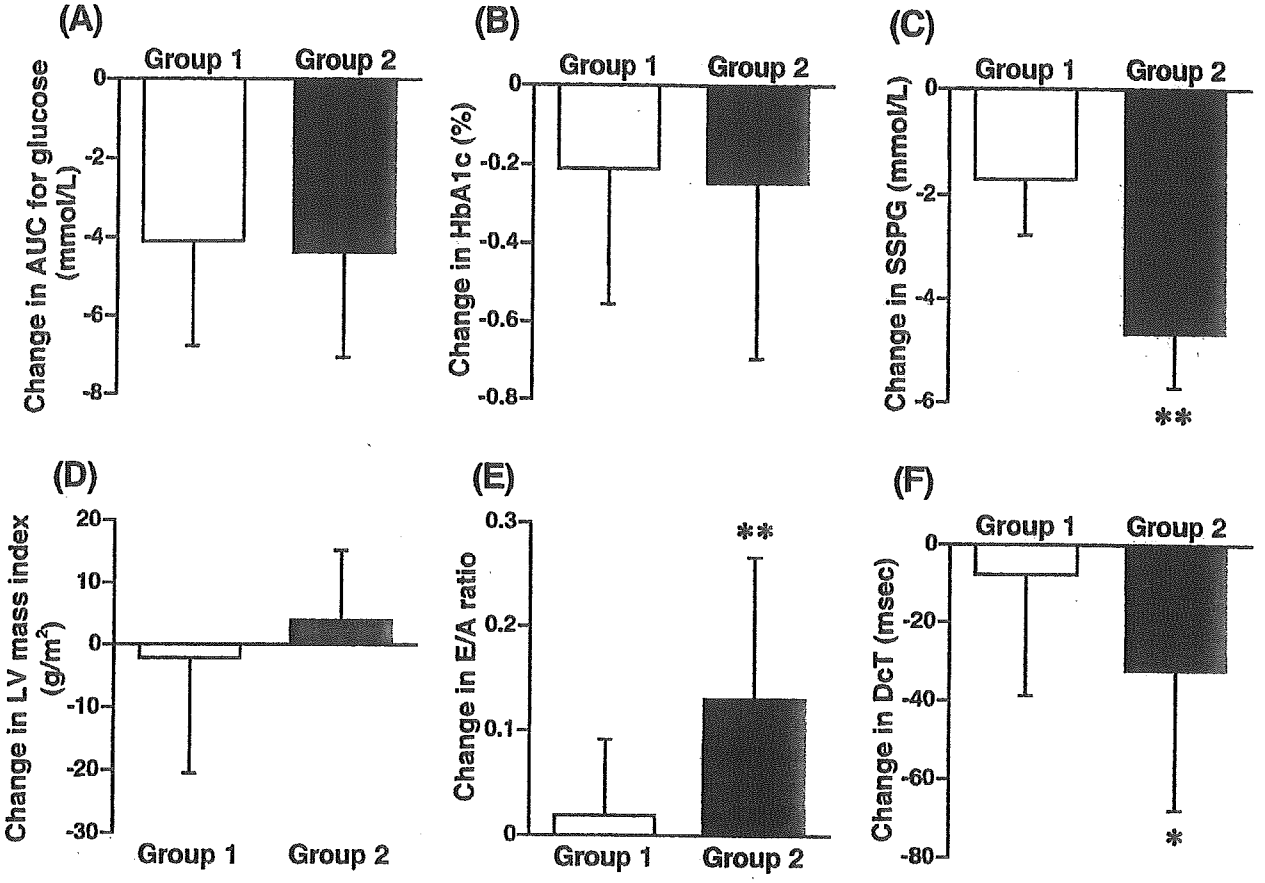

FIG. 2. Comparison of the changes in glucose levels, left ventricular (LV) hypertrophy, and LV diastolic function between the two study groups divided by the extent of improvement in insulin sensitivity after treatment with pioglitazone. Group 1 (n = 19) represents subjects with a modest improvement in insulin sensitivity (modest decrease in steady-state plasma glucose [SSPG]), and group 2 (n = 11) represents those with a greater improvement in insulin sensitivity (greater decrease in SSPG), as shown in (C). The changes in the area under the curve (AUC) for glucose (A), HbA_{1c} (B), and LV hypertrophy (D) did not differ between the two groups. The changes in E/A ratio (E) and DcT (F) were significantly greater in group 2 than in group 1. *$P < .05$ and **$P < .01$ compared with group 1.

hypertrophy and diastolic function in normotensive diabetic patients.[17] To our knowledge, therefore, the present study is the first to report that pioglitazone improves LV diastolic function in hypertensive patients without overt diabetes mellitus, in association with the amelioration of insulin resistance.

There have been many reports that the presence of hyperinsulinemia or insulin resistance is related to LV diastolic dysfunction in hypertensive patients.[4–7,18] On the other hand, some studies showed that abnormal LV relaxation in diabetic and nondiabetic patients with hypertension was associated with glucose levels rather than insulin levels.[19–21] Our previous study also showed that a slight increase in fasting plasma glucose exaggerated LV diastolic dysfunction in treated hypertensive patients.[2] So, the question remains which factor among hyperglycemia, hyperinsulinemia, and insulin resistance has the greatest impact on cardiac diastolic function. In addition, the question is raised as to the improvement of which of them leads to the amelioration of LV diastolic impairment. In the present study, the increase in the E/A ratio and decrease in DcT were significantly greater in subjects with a marked decrease in SSPG by pioglitazone than in those with a modest decrease, although the decrease in glucose levels (AUC for glucose and HbA_{1c}) did not differ between the two groups. Furthermore, the changes in these two indices of diastolic function after pioglitazone treatment were correlated with the extent of change in SSPG, but not with that in the glucose or insulin level. Therefore, our present findings suggest that among metabolic factors, insulin resistance rather than hyperinsulinemia or hyperglycemia is related to the progression of LV diastolic dysfunction in hypertensive patients, and that the impaired diastolic function in such patients is reversed accompanied by an improvement of insulin resistance itself.

Some in vitro studies have shown that PPAR-γ activation by thiazolidinediones and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 inhibits stretch-induced and angiotensin II–induced hypertrophy of cardiac myocytes.[8,9] Asakawa et al[9] also showed that pioglitazone suppressed pressure overload–induced cardiac hypertrophy in mice. In human subjects, one study reported that troglitazone decreased LV mass in a small sample size (n = 12) of normotensive patients with diabetes,[17] but another study using a larger

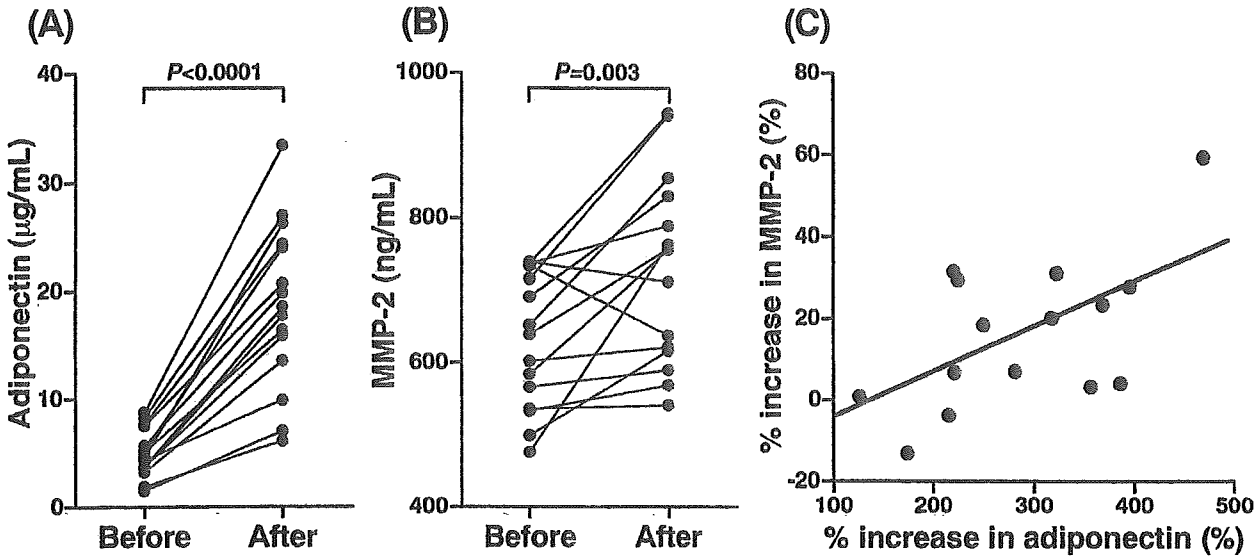

FIG. 3. (A and B) Changes in plasma adiponectin (A) and matrix metalloproteinase-2 (MMP-2) levels (B) before and after treatment with pioglitazone (n = 15). These plasma levels were significantly increased by pioglitazone treatment (adiponectin, $P < .0001$; MMP-2, $P = .003$). (C) Correlation between individual changes in adiponectin and MMP-2 levels before and after treatment with pioglitazone. The percent increase in MMP-2 was positively correlated with the percent increase in adiponectin ($r = 0.588$, $P = .019$).

population (n = 154) of diabetic patients showed that LV mass index did not significantly change 12 to 48 weeks after treatment with troglitazone.[22] Our findings concerning the effect of pioglitazone on LV hypertrophy were consistent with the results of the latter study. In the present study, therefore, we have demonstrated that the amelioration of insulin resistance by pioglitazone selectively improves LV diastolic function without any change in LV mass in hypertensive patients. A recent study showed that cardiac fibrosis, but not hypertrophy, was prevented by treatment with rosiglitazone in DOCA-salt hypertensive rats.[23] Another previous study revealed that pioglitazone improved the LV diastolic function of prediabetic rat hearts in association with a decrease in LV collagen accumulation.[10] These findings suggest the possibility that the suppressive effect of thiazolidinediones on cardiac interstitial fibrosis through the blocking of collagen overproduction, apart from its inhibitory action on myocyte hypertrophy, may result in the improvement of LV diastolic function.

