Adiponectin
An Indispensable Molecule in Rosiglitazone Cardioprotection Following Myocardial Infarction

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Rationale: Patients treated with peroxisome proliferator-activated receptor (PPAR)-γ agonist manifest favorable metabolic profiles associated with increased plasma adiponectin (APN). However, whether increased APN production as a result of PPAR-γ agonist treatment is an epiphenomenon or is causatively related to the cardioprotective actions of PPAR-γ remains unknown.

Objective: To determine the role of APN in rosiglitazone (RSG) cardioprotection against ischemic heart injury.

Methods and Results: Adult male wild-type (WT) and APN knockdown/knockout (APN−/− and APN+/−) mice were treated with vehicle or RSG (20 mg/kg per day), and subjected to coronary artery ligation 3 days after beginning treatment. In WT mice, RSG (7 days) significantly increased adipocyte APN expression, elevated plasma APN levels (2.6-fold), reduced infarct size (17% reduction), decreased apoptosis (0.23±0.02% versus 0.47±0.04% TUNEL-positive in remote nonischemic area), attenuated oxidative stress (48.5% reduction), and improved cardiac function ($P<0.01$). RSG-induced APN production and cardioprotection were significantly blunted ($P<0.05$ versus WT) in APN+/−, and completely lost in APN−/− ($P>0.05$ versus vehicle-treated APN−/− mice).

Moreover, treatment with RSG for up to 14 days significantly improved the postischemic survival rate of WT mice ($P<0.05$ versus vehicle group) but not APN knockdown/knockout mice.

Conclusions: The cardioprotective effects of PPAR-γ agonists are critically dependent on its APN stimulatory action, suggesting that under pathological conditions where APN expression is impaired (such as advanced type 2 diabetes), the harmful cardiovascular effects of PPAR-γ agonists may outweigh its cardioprotective benefits. (Circ Res. 2010;106:409-417.)

Key Words: diabetes ■ myocardial infarction ■ adipocytokine ■ signaling
from cardiovascular causes. Furthermore, a recent study has demonstrated that elderly diabetic patients treated with RSG are at higher risk of developing heart failure and mortality. These results raised a major concern for using RSG in diabetic patients. The etiology of the sizable discrepancy between experimental and clinical studies, as well as between different clinical trials, remains unknown.

Adiponectin (APN), an adipocytokine secreted from adipose tissue, is normally present in plasma at concentrations up to 30 µg/mL. It is markedly downregulated in association with obesity-linked diseases, including coronary artery disease and type 2 diabetes. Clinical observations have revealed that total plasma APN concentrations are inversely correlated with the risk of coronary artery disease and MI. Treatment with PPARγ agonists stimulates APN production in adipocytes, and APN is the mediator through which PPARγ agonists achieve their insulin sensitization and metabolic benefits. Moreover, recent experimental studies have demonstrated that APN plays an essential role in both the hepatic insulin sensitization and the plasminogen activator inhibitor-1 production suppressive effect of PPARγ agonists. However, whether increased APN production as a result of PPARγ agonist treatment is solely an epiphenomenon or is causatively related to the cardioprotective actions of PPAR-γ remains unknown.

Therefore, the aims of the present study were to determine the role of APN in RSG cardioprotection and to investigate how reduced APN production (as seen in both advanced and elderly diabetic patients) may alter the cardiovascular actions of RSG.

**Methods**

Adult male adiponectin knockdown (APN+/−) and knockout (APN−/−) mice and their male wild-type littermate controls (WT) were used in the present study. Generation, breeding, phenotype characteristics, and genotyping of these mice have previously been described in detail. All experiments were performed in adherence with the NIH on the use of laboratory animals and were approved by the Thomas Jefferson University Committee on Animal Care.

