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Cardioprotective Effect of Adiponectin is Partially Mediated by its
AMPK-Independent Anti-Nitrative Action

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Running Title: Anti-apoptotic signaling of adiponectin

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Abstract

Adiponectin (APN) exerts its metabolic regulation largely through AMP-dependent protein kinase (AMPK). However, the role of AMPK in adiponectin's anti-apoptotic effect in ischemic/reperfused (I/R) adult cardiomyocytes remains incompletely understood. The current study was designed to determine the involvement of AMPK in the anti-apoptotic signaling of adiponectin. Cardiomyocytes from adult male mice overexpressing a dominant negative α_2 subunit of AMPK (AMPK-DN) or wild-type (WT) littermates were subjected to simulated I/R (SI/R) and pretreated with 2 μ g/ml gAPN or vehicle. SI/R-induced cardiomyocyte apoptosis was modestly increased in AMPK-DN cardiomyocytes ($P<0.05$). Treatment with gAPN significantly reduced SI/R-induced apoptosis in WT cardiomyocytes as well as in AMPK-DN cardiomyocytes, indicating that the anti-apoptotic effect of gAPN is partially AMPK-independent. Furthermore, gAPN-induced endothelial nitric oxide synthase (eNOS) phosphorylation was significantly reduced in AMPK-DN cardiomyocytes, suggesting the APN-eNOS signaling axis is impaired in AMPK-DN cardiomyocytes. Additional experiments demonstrated that treatment of AMPK-DN cardiomyocytes with gAPN reduced SI/R-induced NADPH oxidase overexpression, decreased superoxide generation, and blocked peroxynitrite formation to the same extent as that observed in WT cardiomyocytes. Collectively, our current study demonstrated that although the metabolic and eNOS activation effects of adiponectin is largely mediated by AMPK, the superoxide-suppressing effect of adiponectin is not mediated by AMPK, and this AMPK-independent anti-oxidant property of adiponectin increased nitric oxide bioavailability, and exerted significant anti-apoptotic effect.

Keywords: Apoptosis; Oxidative Stress; Nitric Oxide; Adipocytokine

61 The incidence of obesity and type 2 diabetes mellitus is increasing worldwide,
62 and both these conditions increase the risk of cardiovascular disease(22).
63 Adiponectin is an adipocyte-produced protein that plays a critical role in glucose and
64 lipid metabolism regulation(23). Prospective studies have demonstrated the
65 association between decreased plasma adiponectin levels and increased cardiovascular
66 morbidity in diabetic patients(27; 34; 41) Clarification of the mechanisms by which
67 adiponectin acts advantageously may contribute to our understanding of diabetic
68 pathophysiology, and provide insight to a potential therapeutic modality.

69 AMP-activated protein kinase (AMPK) is a serine-threonine kinase that acts as an
70 energy sensor in various cell types, and can be activated by hypoxic stress(33). When
71 cellular energy is depleted, AMPK activity leads to downregulation of ATP-utilizing
72 pathways and up-regulation of ATP-generating pathways to restore energy
73 homeostasis(8; 40). Considerable evidence exists that AMPK plays an essential role in
74 adiponectin's metabolic regulatory, anti-inflammatory, and vasculoprotective
75 actions(19). However, recently published studies utilizing AMPK inhibitors in
76 determining the involvement of AMPK in adiponectin's cardioprotective action have
77 yielded inconsistent results(14; 28; 30). The role of AMPK in adiponectin's
78 anti-ischemic and cardioprotective action remains incompletely understood.

79 The aims of the present study were 1) to determine whether exogenous
80 adiponectin supplementation could attenuate myocardial apoptosis in cardiomyocytes
81 subjected to simulated ischemia/reperfusion (SI/R), 2) to determine whether
82 adiponectin exerts its effects in an AMPK-dependent fashion, and 3) to dissect the

83 molecular mechanisms underlying adiponectin's cardioprotective role.