It is suggested that a depressed extracellular degradation of collagen may facilitate cardiac fibrosis in hypertensive patients. In fact, an abnormality in the MMP–TIMP balance (decrease in the ratio of the MMP to TIMP level), which indicates the promotion of collagen synthesis relative to degradation, has been reported in hypertensive subjects with LV diastolic dysfunction (impaired LV relaxation).[24,25] Conversely, some agents that increase levels of MMP including MMP-2 suppress collagen production by cardiac fibroblasts and prevent myocardial fibrosis.[26–28] Furthermore, adiponectin, plasma levels of which are strongly stimulated by PPAR-γ activation, was shown to have antifibrotic effects in the liver.[29] Thus, we examined the changes in plasma adiponectin, MMP-2, and TIMP-1 levels before and after treatment with pioglitazone in some of the present subjects. A marked elevation in the plasma adiponectin concentration was found after administration of pioglitazone in our hypertensive patients, similarly to the previous observation in diabetic patients.[30] In addition, the present study demonstrated for the first time that the observed increase in the plasma adiponectin level caused by pioglitazone was associated with an increase in the plasma MMP-2 level without a change in the TIMP-1 level. Moreover, the increase in these plasma levels was partially linked to the improvement of LV diastolic function. Taken together with the findings mentioned above,[24–29] our results suggest that the antifibrotic actions of adiponectin and MMP induced by PPAR-γ may be partially involved in the beneficial effect of pioglitazone on LV diastolic function. However, we cannot rule out the possibility that LV relaxation is ameliorated by thiazolidinediones directly or indirectly through the improvement of coronary vascular endothelial function, as suggested in some in vitro and in vivo studies.[31,32] Further investigations are needed to clarify the exact mechanism by which pioglitazone favorably affects cardiac diastolic properties.

In the present study, two indices of LV diastolic filling (E/A ratio and DcT) were used as indicators of LV diastolic dysfunction. As a limitation of our study, however, it must be recognized that LV diastolic filling patterns determined by Doppler echocardiographic measurements permit only an indirect estimate of LV diastolic function. Moreover, LV filling is frequently dependent on loading conditions, especially preload-dependent conditions.[14] It is possible that fluid retention induced by the insulin-sensitizing action of pioglitazone increases preload, and it may be not so easy to differentiate a pseudonormalization pattern by preload increase from the improvement of diastolic filling toward a true normal pattern. However, the diame-

ters of the inferior vena cava and left atria, dilations of which are indicators of preload increase, were not altered at all by pioglitazone administration in the present subjects. In addition, our pulmonary venous flow profiles proved that none of the specific patterns observed with preload increase–induced LV diastolic dysfunction (elevation of PVa wave velocity, prolongation of PVad, and shortening of Ad-PVad) were found after pioglitazone treatment. These findings clearly show that the observed changes in LV diastolic filling induced by pioglitazone in the hypertensive patients in the present study truly represent an improvement in diastolic function.

There has been a discrepancy concerning the effects of PPAR-γ activators on the heart between experimental studies and clinical reports. Studies using animal models have demonstrated beneficial effects of thiazolidinediones on cardiac hypertrophy, diastolic function, and remodeling,[9–12,33] with some exceptions.[34,35] In contrast, thiazolidinedione-associated congestive heart failure has been reported in diabetic patients,[36] and a retrospective cohort study evaluating the relationship between thiazolidinedione treatment and heart failure risk in patients with type 2 diabetes showed a significant increase in the development of heart failure with the administration of thiazolidinediones independently of other risk factors.[37] However, the present study demonstrated that pioglitazone improved insulin resistance and cardiac diastolic function in hypertensive patients without overt diabetes mellitus, with no side effects except mild leg edema. Therefore, our results suggest favorable cardiac effects of thiazolidinediones for hypertensive subjects whose symptoms belong to a cluster of the insulin resistance syndrome (metabolic syndrome).

In conclusion, the present study demonstrates that pioglitazone improves LV diastolic function without LV mass regression in hypertensive patients in proportion to the amelioration of insulin resistance. Taken together with various beneficial vascular and cardiac actions of PPAR-γ activation, our findings suggest that thiazolidinediones, apart from antidiabetic drugs, may become therapeutic agents to prevent the progression of cardiovascular complications in hypertensive patients.

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Exacerbation of heart failure in adiponectin-deficient mice due to impaired regulation of AMPK and glucose metabolism

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Abstract

Objective: Insulin resistance (IR) was reported to be associated with chronic heart failure (CHF). Adiponectin, an insulin-sensitizing hormone with anti-inflammatory activity, improves energy metabolism via AMP-activated protein kinase (AMPK). AMPK deficiency is associated with depressed cardiac function under stress conditions. However, it is not clear whether adiponectin plays an important role in CHF. We hypothesize that deficiency of adiponectin might result in deterioration of heart failure.
Methods: Using adiponectin null mice and their littermates, we examined the effects of adiponectin on LV pressure overload-induced cardiac hypertrophy and failure, and investigated the mechanisms involved.
Results: Three weeks after transverse aortic constriction (TAC), cardiac hypertrophy (evaluated from the heart-to-body weight ratio: 7.62±0.27 in wild-type (WT) mice, 9.97±1.13 in knockout (KO) mice, $P<0.05$) and pulmonary congestion (lung-to-body weight ratio: 9.05±1.49 in WT mice, 14.95±2.36 in KO mice, $P<0.05$) were significantly greater in adiponectin KO mice than WT mice. LV dimensions were also increased in KO mice. Compared with WT TAC mice, expression of AMPKα protein was lower, while IR was higher in KO TAC mice.
Conclusion: These findings indicate that adiponectin deficiency leads to progressive cardiac remodeling in pressure overloaded condition mediated via lowing AMPK signaling and impaired glucose metabolism.

Keywords: Adiponectin; Heart failure; Myocardial hypertrophy; Metabolic syndrome

1. Introduction

The metabolic syndrome (MetS) has been identified as a constellation of important risk factors for cardiovascular disease (CVD) [1,2]. The Adult Treatment Panel III report (ATP III)[3] identified insulin resistance (IR)±glucose intolerance as an important component of MetS that is related to CVD. Clinical evidence suggests that LV hypertrophy is associated with either impaired glucose tolerance (IGT) or an increase in IR [4]. An increase in IR is also common in CHF patients with either ischemic heart disease or idiopathic dilated cardiomyopathy [5–7]. These findings lead to the concept that a strategy targeting improvement of IGT or IR should be beneficial for cardiac remodeling.

To date, there is compelling evidence that an impaired myocardial energy metabolism strongly influences cardiac remodeling [8–11]. The important role of the AMP-activated protein kinase (AMPK) in cardiac hypertrophy and failure seems to be deserving of more attention. AMPK

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activity and protein expression were both reported to be increased by pressure overload hypertrophy [8], which should be considered a compensatory mechanism for cardiac remodeling, because the overexpression of mutations of this enzyme leads to deterioration of post-ischemic cardiac dysfunction [10] or experimental glycogen storage cardiomyopathy [11]. Accordingly, we considered that AMPK might play an important role in limiting cardiac remodeling and that an increase of AMPK in the heart might inhibit remodeling by regulation of cellular metabolism to maintain energy homeostasis under stress conditions. Intriguingly, adiponectin, an endogenous adipocyte-derived insulin-sensitizing hormone, has been shown to attenuate inflammation, regulate glucose and lipid metabolism. In addition, adiponectin is able to stimulate glucose utilization and fatty acid oxidation through the activation of AMPK [12]. Furthermore, administration of adiponectin reverses IR in mice with lipoatrophy and diabetes [13,14]. The importance of adiponectin has also been demonstrated by other evidence that it may directly influence the development of cardiovascular disease [15–17]. A recent clinical investigation demonstrated that a high plasma adiponectin concentration was associated with a lower risk of myocardial infarction in men [17]. These lines of evidence strongly suggest that adiponectin might play an important role in the inhibition of cardiac remodeling via its beneficial effects on MetS. Interestingly, a recent experimental study shows that 1 week pressure overload in adiponectin-deficient mice resulted in enhanced concentric cardiac hypertrophy with an increased mortality [18]. However, to our knowledge, no previous study has evaluated the role of AMPK or adiponectin on chronic heart failure (CHF). Therefore, we aimed to test the hypothesis that adiponectin might act as an endogenous protective modulator of chronic cardiac remodeling via regulation of AMPK.

