Jefferson University Committee on Animal Care.

95 Myocardial ischemia/reperfusion: Mice were anesthetized with 2% isoflurane. MI/R was
96 induced by temporarily exteriorizing the heart via a left thoracic incision, and placing a 6-0
97 silk suture slipknot around the left anterior descending coronary artery. Twenty minutes after
98 MI, animals were randomized to receive either vehicle or gAPN (2 µg/g, ip) (24). After 30
99 minutes of MI, the slipknot was released, and the myocardium was reperfused for 3 hours (all
100 assays except cardiac function and infarct size) or 24 hours (cardiac function and infarct size
101 only). All assays were performed utilizing tissue from the ischemic/reperfused area, the area
102 at risk (AAR), identified by Evans blue negative staining. Sham-operated control mice (Sham
103 MI/R) underwent the same surgical procedure, except the suture placed under the left
104 coronary artery was not tied.

105 Inhibition of PKA with *in vivo* siRNAs-mediated knock-down (PKA-KD): To specifically
106 confirm the role of PKA in gAPN' protective effect on MI/R, siRNA gene silencing technique
107 was utilized to knock-down mouse cardiac PKA expression (PKA-KD). In brief, two
108 mouse-specific PKAα subunit-selective siRNA targeting the nucleotide sequences
109 5'-CGTCCTGACCTTTGAGTATCT-3' and 5'-CAGTGTGCTGTTGTAAACATA-3' were
110 employed. siRNA oligos of the same size possessing scrambled nucleotide sequences served
111 as control. All siRNAs were obtained from Integrated DNA Technologies. The siRNAs were
112 diluted in 5% glucose and mixed with *in vivo*-jet PEI (Genesee Scientific). Adult mice were

113 anesthetized with 2% isoflurane. The heart was exposed via left thoracotomy at the fifth
114 intercostal space. 20 μ l PKA-specific siRNAs (dose 0.8 μ g/ μ l) or scrambled control was
115 delivered via three separate intramyocardial injections (by 32G needle), temporarily
116 blanching the left ventricular free wall. 48 hours after siRNAs injection, the mice were
117 subjected to MI/R.

118 Determination of myocardial apoptosis and myocardial infarct size: Myocardial apoptosis
119 was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
120 (TUNEL) staining and caspase-3 activity as described in our previous study (24). Myocardial
121 infarct size was assessed by Evans blue-2,3,5-triphenyl tetrazolium chloride double staining
122 methods (24).

123 Determination of cardiac function: Cardiac function was determined by echocardiography
124 and left ventricular catheterization methods 24 hours after reperfusion before thoracotomy, as
125 described in our previous study(24).

126 Measurement of PKA activity: PKA activity was measured utilizing non-radioactive PepTag
127 assays (Promega, Southampton, Hampshire, UK) reliant upon a change in charge of the
128 PepTag A1 peptide from +1 to -1 following phosphorylation (14). Sample reaction mixtures
129 were incubated at room temperature for 15 minutes. After incubation, samples were separated
130 on a 0.5% (w/v) agarose gel at 100V for 15– 20 minutes. Purified PKA catalytic subunit
131 served as a positive control, while the negative control contained only buffer. Bands were
132 visualized under ultraviolet light.

133 Quantification of Superoxide Production: Superoxide production in I/R heart tissue was
134 measured by lucigenin-enhanced chemiluminescence as previously described(24).
135 Superoxide production was expressed as relative light units (RLU) per second per mg heart
136 weight (RLU/s/mg wet tissue).

137 Determination of Total NOx Content in Cardiac Tissue: Cardiac tissue samples from AAR

were rinsed, homogenized in deionized water (1:10, weight/volume), and centrifuged at 14,000g for 10 minutes. Tissue NO and its in vivo metabolic products (NO₂ and NO₃, collectively known as NO_x) in the supernatant were determined via chemiluminescence NO detector (SIEVER 280i NO Analyzer) as described in our previous study(24).

