Cardioprotective effect of adiponectin is partially mediated by its AMPK-independent antiapoptotic action

Yajing Wang, Ling Tao, Yuexin Yuan, Wayne Bond Lau, Rong Li, Bernard L. Lopez, Theodore A. Christopher, Rong Tian, and Xin-Liang Ma

1Department of Emergency Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania; and 2Nuclear Magnetic Resonance Laboratory for Physiological Chemistry, Brigham and Women’s Hospital, Boston, Massachusetts

Submitted 8 December 2008; accepted in final form 20 May 2009

Wang Y, Tao L, Yuan Y, Lau WB, Li R, Lopez BL, Christopher TA, Tian R, Ma XL. Cardioprotective effect of adiponectin is partially mediated by its AMPK-independent antiapoptotic action. Am J Physiol Endocrinol Metab 297: E384–E391, 2009. Published May 26, 2009; doi:10.1152/ajpendo.90975.2008.—Adiponectin (APN) exerts its metabolic regulation largely through AMPK-dependent protein kinase (AMPK). However, the role of AMPK in APN’s antiapoptotic effect in ischemic-reperfusion (I/R) adult cardiomyocytes remains incompletely understood. The present study was designed to determine the involvement of AMPK in the antiapoptotic signaling of APN. Cardiomyocytes from adult male mice overexpressing a dominant-negative α2 subunit of AMPK (AMPK-DN) or wild-type (WT) littersmates were subjected to simulated I/R (SI/R) and pretreated with 2 μg/ml globular domain of APN (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 antiapoptotic effect of gAPN is partially AMPK independent. Furthermore, gAPN-induced endothelial nitric oxide synthase (eNOS) phosphorylation was significantly reduced in AMPK-DN cardiomyocytes, suggesting that 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 present study demonstrated that although the metabolic and eNOS activation effect of APN is largely mediated by AMPK, the superoxide-suppressing effect of APN is not mediated by AMPK, and this AMPK-independent antioxidant property of APN increased nitric oxide bioavailability and exerted significant antiapoptotic effect.

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

The aims of the present study were 1) to determine whether exogenous adiponectin supplementation could attenuate myocardial apoptosis in cardiomyocytes subjected to simulated ischemia-reperfusion (SI/R), 2) to determine whether adiponectin exerts its effects in an AMPK-dependent fashion, and 3) to dissect the molecular mechanisms underlying adiponectin’s cardioprotective role.

METHODS

Adult mouse cardiomyocyte culture. All experiments were performed on adult (8–10 wk) male mice overexpressing a dominant-negative α2 subunit (D157A) of AMPK (AMPK-DN) or male littermate controls expressing the wild-type α2 subunit of AMPK (WT). Generation, breeding, phenotype characteristics, and genotyping of these mice have previously been described in detail (37). Experiments were performed in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Thomas Jefferson University Committee on Animal Care. Mice were anesthetized with 2% isoflurane, and 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. When the hearts became swollen and hard after ~3 min of digestion, 50 μM Ca^{2+} was added to the enzyme solution. Approximately 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 g for 1 min. The cell pellet was then resuspended in bicarbonate-based buffer containing 125 mM 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 in culture dishes precoated with mouse laminin.

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

Assessment of cardiomyocyte apoptosis. Cardiomyocyte apoptosis was determined by DNA ladder formation, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining, and caspase-3 activity as reported in our previous study (32). For DNA fragmentation assay, total DNA was isolated with the Gentra Puregene Tissue DNA Isolation Kit (Qiagen, Valencia, CA) per manufacturer's instructions. Ten micrograms of DNA was loaded into 1.8% agarose gel containing 0.5 µg/ml ethidium bromide. DNA electrophoresis was carried out at 60 V for 1–2 h. DNA ladder formation, a "hallmark" of tissue apoptosis, was visualized under ultraviolet light and photographed for permanent record. TUNEL assay was performed with the In Situ Cell Death Detection Kit (Roche, Palo Alto, CA). Briefly, cells were fixed with 10% paraformaldehyde and incubated with the TUNEL reaction mixture containing TdT-mediated dUTP nick end labeling. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Samples were visualized on an Olympus BX51 Fluorescence Microscope, and digital images were acquired with IP Lab Imagine Analysis Software (version 3.5, ScanaI,ics, Fairfax, VA). The index of apoptosis (number of TUNEL positively stained nuclei/total number of nuclei × 100) was automatically calculated and exported for further analysis. Assays were performed in a blinded manner. For caspase-3 activity assay, cells were lysed and total protein concentration was determined by the bicinchoninic acid (BCA) method. To each well of a 96-well plate, supernatant containing 200 µg of protein was loaded and incubated with 25 µg Ac-DEVD-pNA at 37°C for 1.5 h. pNA was cleaved from DEVD by activated caspase-3, and the free pNA was quantified with a SpectraMax-Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 405 nm. Caspase-3 activity was expressed as nanomoles of pNA per hour per milligram of protein.