**Experimental Protocols**

On day 0 of the experiment, mice were randomized to receive vehicle (control), RSG (20 mg/kg per day), oral gavage, Cayman Chemical, Ann Arbor, Michigan), MnTE-2-PyP5+/− (a cell-permeable superoxide dismutase mimic, 10 mg/kg per day, IP) or RSG+MnTE-2-PyP5+/− (to determine the possible involvement of superoxide in RSG cardiac signaling). Seventy-two hours after the first drug administration, mice were anesthetized with 2% isoflurane, and MI was produced by temporarily exteriorizing the heart via a left thoracic incision, and ligation of the left anterior descending (LAD) coronary artery with a 6-0 silk suture. Sham-operated control mice underwent the same surgical procedures, except that the suture placed under the left coronary artery was not tied. After 4 days (7 total days of RSG treatment) of MI, animals were reanesthetized, aortic blood (0.5 mL) was collected, and the epididymal fat pad and heart were removed. Adipocyte APN mRNA expression, plasma APN level, myocardial infarct size, myocardial apoptosis, and superoxide production in nonischemic regions were determined according to the below described procedures. To determine the effect of RSG on survival rate, WT, APN+/− or APN−/− mice were continuously treated with RSG for up to 14 days, or until animal death.

**Determination of Adipocyte APN mRNA Expression and Plasma APN Concentration**

Total RNA was isolated from white adipose tissue (epididymal fat pad) using TRIzol reagent (Invitrogen), and cDNA was synthesized from 1 µg of RNA, with the iScript cDNA Synthesis kit (Bio-Rad). Adiponectin mRNA levels was quantified by real-time PCR with the use of SYBR Green (Applied Biosystems, Foster City, Calif) and corrected for GAPDH mRNA level. Total plasma adiponectin concentration was determined with a mouse adiponectin ELISA kit (Phoenix Pharmaceuticals Inc, Belmont, Calif) per the instructions of the manufacturer.

**Determination of Myocardial Apoptosis and Myocardial Infarct Size**

Myocardial apoptosis was determined by TUNEL staining and caspase-3 activity as described previously. Myocardial infarct size was determined by Evans blue-TTC double staining methods and expressed as a percentage of infarct area over ischemic area (area-at-risk).

**Determination of Cardiac Function**

Cardiac function was determined by echocardiography (VisualSonics VeVo 770 imaging system) 4 days after coronary occlusion (7 days after RSG treatment) and by left ventricular (LV) catheterization (1.2-Fr micromanometer, Millar Instruments, Houston, Tex) before animal death (11 days after coronary occlusion/14 days after RSG treatment). Both methods have been described in detail in our previous publications.

**Quantification of Superoxide Production in Cardiac Tissue**

Myocardial superoxide content (nonischemic area) was quantified by lucigenin enhanced luminescence and the cellular origin of reactive oxygen species was determined by dihydroethidium staining (Molecular Probes, Carlsbad, Calif) as described previously.

**Statistical Analysis**

All values in the text and figure are presented as means±SEM of n independent experiments. All data (except survival and Western blot density) were subjected to 2-way ANOVA followed by Bonferroni correction for post hoc t test. Animal survival was evaluated by the Kaplan–Meier method, and the log-rank test was used to compare survival curves between vehicle-treated and RSG-treated groups. Western blot densities were analyzed with the Kruskal–Wallis test, followed by Dunn’s post hoc test. Probabilities of 0.05 or less were considered to be statistically significant.

**Results**

Adipocyte APN mRNA expression and plasma TNFα levels. In WT animals, high levels of adipocyte APN mRNA expression and plasma APN (8.4±1.9 µg/mL) were detected.
As expected, both adipocyte APN expression and plasma APN levels were markedly reduced in APN\textsuperscript{-/-} animals and undetected in APN\textsuperscript{-/-} animals (Figure 1). In WT animals, MI caused a remarkable reduction in adipocyte APN mRNA expression (47.6\% of sham MI control, \(P < 0.01\)), and significantly reduced plasma APN levels (53.3\% of sham MI control, \(P < 0.01\)). In APN\textsuperscript{-/-} mice, both adipocyte APN expression and plasma APN levels were further significantly reduced after MI (Figure 1). Treatment of WT animals with RSG significantly attenuated the inhibitory effect of MI on adipocyte APN mRNA expression (72.4\% of sham MI control, \(P < 0.05\) versus MI vehicle), and increased plasma APN to a level (136.9\% of sham MI control, \(P < 0.01\)) that were even higher than mice subjected to sham MI without RSG treatment (\(P < 0.05\)). Interestingly, the APN stimulatory effect of RSG was almost completely abolished in APN\textsuperscript{-/-} mice. No statistically significant change in either adipocyte APN expression or plasma APN levels were observed in APN\textsuperscript{-/-} mice treated with RSG (Figure 1).