In this study, we evaluated the role of adiponectin in the progression of cardiac hypertrophy and heart failure in a model of LV pressure overload using adiponectin knockout mice, and explored the potential mechanisms involved.

2. Methods

2.1. Adiponectin knockout (KO) mice

Adiponectin KO mice were generated as described previously [19]. Wild-type (WT) littermates served as the control.

2.2. TAC model

All procedures were performed in accordance with our institutional guidelines for animal research and comply with the Declaration of Helsinki and the NIH Guide. Mice (male, 9–10 weeks old, wt 25–29 g) were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg, i.p.), and transverse aortic constriction (TAC) was created as we described previously. In order to confirm that pressure overload was similar between the wild-type and the KO mice, three mice in each group were selected for measurement of the ascending aortic pressure using a 1.4 F Millar pressure catheter on the second day after TAC. The other mice were killed after 3 weeks for morphological analysis. Mice were divided into four groups: WT sham ($n=5$), WT TAC ($n=24$), KO sham ($n=5$), and KO TAC ($n=24$).

2.3. Histology

Hearts were fixed with 10% formalin. The cardiac myocyte cross-sectional surface area was measured using three hearts in each group after images were captured from HE-stained sections as described elsewhere [20]. One hundred myocytes per heart were counted, and the average area was determined. Myocardial and perivascular fibrosis were stained with Azan [21].

2.4. Echocardiography

Transthoracic echocardiography was performed with a Sonos 4500 and a 15–6 L MHz transducer (Philips, the Netherlands). Mice were fixed while conscious and good two-dimensional short-axis LV views were obtained for guided M-mode measurements of the LV posterior wall thickness (LVPW), LV end-diastolic diameter (LVEDd), LV end-systolic diameter (LVESd), LV fractional shortening (LVFS), and LV ejection fraction (EF). $\mathrm{LVFS}=(\mathrm{LVEDd}-\mathrm{LVESd})/\mathrm{LVEDd}*100$, $\mathrm{LVEF}=[(\mathrm{LVEDd})^3-(\mathrm{LVESd})^3]/(\mathrm{LVEDd})^3*100$.

2.5. Measurement of glucose and insulin

Fasting plasma glucose was measured using a blood glucose test meter (Glutestace GT-1640, Arkray Company, Japan). After 14 h withdrawal of food from the cages, whole blood sample (3 μl) was taken from mouse tails with a glucose sensor inserted in Glutestace, and the result of plasma glucose concentration was read-out 30 s later. Serum insulin levels were measured according to the protocols of the manufacturers (EIA-3440 ELISA kit, DRG, German). IR was assessed with the homeostasis model: HOMA-IR = fasting glucose level (mg/dl) × fasting insulin level (ng/ml) ÷ 22.5.

2.6. Western blot analysis

SDS-PAGE was performed with 50 μg of protein extracted from mouse hearts. Blots were incubated with a mouse monoclonal antibody directed against anti-AMPKα_1, anti-AMPKα_2 antibodies (upstate). Signals obtained by Western blotting were quantified using Scion Image software.

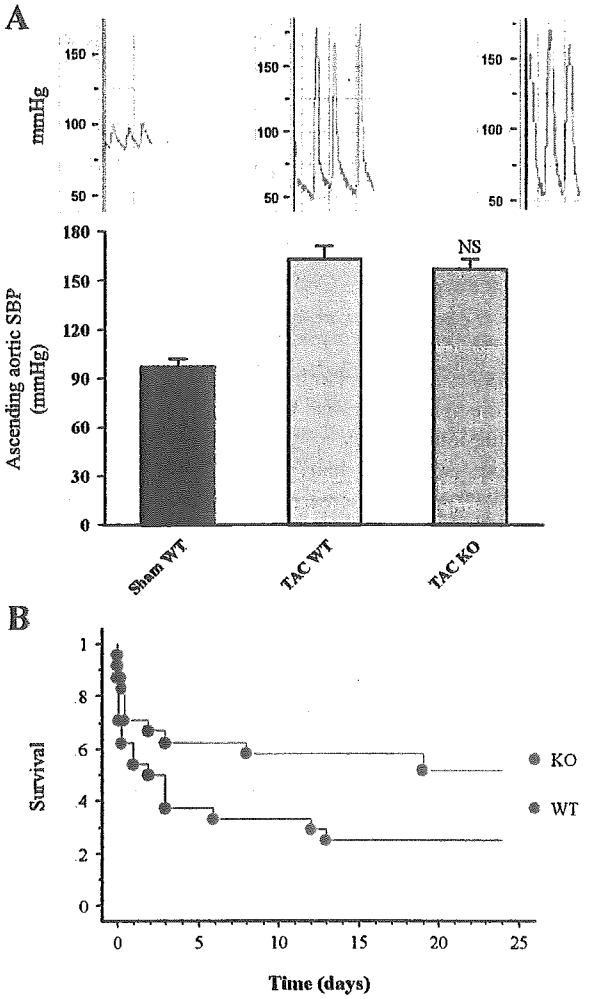

Fig. 1. Left ventricular pressure overload and survival. A) The ascending aortic systolic pressure measured with a 1.4 F catheter was similar in adiponectin KO and WT mice. NS: not significant vs. TAC WT. B) Kaplan–Meier survival analysis showed a significant higher mortality in adiponectin KO mice after TAC (Mantel–Cox test: $P=0.031$, $n=24$ in both WT and KO groups).

2.7. Statistical analysis

For all statistical tests, multiple comparisons were performed by one-way ANOVA with the Tukey–Kramer exact probability test. Survival analysis was performed using the Kaplan–Meier method. Variables with skewed distribution were transformed to logarithmic data. Results are reported as the mean ± SEM and $P<0.05$ was considered statistically significant.

3. Results

3.1. LV pressure overload and survival

To evaluate the role of adiponectin in cardiac remodeling, we used mice lacking the adiponectin/*CRP30* gene. During development up to 16 weeks of age, there were no differences in growth rate and food intake between WT mice and KO (homozygous) mice [19]. The results showed that LV pressure overload was similar in WT and KO mice (Fig. 1A). The mortality after TAC was significantly higher in KO mice than WT mice (Fig. 1B). We found that acute or subacute heart failure was the main cause of death confirmed by postmortem examination (pulmonary edema or hemorrhage was noted in most of the dead mice. Lung-to-body weight ration was 13.1±2.3 mg/g for dead mice in adiponectin KO mice, 11.4±1.9 mg/g for dead mice in WT group). Body weight (BW) and blood pressure (determined by tail cuff measurement) were similar before TAC (BW: 27.1±0.4 g in KO, 27.7±0.4 g in WT) and 3 weeks after TAC (BW: 24.5±1.4 g in KO, 25.5±0.7 g in WT).