Quantification of superoxide production. Superoxide production in viable cardiomyocytes was measured by lucigenin-enhanced chemiluminescence as described previously (8) and expressed as relative light units (RLU) per second per milligram of protein.

Western blot analysis. Posttreatment cultured cardiomyocytes were collected in lysis buffer. Protein was separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and detected with antibodies against endothelial nitric oxide synthase (eNOS), phospho (p)EOS (Upstate, Chicago, IL), acetyl-CoA carboxylase (ACC), pACC, and gp91phox (BD Biosciences Laboratories, San Jose, CA). Nitrocellulose membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G antibody (Cell Signaling, Danvers, MA) for 1 h. The blot was developed with a Supersignal Chemiluminescence Detection Kit (Pierce, Rockford, IL). The band was visualized with a Kodak Image Station 400 (Rochester, NY), and the band densities were analyzed with Kodak 1-Dimensional software.

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

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

Statistical analysis. All values are presented as means ± SE 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 were considered to be statistically significant.

RESULTS

SI/R-induced apoptosis was increased in cardiomyocytes isolated from AMPK-DN mice and gAPN’s anti-apoptotic effects were largely retained in AMPK-DN cardiomyocytes. Adult cardiomyocytes are terminally differentiated, and spontaneous apoptosis occurs even under normal culture conditions. In our system, ~14% of cardiomyocytes were TUNEL positive after 9 h of culture with normal oxygen and glucose (Fig. 1, Sham SI/R). In cardiomyocytes isolated from WT littermates, 3 h of simulated ischemia followed by 6 h of reperfusion markedly stimulated apoptotic cell death, as evidenced by a 2.4-fold increase in TUNEL staining over time-matched control (Fig. 1), clear DNA ladder formation (Fig. 2A), and a 2.3-fold increase in caspase-3 activity (Fig. 2B). Consistent with our previous in vivo experimental results (32), treatment with gAPN significantly reduced SI/R-induced apoptosis (Figs. 1 and 2, P < 0.01). Cardiomyocytes isolated from AMPK-DN mice were morphologically normal and exhibited spontaneous apoptosis in normal culture conditions to an extent comparable to cardiomyocytes isolated from WT mice (Figs. 1 and 2B).

![Image](http://ajpendo.physiology.org.org by 10.220.32.246 on June 23, 2017)
However, when subjected to SI/R, AMPK-DN cardiomyocytes exhibited modestly greater TUNEL positive staining and caspase-3 activity than WT cardiomyocytes (TUNEL: 38.4 ± 0.97% vs. 33.2 ± 2.2%, P < 0.05; caspase-3: 6.2 ± 0.35 vs. 5.3 ± 0.12 nmol pNA·h⁻¹·mg protein⁻¹, P < 0.05). These results indicate that the susceptibility to I/R stress was significantly increased in the presence of impaired AMPK signaling. Most interestingly, the antiapoptotic property of gAPN was reduced but not lost in AMPK-DN cardiomyocytes. Specifically, treatment of AMPK-DN cardiomyocytes with gAPN significantly reduced TUNEL staining (29.5 ± 1.5% vs. 38.4 ± 0.97%, P < 0.01; Fig. 1), partially blocked DNA ladder formation (Fig. 2A), and significantly decreased caspase-3 activity (4.1 ± 0.52 vs. 6.2 ± 0.35 nmol pNA·h⁻¹·mg protein⁻¹, P < 0.01; Fig. 2B). To obtain more evidence in supporting our conclusion that gAPN protects cardiomyocytes partially through AMPK-independent pathway, an additional experiment was performed. Adult cardiomyocytes were isolated from AMPK-DN mice as described above. Compound C, a selective AMPK inhibitor, was added 30 min before APN treatment (20 μmol/l) (31), and AMPK activity was determined by SAMS peptide/[γ-³²P]ATP assay (31). Although AMPK activity was nearly completely inhibited after this combinational treatment (4.4 ± 1.1% of control), the cardioprotective effects of gAPN continued to be significant (TUNEL staining: 30.4 ± 1.2% vs. 40.3 ± 2.1 in vehicle group, P < 0.01; caspase-3 activity: 4.4 ± 0.32 vs. 6.7 ± 0.45 nmol pNA·h⁻¹·mg protein⁻¹, P < 0.01). These results demonstrated that gAPN protects cardiomyocytes from SI/R injury partially in an AMPK-independent fashion.