84

85 **Methods**

86 Adult mouse cardiomyocyte culture: All experiments were performed on adult (8 to 10
87 weeks) male mice overexpressing a dominant negative α_2 subunit (D157A) of AMPK
88 (AMPK-DN) or male littermate controls expressing the wild type of α_2 subunit of
89 AMPK (WT). Generation, breeding, phenotype characteristics, and genotyping of
90 these mice have previously been described in detail(37). Experiments were performed
91 in adherence with the National Institutes of Health Guidelines on the use of
92 Laboratory Animals, and were approved by the Thomas Jefferson University
93 Committee on Animal Care. Mice were anesthetized with 2% isoflurane, and hearts
94 were removed and perfused at 37°C for ~3 min with a Ca^{2+} -free bicarbonate-based
95 buffer. Enzymatic digestion was initiated by adding collagenase type B/D to the
96 perfusion solution. When the hearts became swollen and hard after ~3 min of
97 digestion, 50 μM Ca^{2+} was added to the enzyme solution. Approximately 7 min later,
98 the left ventricle was removed, cut into several sections, and further digested in a
99 shaker for 10 min at 37°C in the same enzyme solution. The supernatant containing
100 the dispersed myocytes was filtered into a sterilized tube and centrifuged at 800 x g
101 for 1 min. The cell pellet was then resuspended in bicarbonate-based buffer containing
102 125 μM Ca^{2+} . After the myocytes were pelleted by gravity for ~10 min, the
103 supernatant was aspirated and the myocytes were resuspended in bicarbonate-based
104 buffer containing 250 μM Ca^{2+} . Myocytes were plated at $0.5\text{-}1 \times 10^4$ cells.cm⁻² in the

105 culture dishes precoated with mouse laminin.

106 After 1 h of culture in a 5% CO₂ incubator at 37° C, cardiomyocytes were
107 subjected to simulated ischemia/reperfusion (SI/R) as originally described by Isner
108 and colleagues(21) and modified in our recently published study(11). In brief,
109 glucose-free culture medium was first gassed for 5 minutes with a hypoxic gas
110 mixture (95%N₂/5%CO₂). Normal culture medium was quickly replaced with the
111 hypoxia-hypoglycemic medium containing either vehicle or globular domain of
112 adiponectin (gAPN, 2 µg/ml), and cardiomyocytes were placed in a Napco 8000WJ
113 hypoxia (1%O₂/5%CO₂/94%N₂) incubator (Thermo Scientific, Waltham, MA). The
114 concentration of gAPN was selected based on our previous publication(32). After 3
115 hours of hypoxia-hypoglycemic culture, the hypoxia/hypoglycemic medium was
116 replaced with normal culture medium (containing same concentration of vehicle or
117 gAPN). Cells were then incubated under normoxic conditions in a CO₂ incubator for
118 an additional 6 hours.

119 Assessment of cardiomyocyte apoptosis: Cardiomyocyte apoptosis was determined by
120 DNA ladder formation, terminal deoxynucleotidyl transferase-mediated dUTP
121 nick-end labeling (TUNEL) staining, and caspase 3 activity as reported in our
122 previous study(32). For DNA fragmentation assay, total DNA was isolated with the
123 Gentra Puregene Tissue DNA Isolation Kit (QIAGEN, Valencia, CA) per
124 manufacturer's instructions. 10 µg of DNA was loaded into 1.8% agarose gel
125 containing 0.5 µg ml⁻¹ ethidium bromide. DNA electrophoresis was carried out at 60
126 V for 1 to 2 hours. DNA ladder formation, a "hallmark" of tissue apoptosis, was

127 visualized under ultraviolet light and photographed for permanent record. TUNEL
128 assay was performed utilizing the In Situ Cell Death Detection Kit (Roche, Palo Alto,
129 CA). Briefly, cells were fixed with 10% paraformaldehyde, and incubated with the
130 TUNEL reaction mixture containing TdT-mediated dUTP nick end labeling. Nuclei
131 were counter-stained with 4,6-diamidino-2-phenylindole (DAPI). Samples were
132 visualized on an Olympus BX51 Fluorescence Microscope, and digital images were
133 acquired with IP Lab Image Analysis Software (Version 3.5, Scanalytics, Fairfax,
134 VA, USA). The index of apoptosis (number of TUNEL positively stained nuclei/total
135 number of nuclei x 100%) was automatically calculated and exported for further
136 analysis. Assays were performed in a blinded manner. For caspase-3 activity assay,
137 cells were lysed and total protein concentration was determined by the bicinchoninic
138 acid (BCA) method. To each well of a 96-well plate, supernatant containing 200 µg of
139 protein was loaded and incubated with 25 µg Ac-DEVD-pNA at 37° C for 1.5 hours.
140 pNA was cleaved from DEVD by activated caspase-3, and the free pNA was
141 quantified using a SpectraMax-Plus microplate spectrophotometer (Molecular
142 Devices, Sunnyvale, CA) at 405 nm. Caspase-3 activity was expressed as nmol
143 pNA/h/mg protein.