Previous studies have demonstrated that there exists a reciprocal inhibitory relationship between APN and TNF\(\alpha\).\textsuperscript{30} To determine whether TNF\(\alpha\) concentration was elevated in APN\textsuperscript{-/-} mice, and thereby potentially counteractive of the RSG APN stimulatory effect, serum TNF\(\alpha\) levels were determined by ELISA. There was no significant difference in basal serum TNF\(\alpha\) levels between WT, APN\textsuperscript{+/+}, and APN\textsuperscript{-/-} mice (7.6\% ± 0.81, 7.4\% ± 1.1 and 9.1\% ± 1.0 pg/mL). However, MI-induced TNF\(\alpha\) overproduction was significantly potentiated in APN deficient mice (WT: 28.4\% ± 2.1 pg/mL; APN\textsuperscript{+/+}: 86.1\% ± 4.3 pg/mL, \(P < 0.01\) versus WT; APN\textsuperscript{-/-}: 138\% ± 5.6 pg/mL, \(P < 0.01\) versus WT and APN\textsuperscript{+/+} groups).

Loss of RSG Acute Cardioprotection in APN Knockout Mice

Four days of permanent coronary occlusion caused more than 80\% cell death in the ischemic area (Figure 2). Treatment of WT mice with RSG modestly reduced myocardial infarct size in this severe, permanent myocardial ischemic model (Figure 2A). In APN knockout mice (both APN\textsuperscript{+/+} and APN\textsuperscript{-/-} mice), infarct size was slightly increased compared to WT animals. However, this difference was not statistically significant. This result is consistent with a recent report demonstrating that permanent coronary occlusion for 4 weeks resulted in comparable myocardial infarct size in WT and APN\textsuperscript{-/-} mice.\textsuperscript{31} More importantly, treatment with RSG failed to reduce infarct size in APN\textsuperscript{+/+} and APN\textsuperscript{-/-} mice, and a very significant difference was observed between RSG-treated WT and APN\textsuperscript{-/-} mice (Figure 2A).

In contrast to the abovementioned results demonstrating no significant infarct size difference between APN\textsuperscript{-/-} and WT mice subjected to permanent ischemia, previous studies by Shibata et al\textsuperscript{30} and our group\textsuperscript{29} have demonstrated signifi-
significantly larger infarct size during temporary ischemia followed by reperfusion in APN−/− than WT mice. Additional experiments were performed to further determine whether APN is also required for RSG cardioprotection in the ischemia/reperfusion model. As summarized in Figure 2B, 30 minutes of ischemia followed by 4 days of reperfusion caused approximately 50% cell death in WT mice. Infarct size was significantly larger in APN+/− (P<0.05) and APN−/− (P<0.01) mice. Moreover, treatment with RSG (in an identical manner as described for the permanent coronary occlusion model) caused a 46% infarct size reduction in WT mice (P<0.01). The protective effect of RSG was significantly attenuated in APN+/− (P<0.05 versus WT+RSG) and completely lost in APN−/− (P<0.01 versus WT+RSG) mice.

Apoptotic cell death in remote, nonischemic areas of the heart plays a critical role in post-MI left ventricular remodeling and greatly influences cardiac function deterioration. We thus determined whether RSG may attenuate apoptotic cell death in nonischemic cardiac regions in the permanent ischemic model and, more importantly, whether APN is requisite for the antiapoptotic effects of RSG. As illustrated in Figure 3, no TUNEL-positive cells were detected in hearts subjected to sham MI, and caspase-3 activity was very low. In WT mice, 4 days of permanent coronary occlusion caused 0.47±0.04% cells in nonischemic regions to become TUNEL-positive and a 3.4-fold increase of caspase-3 activity (P<0.01). RSG treatment significantly reduced apoptosis, as determined by TUNEL staining and caspase-3 activity (Figure 3). Although similar levels of TUNEL-positive staining and caspase-3 activation were observed in APN+/− mice, RSG treatment failed to attenuate cardiomyocyte apoptosis in these animals. In APN−/− mice, permanent coronary occlusion caused greater apoptosis in nonischemic areas, as evidenced by more TUNEL-positive staining (0.63±0.05%, P<0.05 versus WT) and higher caspase-3 activity (0.74±0.09 nmol PNA/h/mg protein, P<0.05 versus WT). Moreover, there is a trend of increasing TUNEL index and caspase-3 activity in the nonischemic area in RSG treated APN+/− mice, although the difference was not statistically significant. The difference in apoptotic cell death between RSG-treated WT and APN−/− mice was highly significant (P<0.01).