Adiponectin-induced activation of ACC, a primary downstream molecule of AMPK, was lost in AMPK-DN cardiomyocytes. The observation that the antiapoptotic effect of gAPN was largely retained in AMPK-DN cardiomyocytes is somewhat surprising, because this is opposite of previously published results demonstrating the abolishment of metabolic regulatory and vascular protective effects of adiponectin when AMPK is pharmacologically or genetically inhibited (4, 38). One possible explanation for this discrepancy is that our AMPK-DN approach failed to effectively block adiponectin-induced AMPK activation. To directly address this issue, ACC phosphorylation, a major signaling step through which AMPK exerts its metabolic regulation, was determined. As summarized in Fig. 3A, treatment with gAPN significantly increased ACC phosphorylation in cardiomyocytes isolated from WT (Fig. 3A, left) but not from AMPK-DN (Fig. 3A, right) heart. Moreover, a 1.8-fold increase in ACC phosphorylation was...
observed in WT cardiomyocytes after SI/R, and treatment with gAPN further enhanced (1.2-fold over SI/R + vehicle, \( P < 0.05 \)) ACC phosphorylation modestly (Fig. 3B). However, SI/R-induced ACC phosphorylation was lost in AMPK-DN cardiomyocytes. More importantly, treatment with gAPN failed to stimulate ACC phosphorylation in these cells (Fig. 3B, right). These results demonstrated that the AMPK signaling system was indeed blocked in our AMPK-DN cardiomyocytes.

Adiponectin-induced eNOS phosphorylation was significantly reduced in AMPK-DN cardiomyocytes. Substantial evidence exists that the physiological or pharmacological concentration of NO produced by eNOS or NO donors is cardioprotective. Moreover, previous studies have demonstrated that adiponectin possesses strong eNOS phosphorylation ability and stimulates NO production in cultured endothelial cells. We thus explored the possibility that gAPN may cause eNOS phosphorylation in cardiomyocytes independent of AMPK, and thus reduce cardiomyocyte apoptosis in AMPK-DN cardiomyocytes. As summarized in Fig. 4, treatment of WT cardiomyocytes subjected to sham SI/R (Fig. 4A) or SI/R (Fig. 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 gp91phox expression in largely AMPK-independent fashion. Data presented in Figs. 1 and 2 indicated that >70% of the antiapoptotic effect of gAPN was retained in cardiomyocytes isolated from AMPK-DN heart. In contrast, >70% of eNOS activation effect and >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 antiapoptotic effect of gAPN in AMPK-DN cardiomyocytes. It is well recognized that overproduction of reactive oxygen species plays a critical pathogenic role in myocardial I/R 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 Fig. 5, compared with 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 (Fig. 5).

Previous studies from other investigators and our laboratory have demonstrated that NADPH oxidase is a major cytosolic source of I/R-induced superoxide overproduction in the heart. Having demonstrated that gAPN inhibits superoxide overproduction largely in an AMPK-independent fashion, we next determined its effect on SI/R-induced upregulation of gp91phox, the membrane component of NADPH oxidase. Compared with WT, mRNA and protein expression of gp91phox were slightly increased in AMPK-DN cardiomyocytes before SI/R (\( P > 0.05 \)). However, SI/R-induced gp91phox mRNA (2.69 ± 0.14-fold over basal expression in AMPK-DN cardiomyocytes vs. 2.11 ± 0.15-fold over basal expression in WT cardiomyocytes, \( P < 0.05 \)) and protein overexpression (Fig. 5B, \( P < 0.05 \)) were significantly increased in AMPK-DN cardiomyocytes. Most interestingly, treatment of AMPK-DN cardiomyocytes with gAPN inhibited SI/R-induced gp91phox mRNA (79.1 ± 3.8% and 77.9 ± 3.1% reduction from vehicle-treated group in AMPK-DN and WT cardiomyocytes, respectively) and protein overexpression (Fig. 5B) to a level comparable to that seen in WT cardiomyocytes. Collectively, these results demonstrate that gAPN inhibited NADPH oxidase overexpression and subsequent superoxide overproduction in an AMPK-independent fashion.