144 Quantification of superoxide production: Superoxide production in viable
145 cardiomyocytes was measured by lucigenin-enhanced chemiluminescence as
146 described previously(8), and expressed as relative light units (RLU) per second per
147 milligram protein (RLU/s/mg protein).

148 Western blot analysis: The post-treatment cultured cardiomyocytes were collected

149 in lysis buffer. Protein was separated on SDS-PAGE gels, transferred to
150 nitrocellulose membranes, and detected with antibodies against eNOS, peNOS
151 (Upstate, Chicago, IL), acetyl-CoA carboxylase (ACC), pACC, and gp91^{phox} (BD
152 Bioscience Laboratories, San Jose, CA). Nitrocellulose membranes were then
153 incubated with HRP-conjugated anti-rabbit immunoglobulin G antibody (Cell
154 Signaling, Danvers, MA) for 1 h. The blot was developed with a Supersignal
155 Chemiluminescence Detection Kit (Pierce, Rockford, IL). The band was visualized
156 with a Kodak Image Station 400 (Rochester, NY), and the band densities were
157 analyzed with Kodak 1-Dimensional software.

158 Immunocytochemistry: At the end of experiments, cardiomyocytes were fixed with
159 formaldehyde (4% in PBS) and stained with an anti-nitrotyrosine antibody (Upstate,
160 Chicago, IL). Immunostaining was developed with a Vectastain ABC kit (Vector
161 Laboratories, Burlingame, CA), and visualized with an Olympus BX51 Fluorescence
162 Microscope.

163 Quantification of tissue nitrotyrosine content: Cardiomyocyte nitrotyrosine content,
164 the footprint of peroxynitrite (ONOO⁻) formation and an index of nitric oxide (NO)
165 inactivation by superoxide, was determined using a nitrotyrosine ELISA kit (Cell
166 Sciences, Canton, MA) per manufacturer's instructions.

167 Statistical Analysis: All values in the text and figures are presented as means $\pm$ SEM
168 of *n* independent experiments. All data (except Western blot density) were subjected
169 to ANOVA followed by Bonferroni correction for *post-hoc t* test. Western blot

170 densities were analyzed with the Kruskal-Wallis test followed by Dunn's post-hoc test.
171 Probabilities of 0.05 or less were considered to be statistically significant.

172 **Results**

173 SI/R-induced apoptosis was increased in cardiomyocytes isolated from AMPK-DN
174 mice and gAPN's anti-apoptotic effects were largely retained in AMPK-DN

175 cardiomyocytes: Adult cardiomyocytes are terminally differentiated, and spontaneous
176 apoptosis occurs even under normal culture conditions. In our system, approximately
177 14% cardiomyocytes were TUNEL positive after 9 hours of culture with normal
178 oxygen and glucose (Figure 1, Sham SI/R). In cardiomyocytes isolated from WT
179 littermates, 3 hours of SI followed by 6 hours of reperfusion markedly stimulated
180 apoptotic cell death, as evidenced by a 2.4-fold increase in TUNEL staining over
181 time-matched control (Figure 1), clear DNA ladder formation (Figure 2 A), and a
182 2.3-fold increase in caspase 3 activity (Figure 2B). Consistent with our previous *in*
183 *vivo* experimental results(32), treatment with gAPN significantly reduced
184 SI/R-induced apoptosis (Figure 1 and 2, $P<0.01$). Cardiomyocytes isolated from
185 AMPK-DN mice were morphologically normal, and exhibited spontaneous apoptosis
186 in normal culture conditions to an extent comparable to cardiomyocytes isolated from
187 WT mice (Figures 1 and 2B). However, when subjected to SI/R, AMPK-DN
188 cardiomyocytes exhibited modestly greater TUNEL positive staining and caspase 3
189 activity than WT cardiomyocytes (TUNEL: $38.4\pm0.97\%$ vs. $33.2\pm2.2\%$, $P<0.05$;
190 caspase 3: 6.2 ± 0.35 vs. 5.3 ± 0.12 nmol pNA/h/mg protein, $P<0.05$). These results
191 indicate that the susceptibility to ischemia/reperfusion stress was significantly

192 increased in the presence of impaired AMPK signaling. Most interestingly, the
193 anti-apoptotic property of gAPN was reduced but not lost in AMPK-DN
194 cardiomyocytes. Specifically, treatment of AMPK-DN cardiomyocytes with gAPN
195 significantly reduced TUNEL staining ($29.5 \pm 1.5\%$ vs. $38.4 \pm 0.97\%$, $P < 0.01$, Figure 1),
196 partially blocked DNA ladder formation (Figure 2A), and significantly decreased
197 caspase 3 activity (4.1 ± 0.52 vs. 6.2 ± 0.35 nmol pNA/h/mg protein, $P < 0.01$, Figure 2B).

