Adiponectin Cardioprotection After Myocardial Ischemia/Reperfusion Involves the Reduction of Oxidative/Nitrative Stress

Ling Tao, MD, PhD; Erhe Gao, MD, PhD; Xiangying Jiao, MD, PhD; Yuexing Yuan, PhD; Shuzhuang Li, MD; Theodore A. Christopher, MD; Bernard L. Lopez, MD; Walter Koch, PhD; Lawrence Chan, MBBS, DSc; Barry J. Goldstein, MD, PhD; Xin L. Ma, MD, PhD

Background—Several clinical studies have demonstrated that levels of adiponectin are significantly reduced in patients with type 2 diabetes and that adiponectin levels are inversely related to the risk of myocardial ischemia. The present study was designed to determine the mechanism by which adiponectin exerts its protective effects against myocardial ischemia/reperfusion.

Methods and Results—Adiponectin−/− or wild-type mice were subjected to 30 minutes of myocardial ischemia followed by 3 hours or 24 hours (infarct size and cardiac function) of reperfusion. Myocardial infarct size and apoptosis, production of peroxynitrite, nitric oxide (NO) and superoxide, and inducible NO synthase (iNOS) and gp91phox protein expression were compared. Myocardial apoptosis and infarct size were markedly enhanced in adiponectin−/− mice (P<0.01). Formation of NO, superoxide, and their cytotoxic reaction product, peroxynitrite, were all significantly higher in cardiac tissue obtained from adiponectin−/− than from wild-type mice (P<0.01). Moreover, myocardial ischemia/reperfusion–induced iNOS and gp91phox protein expression was further enhanced, but endothelial NOS phosphorylation was reduced in cardiac tissue from adiponectin−/− mice. Administration of the globular domain of adiponectin 10 minutes before reperfusion reduced myocardial ischemia/reperfusion–induced iNOS/gp91phox protein expression, decreased NO/superoxide production, blocked peroxynitrite formation, and reversed proapoptotic and infarct-enlargement effects observed in adiponectin−/− mice.

Conclusion—The present study demonstrates that adiponectin is a natural molecule that protects hearts from ischemia/reperfusion injury by inhibition of iNOS and nicotinamide adenine dinucleotide phosphate-oxidase protein expression and resultant oxidative/nitrative stress. (Circulation. 2007;115:&NA;-.)

Key Words: apoptosis ■ diabetes mellitus ■ myocardial infarction

Cardiovascular disease is the most prevalent cause of morbidity and mortality in patients with type 2 diabetes. Experimental results have demonstrated that hyperglycemia and hyperlipidemia not only cause vascular injury that leads to ischemic heart disease but also have a direct adverse impact on ischemic cardiomyocytes, which results in larger infarct size1,2 and more severe heart failure after myocardial ischemia. In recent years, enormous efforts have been made to identify the mechanisms responsible for diabetic vascular injury, and many signaling pathways have been reported.3 The molecular basis that links diabetes with increased vulnerability of diabetic hearts to ischemic injury and resultant higher mortality has not been established, however.

Adiponectin is an adipocytokine secreted from adipose tissue.4 It contains a stalk with 22 collagen repeats and a highly conserved globular domain (gAd). Adiponectin is normally present in plasma at concentrations up to 30 μg/mL, but it is markedly downregulated in association with obesity-linked diseases such as coronary artery disease and type 2 diabetes.5 Clinical observations have revealed that plasma total adiponectin concentrations are inversely correlated with the risk of myocardial infarction.6,7 Moreover, Shibata et al recently reported that myocardial ischemic/reperfusion (I/R) injury is amplified in adiponectin knockout (adiponectin−/−) mice that carry significant metabolic phenotypes such as delayed clearance of free fatty acid in plasma, decreased muscle fatty acid-transport protein-1 expression, and increased tumor necrosis factor (TNF)-α concentration.8 This result provided the first direct evidence that adiponectin is an
essential intrinsic molecule that protects the heart from I/R injury.