Chronic RSG Treatment Improved Post-MI Survival Rate in WT but Not in APN Knockout Mice

Having demonstrated that the acute antiapoptotic and infarct-sparing effects of RSG were critically dependent on its APN stimulatory effects, we extended RSG administration for another 7 days and determined whether APN is required in the ultimate cardioprotective effects of RSG after MI. As summarized in Figure 4, approximately 35% of WT animals receiving vehicle died 11 days after MI. No significant difference in survival rate was observed between WT and APN+/− or between WT and APN−/− mice when treated with vehicle. RSG treatment significantly increased survival rate in WT (91.3% versus 70.0%, P<0.05) but had no significant effect in APN+/− and APN−/− mice. The survival rate difference between RSG-treated WT and APN−/− mice was highly significant (P<0.01).

Treatment With RSG Improved Cardiac Function in WT Mice but Not in APN Knockout Mice

Four days of permanent coronary occlusion in WT and APN knockout mice caused severe cardiac dysfunction (>50% reduction in left ventricular ejection fraction, Figure 5). Although comparable reduction in LVEF was observed in both vehicle-treated WT and APN knockout mice at this relatively early time point, the cardiac response to RSG progressively diminished as APN production decreased. Specifically, RSG significantly increased LVEF in WT mice (P<0.01 versus vehicle group; Figure 5), slightly but insignificantly improved LVEF in APN+/− mice, and became completely ineffective in APN−/− mice. When MI and RSG treatment were extended for another 7 days, the diverse effect of RSG on cardiac function became more evident. As summarized in Figure 6, dP/dt max was markedly reduced, and LV end diastolic pressure (LVEDP) was significantly in-
creased in all three groups of MI animals treated with vehicle. Significantly worse cardiac function was observed in APN/H11002/H11002 mice when compared with WT mice at this late time point (Figure 6). Treatment with RSG significantly increased dP/dt\text{max} and reduced LVEDP in WT mice but not in APN/H11002/H11002 mice. Most surprisingly, 14 days of RSG treatment of APN/H11002/H11002 mice further worsened cardiac function, evidenced by lower dP/dt\text{max} and higher LVEDP (Figure 6). The difference between RSG-treated WT and APN/H11002/H11002 mice in all cardiac functional measurements (LVEF, dP/dt\text{max} and LVEDP) was highly significant (P<0.01).

Treatment With RSG Reduced Oxidative Stress in WT Mice but Further Enhanced Superoxide Production in APN/H11002/H11002 Mice

The aforementioned experimental results demonstrated that partial APN loss attenuated, and complete APN absence abrogated, RSG cardioprotection against ischemic heart injury. However, it is unlikely that RSG cardioprotective mechanisms reside exclusively in the APN signaling pathway. A more likely explanation for these results might be that RSG has both harmful and protective effects on the ischemic heart, and a balance of these factors determines the final outcome. In a recent study, it has been reported that the effect of RSG on myocardial ischemia/reperfusion injury depends on the dose and timing of administration and that the deleterious effect of high dose RSG administration before ischemia is reversed by an antioxidant (N-acetylcysteine). This result suggests that RSG may enhance oxidative stress in the ischemic heart. To directly examine this possibility, the effect of RSG treatment on cardiac oxidative stress in WT and APN/H11002/H11002 mice was observed. RSG had no effect on basal
superoxide production in sham-operated animals (data not shown). As summarized in Figure 7A, superoxide production (assessed by dihydroethidium staining and lucigenin-enhanced luminescence) in remote, nonischemic region 4 days after permanent LAD occlusion (n=14 to 16 mice/group). *P<0.05, **P<0.01 vs respective vehicle group; $P<0.05$, $$$P<0.01$ vs WT animals in the same treatment group.