198 To obtain more evidence in supporting our conclusion that gAPN protects
199 cardiomyocyte partially through AMPK-independent pathway, an additional
200 experiment was performed. Adult cardiomyocytes were isolated from AMPK-DN
201 mice as described above. Compound C, a selective AMPK inhibitor, was added 30
202 minutes before APN treatment ($20 \mu\text{M}$)(31), and AMPK activity was determined
203 by SAMS peptide/ $(\gamma\text{-}^{32}\text{P})\text{ATP}$ assay(31). Although AMPK activity was nearly
204 completely inhibited after this combinational treatment ($4.4 \pm 1.1\%$ of control), the
205 cardioprotective effects of gAPN remained to be significant (TUNEL staining:
206 $30.4 \pm 1.2\%$ vs. 40.3 ± 2.1 in vehicle group, $P < 0.01$; Caspase-3 activity: 4.4 ± 0.32 vs.
207 6.7 ± 0.45 nmol pNA/h/mg protein, $P < 0.01$). These results demonstrated that gAPN
208 protects cardiomyocytes from SI/R injury partially in an AMPK-independent fashion.

209 *Adiponectin-induced activation of ACC, a primary downstream molecule of AMPK,*

210 *was lost in AMPK-DN cardiomyocytes:* The observation that the anti-apoptotic effect
211 of gAPN was largely retained in AMPK-DN cardiomyocytes is somewhat surprising,
212 because this is opposite of previously published results demonstrating the abolishment
213 of metabolic regulatory and vascular protective effects of adiponectin when AMPK is

214 pharmacologically or genetically inhibited (4; 38). One possible explanation for this
215 discrepancy is that our AMPK-DN approach failed to effectively block
216 adiponectin-induced AMPK activation. To directly address this issue, Acetyl-CoA
217 carboxylase (ACC) phosphorylation, a major signaling step through which AMPK
218 exerts its metabolic regulation, was determined. As summarized in Figure 3A,
219 treatment with gAPN significantly increased ACC phosphorylation in cardiomyocytes
220 isolated from WT (left panel), but not from AMPK-DN heart (right panel). Moreover,
221 a 1.8-fold increase in ACC phosphorylation was observed in WT cardiomyocytes
222 following SI/R, and treatment with gAPN further enhanced (1.2-fold over SI/R +
223 vehicle, $P < 0.05$) ACC phosphorylation modestly (Figure 3B). However, SI/R-induced
224 ACC phosphorylation was lost in AMPK-DN cardiomyocytes. More importantly,
225 treatment with gAPN failed to stimulate ACC phosphorylation in these cells (Figure
226 3B, right panel). These results demonstrated that the AMPK signaling system was
227 indeed blocked in our AMPK-DN cardiomyocytes.

228 Adiponectin-induced eNOS phosphorylation was significantly reduced in AMPK-DN

229 cardiomyocytes: Substantial evidence exists that the physiological or pharmacological
230 concentration of NO produced by eNOS or NO donors is cardioprotective. Moreover,
231 previous studies have demonstrated that adiponectin possesses strong eNOS
232 phosphorylation ability, and stimulates NO production in cultured endothelial cells.
233 We thus explored the possibility that gAPN may cause eNOS phosphorylation in
234 cardiomyocytes independent of AMPK, and thus reduce cardiomyocyte apoptosis in
235 AMPK-DN cardiomyocytes. As summarized in Figure 4, treatment of WT

cardiomyocytes subjected to sham SI/R (4A) or SI/R (4B) with gAPN resulted in significant eNOS phosphorylation. However, gAPN-induced eNOS phosphorylation was significantly blunted, although not completely lost, in AMPK-DN cardiomyocytes. These results indicate that adiponectin induces cardiomyocyte eNOS phosphorylation predominantly by the AMPK-mediated pathway, and a relatively weaker AMPK-independent eNOS phosphorylation pathway exists in this cell type.