In human and mouse plasma, adiponectin exists in 3 major oligomeric forms: trimers, hexamers, and a high-molecular-weight form.12 A proteolytic cleavage product of adiponectin, which includes gAd, may occur in human and mouse plasma.13 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 gAd.14 However, the physiological or pathophysiological role of this proteolytic cleavage product remains largely unknown, and its relationship to cardiovascular disease is unclear.15

Considerable evidence indicates that apoptosis, a special form of cell death that differs from necrosis in many aspects, plays an essential role in cardiomyocyte death induced by myocardial I/R.16 In a recent study, Lin et al demonstrated that angiotensin II–induced endothelial cell apoptosis in vitro is significantly inhibited by gAd,17 which suggests that gAd may exert cardiovascular protective effects by its antiapoptotic property. Whether gAd may protect I/R-induced cardiomyocyte apoptosis and thus improve cardiac function has never been previously investigated. More important, the mechanisms by which gAd exerts its antiapoptotic effect remain unknown.

Therefore, the purposes of the present study were to determine whether myocardial I/R injury is similarly amplified in a different strain of adiponectin−/− mice that carries minimal metabolic phenotype18; if so, to investigate whether the enhanced I/R injury observed in these animals can be rescued by a bolus administration of gAd immediately before reperfusion; and to delineate mechanisms by which adiponectin may confer its cardioprotective effects after myocardial I/R.

Methods

Experimental Protocols

The experiments were performed in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Thomas Jefferson University Committee on Animal Care. Homozygous (adiponectin−/−) and heterozygous adiponectin knockout (adiponectin+/−) mice were described previously.19 Male adult mice (wild-type [WT; Taconic Farms, Inc, Germantown, NY], adiponectin−/−, and adiponectin+/−) were anesthetized with 2% isoflurane, and myocardial infarction (MI) was produced by temporarily exteriorizing the heart via a left thoracic incision and placing a 6–0 silk suture slipknot around the left anterior descending coronary artery. After 30 minutes of MI, the slipknot was released, and the myocardium was reperfused for 3 hours or 24 hours (for cardiac function and infarct size determination). Sham-operated control mice (sham MI/R) underwent the same surgical procedures except that the suture placed under the left coronary artery was not tied.

Determination of Myocardial Apoptosis

Myocardial apoptosis was determined by TUNEL staining and caspase-3 activity assay, which includes the entire I/R area commonly termed as area at risk as described in our previous study.19

Determination of Cardiac Function and Myocardial Infarct Size

At the end of the 24-hour reperfusion period, mice were re-anesthetized and cardiac function was determined by invasive hemodynamic evaluation methods. The left ventricular systolic pressure, left ventricular end-diastolic pressure, first derivative of the left ventricular pressure (+dP/dt max and −dP/dt min) and heart rate were obtained by use of computer algorithms and an interactive videographics program (Po-Ne-Mah Physiology Platform P3 Plus, Gould Instrument Systems, Valley View, Ohio). After completion of functional determination, the ligature around the coronary artery was retied, and MI size was determined by the Evans blue/TTC double staining method as described previously.19

Determination of Total Nitric Oxide and Nitrotyrosine Content in Cardiac Tissue

Cardiac tissue (area at risk) was rinsed and homogenized. The tissue nitric oxide (NO) and its metabolic products (NO2 and NO3) in the supernatant, collectively known as NOx, were determined by use of a chemiluminescence NO detector (Siever 280i NO Analyzer) as described previously.20 Nitrotyrosine content in the I/R cardiac tissue, a footprint of in vivo peroxynitrite formation and an index for nitrative stress,21 was determined by use of an ELISA method described in our previous publication.22

Figure 1. A, Lack of adiponectin increases MI size. Evans blue–stained areas (black) indicate nonischemic/reperfused area; 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) stained areas (red staining) indicate ischemic but viable tissue; Evans blue/TTC double staining indicates ischemic but viable tissue; Evans blue/TTC staining negative areas indicate infarcted myocardium. B, gAd reduces MI size in a dose-dependent fashion. AAR indicates area at risk; Adp, adiponectin; PT, pretreatment. N=10 to 12/group. *P<0.05, **P<0.01 versus WT mice. §§P<0.01 versus Adp−/− mice. §§§P<0.001 versus 1 µg/g gAd.