Quantitation of Tissue Nitrotyrosine Content: Ischemic/reperfused cardiac tissue nitrotyrosine content (pmol of nitrotyrosine/mg protein), an accepted footprint of in vivo peroxynitrite formation and a reliable index of nitrative stress, was determined via ELISA, described in our previous publication(22; 26).

Measurement of nuclear translocation of NF-kB p65: The relative increase of NF-kB p65 translocation into the nuclei of the ischemic/reperfused cardiomyocytes was determined via ELISA per manufacturer's protocol (IMGENEX, San Diego, CA). In brief, cardiac tissue was cut into small pieces and homogenized. The mixture was centrifuged for 10 minutes at 10,000 rpm at 4°C. The supernatant represents the cytoplasmic fraction. The pellet was resuspended in nuclear lysis buffer, and subsequently centrifuged for 10 minutes at 14,000 rpm at 4°C. This supernatant represents the nuclear fraction. A plate coated with anti-p65 antibody captured free p65 (both nuclear or cytoplasmic). Bound p65 was detected by adding a secondary antibody followed by alkaline phosphatase-conjugated secondary antibody. Each well's absorbance value was determined at 405 nm by a microplate reader (Molecular Devices). The relative ratio of nuclear to cytoplasmic p65 was calculated by: (absorbance value of nucleus)/(absorbance value of cytoplasm) (16).

Western blot analysis: Protein from tissue homogenates was separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and incubated with primary antibodies (against PKA C- α Antibody, IKK β , Phospho-IKK β , I κ B α , Phospho-I κ B α [Cell Signaling Technology, Danvers, MA], iNOS [Upstate, Chicago, IL], gp91phox [Transduction Laboratories, San Jose, CA]) and horseradish peroxidase-conjugated secondary antibody. The blot was developed

with a Supersignal Chemiluminescence detection kit (Pierce, Rockford, IL), and observed with a Kodak Image Station 400 (Rochester, NY).

Statistical analysis: All values in the text and figures are presented as mean±standard error of the mean of n independent experiments. All data (except Western blot density) were subjected to one-way ANOVA followed by Bonferoni 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

PKA deficiency blocked gAPN inhibition of MI/R-induced myocardial infarct, apoptosis, and cardiac dysfunction. MI/R did not alter PKA expression and slightly reduced PKA activity. Administration of APN 10 min before reperfusion had no effect upon PKA expression but significantly increased PKA activity (Figure 1) and reduced myocardial infarct size (Figure 2). To determine whether APN activation of PKA plays a causative role in APN cardioprotection, PKA expression was downregulated via intramyocardial siRNA injection. Pretreatment with the PKA-selective siRNAs reduced PKA expression by 70% ($P<0.01$ relative to scramble group, $n=6$, Figure 2A). Cardiac specific PKA knockdown blocked APN-induced PKA activation (Figure 2B) and attenuated APN's infarct reduction effects (Figure 2C).

MI/R resulted in significant cardiac injury including cardiac systolic and diastolic dysfunction (Figure 3) and myocardial apoptotic cell death (Figure 4). Consistent with our previous observations (22), gAPN administration significantly improved cardiac function after reperfusion (Figure 3, Scramble) and reduced apoptosis (Figure 4, Scramble). The knockdown of cardiac PKA expression (PKA siRNA) slightly worsened MI/R-induced cardiac dysfunction measured by LVEF and LVEDP (Figure 3A, B), but significantly increased infarct size (Figure 2C) and apoptosis (Figure 4A). More importantly, cardioprotective effect of APN was either completely abolished (anti-apoptotic effect on Figure 4) or significantly attenuated (Figure 2C, Figure 3) in the PKA-KD mice. Taken together, these results demonstrated that PKA activation plays a critical role in APN cardioprotection after MI/R.