Adiponectin reduced superoxide production and attenuated gp91^{phox} expression in largely AMPK-independent fashion: Data presented in Figures 1 and 2 indicated that greater than 70% of the anti-apoptotic effect of gAPN was retained in cardiomyocytes isolated from AMPK-DN heart. In contrast, greater than 70% of eNOS activation effect, and greater than 95% of ACC activation effect of gAPN were blocked in AMPK-DN cardiomyocytes. These results strongly suggest that mechanisms other than the metabolic benefit and eNOS activation are active and responsible for the anti-apoptotic effect of gAPN in AMPK-DN cardiomyocytes. It is well-recognized that overproduction of reactive oxygen species plays a critical pathogenic role in myocardial ischemia/reperfusion injury. We next determined the effect of gAPN on SI/R-induced superoxide overproduction, and the involvement of AMPK in this action. As summarized in Figure 5, when compared to WT cardiomyocytes, AMPK-DN cardiomyocytes exhibited slightly increased (not statistically significant) basal superoxide production, with significantly increased SI/R-induced superoxide overproduction ($P<0.05$). Most interestingly, treatment with gAPN markedly reduced superoxide overproduction in WT as well as AMPK-DN cardiomyocytes (Figure 5).

258 Previous studies from other investigators and our laboratory have demonstrated
259 that NADPH oxidase is a major cytosolic source of ischemia/reperfusion-induced
260 superoxide overproduction in the heart. Having demonstrated that gAPN inhibits
261 superoxide overproduction largely in an AMPK-independent fashion, we next
262 determined its effect on SI/R-induced upregulation of gp91^{phox}, the membrane
263 component of NADPH oxidase. Compared to WT, mRNA and protein expression of
264 gp91^{phox} were slightly increased in AMPK-DN cardiomyocytes before SI/R (P>0.05).
265 However, SI/R-induced gp91^{phox} mRNA (2.69±0.14-fold over basal expression in
266 AMPK-DN cardiomyocytes vs. 2.11±0.15-fold over basal expression in WT
267 cardiomyocytes, P<0.05) and protein overexpression (Figure 5B, second bar vs. fifth
268 bar, P<0.05) were significantly increased in AMPK-DN cardiomyocytes. Most
269 interestingly, treatment of AMPK-DN cardiomyocytes with gAPN inhibited
270 SI/R-induced gp91^{phox} mRNA (79.1±3.8% and 77.9±3.1% reduction from
271 vehicle-treated group in AMPK-DN and WT cardiomyocytes, respectively) and
272 protein overexpression (Figure 5B) to a comparable level seen in WT cardiomyocytes.
273 Collectively, these results demonstrate that gAPN inhibited NADPH oxidase
274 overexpression and subsequent superoxide overproduction in an AMPK-independent
275 fashion.

276 Inducible nitric oxide synthase (iNOS) is responsible for peroxynitrite overproduction
277 in cardiomyocytes subjected to SI/R: Inactivation of nitric oxide with subsequent
278 formation of peroxynitrite is the most pathologically relevant mechanism through
279 which superoxide exerts its cytotoxic effects. Superoxide reacts with NO in 1:1 ratio

280 and peroxynitrite is maximally produced when both superoxide and NO production
281 are simultaneously elevated. Although eNOS and nNOS are expressed in
282 cardiomyocytes, basal NO production from these forms of NOS is very low.
283 Moreover, eNOS phosphorylation is reduced in AMPK-DN cardiomyocytes whereas
284 peroxynitrite is increased in these cells, indicating that eNOS is not responsible for
285 peroxynitrite formation in AMPK-DN cardiomyocytes. We thus determined iNOS
286 expression in SI/R cardiomyocytes, and elucidated its role in SI/R-induced
287 peroxynitrite production. As illustrated in Figure 6, no significant iNOS expression
288 was detected in cardiomyocytes isolated from either WT or AMPK-DN hearts, if not
289 subjected to SI/R. However, SI/R caused significant iNOS expression, which was
290 markedly inhibited by gAPN in WT or AMPK-DN cardiomyocytes (Figure 6A). In
291 addition, treatment with 1400W (20 µg/ml), a selective iNOS inhibitor, at the time of
292 reperfusion significantly inhibited peroxynitrite formation in ischemia/reperfused
293 cardiomyocytes (Figure 6B). These results demonstrated that NO generated from
294 iNOS is responsible for SI/R-induced peroxynitrite formation, which was inhibited by
295 gAPN in an AMPK-independent fashion.