Quantification of Superoxide Production

Myocardial superoxide content was determined by lucigenin-enhanced luminescence as described in our previous study.23 In situ
superoxide detection was performed with dihydroethidium staining (DHE, Molecular Probes, Carlsbad, Calif) as described previously.24

Immunoblotting
Protein from I/R tissue homogenates was separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and Western blotted with monoclonal antibody against endothelial NO synthase (eNOS), phosphorylated eNOS, inducible NOS (iNOS; Upstate, Chicago, Ill) or gp91phox (Transduction Laboratories, San Jose, Calif). Nitrocellulose membranes were then incubated with HRP-conjugated antimouse immunoglobulin G antibody (1:2000, Cell Signaling, Danvers, Mass) for 1 hour, and the blot was developed with a Supersignal chemiluminescence detection kit (Pierce, Rockford, Ill). The immunoblotting was visualized with a Kodak Image Station 400 (Rochester, NY), and the blot densities were analyzed with Kodak 1-dimensional software.

Immunohistochemistry
Paraformaldehyde-fixed tissues were cut into semithin sections 4 to 5 μm thick, and stained with an antibody against nitrotyrosine (Upstate). Immunostaining was developed with a Vectastain ABC kit (Vector Laboratories, Burlingame, Calif).

Determination of Plasma Adiponectin Concentration
Endogenous plasma adiponectin level was determined with mouse adiponectin ELISA kit and exogenous gAd was determined with human adiponectin ELISA kit (Phoenix Pharmaceuticals, Inc., Bel- mont, Calif) in accordance with the manufacturer’s instructions.

Adult Mouse Cardiomyocyte Culture
Adult mouse cardiomyocytes were isolated and subjected to 3 hours of simulated ischemia (SI) and 6 hours of reperfusion (SIR) as described in our previous studies.25,26 Effects of adiponectin on SI/R-induced iNOS and gp91phox protein expression were determined.

Statistical Analysis
All values in the text and figures are presented as mean±SEM of n independent experiments. All data (except Western blot density) were subjected to ANOVA followed by Bonferroni correction for post hoc r test. Western blot densities were analyzed with the Kruskal-Wallis test followed by Dunn post hoc test. P≤0.05 was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Myocardial Reperfusion Injury Is Markedly Increased in Adiponectin−/− Mice
Thirty minutes of ischemia followed by 24 hours of reperfusion resulted in significant cardiomyocyte death (>45% of area at risk) and impaired cardiac function. Compared with WT mice, MI size was enlarged and cardiac function was further depressed in adiponectin−/− mice whose plasma adiponectin was undetectable (P<0.01) (Figures 1 and 2). To obtain more evidence to support a causative role of adiponec- tin deficiency and increased myocardial reperfusion injury, 2 additional experiments were performed. In the first series of experiments, heterozygous knockout mice (adiponectin+/−) whose plasma adiponectin concentration was significantly reduced (4.2±0.18 versus 14.4±1.1 μg/mL in WT, P<0.01) were subjected to MI/R. Adiponectin−/− mice also had an increased MI size and impaired cardiac function, although to a lesser extent than that seen in adiponectin−/− animals (Figures 1 and 2). In the second series of experiments, human recombinant gAd (amino acids 108 to 244) was prepared as described27 and administered to the adiponectin−/− mice 10 minutes before reperfusion (1 to 4 μg/g, intraperitoneally). Our preliminary experiments demonstrated that no human adiponectin was detected before gAd administration by use of a human adiponectin ELISA kit. Intraperitoneally adminis- tered gAd (2 μg/g) was partially absorbed and human adiponectin was detected in adiponectin−/− and WT mice at 10, 60, and 120 minutes postinjection (adiponectin−/−: 9.9±0.7, 23.6±1.2, and 20.1±1.9 ng/mL; WT: 11.2±0.7, 21.8±1.7, and 20.1±1.9 ng/mL). Treatment with gAd re-duced MI size in a dose-dependent fashion (Figure 1), and administration of 2 μg/g gAd either 10 minutes before MI or 10 minutes before reperfusion reduced MI size to a level not...
only significantly less than that in the adiponectin \(^{-/-}\) group but also less than that in the WT mice that were administered vehicle (Figure 1). Moreover, cardiac dysfunction in adiponectin \(^{-/-}\) mice was reversed by treatment with gAd (Figure 2).