PKA deficiency abolished the anti-oxidative and inhibited anti-nitrative effects of gAPN post I/R. Having demonstrated that gAPN protects against reperfusion injury in a partially PKA-dependent manner, we investigate the underlying mechanisms potentially responsible. We report previously gAPN cardioprotection involves reduction of oxidative/nitrative stress

(22). We performed two series of experiments assessing the relationship between PKA signaling and oxidative/nitrative stress in MI/R hearts. In the first experiment series, MI/R-induced oxidative stress was detected. Under basal conditions, no significant difference existed between superoxide production in scramble and PKA-KD mice. MI/R induced greater increases in superoxide production in PKA-KD animals (Figure 5A). APN administration significantly inhibited MI/R-induced superoxide production in scramble animals (Figure 5A). However, APN treatment did not reduce superoxide production in PKA-KD animals (Figure 5A). We next identified potentially responsible molecular sources for the impaired antioxidant actions of gAPN in PKA-KD mice. Expression of gp91^{phox}, the primary isoform of NADPH oxidase expressed in heart (1; 2; 29) was significantly increased after MI/R in both scramble and PKA-KD heart (Figure 5B). gAPN treatment of scramble animals subjected to MI/R significantly inhibited gp91^{phox} expression. In contrast, the inhibitory effect of APN upon MI/R-induced gp91^{phox} overexpression was abolished in PKA-KD mice, in consistent fashion with superoxide generation trends in PKA-KD mice (Figure 5B). Together, these results demonstrate that MI/R-induced oxidative stress was modestly increased in PKA-KD mice, and the anti-oxidant effect of gAPN was completely abolished by PKA signaling deficiency.

In the second experiment series, we assessed MI/R-induced myocardial nitrative stress. There was no significant total nitric oxide (NOx) content difference between PKA-KD and scramble animals during basal conditions or after MI/R (Figure 6A). Administration gAPN significantly inhibited MI/R-induced NOx overproduction in both scramble and PKA-KD mice (Figure 6A). Inducible nitric oxide synthase (iNOS) expression was significantly increased after MI/R-induced in scramble and PKA-KD animals (Figure 6B). Treatment with gAPN significantly inhibited iNOS expression, an effect not inhibited by PKA-KD (Figure 6B). These results demonstrate that although the anti-oxidant effect of gAPN is mediated by

PKA, iNOS-inhibitory effect of gAPN is not mediated by PKA.

Superoxide rapidly reacts with NO to form the cytotoxic molecule peroxynitrite. Nitrotyrosine (NT) content, the accepted footprint of peroxynitrite production, was increased by MI/R injury both in scramble and PKA-KD mice (Figure 6C). Administration of gAPN significantly inhibited MI/R-induced NT generation in scramble animals, an effect that was partially blocked in PKA-KD animals (Figure 6C).

gAPN decreased IKK/I κ B/NF- κ B signaling activation in I/R myocardium, which was blocked by PKA deficiency. Decreased I κ B phosphorylation preventing nuclear NF- κ B translocation attenuates myocardial reperfusion injury (17) and preserves cardiac function post MI. PKA signaling is involved in gAPN-suppressed IKK-I κ B-NF κ B activation in endothelial cells subjected to tumor necrosis factor- α or high glucose treatment (27). We investigated the possible mechanism underlying the PKA-dependent cardioprotective effects of gAPN, and determined activation of IKK-I κ B-NF κ B by MI/R. Consistent with others' previous results(15; 30), MI/R induced significant IKK phosphorylation (Figure 7A), resulting in I κ Ba phosphorylation (Figure 7B) and NF- κ B activation (Figure 7C) in both scramble and PKA-KD mice. MI/R-induced NF κ B activation by IKK/I κ B phosphorylation was significantly inhibited by gAPN in scramble animals (Figures 7). However, gAPN did not inhibit NF κ B activity during PKA-knockdown (Figure 7). These data suggest the cardioprotective effect of gAPN was partially due to inhibition of IKK/I κ B/NF- κ B activation, and such effect was dependent upon PKA signaling.