296 Diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, significantly reduced
297 peroxynitrite formation and attenuated caspase 3 activation after SI/R: Results
298 presented above strongly suggest that gAPN attenuates post-ischemic cardiomyocyte
299 apoptosis by inhibiting NADPH oxidase-derived superoxide production and
300 subsequent peroxynitrite formation. To obtain more evidence supporting this
301 conclusion, additional experiments were performed. As summarized in Figure 7,

302 treatment with DPI, a selective NADPH oxidase inhibitor, reduced peroxynitrite
303 formation (7A) and cardiomyocytes apoptosis (7B) to a comparable level seen with
304 gAPN treatment. Moreover, no additive effect in either peroxynitrite inhibition or
305 caspase-3 suppression was observed when SI/R cardiomyocytes were treated with
306 gAPN plus DPI. These new results further support a conclusion that inhibiting
307 NADPH oxidase-derived superoxide overproduction is largely responsible for
308 gAPN's anti-oxidant/anti-nitrative effects.

309 **Discussion**

310 In a recent *in vivo* animal study, we have demonstrated that AMPK deficiency
311 enhanced myocardial ischemia/reperfusion injury but has minimal effect on
312 adiponectin's anti-oxidant/anti-nitrative protection(36). Our current study not only
313 confirmed our previous finding in a different experimental model, but more
314 importantly, provided new evidence demonstrating that iNOS/NADPH oxidase
315 overexpression is sufficient to increase nitrative/oxidative stress and that gAPN exerts
316 its AMPK-independent cardioprotection partially through iNOS/NADPH oxidase
317 suppression.

318 Adiponectin, also known as Acrp30, is an adipocytokine originally thought to be
319 exclusively secreted from adipose tissue(2; 5). However, growing evidence indicates
320 that adiponectin is synthesized and secreted by adult human and murine
321 cardiomyocytes(7; 15; 26), although its physiological and pathologic significance
322 remains completely unknown. In human and mouse plasma, adiponectin exists in
323 three major oligomeric forms: trimers, hexamers, and a high-molecular weight

324 form(25). A proteolytic cleavage product of adiponectin that includes its globular
325 domain, known as globular adiponectin or gAPN, may occur in human and mouse
326 plasma(10; 39). Recently, it has been shown that the cleavage of adiponectin by
327 leukocyte elastase secreted from activated monocytes and/or neutrophils could be
328 responsible for the generation of gAPN(35). However, the physiological or
329 pathophysiological role of this proteolytic cleavage product remains largely unknown,
330 and its relationship to cardiovascular disease is unclear(13).

331 After approximately 10 years of extensive experimental and clinical investigation,
332 it is now clear that adiponectin has 3 major biologic functions, including an insulin
333 sensitization/metabolic regulatory function (in the liver and muscle), an
334 anti-inflammatory/vascular protective function, and an anti-ischemic/cardioprotective
335 function(12). Substantial accumulated evidence supports the concept that AMPK
336 plays an essential role in adiponectin's metabolic regulatory and vascular protective
337 actions. Specifically, pharmacological inhibition of AMPK activity or genetic
338 inhibition of AMPK expression virtually abolishes the metabolic(3; 38),
339 anti-inflammatory(17), and vasculoprotective(24; 29) effects of adiponectin. However,
340 although the evidence for the anti-ischemic/cardioprotective effects of adiponectin is
341 strong, whether AMPK is the central mediator of this effect remains uncertain.(9)

342 Two ex vivo studies reported that AMPK is essential for the cardioprotective
343 effects of full length adiponectin (fAPN). The study by Shinmura(30) et al
344 demonstrated that short-term caloric restriction protected the heart by increasing
345 serum total adiponectin levels with subsequent AMPK activation. In a more recent