**Adiponectin Deficiency Markedly Increases I/R-Induced Cardiomyocyte Apoptosis That Is Rescued by Administration of gAd**

Apoptosis is the major form of cell death after a short period of ischemia followed by reperfusion. To investigate whether the enlarged MI size in adiponectin \(^{-/-}\) animals is associated with increased cardiomyocyte apoptosis in these animals, TUNEL staining and caspase-3 activity in I/R cardiac tissue were determined. As illustrated in Figure 3, I/R-induced cardiomyocyte apoptosis was significantly increased in adiponectin \(^{-/-}\) mice, evidenced by increased TUNEL staining and caspase-3 activity. Consistent with MI size, myocardial apoptosis was also increased in adiponectin \(^{-/-}\) mice, although to a lesser extent than that seen in adiponectin \(^{-/-}\) mice. Treatment with gAd completely blocked additional cardiomyocyte apoptosis in adiponectin \(^{-/-}\) mice (Figure 3).

**Lack of Adiponectin Increases Total NO Production via Upregulation of iNOS Expression in the I/R Heart**

Considerable evidence exists that the low concentration of NO released from eNOS or NO donors is antiapoptotic and cardioprotective, and that adiponectin has been shown to stimulate NO production via Akt-mediated eNOS phosphorylation. Therefore, we initially hypothesized that lack of adiponectin may reduce NO production, and thus render cardiomyocytes to be more susceptible to I/R injury. To our surprise, total NO production was markedly increased, rather than decreased, in I/R tissue obtained from adiponectin \(^{-/-}\) mice (Figure 4). To identify the molecular sources responsible for this increased NO production, eNOS/iNOS protein expression and eNOS phosphorylation were determined. There was no change in eNOS expression in adiponectin \(^{-/-}\) mice.
animals. However, eNOS phosphorylation was reduced in cardiac tissues obtained from adiponectin $^{-/-}$ mice, and treatment with gAd increased eNOS phosphorylation (Figure 4). These results are consistent with previous reports that adiponectin is an upstream molecule that stimulates NO production via eNOS phosphorylation. In contrast to eNOS phosphorylation, the iNOS expression was further enhanced in adiponectin $^{-/-}$ mice, and treatment with gAd blocked iNOS expression in these animals (Figure 4).

Lack of Adiponectin Increases $\cdot O_2^-$ Production via Upregulation of gp91phox in the I/R Heart

Our previous study demonstrated that oxLDL-induced $\cdot O_2^-$ production in endothelial cells is inhibited by a NADPH inhibitor as well as gAd, which suggests that adiponectin may inhibit $\cdot O_2^-$ production from NADPH oxidase. Because genes that encode NADPH oxidase and iNOS belong to the same inflammatory gene family, the aforementioned results (adiponectin inhibits iNOS expression) further suggest that adiponectin may inhibit NADPH oxidase expression and subsequent $\cdot O_2^-$ production. To directly test this hypothesis, $\cdot O_2^-$ production and gp91phox protein expression in the I/R cardiac tissue were determined. As illustrated in Figure 5, I/R-induced superoxide overproduction was intensified in cardiac tissue obtained from adiponectin $^{-/-}$ animals. However, eNOS phosphorylation was reduced in adiponectin $^{-/-}$ mice, and replenishment with gAd before reperfusion attenuated superoxide production. Moreover, I/R induced overexpression of gp91phox, a major component of NADPH oxidase, was further increased in adiponectin $^{-/-}$ mice, and administration of gAd before reperfusion blocked this adverse effect.