PKA-mediated cardioprotective effect of APN against MI/R injury is AMPK-independent:

Despite considerable evidence supporting the essential role of AMPK in adiponectin intracellular signaling(4; 5; 7; 8; 11), we recently provided direct evidence of AMPK-independent gAPN-mediated cardioprotection in the intact animal (24; 25). The current study suggests gAPN is cardioprotective in mice subjected to MI/R, with involvement

246 of PKA signaling. We next investigated whether there is crosstalk between PKA and AMPK
247 pathways. Adult male mice overexpressing a dominant negative $\alpha 2$ subunit (D157A) of
248 AMPK (AMPK-DN) mice were pretreated with PKA siRNAs, and the effects of gAPN
249 administration upon MI/R injury were determined. Consistent with our previous data (24),
250 gAPN inhibited MI/R-induced infarction (Figure 8A) and improved cardiac function (Figure
251 8B) in AMPK-DN animals. Importantly, PKA knockdown significantly attenuated this
252 protective effect, consistent with wild type (WT) mice results presented in Figures 1-3.
253 Moreover, PKA deficiency blocked the anti-oxidative/antinitrative effects of gAPN,
254 evidenced by augmented superoxide (Figure 8C) and peroxynitrite (Figure 8D) generation.
255 Together, these results support PKA-mediated gAPN cardioprotective effect against MI/R
256 injury is independent of AMPK signaling.

Discussion

Several important observations have been made in the present study. Firstly, we demonstrate gAPN could not inhibit post-ischemic myocardial apoptosis, infarction, or cardiac dysfunction in cardiac specific PKA knockdown animals. This is the first evidence that PKA signaling mediates the cardioprotective effects of APN in ischemic heart disease in vivo. Secondly, we provide the first direct evidence that the anti-oxidative and antinitrative effects of gAPN in ischemic/reperfused hearts are mediated by PKA. Thirdly, we demonstrate PKA signaling is involved with adiponectin-mediated inhibition of IKK-I κ B-NF κ B activation after MI/R. Finally, we provide evidence that PKA-mediated gAPN's cardioprotective effect against MI/R injury is independent of AMPK signaling.

APN is an abundant circulating adipocytokine secreted primarily from adipose tissue, exerting at least three major functions, including an insulin sensitization/metabolic-regulatory function (in the liver and muscle), an anti-inflammatory/vasculoprotective function, and an anti-ischemic/cardioprotective function. Numerous epidemiological studies underline the correlation between hypoadiponectinemic states and increased morbidity/mortality of cardiovascular ischemic diseases(6; 12; 13; 18). Consistent with our and others' previous results, the present study confirms exogenous APN supplementation can significantly mitigate myocardial apoptosis, infarct size, and cardiac dysfunction.

The signal transduction pathway mediating the antioxidant effect of adiponectin remains intensely investigated. PKA is involved in adiponectin-mediated suppression of ROS and apoptosis in endothelial cells(9; 19). Therefore, the current study sought to determine the role of PKA in gAPN's cardioprotective effect against MI/R injury, utilizing in vivo gene silencing techniques. In wild type mice, we demonstrate gAPN reversed MI/R-induced myocardial infarction, cardiomyocytes apoptosis, and cardiac dysfunction. However, in mice subjected to intramyocardial PKA knockdown, such protective effects of gAPN were either

completely abolished or significantly attenuated. This is direct evidence that a significant portion of gAPN-mediated cardioprotection is PKA-dependent in intact animals.

Simultaneous overproduction of superoxide and NO not only causes inactivation of the cytoprotective NO, but generates the highly cytotoxic molecule peroxynitrite. Therefore, those therapeutic interventions preventing concomitant NO/superoxide overproduction effectively block peroxynitrite formation, and offer great tissue protection. The current study's results demonstrate that gAPN administration reduced synchronized NO/superoxide overproduction from NADPH oxidase and iNOS, and effectively block peroxynitrite formation in ischemic/reperfused hearts. Here, we demonstrate for the first time that gAPN inhibits I/R-induced oxidative in a PKA-dependent fashion, which may be the mechanism responsible for reduced cardioprotection by gAPN observed in PKA-deficient animals.