3.2. Earlier transition from hypertrophy to heart failure in KO mice

Serial echocardiographic examinations showed that the heart function evaluated by LVEF and LVFS progressively

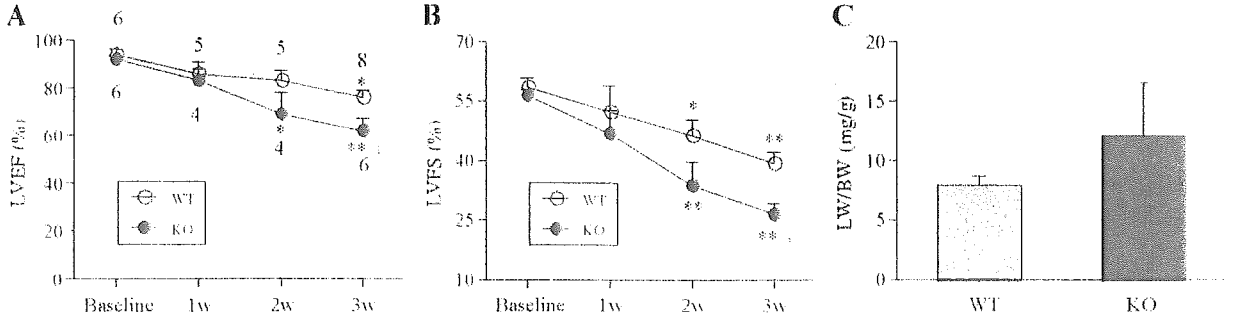

Fig. 2. The transition from hypertrophy to heart failure. A) Left ventricular ejection fraction (LVEF) and B) left ventricular fractional shortening (LVFS) were progressively depressed in adiponectin KO mice after 1 week of TAC, and the transition to heart failure occurred at 2 weeks after TAC in KO mice, which was confirmed by sacrifice to show an significant increase of lung-to-body weight ratio (C, $n=4$ for both WT and TAC mice). The number of mice in each time point for echocardiographic examination is indicated above or under the data points. *$P<0.05$, **$P<0.01$ vs. baseline. †$P<0.05$ vs. WT mice.

depressed in both adiponectin KO and WT mice over the course of 3 weeks (Fig. 2A, B). Two weeks after TAC, a significant reduction of LVEF and LVFS was noted in KO mice, indicating a proceeded transition to heart failure. To confirm the occurrence of heart failure, we sacrificed four mice in both KO and WT groups at 2 weeks after TAC and found a marked pulmonary congestion in KO mice (Fig. 2C).

3.3. Greater cardiac hypertrophy in KO mice

Three weeks after TAC, mice were sacrificed after echocardiographic examination. The wet heart-to-body weight ratio (HW/BW) was increased by 53% in TAC WT mice compared with sham WT mice, whereas HW/BW was dramatically increased by 110% in adiponectin TAC KO mice vs. sham KO mice. There was a significant difference of HW/BW between WT and KO TAC mice (Fig. 3A–C, E). The cross-sectional surface area of cardiac myocytes was significantly larger in KO mice than WT mice (Fig. 3F). There were no significant differences of HW/BW and cardiac myocyte cross-sectional surface area between WT and KO sham mice. These findings indicate that cardiac hypertrophy was far more extensive in adiponectin KO mice. We also examined myocardial and perivascular fibrosis and did not find significant difference between WT and KO TAC mice (Fig. 3D).

3.4. Worse pulmonary congestion in KO mice

We confirmed in previous studies that pulmonary edema is a reliable index of cardiac function in this model [22–24]. Severe pulmonary congestion was found in adiponectin KO mice. Compared with sham mice, the lung-to-body weight ratio (LW/BW) was increased by 170% in KO TAC mice,

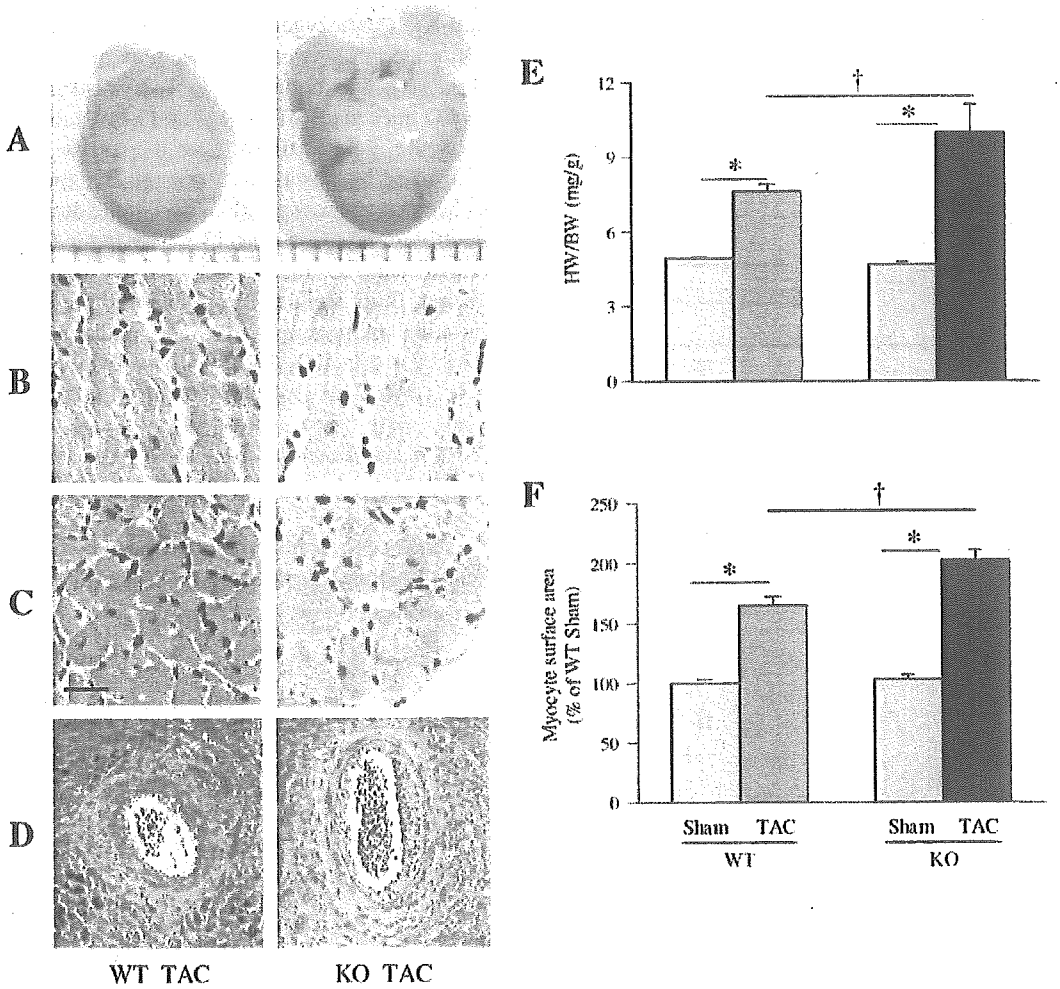

Fig. 3. Cardiac remodeling was more severe in KO mice. A) Representative pictures of cardiac hypertrophy in WT and KO mice at 3 weeks after TAC. B and C) Represent long-axis and cross-sectional views of cardiac myocytes with HE staining. D) Represents cardiac fibrosis with Azan staining (×100 magnification). HW/BW (E, $n=5$ in both sham groups, $n=8$ in WT TAC group, and $n=6$ in KO TAC group) and the cardiac myocyte cross-sectional surface area (F, $n=2$ in each sham group and $n=3$ in each TAC group) were increased significantly in KO mice compared with their wild-type (WT) littermates. $^{*}P<0.01$, $\dagger P<0.05$. Bar=20 μm for B and C.