Lack of Adiponectin Increases TNF-α Production in the I/R Heart

Adiponectin has been reported to protect the heart partially by inhibition of TNF-α production. Because the adiponectin $^{-/-}$ mice used in the present study differ in their metabolic phenotype from those used by Shibata et al., we thus determined whether TNF-α production is also increased in this particular strain of adiponectin $^{-/-}$ mice. In cardiac tissues obtained from nonischemic control animals, TNF-α levels were only slightly increased in our adiponectin $^{-/-}$ mice compared with WT mice (1.18±0.08 versus 1.04±0.11 pg/mg protein, $P>0.05$). However, I/R-induced TNF-α overproduction was significantly enhanced in adiponectin $^{-/-}$ mice (1.86±0.16 versus 1.59±0.09 pg/mg protein in WT, $P<0.05$). Treatment with gAd markedly reduced I/R-induced TNF-α production in these animals (1.39±0.15 pg/mg protein, $P<0.01$).

Overproduction of Peroxynitrite in Adiponectin $^{-/-}$ Animals

Studies from other investigators as well as our group have demonstrated that overproduction of peroxynitrite and resultant nitrative stress play a causative role in postischemic myocardial apoptosis. After demonstration of production of NO and $\cdot O_2^-$, the 2 free radicals that react at a diffusion-limited rate to form the highly cytotoxic molecule peroxynitrite, we then tested a hypothesis that lack of adiponectin may increase peroxynitrite formation, and thus render cardiomyocytes more susceptible to I/R injury. Consistent with our previous findings, I/R resulted in significant peroxynitrite production as evidenced by a $>2.3$-fold increase in nitrotyrosine content in the I/R cardiac tissue. Most important, peroxynitrite formation was further increased in adiponectin $^{-/-}$ mice, and replenishment with gAd before reperfusion markedly attenuated nitrotyrosine formation (Figure 6).

Inhibition of Peroxynitrite Formation Preferentially Attenuated Cardiac Injury in Adiponectin $^{-/-}$ Animals

Our results suggest that adiponectin may exert its cardioprotective effects by inhibition of the overproduction of per-
oxynitrite. To obtain more evidence to support this conclusion, an additional series of experiments was performed. Male adult adiponectin−/− mice or WT mice were subjected to MI/R as described above and treated with either 1400W (2 mg/kg), a selective iNOS inhibitor, or M40401 (0.25 mg/kg), a peroxynitrite scavenger,32 10 minutes before reperfusion. As summarized in Figure 7, treatment with 1400W or M40401 significantly reduced nitrotyrosine content in I/R tissue, which indicates that blockage of excess NO production from iNOS or enhancement of peroxynitrite decomposition effectively reduced peroxynitrite-induced protein nitration in MI/R cardiac tissue. These treatments also markedly attenuated myocardial apoptosis as determined by caspase-3 activity (Figure 7). It is noteworthy that although nitrotyrosine content and caspase-3 activity were markedly higher in I/R tissue from adiponectin−/− mice administrated vehicle than tissue from WT mice (Figure 7), treatment with 1400W or M40401 reduced MI/R-induced nitrotyrosine formation and caspase-3 activation to comparable levels in adiponectin−/− mice and WT mice (Figure 7). These results demonstrate that blocking iNOS activity or scavenging peroxynitrite preferentially protected hearts against MI/R-induced cardiomyocyte apoptosis in adiponectin−/− mice, and further suggest that increased oxidative and nitrative stress in adiponectin−/− mice plays a causative role in the accelerated MI/R injury observed in these animals.