346 study, Gonon et al reported that full length adiponectin protected against myocardial
347 ischemia-reperfusion injury via the AMPK/Akt/NO signaling axis(14). However,
348 although the data clearly demonstrated that administration of fAPN in isolated
349 perfused hearts resulted in AMPK phosphorylation, it was not determined whether the
350 protective effects of adiponectin were blocked when AMPK was inhibited, and no
351 cause-effect relationship was established. It thus remains unclear whether AMPK
352 phosphorylation is a necessary step for adiponectin to exert
353 anti-ischemic/cardioprotective effects. In the first study reporting adiponectin's
354 cardioprotective effect, Shibata et al(28) demonstrated the varying importance of
355 AMPK in fAPN's cardioprotective effect in different models. Specifically, fAPN
356 blocks hypoxia/reoxygenation-induced neonatal cardiomyocyte apoptosis largely
357 through AMPK activation. In contrast, its inhibitory effect on LPS-induced TNF α
358 production is completely AMPK-independent. Moreover, in vivo administration of
359 NS398, a COX-2 inhibitor, partially blocked the infarct-sparing action of exogenous
360 fAPN, suggesting that COX-2 plays a significant role in fAPN's cardioprotection after
361 ischemia/reperfusion. These seemingly conflicting results underscore the complexity
362 of adiponectin signaling, and suggest that multiple signaling mechanisms are involved
363 in adiponectin's cardioprotection in adult animals.

364 AMPK is activated in response to many types of cellular stress, particularly to
365 elevated ratios of AMP to ATP, which occurs during myocardial ischemia(16). Indeed,
366 our current experimental results (Figure 3) demonstrated that ACC phosphorylation,
367 an index for AMPK activity, was markedly increased when cardiomyocytes were

368 subjected to SI/R. However, treatment with gAPN under this particular pathologic
369 condition only elicited moderate additional AMPK activation. These results strongly
370 suggest that the degree of AMPK involvement in adiponectin's biological functions is
371 determined by the intracellular environment, particularly AMP concentration. Under
372 those pathologic conditions where intracellular AMP concentrations are not elevated
373 (i.e., muscular insulin resistance and vascular inflammation), AMPK might be the
374 most important molecule in adiponectin intracellular signaling, as previously reported.
375 In contrast, under those pathologic conditions where intracellular AMP concentrations
376 are elevated, and AMPK has already been significantly activated (such as myocardial
377 ischemia), adiponectin may exert its biological actions largely through signaling
378 molecules other than AMPK.

379 Numerous studies have demonstrated that nitric oxide at physiologic or
380 pharmacologic concentrations is anti-apoptotic and cardioprotective(18). Moreover,
381 molecules capable of stimulating eNOS phosphorylation (e.g., insulin) reduce
382 apoptotic cell death in the ischemic/reperfused heart, attenuating myocardial
383 reperfusion injury(11). As first reported by Chen et al(6), and subsequently confirmed
384 by other investigators(20), adiponectin stimulates eNOS phosphorylation and
385 increases NO production in cultured endothelial cells. However, the
386 AMPK-independent anti-apoptotic action of gAPN as observed in the current study
387 cannot be attributed entirely to gAPN's eNOS stimulatory and NO productive effects,
388 because >70% eNOS phosphorylation by gAPN was lost in AMPK-DN
389 cardiomyocytes. In contrast, our current study provided clear evidence that

390 SI/R-induced NADPH oxidase upregulation and superoxide overproduction are
391 inhibited by gAPN in an AMPK-independent fashion. Although the precise signaling
392 mechanisms by which gAPN may inhibit NADPH oxidase expression cannot be
393 addressed in the current study, the AMPK-independent anti-oxidant effect of gAPN
394 suggests a likely mechanism responsible for gAPN's anti-apoptotic and
395 cardioprotective effects in AMPK-DN cardiomyocytes.

396 The downregulation of NADPH oxidase expression and suppression of
397 superoxide production by gAPN can provide cardioprotection by multiple
398 mechanisms. First, reduction in superoxide production in ischemic/reperfused
399 cardiomyocytes will reduce the production of the hydroxyl radical, thus attenuating
400 oxidative cell injury. Second, superoxide inactivates nitric oxide in a nearly
401 diffusion-limited rate reaction(1). By reducing superoxide production, gAPN
402 increases bioactive NO concentration, thus preserving the anti-apoptotic and
403 cytoprotective function of NO. Finally, by reducing the superoxide/NO reaction,
404 gAPN blocks the formation of peroxynitrite, a highly cytotoxic species that causes
405 cell death by multiple mechanisms, thus attenuating peroxynitrite-induced cell death.