Inducible nitric oxide synthase is responsible for peroxynitrite overproduction in cardiomyocytes subjected to SI/R. Inactivation of NO with subsequent formation of peroxynitrite is the most pathologically relevant mechanism through which superoxide exerts its cytotoxic effects. Superoxide reacts with NO in a ratio of 1:1, and peroxynitrite is maximally produced when both superoxide and NO production are simultaneously elevated. Although eNOS and neuronal NOS (nNOS) are expressed in cardiomyocytes, basal NO production from these forms of NOS is very low. Moreover, eNOS phosphorylation is reduced in AMPK-DN cardiomyocytes, whereas peroxynitrite is increased in these cells, indicating that eNOS is not responsible for peroxynitrite formation in AMPK-DN cardiomyo-
cytes. We thus determined inducible NOS (iNOS) expression in SI/R cardiomyocytes and elucidated its role in SI/R-induced peroxynitrite production. As illustrated in Fig. 6, no significant iNOS expression was detected in cardiomyocytes isolated from either WT or AMPK-DN hearts if not subjected to SI/R. However, SI/R caused significant iNOS expression, which was markedly inhibited by gAPN in WT or AMPK-DN cardiomyocytes. These results demonstrated that NO generated from iNOS is responsible for SI/R-induced peroxynitrite formation, which was inhibited by gAPN in an AMPK-independent fashion.

Diphenyleneiodonium, an NADPH oxidase inhibitor, significantly reduced peroxynitrite formation and attenuated caspase-3 activation after SI/R. Results presented above strongly suggest that gAPN attenuates postischemic cardiomyocyte apoptosis by inhibiting NADPH oxidase-derived superoxide production and subsequent peroxynitrite formation. To obtain more evidence supporting this conclusion, additional experiments were performed. As summarized in Fig. 7, treatment with diphenyleneiodonium (DPI), a selective NADPH oxidase inhibitor, reduced peroxynitrite formation (Fig. 7A) and cardiomyocyte apoptosis (Fig. 7B) to a level comparable to that seen with gAPN treatment. Moreover, no additive effect in either peroxynitrite inhibition or caspase-3 suppression was observed when SI/R cardiomyocytes were treated with gAPN plus DPI. These new results further support a conclusion that inhibiting...
NADPH oxidase-derived superoxide overproduction is largely responsible for gAPN’s antioxidant/antinitrative effects.

**DISCUSSION**

In a recent in vivo animal study, we demonstrated (36) that AMPK deficiency enhanced myocardial I/R injury but had minimal effect on adiponectin’s antioxidant/antinitrative protection. The present study not only confirmed our previous finding in a different experimental model but, more importantly, provided new evidence demonstrating that iNOS/NADPH oxidase overexpression is sufficient to increase nitrate/oxidative stress and that gAPN exerts its AMPK-independent cardioprotection partially through iNOS/NADPH oxidase suppression.

Adiponectin, also known as Acrp30, is an adipocytokine originally thought to be exclusively secreted from adipose tissue (2, 5). However, growing evidence indicates that adiponectin is synthesized and secreted by adult human and murine cardiomyocytes (7, 15, 26), although its physiological and pathological significance remains completely unknown. In human and mouse plasma, adiponectin exists in three major oligomeric forms: trimers, hexamers, and a high-molecular-weight form (25). A proteolytic cleavage product of adiponectin that includes its globular domain, known as globular adiponectin or gAPN, may occur in human and mouse plasma (10, 39). Recently, it has been shown that the cleavage of adiponectin by leukocyte elastase secreted from activated monocytes and/or neutrophils could be responsible for the generation of gAPN (35). However, the physiological or pathophysiological role of this proteolytic cleavage product remains largely unknown, and its relationship to cardiovascular disease is unclear (13).