Treatment With gAd Attenuated SI/R-Induced iNOS Expression and gp91phox Expression

Having demonstrated that in vivo administration of gAd reduces iNOS and gp91phox expression in the I/R heart, we further examined whether gAd may exert this antioxidative/antinitrative effect directly on cardiomyocytes. As illustrated in Figure 8, exposure of adult mouse cardiomyocytes to SI/R caused an upregulation of iNOS and gp91phox. Treatment with gAd (2 µg/mL) significantly attenuated SI/R-induced iNOS and gp91phox protein expression (P<0.01). These results provide direct evidence that iNOS and gp91phox protein expression is upregulated in I/R cardiomyocytes themselves.
and that gAd is capable of inhibition of their expression in this particular cell type.

Discussion

We have made several important observations in the present study. First, with an adiponectin \(^{-/-}\) mouse strain that exhibits minimal metabolic phenotype,\(^{18}\) we have provided additional evidence that adiponectin possesses cardioprotective properties. Second, we have demonstrated for the first time that acute administration of gAd shortly before reperfusion significantly attenuates MI/R injury. Most important, we have obtained the first direct evidence that adiponectin protects against MI/R injury, at least in part by a novel antioxidative and antinitrative mechanism that has never been previously reported.

Compared with other forms of adiponectin, the proteolytically cleaved product of adiponectin (ie, gAd) presents in the circulation at an extremely lower concentration, and its biological significance therefore remains uncertain. Interestingly, it has been recently reported that gAd exhibits much more extensive (>20-fold) biological activity than the full-length form.\(^{33,34}\) Moreover, Fruebis and colleagues reported that gAd is not only much more potent in the stimulation of fatty acid oxidation in skeletal muscles than full-length adiponectin but also has much more rapid action than full-length adiponectin after a single in vivo dose.\(^{13}\) These authors thus speculated that adiponectin circulates in plasma as an inactive precursor of a regulatory protein. In a more recent study, Pajvani and colleagues\(^{12}\) demonstrated that formation of the oligomer form of adiponectin depends on disulfide bond formation mediated by Cys-39. Mutation of Cys-39 results in trimers that are subject to proteolytic cleavage in the collagenous domain. The proteolytic cleavage products are significantly more bioactive than the higher order oligomeric forms of the protein (>30-fold). On the basis of these results and results published by others, the authors proposed that high-molecular-weight adiponectin complexes that circulate in serum represent a precursor pool that can be activated by a serum reductase. Reduction of high-molecular-weight adiponectin results in its dissociation and the transient appearance of the bioactive trimer. Once reduced to the basic trimer, adiponectin may be subject to proteolysis by membrane-bound proteases found on the cell surface of target cells, which leads to the formation of more biologically active gAd. Therefore, although the level of gAd is extremely low in circulation, gAd may represent the final active ligand of adiponectin at its target cells. In the present study, we have provided the first direct evidence that administration of recombinant gAd as a bolus 10 minutes before reperfusion reversed the adverse effects of adiponectin knockout on MI/R injury. These results suggest that gAd may have broad clinical applications in a variety of cardiovascular diseases where apoptotic cell death is increased, such as heart failure, atherosclerosis, and hypertension. However, the most potent oligomeric form of adiponectin for cardioprotection remains unknown and warrants further investigation.