Nuclear factor kappa B (NF- κ B) is a crucial transcription factor in the induction of genes involved in various physiological processes, including response to injury and inflammation. Activation of NF- κ B requires phosphorylation of the inhibitor of NF- κ B (I κ B) by I κ B kinase (IKK), resulting 26S proteasome-mediated degradation of I κ B. This allows the translocation of NF κ B from the cytosol to the nucleus, where the heterodimer binds the promotor region of specific target genes. Activation of NF- κ B leads to the transcription of factors promoting inflammation (i.e. adhesion molecules, cytokines, and chemokines), but may also contribute to tissue remodeling, inflammation resolution, and the transcription of genes resulting in anti-inflammatory effects. Notably, NF- κ B activation is implicated in the pathophysiology underlying acute myocardial infarction, heart failure, endothelial dysfunction, and unstable angina pectoris(3; 10; 30). Furthermore, IKK inhibition attenuates myocardial injury and dysfunction following acute I/R (30), and inhibition of I κ B phosphorylation and resultant NF- κ B activation attenuates MI/R injury in cardiomyocytes. We currently demonstrate gAPN inhibits IKK/I κ B/NF κ B activation in the scramble heart.

307 However, gAPN could not block MI/R-induced IKK and I κ B phosphorylation and resultant
308 NF κ B activation in PKA-deficient mice. Together, these results indicate gAPN confers
309 cardioprotection via inhibition of IKK/I κ B/NF κ B-mediated inflammation, and intact PKA
310 signaling is necessary for such gAPN-mediated anti-inflammatory protection against I/R
311 injury.

312 Substantial evidence supports the essential role AMPK plays in adiponectin
313 intracellular signaling. Pharmacological or genetic inhibition of AMPK activity virtually
314 abolishes the metabolic, anti-inflammatory and vasculoprotective effects of adiponectin.
315 However, we previously demonstrate APN-cardioprotection against MI/R injury is retained
316 in AMPK-DN mice. APN administration to AMPK-DN mice reduces MI/R-induced
317 oxidative and nitrative stress, indicating that AMPK-independent mechanisms mediate
318 gAPN's cardioprotective effect (24). Our present study data is consistent with our previous
319 findings. In AMPK-DN mice, the antioxidative/antinitrative effects (and consequent
320 cardioprotective effects) of gAPN were dependent upon whether PKA was silenced or not.
321 Our data supports the responsibility of PKA signaling in part for the protective actions of
322 adiponectin against MI/R injury.

323 In summary, we demonstrate for the first time that APN inhibits oxidative/nitrative
324 stress during MI/R via PKA signaling, in an AMPK independent fashion. This represents new
325 understanding in the intracellular signaling mechanisms of APN, as we continue to identify
326 novel therapeutic targets in the treatment of diabetic ischemic/reperfusion injury.

327

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333

334

335 **Author Disclosure Statement**

336 No conflicts of interest, financial or otherwise, are declared by the authors.

337

Figure Legend

Figure 1. The influence of APN upon PKA expression and activation. (A) Cardiac PKA expression in control (Sham MI/R) or MI/R treated with vehicle or gAPN. (B) Cardiac PKA activity in control or MI/R treated with vehicle or gAPN. n=6-9 hearts/group. * $P<0.05$ vs. vehicle.

Figure 2. The influence of PKA signaling blockade by PKA-selective siRNAs upon myocardial PKA expression and activity and the resultant effect on myocardial infarct size. (A) Cardiac specific PKA knockdown decreased PKA expression. (B) PKA siRNAs significantly inhibited PKA expression. (C) PKA-specific siRNA significantly blocked gAPN inhibition of MI/R-induced myocardial infarction. n=9-12 hearts/group. * $P<0.05$, ** $P<0.01$ vs. vehicle; # $P<0.05$, ## $P<0.01$ vs. scramble with the same treatment.