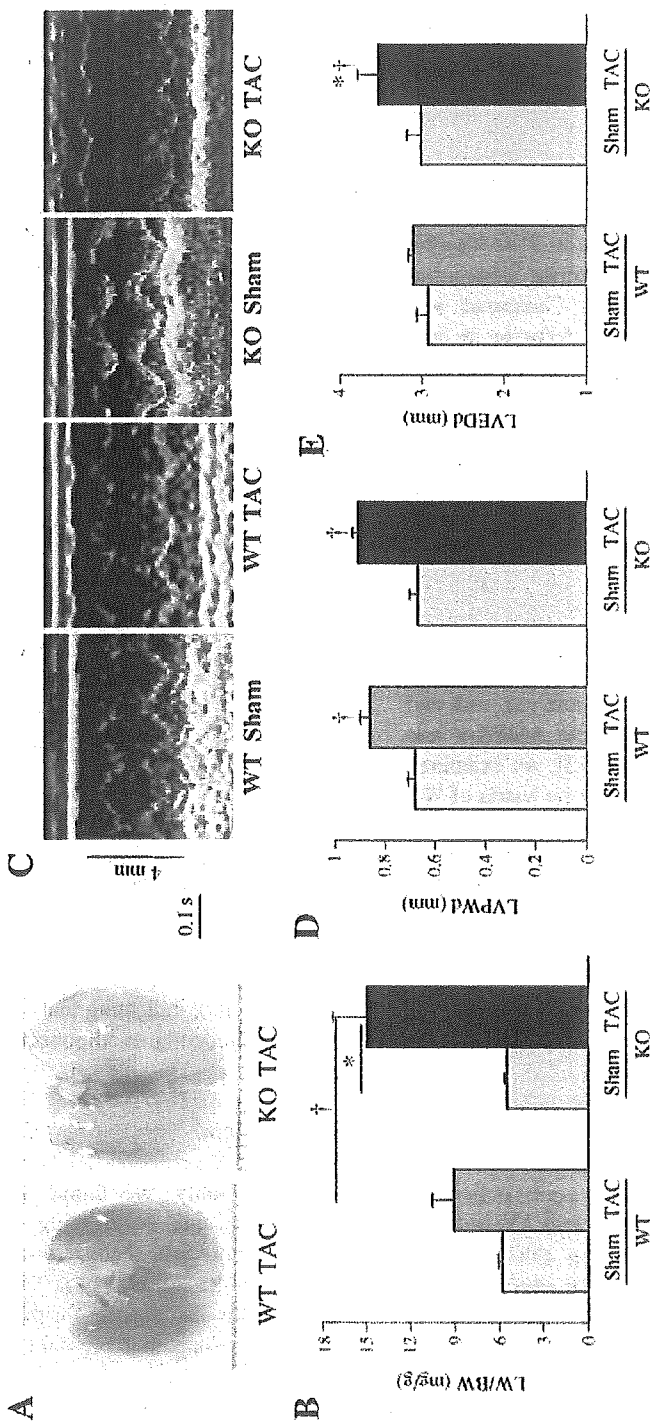

Fig. 4. Pulmonary congestion and echocardiographic findings at 3 weeks after TAC. The lungs of an adiponectin KO mouse were markedly enlarged compared with those of WT mice (A). The lung-to-body weight ratio (LW/BW) was markedly increased in KO mice compared with WT mice (B). $*P<0.01$. $\dagger P<0.05$. Echocardiography (C) shows that the LV posterior wall diastolic thickness (LVPWd) (D) is similar in KO and WT TAC mice. The LV end-diastolic dimension (LVEDd) (E) is significantly increased in KO mice compared with WT mice. $*P<0.05$ vs. TAC WT. $\dagger P<0.01$ vs. responding sham mice. The number of animals is the same as Fig. 3 in each group for analysis of LW/BW and echocardiography.

whereas there was only a 55% increase in WT TAC littermates (Fig. 4A, B). There was no significant difference in LW/BW between KO and WT sham mice. We did not evaluate LV hemodynamics using a Millar pressure catheter because most of the KO mice appeared to be too weak to endure this procedure (including anesthesia) at 3 weeks after TAC.

3.5. Echocardiography findings

Because anesthesia has a significant influence on echocardiography data in mice [25] and most of the KO TAC mice were too weak for anesthesia at 3 weeks after TAC, we developed a method of performing echocardiographic examination in conscious mice. Compared with WT TAC mice, there was a significant decrease in both LV fractional shortening (LVFS) and the LV ejection fraction (LVEF) in KO TAC mice (Fig. 2A, B), and marked LV chamber dilation was observed in KO TAC mice (Fig. 4C, D). In contrast, there were no significant differences in these parameters between WT sham and KO sham mice. These findings indicate an increase in cardiac remodeling under pressure overload in adiponectin KO mice.

3.6. Myocardial AMPK expression

AMPK consists of one catalytic subunit (α) and two noncatalytic subunits (β and γ). Because AMPKα was reported to be activated by adiponectin [12], we examined the AMPKα_1 and α_2 protein expression in the hearts of WT and KO mice. As shown in Fig. 5, in the presence of LV pressure overload, AMPKα expression increased significantly, but the increment of AMPKα protein was less in KO than in WT hearts. These findings suggested that adiponectin deficiency means that the expression of AMPK cannot be increased sufficiently enough to provide adequate cardiac protection under stress conditions.

3.7. Increase of fasting glucose and IR

As IR is closely associated with cardiac remodeling [4–7] and adiponectin deficiency can lead to diet-induced IR [19], we determined the influence of adiponectin deficiency on glucose metabolism and IR in mice with LV pressure overload. As shown in Fig. 6A, fasting glucose levels increased by 40% in KO mice at 3 weeks after TAC, but rose by only about 20% in WT littermates, suggesting that the glucose metabolisms were more impaired in the adiponectin KO mice. Meanwhile, a similar increase in serum insulin was noted in both WT and KO TAC mice (Fig. 6B). As an index of IR, HOMA-IR was more increased in adiponectin KO mice than in WT mice at three weeks after TAC (Fig. 6C). Furthermore, we found a significant positive correlation between IR and the heart weight-to-body weight ratio in adiponectin KO mice rather than in WT

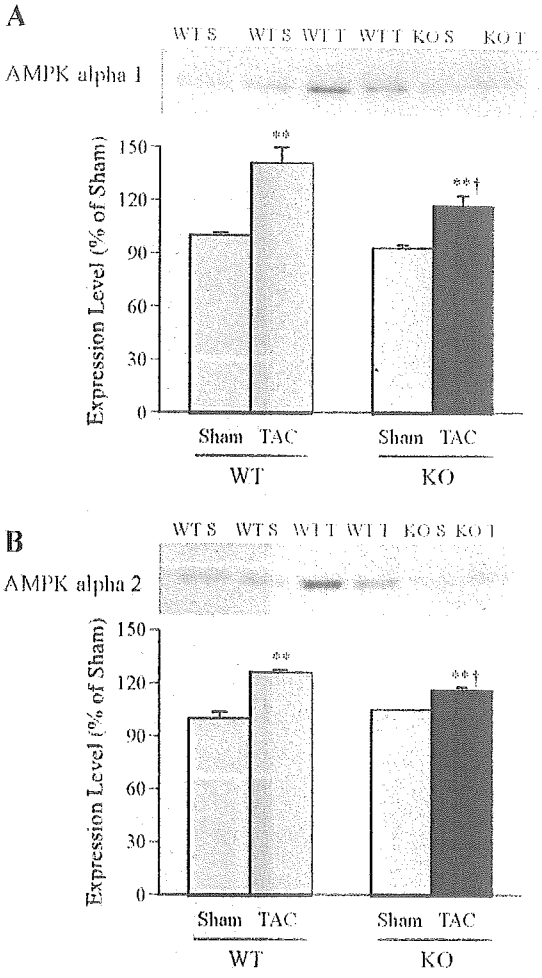

Fig. 5. Myocardial expression of AMPK. AMPKα_1 (A), α_2 (B) were increased in TAC mice, but the change was smaller in KO mice ($n=3$ in each group, $^{**}P<0.01$ vs. responding sham mice; $\dagger P<0.05$ vs. WT TAC). S: sham, T: TAC.

mice (Fig. 6D), indicating that IR might also be involved in cardiac remodeling in adiponectin KO mice.

4. Discussion

In this study, we found that adiponectin deficiency worsens cardiac remodeling induced by LV pressure overload, and this change was associated closely with a decrease in the expression of AMPK, and an increase in IR. These results are consistent with a recent study by Shibata et al. [18] showing that pressure overload for one week in adiponectin KO mice resulted in greater cardiac hypertrophy and higher mortality. Differently, this study further investigated the potential role of adiponectin-deficiency on the development of cardiac hypertrophy and chronic heart failure. We demonstrated that the transition from hypertrophy to heart failure proceeded in adiponectin KO mice. Additionally, we investigated the influence of adiponectin