Reactive oxygen species (ROS) have long been recognized to cause oxidative stress and act as the major mediators of I/R injury. We have previously demonstrated that treatment with gAd significantly inhibited basal \(\cdot O_2^-\) release by bovine aortic endothelial cells, and abolished the oxidized low-density lipoprotein–induced increase in \(\cdot O_2^-\) production.\(^{31}\) Moreover, our most recent study demonstrated that adiponectin blocked high glucose–induced endothelial \(\cdot O_2^-\) generation in vascular endothelial cells, which indicates that the \(\cdot O_2^-\) suppression effect of adiponectin is a general property of this novel molecule, rather than a specific effect on oxidized LDL–induced oxidative injury.\(^{35}\) In the present study, we have demonstrated that I/R-induced gp91phox protein expression and resultant \(\cdot O_2^-\) production were markedly increased in the adiponectin \(^{-/-}\) animals. We have also shown that administration of gAd reversed gp91phox overexpression and \(\cdot O_2^-\) production observed in adiponectin \(^{-/-}\) animals. These results provide the first direct evidence to demonstrate that adiponectin is a natural molecule that inhibits gp91phox overexpression in I/R cardiac tissue, and thus attenuates oxidative stress–induced tissue injury.

The most interesting finding of the present study is that adiponectin differentially regulates NO production from eNOS and iNOS. Several previous studies have demonstrated that adiponectin increases NO production by activation of the AMPK-Akt-eNOS phosphorylation pathway.\(^{29,30}\) Consistent with these reports, our present study demonstrated that eNOS phosphorylation was reduced in I/R cardiac tissue obtained from adiponectin \(^{-/-}\) mice. Surprisingly, total NO production in the I/R heart in adiponectin \(^{-/-}\) mice was markedly increased, rather than decreased. This paradoxical result strongly suggests that other forms of NO might be increased in these animals. Indeed, we have provided direct evidence that I/R-induced iNOS expression was further increased in adiponectin \(^{-/-}\) mice and that treatment with gAd significantly reduced iNOS expression in I/R cardiac tissue. Taken together with previous studies, our present study suggests that adiponectin increases NO production from eNOS under physiological conditions, and thus contributes to adiponectin’s vasodilatory/cardioprotective effects. In contrast, under pathological conditions where iNOS expression is stimulated, adiponectin inhibits NO overproduction by inhibition of iNOS expression and thus protects tissues from nitrative stress.

NO itself is not toxic and does not produce significant tissue injury even at a very high concentration.\(^{36}\) However, the NO/ \(\cdot O_2^-\) reaction and the subsequent production of peroxynitrite reverses its biological protective properties and results in oxidative/nitrative tissue injury.\(^{37}\) In the present study, we have demonstrated that I/R-induced peroxynitrite formation was markedly potentiated in adiponectin \(^{-/-}\) mice and that treatment with gAd attenuated peroxynitrite production. Numerous studies have demonstrated that peroxynitrite is the most important nitrative species that causes severe nitrative stress and tissue injury.\(^{37,39}\) The present study has provided the first evidence that adiponectin is an endogenous molecule that inhibits the formation of peroxynitrite by inhibition of NADPH oxidase–derived \(\cdot O_2^-\) and iNOS–derived NO.

Physiological or pharmacological concentrations of NO produced from eNOS or NO donors exert significant cardioprotective effects, whereas the reaction product between NO and \(\cdot O_2^-\), peroxynitrite, is extremely cytotoxic.\(^{36,37}\) Theoretically, a signaling system that possesses dual actions (ie, stimulation of NO production and inhibition of \(\cdot O_2^-\) production) would provide the most protection against I/R injury. Unfortunately, most, if not all cytokines studied to date stimulate both NO and \(\cdot O_2^-\) production simultaneously, and result in peroxynitrite production and subsequent tissue injury. To our knowledge, advi...
Adiponectin is the first cytokine that has been shown to increase physiological NO production (via eNOS phosphorylation), inhibit \( \cdot \mathrm{O}_2^- \) production, and block peroxynitrite formation. These novel findings suggest that reduced adiponectin concentration, like that observed in type 2 diabetes, may render cardiomyocytes more susceptible to I/R injury by deregulation of expression of inflammatory genes and enhancement of ROS and reactive nitrogen species production.