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

Two ex vivo studies reported that AMPK is essential for the cardioprotective effects of full-length adiponectin (fAPN). The study by Shinmura et al. (30) demonstrated that short-term caloric restriction protected the heart by increasing serum total adiponectin levels with subsequent AMPK activation. In a more recent study, Gonon et al. (14) reported that fAPN protected against myocardial ischemia-reperfusion injury via the AMPK/Akt/NO signaling axis. However, although the data clearly demonstrated that administration of fAPN in isolated perfused hearts resulted in AMPK phosphorylation, it was not determined whether the protective effects of adiponectin were blocked when AMPK was inhibited, and no cause-effect relationship was established. It thus remains unclear whether AMPK phosphorylation is a necessary step for adiponectin to exert anti-ischemic/cardioprotective effects. In the first study reporting adiponectin’s cardioprotective effect, Shibata et al. (28) demonstrated the varying importance of AMPK in fAPN’s cardioprotective effect in different models. Specifically, fAPN blocks hypoxia/reoxygenation-induced neonatal cardiomyocyte apoptosis largely through AMPK activation. In contrast, its inhibitory effect on LPS-induced TNF-α production is completely AMPK independent. Moreover, in vivo administration of NS398, a cyclooxygenase (COX)-2 inhibitor, partially blocked the infarct-sparing action of exogenous fAPN, suggesting that COX-2 plays a significant role in fAPN’s cardioprotection after I/R. These seemingly conflicting results underscore the complexity of adiponectin signaling and suggest that multiple signaling mechanisms are involved in adiponectin’s cardioprotection in adult animals.

AMPK is activated in response to many types of cellular stress, particularly elevated ratios of AMP to ATP, which occur during myocardial ischemia (16). Indeed, our present experimental results (Fig. 3) demonstrated that ACC phosphorylation, an index for AMPK activity, was markedly increased when cardiomyocytes were subjected to SI/R. However, treatment with gAPN under this particular pathological condition only elicited moderate additional AMPK activation. These results strongly suggest that the degree of AMPK involvement in adiponectin’s biological functions is determined by the intracellular environment, particularly AMP concentration. Under those pathological conditions in which intracellular AMP concentrations are not elevated (i.e., muscular insulin resistance and vascular inflammation), AMPK might be the most important molecule in adiponectin intracellular signaling, as previously reported. In contrast, under those pathological conditions in which intracellular AMP concentrations are elevated, and AMPK has already been significantly activated (such as myocardial ischemia), adiponectin may exert its biological actions largely through signaling molecules other than AMPK.

Numerous studies have demonstrated that NO at physiological or pharmacological concentrations is antiapoptotic and cardioprotective (18). Moreover, molecules capable of stimulating eNOS phosphorylation (e.g., insulin) reduce apoptotic cell death in the I/R heart, attenuating myocardial reperfusion injury (11). As first reported by Chen et al. (6), and subsequently confirmed by other investigators (20), adiponectin stimulates eNOS phosphorylation and increases NO production in cultured endothelial cells. However, the AMPK-independent antiapoptotic action of gAPN as observed in the present study cannot be attributed entirely to gAPN’s eNOS stimulatory and NO productive effects, because ~70% eNOS phosphorylation by gAPN was lost in AMPK-DN cardiomyocytes. In contrast, our present study provided clear evidence that SI/R-induced NADPH oxidase upregulation and superoxide overproduction are inhibited by gAPN in an AMPK-independent fashion. Although the precise signaling mechanisms by which gAPN may inhibit NADPH oxidase expression cannot be addressed in the present study, the AMPK-independent anti-oxidant effect of gAPN suggests a likely mechanism responsible for gAPN’s antiapoptotic and cardioprotective effects in AMPK-DN cardiomyocytes.

Downregulation of NADPH oxidase expression and suppression of superoxide production by gAPN can provide car-
dioprotection by multiple mechanisms. First, reduction in superoxide production in I/R cardiomyocytes will reduce the production of the hydroxyl radical, thus attenuating oxidative cell injury. Second, superoxide inactivates NO in a nearly diffusion-limited rate reaction (1). By reducing superoxide production, gAPN increases bioactive NO concentration, thus preserving the antiapoptotic and cytoprotective function of NO. Finally, by reducing the superoxide/NO reaction, gAPN blocks the formation of peroxynitrite, a highly cytotoxic species that causes cell death by multiple mechanisms, thus attenuating peroxynitrite-induced cell death.

It should be indicated that the globular domain of adiponectin (gAPN) was investigated in the present study. Whether the AMPK-independent antiapoptotic effect and responsible signaling mechanisms identified in this study are unique to this particular isoform of adiponectin, or are shared by other adiponectin isoforms, remains unknown. Additional experiments addressing this question are currently under investigation.

**REFERENCES**

33. Tschritter O, Fritsche A, Haap M, Shirkaev F, Rahe S, Stainier H, Maerker E, Haring H, Stumvoll M. Plasma adiponectin...