Figure 3. PKA deficiency blocked gAPN inhibition of MI/R-induced myocardial dysfunction. Cardiac function determined by echocardiography (A, LVEF) and left ventricular catheterization hemodynamic assay (B, LVEDP; C, +dp/dtmax). n=12-14 animals/group. * $P<0.05$, ** $P<0.01$ vs. vehicle; # $P<0.05$, ## $P<0.01$ vs. scramble with the same treatment.

Figure 4. PKA deficiency blocked gAPN inhibition of MI/R-induced cardiomyocyte apoptosis. (A) TUNEL staining; (B) caspase-3 activity. n=5-6 hearts/group. ** $P<0.01$ vs. vehicle; # $P<0.05$, ## $P<0.01$ vs. scramble with the same treatment.

Figure 5. PKA deficiency blocked the anti-oxidative effects of gAPN post MI/R. (A) Production of superoxide (n=6-7 hearts/group); (B) gp91^{phox} expression, representative

Western blots shown (n=5–6 hearts/group). ** $P<0.01$ vs. vehicle; # $P<0.05$, ## $P<0.01$ vs. scramble with the same treatment.

Figure 6. PKA deficiency partially blocked the anti-nitrative effects of gAPN post MI/R
(A) NO_x content (n=5-6 hearts/group); (B) Inducible nitric oxide synthase (iNOS) expression, representative Western blots shown (n=5-6 hearts/group); (C) Nitrotyrosine content, determined by ELISA (n=6-8 hearts/group). * $P<0.05$, ** $P<0.01$ vs. vehicle; # $P<0.05$, ## $P<0.01$ vs. scramble with the same treatment.

Figure 7. gAPN decreased IKK/I κ B/NF- κ B signaling activation in MI/R myocardium, which was blocked by PKA deficiency. (A) Phosphorylated IKK expression, representative Western blots shown (n=6 hearts/group); (B) Phosphorylated I κ B expression, representative Western blots shown (n=5-6 hearts/group); (C) Activated NF κ B, determined by ELISA (n=6-8 hearts/group). * $P<0.05$, ** $P<0.01$ vs. vehicle; # $P<0.05$, ## $P<0.01$ vs. scramble with the same treatment.

Figure 8. PKA-mediated cardioprotective effect of APN against MI/R injury is AMPK-independent (A) Myocardial infarction, determined by Evans blue/TTC double staining (n=9-12 hearts/group); (B) Cardiac function, determined by echocardiography assay (n=12-15 animals/group); (C) Superoxide production (n=6-8 hearts/group); (D) Nitrotyrosine content, determined by ELISA (n=6-8 hearts/group). * $P<0.05$, ** $P<0.01$ vs. vehicle; # $P<0.05$, ## $P<0.01$ vs. scramble with the same treatment.

References

1. **Bendall JK, Cave AC, Heymes C, Gall N and Shah AM.** Pivotal role of a gp91phox-containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice. *Circulation* 105: 293-296, 2002.
2. **Borchi E, Parri M, Papucci L, Becatti M, Nassi N, Nassi P and Nediani C.** Role of NADPH oxidase in H9c2 cardiac muscle cells exposed to simulated ischaemia-reperfusion. *J Cell Mol Med* 13: 2724-2735, 2009.
3. **Chandrasekar B, Smith JB and Freeman GL.** Ischemia-reperfusion of rat myocardium activates nuclear factor-KappaB and induces neutrophil infiltration via lipopolysaccharide-induced CXC chemokine. *Circulation* 103: 2296-2302, 2001.
4. **Chandrasekar B, Boylston WH, Venkatachalam K, Webster NJG, Prabhu SD and Valente AJ.** Adiponectin Blocks Interleukin-18-mediated Endothelial Cell Death via APPL1-dependent AMP-activated Protein Kinase (AMPK) Activation and IKK/NF- κ B/PTEN Suppression. *J Biol Chem* 283: 24889-24898, 2008.
5. **Cheng KK, Lam KS, Wang Y, Huang Y, Carling D, Wu D, Wong C and Xu A.** Adiponectin-induced endothelial nitric oxide synthase activation and nitric oxide production are mediated by APPL1 in endothelial cells. *Diabetes* 56: 1387-1394, 2007.
6. **Combs TP, Pajvani UB, Berg AH, Lin Y, Jelicks LA, Laplante M, Nawrocki AR, Rajala MW, Parlow AF, Cheeseboro L, Ding YY, Russell RG, Lindemann D, Hartley A, Baker GR, Obici S, Deshaies Y, Ludgate M, Rossetti L and Scherer PE.** A transgenic mouse with a deletion in the collagenous domain of adiponectin displays elevated circulating adiponectin and improved insulin sensitivity. *Endocrinology* 145: 367-383, 2004.