406 It should be indicated that the globular domain of adiponectin was investigated in
407 the current study. Whether the AMPK-independent anti-apoptotic effect and
408 responsible signaling mechanisms identified in this study are unique to this particular
409 isoform of adiponectin, or are shared by other adiponectin isoforms, remains
410 unknown. Additional experiments addressing this question are currently under
411 investigation.

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539

540 **Figure Legend**

541

542 Figure 1. Effect of gAPN on SI/R-induced apoptosis in cardiomyocytes isolated from
543 wild type mice (Left panel, WT) and AMPK-DN mice (right panel). A:
544 Representative photomicrographs of TUNEL staining from cardiomyocytes subjected
545 to sham SI/R or 3h SI followed by 6h R treated with vehicle or gAPN (green:
546 TUNEL-positive nuclei; blue: total nuclei). B: Quantification summary of apoptotic
547 index calculated as TUNEL positive/total x 100%. *P<0.05, **P<0.01 vs. sham SI/R;
548 ^{##}P<0.01 vs. SI/R+V. N=14-16 wells/group with cardiomyocytes isolated from 6 to 8
549 mice. Assays were performed at the end of 6 hours of reperfusion or sham
550 reperfusion.

551

552 Figure 2. Effect of gAPN on SI/R-induced DNA fragmentation (A) and caspase 3
553 activation (B). *P<0.05, **P<0.01 vs. sham SI/R; ^{##}P<0.01 vs. SI/R+V. N=4-6
554 wells/group for DNA fragmentation; N=14-16 wells/group for caspase 3 activity
555 assay. Cells are isolated from 6 to 8 mice. Assays were performed at the end of 6
556 hours of reperfusion or sham reperfusion.

557

558 Figure 3. Effect of gAPN on ACC phosphorylation in cardiomyocytes isolated from
559 WT (left panel) and AMPK-DN mice (right panel) with (B) or without (A) SI/R.
560 **P<0.01 vs. vehicle (A) or sham SI/R (B); [#]P<0.05 vs. SI/R+V. N=8-10 wells/group
561 with cardiomyocytes isolated from 4 to 6 mice. Assays were performed 3 hours after
562 reperfusion or sham reperfusion.

563

564 Figure 4. Effect of gAPN on eNOS phosphorylation in cardiomyocytes isolated from
565 WT (left panel) and AMPK-DN mice (right panel) with (B) or without (A) SI/R.
566 *P<0.05, **P<0.01 vs. vehicle (A) or sham SI/R (B); [#]P<0.05, ^{##}P<0.01 vs. SI/R+V.
567 N=8-10 wells/group with cardiomyocytes isolated from 4 to 6 mice. Assays were
568 performed 3 hours after reperfusion or sham reperfusion

569

570 Figure 5. Effect of gAPN on SI/R-induced superoxide production determined with
571 lucigenin-enhanced chemiluminescence (A) and gp91^{phox} overexpression (B) in
572 cardiomyocytes isolated from WT (left panel) and AMPK-DN mice (right panel).
573 *P<0.05, **P<0.01 vs. sham SI/R; [#]P<0.05, ^{##}P<0.01 vs. SI/R+V. N=14-16

574 wells/group for superoxide production assay. N=8-10 wells/group for gp91^{phox}
575 expression. Assays were performed 3 hours after reperfusion or sham reperfusion.

576

577 Figure 6. Effect of gAPN on SI/R-induced iNOS expression (A) and effect of 1400W,
578 a selective iNOS inhibitor on SI/R-induced peroxynitrite formation (B). **P<0.01 vs.
579 sham SI/R; ^{###}P<0.01 vs. SI/R+V. N=14-16 wells/group with cardiomyocytes isolated
580 from 6 to 8 mice for nitrotyrosine assay. N=8-10 wells/group for iNOS expression.
581 Assays were performed 3 hours after reperfusion or sham reperfusion.

582

583 Figure 7. Effect of gAPN and DPI (an NADPH oxidase inhibitor) or their
584 combination on SI/R-induced peroxynitrite formation (A) and caspase-3 activation
585 (B). P<0.05, **P<0.01 vs. sham SI/R; ^{###}P<0.01 vs. SI/R+V. N=14-16 wells/group
586 with cardiomyocytes isolated from 6 to 8 mice. Assays were performed 3 hours after
587 reperfusion or sham reperfusion.

588

589

590

591

A

B

Figure 1

A **B** **Figure 2**

Figure 3

A **B** **Figure 5**

Figure 6

Figure 7