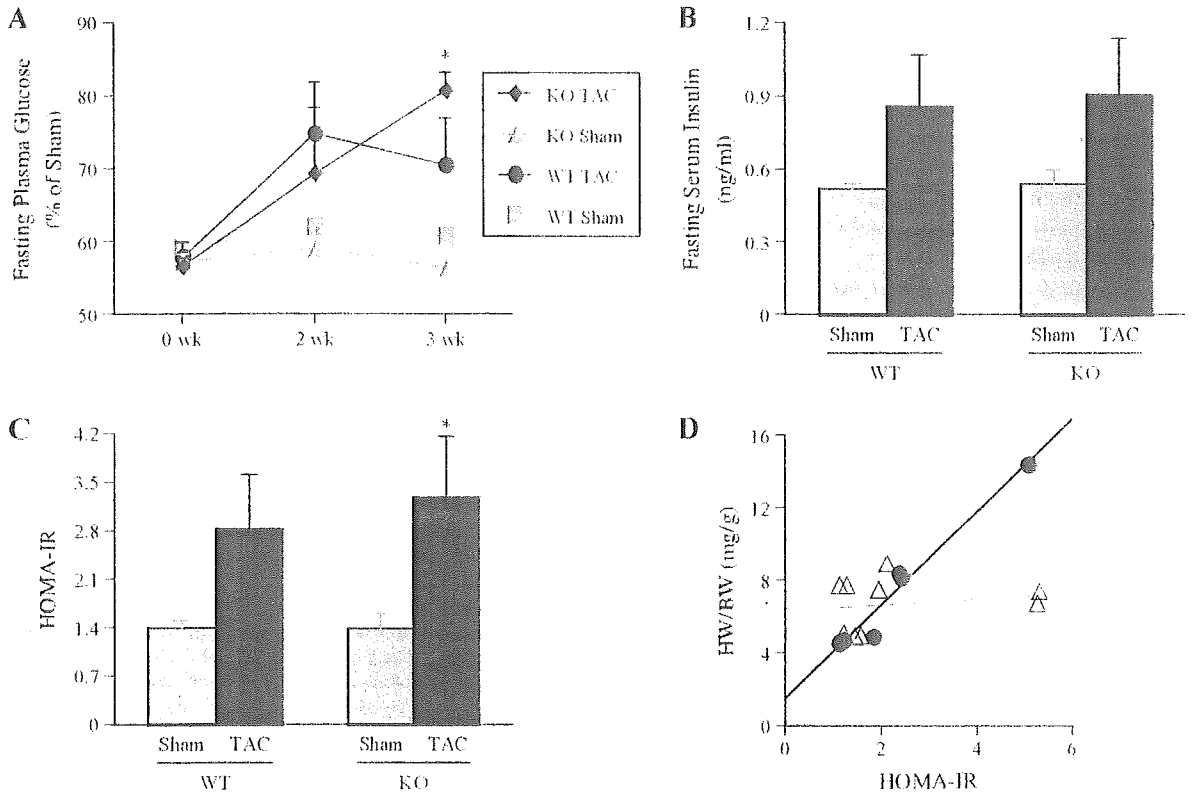

Fig. 6. Changes in glucose metabolism. Fasting glucose levels (A) were increased in adiponectin KO mice at 3 weeks after the onset of TAC, $^{*}P < 0.01$ vs. WT TAC ($n = 5$ for all the groups at 0 week and for both sham groups at other two time points; $n = 4$ for WT and KO TAC mice at 2 weeks, and $n = 5$ and 3 for WT and KO TAC mice at 3 weeks, respectively). Serum insulin (B) was increased after TAC, but no significant difference was found between WT and KO mice, while the insulin resistance index HOMA-IR (C) was increased in KO mice $^{\dagger}P < 0.05$ vs. KO sham ($n = 3$ in both KO sham and TAC groups, $n = 3$ in WT sham and $n = 6$ in TAC groups). Linear correlation between HOMA-IR and HW/BW in both WT and KO mice groups (D) irrespective of TAC, $r = 0.982$, $P < 0.0001$, $n = 6$ for KO mice (solid circle), while no significant correlation was found for WT mice ($n = 9$, open triangle).

on glucose metabolism and addressed the important relation between metabolism and cardiac remodeling.

An increase in IR, glucose intolerance, and a proinflammatory state are among the six components of the MetS related to CVD, which is viewed as the primary outcome of this syndrome. In the present study, we noted that adiponectin deficiency induced an increase in IR and fasting glucose levels in the presence of pressure overload, suggesting that adiponectin has a strong influence on MetS and subsequently on cardiac remodeling. An increase in IR appears to downregulate adiponectin receptor expression via the phosphoinositide 3-kinase/Foxo1-dependent pathway [26]. In addition, Foxo1 is recognized as a negative regulator of insulin sensitivity [27], so it is theoretically acceptable that adiponectin knockout leads to MetS or that adiponectin KO mice are more susceptible to MetS under pathological stress. Although the exact relationship between MetS and CVD is not clear, both genetic and environmental factors may be involved. There is evidence that neuroendocrine factors [28] or the RAS (review [29]) may play an important role in MetS. We previously showed that plasma concentrations of catecholamines and renin were increased by LV pressure overload in mice [23]. In the present study, in addition to endogenous adiponectin deficiency, activation of the sympathoadrenal system and renin–angiotensin system (RAS) may have contributed to the onset of MetS.

The impact of MetS on CVD mortality has been investigated in several clinical studies [30–32]. It is generally agreed that CVD mortality is higher in subjects with MetS than in those without it. We found a positive correlation between IR and cardiac hypertrophy in adiponectin KO mice rather than in WT mice in this study, with both IR and HW/BW higher in adiponectin KO mice than in WT mice, suggesting that deficiency of adiponectin contributed to enhanced cardiac remodeling. Consistent with our results, a recent case-control study found that abnormal LV geometry and LV dysfunction were related to MetS [33]. Additionally, it is well known that type 2 diabetic patients are susceptible to diabetic cardiomyopathy, and the fasting plasma insulin level was reported to be the strongest independent predictor of LV mass in type 2 diabetes [34]. Taken together, these findings support the concept that MetS has an impact on cardiac remodeling. Although IR is known to be an important contributor to the

progression of heart failure, our data reported here are not enough to delineate the causal relationship between IR and cardiac remodeling. In spite of an increase tendency of IR showing in mice with cardiac hypertrophy, we did not find a significant correlation between IR and heart-to-body weight ratio in a relatively small sample of wild-type mice. In accordance with this study, previous clinical observations have shown IR to be related to the thickness of LV walls rather than LVH [35,36].

Adiponectin was reported to reduce the production of TNFα, and to improve both glucose metabolism and IR via the AMPK signaling pathway [12], suggesting that it may improve MetS. Evidence is emerging to demonstrate a critical role of AMPK in cardiac remodeling. Mutation of the gamma 2 subunit of AMPK has been shown to cause glycogen storage cardiomyopathy, and the influence of AMPKα on cardiac remodeling is another attractive research field. Both AMPKα_1 and AMPKα_2 expression were increased in hypertrophied hearts in the present study, which is only partially consistent with a previous investigation by Tian et al. [8]. They reported that α_1 was increased, α_2 expression was decreased, whereas activity of both AMPKα_1 and α_2 was increased in pressure overload rats. The reasons for this discrepancy are not clear. Generally, the activity of both AMPKα_1 and α_2 was reported to increase under stress conditions such as ischemia and pressure overload [8,10,18]. The protein expression of myocardial AMPK was seldom investigated and the reports are inconsistent. Acute ischemia [37] or short-term pressure overload [18] stimulates activity of myocardial AMPK without changing the AMPK protein expression, whereas both AMPKα_2 activity and expression were decreased at three weeks following volume-overload [38]. AMPK deficiency is reported to result in depressed LV function, increased myocardial necrosis, and apoptosis following ischemia/reperfusion injury [10]. The finding that AMPKα protein expression was increased in WT mice after TAC suggests that the augmentation of AMPKα signaling is a compensatory mechanism that attempts to maintain energy homeostasis in the heart under pressure overload. This mechanism may be partly controlled by adiponectin, because AMPK signaling was impaired in adiponectin KO mice and there was consequent progression of cardiac remodeling. Thus, this study provided a new link between adiponectin and AMPK in the process of cardiac remodeling. Apart from its influence on IR, AMPK, and TNFα, other mechanisms may also be involved in the beneficial effect of adiponectin on cardiac remodeling. Adiponectin has been reported to suppress superoxide generation and enhance eNOS activity [39], to have an antiproliferative effect [40], and to counteract beta adrenergic stimulation [41], all of which are closely related to cardiac remodeling [42]. Interestingly, AMPK and eNOS co-localize in hearts and AMPK was reported to activate eNOS [43,44]. Thus, it is reasonable for adiponectin deficiency to lead to progressive cardiac remodeling in response to pressure overload, as we showed in this study.

Acknowledgments

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nature medicine

Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2–dependent mechanisms

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Obesity-related disorders are associated with the development of ischemic heart disease. Adiponectin is a circulating adipose-derived cytokine that is downregulated in obese individuals and after myocardial infarction. Here, we examine the role of adiponectin in myocardial remodeling in response to acute injury. Ischemia-reperfusion in adiponectin-deficient (APN-KO) mice resulted in increased myocardial infarct size, myocardial apoptosis and tumor necrosis factor (TNF)-α expression compared with wild-type mice. Administration of adiponectin diminished infarct size, apoptosis and TNF-α production in both APN-KO and wild-type mice. In cultured cardiac cells, adiponectin inhibited apoptosis and TNF-α production. Dominant negative AMP-activated protein kinase (AMPK) reversed the inhibitory effects of adiponectin on apoptosis but had no effect on the suppressive effect of adiponectin on TNF-α production. Adiponectin induced cyclooxygenase (COX)-2–dependent synthesis of prostaglandin E_2 in cardiac cells, and COX-2 inhibition reversed the inhibitory effects of adiponectin on TNF-α production and infarct size. These data suggest that adiponectin protects the heart from ischemia-reperfusion injury through both AMPK- and COX-2–dependent mechanisms.

Ischemic heart disease including myocardial infarction is the major cause of death in industrial countries[1,2]. Obesity-linked disorders are thought to be involved in the severity and outcome of ischemic heart disease[3,4], but the link between obesity and the development of heart disease is poorly understood at the molecular level. Adiponectin, also referred to as ACRP30, AdipoQ and gelatin-binding protein-28 (refs. 5–7), is an adipocyte-specific cytokine. Circulating adiponectin levels are diminished in obese individuals[8] and are inversely correlated with cardiovascular risk factors including hyperlipidemia, blood pressure and C-reactive protein (CRP) levels[9,10]. It has also been shown that hypoadiponectinemia is an independent risk factor for developing type 2 diabetes[11], hypertension[12] and coronary artery disease[13]. Adiponectin-knockout (APN-KO) mice have diet-induced insulin resistance[14], impaired angiogenic responses to ischemia[15] and excessive cardiac remodeling after pressure overload[16]. Conversely, overexpression of adiponectin promotes insulin sensitivity and angiogenesis, and inhibits cardiac hypertrophy. These data suggest that adiponectin functions as a mediator of obesity-linked cardiovascular and metabolic disorders, and that it may have utility for the treatment of a number of chronic diseases including type 2 diabetes, peripheral artery disease and hypertrophic cardiomyopathy.