- 410 7. **Dyck JRB.** The Ischemic Heart: Starving to Stimulate the Adiponectin-AMPK Signaling Axis.
411 *Circulation* 116: 2779-2781, 2007.

- 412 8. **Fang X, Palanivel R, Cresser J, Schram K, Ganguly R, Thong FS, Tuinei J, Xu A, Abel ED and**
413 **Sweeney G.** An APPL1-AMPK signaling axis mediates beneficial metabolic effects of adiponectin in the
414 heart. *Am J Physiol Endocrinol Metab* 299: E721-E729, 2010.

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

- 417 10. **Grabellus F, Levkau B, Sokoll A, Welp H, Schmid C, Deng MC, Takeda A, Breithardt G and Baba**
418 **HA.** Reversible activation of nuclear factor-kappaB in human end-stage heart failure after left ventricular
419 mechanical support. *Cardiovasc Res* 53: 124-130, 2002.

- 420 11. **Hattori Y, Nakano Y, Hattori S, Tomizawa A, Inukai K and Kasai K.** High molecular weight
421 adiponectin activates AMPK and suppresses cytokine-induced NF-kappaB activation in vascular
422 endothelial cells. *FEBS Lett* 582: 1719-1724, 2008.

- 423 12. **Hui X, Lam KS, Vanhoutte PM and Xu A.** Adiponectin and Cardiovascular Health: an Update. *Br J*
424 *Pharmacol* 165: 574-590, 12 A.D.

- 425 13. **Kawano T, Saito T, Yasu T, Saito T, Nakamura T, Namai K, Tamemoto H, Kawakami M, Saito M**
426 **and Ishikawa SE.** Close association of hypoadiponectinemia with arteriosclerosis obliterans and
427 ischemic heart disease. *Metabolism* 54: 653-656, 2005.

- 428 14. **Khaliulin I, Parker JE and Halestrap AP.** Consecutive pharmacological activation of PKA and PKC
429 mimics the potent cardioprotection of temperature preconditioning. *Cardiovasc Res* 88: 324-333, 2010.

- 430 15. **Li C, Kao RL, Ha T, Kelley J, Browder IW and Williams DL.** Early activation of IKK{beta} during in

431 vivo myocardial ischemia. *Am J Physiol Heart Circ Physiol* 280: H1264-H1271, 2001.

432 16. **Ohsugi T, Horie R, Kumasaka T, Ishida A, Ishida T, Yamaguchi K, Watanabe T, Umezawa K and**
433 **Urano T.** In vivo antitumor activity of the NF-kappaB inhibitor dehydroxymethylepoxyquinomicin in a
434 mouse model of adult T-cell leukemia. *Carcinogenesis* 26: 1382-1388, 2005.

435 17. **Onai Y, Suzuki J, Kakuta T, Maejima Y, Haraguchi G, Fukasawa H, Muto S, Itai A and Isobe M.**
436 Inhibition of IkappaB phosphorylation in cardiomyocytes attenuates myocardial ischemia/reperfusion
437 injury. *Cardiovasc Res* 63: 51-59, 2004.