Although ischemia stimulates superoxide production from multiple sources including mitochondria and the xanthine/xanthine oxidase system, NOX-2(gp91phox)–containing NADPH oxidase has been increasingly recognized as the most significant molecule responsible for superoxide overproduction in cardiomyocytes. As summarized in Figure 7B, ischemia-induced NOX-2 overexpression was further increased in APN−/− mice, and the inhibitory effect of RSG on NOX-2 overexpression was completely lost in these animals.

**Treatment With a Superoxide Dismutase Mimic (SODM) Preferentially Improved Cardiac Function in APN−/− Mice Versus WT Mice and Reversed the Detrimental Effect of RSG on Cardiac Function in APN−/− Mice**

Experimental results summarized in Figures 6 and 7 suggest that, in APN knockout mice, increased oxidative stress may contribute to the detrimental cardiovascular actions of RSG. To obtain more supporting evidence of this hypothesis, we determined whether treatment with a cell membrane–permeable SODM, MnTE-2-PyP5+, might reverse or attenuate the adverse effects of RSG on APN−/− mice cardiac function. WT or APN−/− mice were subjected to MI and treated with vehicle, RSG, SODM, or RSG+SODM for 14 days. As summarized in Figure 8, daily administration of SODM significantly improved cardiac function in WT, as well as in APN−/− mice, but a preferential effect was observed. Specifically, treatment of WT mice with SODM caused a 36.5% increase in dP/dtmax and a 33% reduction in LVEDP (Figure 8A). However, treatment of APN−/− mice with SODM caused a 74.6% increase in dP/dtmax and a 53.6% reduction in LVEDP (Figure 8B). Furthermore, cotreatment of SODM with RSG completely reversed the detrimental effect of RSG on cardiac function in APN−/− mice.

**APN Knockout Mice Displayed Reduced Myocardial Capillary Density and Lost Response to RSG**

In a permanent myocardial ischemic model, APN has been shown to improve cardiac function by protecting against MI-induced capillary loss in the border zone. To determine whether RSG-stimulated APN may protect heart by a similar mechanism, capillary density was assessed by CD31 staining of tissue sections in the peri-infarct zone, as described by Shibata et al. No difference in capillary density was observed in sham-operated WT mice and APN−/− mice (2769 ± 125 and 2718 ± 149 capillaries/mm²). Consistent with the reports of Shibata et al, capillary density in peri-infarct zone was significantly lower in APN−/− mice than WT mice (2016 ± 133 and 2418 ± 118 capillaries/mm², P<0.05). Treatment of WT with RSG significantly attenuated MI-induced capillary loss (2721 ± 109 capillaries/mm², P<0.05 versus vehicle). This is similar to a recent study demonstrating that pioglitazone restores ischemia-induced angiogenesis. However, RSG failed to restores ischemia-induced capillary loss in APN−/− mice (2138 ± 110 capillaries/mm²).

**Discussion**

We have made several important observations in the present study. First, we have demonstrated that MI caused significant reduction in APN mRNA expression and plasma APN concentration. RSG treatment of WT mice, but not APN−/− mice, successfully recovered adipocyte APN mRNA expression and increased plasma APN. Our findings are consistent with previous clinical and experimental model data reporting plasma APN reduction following MI and RSG stimulation of APN production. However, to our knowledge, our present study is the first reporting RSG treatment efficacy in reversing MI-induced APN inhibition. This result revealed a novel molecular mechanism that may contribute to the previously reported antiischemic and cardioprotective effects of RSG. Interestingly, treatment with RSG increased plasma APN to a level not only significantly greater than that of vehicle-treated MI animals, but also higher than that seen in sham MI animals. The greater effect of RSG on plasma APN level than its effect on adipocyte mRNA expression indicates
that RSG may regulate APN protein production at multiple levels, including traditional transcriptional regulation,\textsuperscript{16} and recently reported posttranscriptional regulation.\textsuperscript{36}

Second, we have demonstrated that, even in a model of very severe permanent coronary artery occlusion, RSG treatment was capable of modestly reducing infarct size, significantly decreasing apoptotic cell death in remote, nonischemic heart regions, and improving cardiac function in WT mice. However, in APN knockout mice, the infarct-sparing effect of RSG was completely lost, and slightly higher apoptotic cell death and worse cardiac function were observed. These results further emphasize APN as an indispensable molecule playing a crucial role in the antiischemic, antiapoptotic, and cardioprotective effects of RSG. Substantial evidence now exists that apoptosis is a major player in post-MI cardiac remodeling.\textsuperscript{37} Our present experimental results demonstrated that RSG modestly reduced infarct size but significantly reduced apoptosis in nonischemic regions. These results suggest that RSG, via its APN stimulatory effects and antiapoptotic effect, may have therapeutic application in reducing cardiac remodeling and improving cardiac function in MI patients.