Recently, it was shown that high plasma adiponectin levels are associated with a lower risk of myocardial infarction independent of CRP levels and glycemic status[17]. Furthermore, it is recognized that adiponectin levels rapidly decline after acute myocardial infarction[18]. Here, we investigate whether adiponectin confers resistance to acute ischemic injury in the heart. We tested the effects of adiponectin on myocardial infarct size, apoptotic activity and inflammation with loss- and gain-of-function genetic manipulations. Our observations indicate that adiponectin is cardioprotective in the context of ischemia-reperfusion injury through both AMPK-dependent antiapoptotic actions and cyclooxygenase (COX)-2–dependent anti-inflammatory actions on cardiac cells.

RESULTS

Increased myocardial infarct size in APN-KO mice

We subjected wild-type and APN-KO mice to 30 min of left anterior descending (LAD) vessel ligation followed by 48 h of reperfusion. All mice survived the surgical induction of ischemia and reperfusion. Body weight and blood pressure did not differ between wild-type and APN-KO mice (data not shown). Representative photographs of myocardial tissues after staining with Evans blue dye to delineate area at risk (AAR) and 2,3,5-triphenyltetrazolium chloride to delineate infarct area in wild-type and APN-KO mice are shown in **Figure 1a**. The ratio of AAR to left ventricular area was the same in APN-KO and wild-type mice (**Fig. 1b**). But the ratios of infarct area to AAR

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and infarct area to left ventricular area were increased 78% and 76%, respectively, in APN-KO mice compared with those of wild-type mice. Serum creatine phosphokinase (CPK) level, an index of myocyte injury, was also significantly higher in APN-KO mice compared with wild-type mice after ischemia and 6 h of reperfusion (**Fig. 1c**).

Increased myocardial apoptosis in APN-KO mice

To investigate the extent of apoptosis in the AAR regions, we performed TUNEL staining on the different experimental groups. Representative photographs of TUNEL-positive nuclei in the heart are shown in **Figure 1d**. Quantitative analysis showed a significantly higher proportion of TUNEL-positive cells in the myocardium of APN-KO mice compared with wild-type mice after ischemia-reperfusion injury, whereas little or no TUNEL-positive cells could be detected in the hearts of wild-type or APN-KO mice after sham operation (**Fig. 1e**).

Because AMP-activated protein kinase (AMPK) is reported to protect myocytes from ischemia-reperfusion injury[19], we assessed the phosphorylation status of AMPK at threonine residue 172 in heart tissue by western blotting (**Fig. 1f**). Ischemia-reperfusion increased phosphorylation of AMPK in wild-type hearts, but this induction was markedly attenuated in the APN-KO hearts. Basal levels of AMPK phosphorylation were also reduced in the sham-operated hearts of APN-KO compared to wild-type mice. Adiponectin-activated AMPK signaling in endothelial cells is proangiogenic[20], but no difference in capillary density was detected between wild-type and APN-KO 2 d after injury (**Supplementary Fig. 1** online).

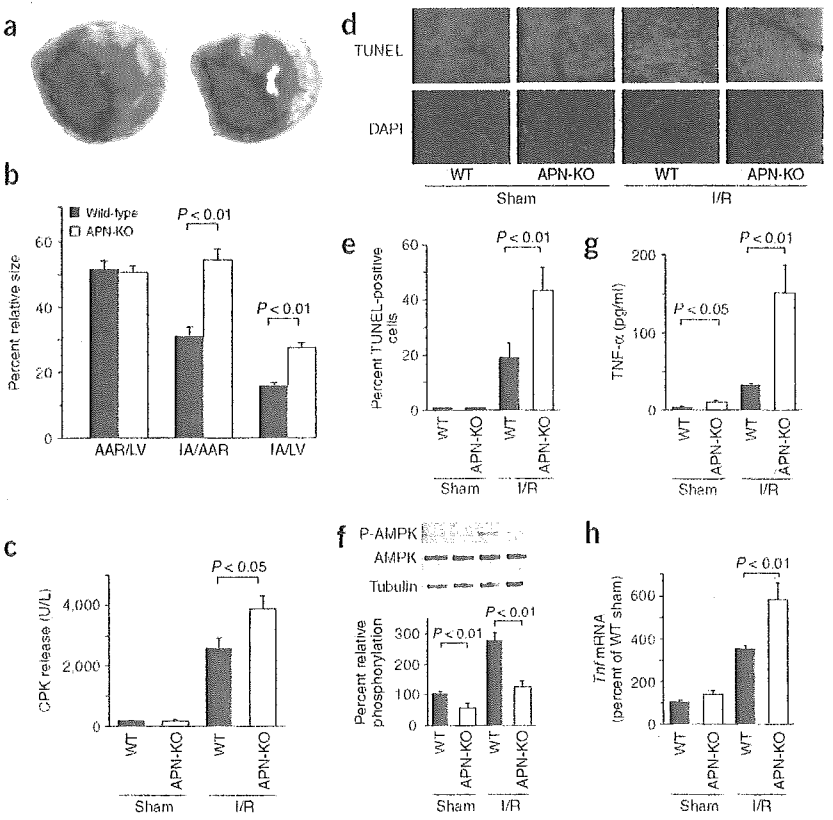

Figure 1 Increased myocardial infarction, myocardial apoptosis and TNF-α expression in APN-KO mice subjected to ischemia-reperfusion injury. **(a)** Representative pictures of myocardial tissues from wild-type (left) and APN-KO (right) mice at 48 h after ischemia-reperfusion. The nonischemic area is indicated by blue, the AAR by red and the infarct area by white. **(b)** Quantification of infarct size in wild-type ($n = 7$) and APN-KO ($n = 7$) mice. AAR/LV, ratio of AAR to left ventricular area; IA/AAR, ratio of infarct area to AAR; IA/LV, ratio of infarct area to left ventricular area. **(c)** Release of CPK from wild-type ($n = 4$) and APN-KO mice ($n = 4$) after sham operation or ischemia-reperfusion. **(d)** Representative photographs of TUNEL-stained heart sections from wild-type and APN-KO mice at 48 h after sham operation or ischemia-reperfusion. Apoptotic nuclei were identified by TUNEL staining (green) and total nuclei by DAPI counterstaining (blue). **(e)** Quantitative analysis of apoptotic nuclei from wild-type ($n = 5$) and APN-KO mice ($n = 5$) hearts after sham operation or ischemia-reperfusion. TUNEL-positive nuclei are expressed as a percentage of the total number of nuclei. **(f)** Phosphorylation of AMPK in heart tissues from wild-type and APN-KO mice at 48 h after sham operation or ischemia-reperfusion. ($n = 4$ hearts per group). **(g)** Serum levels of TNF-α, determined by ELISA, in wild-type ($n = 5$) and APN-KO ($n = 5$) mice at 48 h after sham operation or ischemia-reperfusion. **(h)** Myocardial levels of *Tnf* transcript in wild-type ($n = 5$) and APN-KO ($n = 5$) mice. *Tnf* mRNA in myocardium of wild-type and APN-KO mice were quantified by RT-PCR. Results are presented as mean ± s.d. WT, wild-type; I/R, ischemia-reperfusion.

Elevated production of TNF-α in APN-KO mice

Because inflammation contributes to myocardial injury after ischemia and reperfusion[21], we assessed serum and cardiac levels of TNF-α in each experimental group (**Fig. 1g,h**). Ischemia-reperfusion led to an increase in serum TNF-α in wild-type mice, consistent with previous reports[21], but the magnitude of this induction was greater in APN-KO than in wild-type mice (**Fig. 1g**). Basal serum TNF-α levels in sham-operated mice were mildly elevated in APN-KO compared with wild-type mice. We quantified *Tnf* mRNA (which encodes TNF-α) in heart by real-time PCR. Cardiac *Tnf* mRNA was elevated by ischemia-reperfusion injury to a greater degree in APN-KO mice than in wild-type mice (**Fig. 1h**). We also measured serum levels of interleukin (IL)-1β and IL-6 in each experimental group. Both cytokines were upregulated after ischemia-reperfusion but, in contrast to TNF-α, levels of serum IL-1β and IL-6 did not differ between APN-KO and wild-type mice (**Supplementary Fig. 2** online).