438 18. **Ouchi N, Shibata R and Walsh K.** Cardioprotection by adiponectin. *Trends Cardiovasc Med* 16:
439 141-146, 2006.

440 19. **Ouedraogo R, Wu X, Xu SQ, Fuchsel L, Motoshima H, Mahadev K, Hough K, Scalia R and**
441 **Goldstein BJ.** Adiponectin suppression of high-glucose-induced reactive oxygen species in vascular
442 endothelial cells: evidence for involvement of a cAMP signaling pathway. *Diabetes* 55: 1840-1846, 2006.

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

446 21. **Simons M, Annex BH, Laham RJ, Kleiman N, Henry T, Dauerman H, Udelson JE, Gervino EV,**
447 **Pike M, Whitehouse MJ, Moon T and Chronos NA.** Pharmacological treatment of coronary artery
448 disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial.
449 *Circulation* 105: 788-793, 2002.

450 22. **Tao L, Gao E, Jiao X, Yuan Y, Li S, Christopher TA, Lopez BL, Koch W, Chan L, Goldstein BJ and**
451 **Ma XL.** Adiponectin cardioprotection after myocardial ischemia/reperfusion involves the reduction of

oxidative/nitrative stress. *Circulation* 115: 1408-1416, 2007.

23. **Tishinsky JM, Robinson LE and Dyck DJ.** Insulin-sensitizing properties of adiponectin. *Biochimie* 94: 2131-2136, 2012.

24. **Wang Y, Gao E, Tao L, Lau WB, Yuan Y, Goldstein BJ, Lopez BL, Christopher TA, Tian R, Koch W and Ma XL.** AMP-activated protein kinase deficiency enhances myocardial ischemia/reperfusion injury but has minimal effect on the antioxidant/antinitrative protection of adiponectin. *Circulation* 119: 835-844, 2009.

25. **Wang Y, Tao L, Yuan Y, Lau WB, Li R, Lopez BL, Christopher TA, Tian R and Ma XL.** Cardioprotective effect of adiponectin is partially mediated by its AMPK-independent antinitrative action. *Am J Physiol Endocrinol Metab* 297: E384-E391, 2009.

26. **Wang Y, Wang X, Jasmin JF, Lau WB, Li R, Yuan Y, Yi W, Chuprun K, Lisanti MP, Koch WJ, Gao E and Ma XL.** Essential role of caveolin-3 in adiponectin signalsome formation and adiponectin cardioprotection. *Arterioscler Thromb Vasc Biol* 32: 934-942, 2012.

27. **Wu X, Mahadev K, Fuchsel L, Ouedraogo R, Xu SQ and Goldstein BJ.** Adiponectin suppresses I κ B kinase activation induced by tumor necrosis factor- α or high glucose in endothelial cells: role of cAMP and AMP kinase signaling. *Am J Physiol Endocrinol Metab* 293: E1836-E1844, 2007.

28. **Xing Y, Musi N, Fujii N, Zou L, Luptak I, Hirshman MF, Goodyear LJ and Tian R.** Glucose Metabolism and Energy Homeostasis in Mouse Hearts Overexpressing Dominant Negative α 2 Subunit of AMP-activated Protein Kinase. *J Biol Chem* 278: 28372-28377, 2003.

29. **Yi W, Sun Y, Gao E, Wei X, Lau WB, Zheng Q, Wang Y, Yuan Y, Wang X, Tao L, Li R, Koch W and Ma XL.** Reduced Cardioprotective Action of Adiponectin in High-Fat Diet-Induced Type II Diabetic

473 Mice and Its Underlying Mechanisms. *Antioxid Redox Signal* 15: 1779-1788, 2011.

474 30. **Zingarelli BF, - Hake PW FAU - Yang Z, Yang ZF, O'Connor MF, Denenberg AF and Wong HR. -**

475 Absence of inducible nitric oxide synthase modulates early reperfusion-induced NF-kappaB and AP-1

476 activation and enhances myocardial damage. - *FASEB J* 2002 Mar;16(3):327-42 327-342.

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