**Limitations and Future Direction**

A study by Shibata et al demonstrated that adiponectin confers its cardioprotective effects through 2 distinct pathways: the AMPK signaling pathway and the COX-2 signaling pathway.\(^{10}\) The present study demonstrated that adiponectin cardioprotection after MI/R involves the reduction of oxidative/nitrative stress. However, the signaling hierarchy between activation of AMPK/COX-2 reported by Shibata et al\(^{10}\) and the reduction of ROS/reactive nitrogen species observed in the present study remains unclear. Treatment with metformin has been reported to inhibit hyperglycemia-induced superoxide production in endothelial cells, which is blocked by the dominant negative form of AMPK transfection.\(^{40}\) Activation of AMPK by insulin-sensitizing drugs markedly inhibits endotoxin-induced iNOS expression and NO production in muscle and adipose tissues, which is blocked by AMPK small interfering RNA.\(^{41}\) It is therefore possible that reduction of oxidative/nitrative stress functions as a downstream mechanism by which adiponectin-activated AMPK exerts its cardioprotective effects. On the other hand, adenovirus-mediated transfer of COX-2 blocks sanguinarine-induced NO/superoxide overproduction in LNCaP cells and reduces apoptosis,\(^{42}\) which suggests that COX-2 activation may be an upstream mechanism by which adiponectin reduces oxidative/nitrative stress. In addition, we have recently demonstrated that gAd suppresses high-glucose-induced ROS production in vascular endothelial cells by a cAMP-mediated signaling pathway.\(^{35}\) Taken together, current experimental evidence suggests that adiponectin may reduce ROS/reactive nitrogen species overproduction by multiple upstream signaling pathways, and that reduction of oxidative/nitrative stress observed in our present study may be a final common pathway by which adiponectin exerts its cardioprotective effects. Moreover, it is possible that different form of adiponectin may block ROS/reactive nitrogen species production induced by different stimulation in different cell type by different signaling pathway. Clarification of the individual signaling mechanism is of scientific and clinical importance and thus warrants future investigations.

**Sources of Funding**

This research was supported by the following grants: National Institutes of Health (NIH) 2R01HL-63828, American Diabetes Association 7-05-RA-83, and Commonwealth of Pennsylvania Department of Health (to Dr Ma); American Diabetes Association 7-6-JF59 (to Dr Tao); NIH DK63018, NIH DK71360, and American Diabetes Association (to Dr Goldstein); and NIH HL51586 and NIH DK68037 (to L. Chan). Dr Christopher has received a grant from the American Heart Association; Dr Koch has received a grant from NIH.

**Disclosures**

None.

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Adiponectin and Myocardial Reperfusion Injury

Cardiovascular disease is the major cause of morbidity and mortality in patients with diabetes mellitus. The molecular links between diabetes and cardiovascular disease have not been fully clarified. Adiponectin is an adipocytokine secreted by adipose tissue that is normally present in plasma, but is reduced with obesity-linked diseases such as coronary artery disease and type 2 diabetes. Clinical observations have revealed that total adiponectin concentrations are inversely correlated with the risk of myocardial infarction, which suggests that reduced adiponectin plays a pathogenic role in the development of cardiovascular disease. The present study demonstrates that cardiac injury associated with myocardial ischemia/reperfusion is marked enhanced in adiponectin knockout animals. Acute administration of the global domain of adiponectin shortly before reperfusion attenuates myocardial injury by a reduction of oxidative/nitritative stress and subsequent apoptotic cell death. The reduced adiponectin found in patients with type 2 diabetes may increase their susceptibility to ischemia/reperfusion injury by deregulation of expression of inflammatory genes and enhancement of production of reactive oxygen/nitrogen species. Restoration of the production of endogenous adiponectin or supplementation with exogenous adiponectin may have therapeutic value in the reduction of myocardial ischemia/reperfusion injury in patients with type 2 diabetes.
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Circulation. published online March 5, 2007;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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