Third, we have demonstrated that RSG influence on post-MI survival rate (the ultimate measurement of cardioprotection) is related to APN production. Specifically, RSG treatment significantly improved post-MI survival rate in WT mice, whereas the identical treatment failed to improve the final outcome of MI in APN-deficient mice. Although these results do not assert that APN stimulation is the only cardioprotective pathway through which RSG achieves its cardiovascular benefits, it is safe to conclude that APN is indispensable in the overall cardioprotective function of RSG. Moreover, our results suggest that RSG has diverse effects on the cardiovascular system, and its beneficial effects are predominantly achieved through stimulation of APN production. In the absence of APN, the balance between the protective and detrimental actions of RSG is tilted, and the remaining cardioprotective forces of RSG are not strong enough to overcome its cardiac-detrimental side effects. Given that plasma APN is markedly reduced in advanced type 2 diabetic patients,\textsuperscript{20} it is possible that adipocytes may have lost their response to RSG as observed in APN knockout mice in the present study. Under such conditions, the adverse side effects of RSG would predominate unchecked, with the risk of unfavorable cardiovascular outcomes. Although it is impossible to directly extrapolate experimental findings to clinical patients, our present experimental results offer a possible explanation for seemingly divergent results from experimental and clinical RSG studies, as well as between different clinical trials.

Finally, we have demonstrated that treatment with RSG exerted significant antioxidant effects in WT mice. This is consistent with a recent report showing that RSG improves myocardial diastolic function in type 2 diabetic patients through oxidative stress reduction.\textsuperscript{38} However, we have demonstrated for the first time that treatment of APN-deficient animals with RSG significantly increased superoxide production, and that coadministration of a cell membrane

Figure 8. Effect of RSG, SODM, and their combination on cardiac function determined by Millar Mikro-Tip pressure transducer catheter 11 days after permanent LAD occlusion in WT (A) and APN\textsuperscript{-/-} (B) mice. *P<0.05, **P<0.01 vs MI+vehicle; $$$P<0.01 vs MI+RSG.
permeable superoxide dismutase mimic completely reversed unfavorable effect of chronic RSG treatment on cardiac function. These results indicate that the antioxidant effect of RSG reported in previous studies\textsuperscript{10,19} is achieved through APN and that the unfavorable action of RSG on postischemic oxidative stress is likely responsible for its cardiodepressive effects observed when APN production is diminished.

In summary, our present study demonstrated that APN is an indispensable molecule in the antioxidative, antiischemic, antiapoptotic, and cardioprotective actions of RSG. Under pathological conditions in which plasma APN is markedly reduced and/or APN stimulatory action of RSG is impaired, RSG treatment may upregulate superoxide production, culminating in unfavorable cardiovascular outcomes. These results create an impetus to identify alternative treatments, such as combining RSG with antioxidant agents, in a fashion potentially circumventing the adverse PPAR\textgamma agonist side effects, achieving maximal cardiovascular benefits in both advanced and elderly type 2 diabetic patients requiring extended PPAR\textgamma agonist treatment.

**Limitations**

More than 90% of animals died when no laboratory personnel were present, and we were unable to precisely determine the cause of death. Additional studies with a continuous animal monitoring system are required. In addition, the signaling mechanism responsible for RSG enhancement of superoxide production in the APN\textsuperscript{−/−} animals remains unclear. Possible involvement of COX-2\textsuperscript{−/−} and other cardioprotective signaling molecules,\textsuperscript{41} specifically in relation to complicating disease states such as diabetes,\textsuperscript{42} warrants further investigation.

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**Disclosures**

None.

**References**


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