Adiponectin supplementation is cardiac protective

To test whether increased expression of adiponectin could minimize infarct area, we pretreated wild-type and APN-KO mice with adenoviral vectors expressing either adiponectin (Ad-APN) or β-galactosidase (Ad-βgal) as a control. When mice were killed, circulating adiponectin levels were 11.1 ± 1.8 μg/ml in wild-type control, 25.2 ± 4.5 μg/ml in wild-type Ad-APN–treated, <0.05 μg/ml in APN-KO control and 14.8 ± 8.1 μg/ml in APN-KO Ad-APN–treated mice. Both wild-type and APN-KO mice treated with Ad-APN showed a significant decrease in infarct area after ischemia-reperfusion compared with mice receiving the control vector (**Fig. 2a**). These data indicate that adiponectin replacement can rescue the increase in infarct size

seen in APN-KO mice and that overexpression of adiponectin can protect against myocardial injury after ischemia-reperfusion in wild-type mice.

To examine whether an increase in adiponectin levels has antiapoptotic actions *in vivo*, we assessed the viability of myocardial cells by TUNEL assay in tissue sections after ischemia-reperfusion in both wild-type and APN-KO mice treated with Ad-APN or Ad-βgal. Ad-APN treatment decreased the frequency of TUNEL-positive cells in both the wild-type and APN-KO mice (**Fig. 2b**). The reduction in myocyte apoptosis by adiponectin was associated with increases in the regulatory phosphorylation of AMPK at Thr172 in wild-type and APN-KO mice (**Fig. 2c**).

To test whether increased expression of adiponectin could decrease production of TNF-α after infarction, we determined serum levels of TNF-α and myocardial levels of *Tnf* mRNA after ischemia-reperfusion in wild-type and APN-KO mice treated with Ad-APN or Ad-βgal. Ad-APN treatment markedly decreased serum TNF-α after infarction in both wild-type and APN-KO mice (**Fig. 2d**). Ad-APN treatment also decreased *Tnf* mRNA in the hearts of both wild-type and APN-KO mice (**Fig. 2e**).

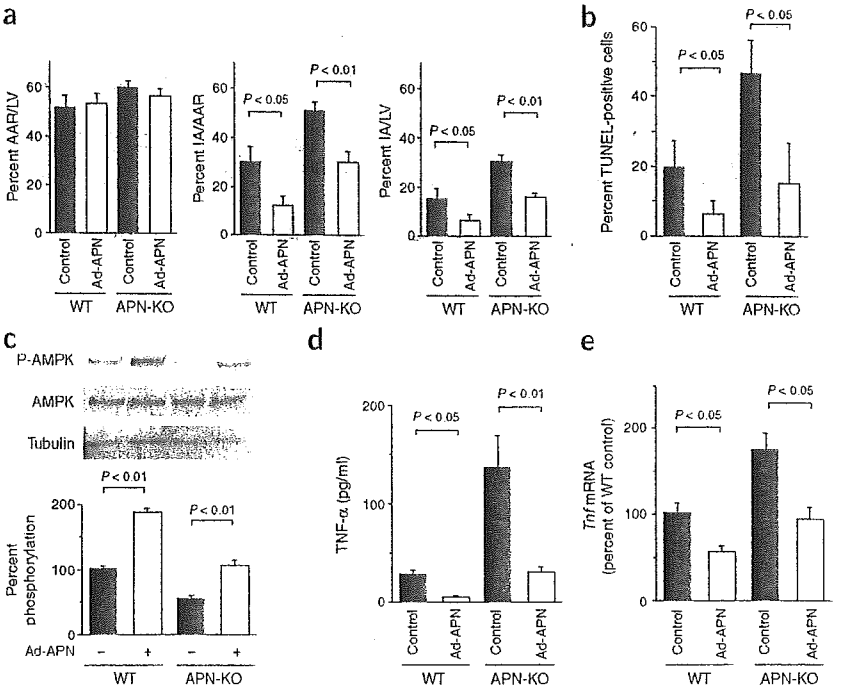

Figure 2 Adenovirus-mediated expression of adiponectin diminishes infarct size, apoptosis and TNF-α production after ischemia-reperfusion in wild-type (WT) and APN-KO mice. Ad-APN (2×10^8 plaque-forming units total) was delivered intravenously through the jugular vein 3 d before ischemia-reperfusion injury. (**a**) Quantification of infarct size in wild-type ($n = 4$) and APN-KO ($n = 4$) treated with Ad-APN or Ad-βgal (control) at 48 h after surgery. AAR/LV, ratio of AAR to left ventricular area; IA/AAR, ratio of infarct area to AAR; IA/LV, ratio of infarct area to left ventricular area. (**b**) Quantitative analysis of apoptotic nuclei from wild-type ($n = 4$) and APN-KO ($n = 4$) mice hearts treated with Ad-APN or Ad-βgal after ischemia-reperfusion. TUNEL-positive nuclei were counted in several randomly selected fields and expressed as a percentage of the total number of nuclei. (**c**) Phosphorylation of AMPK in heart tissues of wild-type and APN-KO mice treated with Ad-APN or Ad-βgal at 48 h after sham operation or ischemia-reperfusion. AMPK phosphorylation (P-AMPK) and total AMPK in myocardium was analyzed by western blotting. Phosphorylation levels of AMPK were quantified and expressed relative to untreated WT. Immunoblots were normalized to total loaded protein ($n = 4$ hearts per experimental group). (**d**) Serum levels of TNF-α, in wild-type ($n = 4$) and APN-KO ($n = 4$) mice treated with Ad-APN or Ad-βgal at 48 h after ischemia-reperfusion. (**e**) Myocardial levels of *Tnf* transcript in wild-type ($n = 4$) and APN-KO ($n = 4$) mice treated with Ad-APN or Ad-βgal at 48 h after ischemia-reperfusion. Results are presented as mean ± s.d.

Adiponectin inhibits apoptosis through AMPK

To analyze the antiapoptotic actions of adiponectin at a cellular level, primary cultures of neonatal rat ventricular myocytes or fibroblasts were deprived of serum under conditions of normoxia or hypoxia-reoxygenation in the presence or absence of recombinant adiponectin. We examined TUNEL-positive cells after 48 h of serum deprivation or after 12 h of hypoxia followed by 24 h of reoxygenation under conditions of serum deprivation (**Fig. 3a**). Pretreatment with adiponectin diminished the frequency of TUNEL-positive cells under normoxic conditions by 69% in cardiomyocytes and by 45% in cardiac fibroblasts (**Fig. 3b**). Hypoxia-reoxygenation increased the frequency of TUNEL-positive cardiomyocytes and cardiac fibroblasts. Pretreatment with adiponectin suppressed the frequency of TUNEL-positive cells under conditions of hypoxia-reoxygenation by 47% in cardiomyocytes and by 62% in cardiac fibroblasts. To test whether AMPK signaling was involved in the antiapoptotic actions of adiponectin, we pretreated cultured cardiac cells with an adenoviral vector expressing a dominant negative mutant of AMPK (Ad-dnAMPK). Transduction with Ad-dnAMPK effectively suppressed adiponectin-induced phosphorylation of acetyl-CoA carboxylase, a downstream target of AMPK, in both cardiac myocytes and fibroblasts (data not shown), and reversed the inhibitory effects of adiponectin on apoptosis under conditions of serum deprivation and hypoxia-reoxygenation in both cell types (**Fig. 3c**). In contrast, treatment with the COX-2 inhibitor NS398 had no effect on the protective actions of adiponectin on apoptosis of cardiac myocytes or fibroblasts (**Supplementary Fig. 3** online).

Adiponectin inhibits TNF-α production through COX-2

Cardiac cells produce TNF-α when stimulated with lipopolysaccharide (LPS)[22]. To test the effects of adiponectin on production of TNF-α, we subjected cultured neonatal cardiac myocytes and fibroblasts to stimulation with LPS for 6 h in the presence or absence of adiponectin. We determined the accumulation of TNF-α in the culture medium by ELISA. Exposure to LPS increased the secretion of TNF-α by 42-fold in cardiomyocytes and 15-fold in cardiac fibroblasts, and pretreatment with adiponectin markedly inhibited LPS-induced production of TNF-α in both cell types (**Fig. 4a** and **Supplementary Fig. 4** online). In contrast to the effects on cell viability, transduction with Ad-dnAMPK had little or no effect on LPS-induced production of TNF-α in either cell type.

Prostaglandin E_2 (PGE_2) inhibits LPS-induced production of TNF-α in monocytic cells[23,24]. Therefore, we assessed whether adiponectin regulates the synthesis of PGE_2 in cardiac cells. Adiponectin stimulated the production of PGE_2 in both myocytes and fibroblasts (**Fig. 4b** and **Supplementary Fig. 4** online). Adiponectin-stimulated production of PGE_2 was inhibited by the selective COX-2 inhibitor

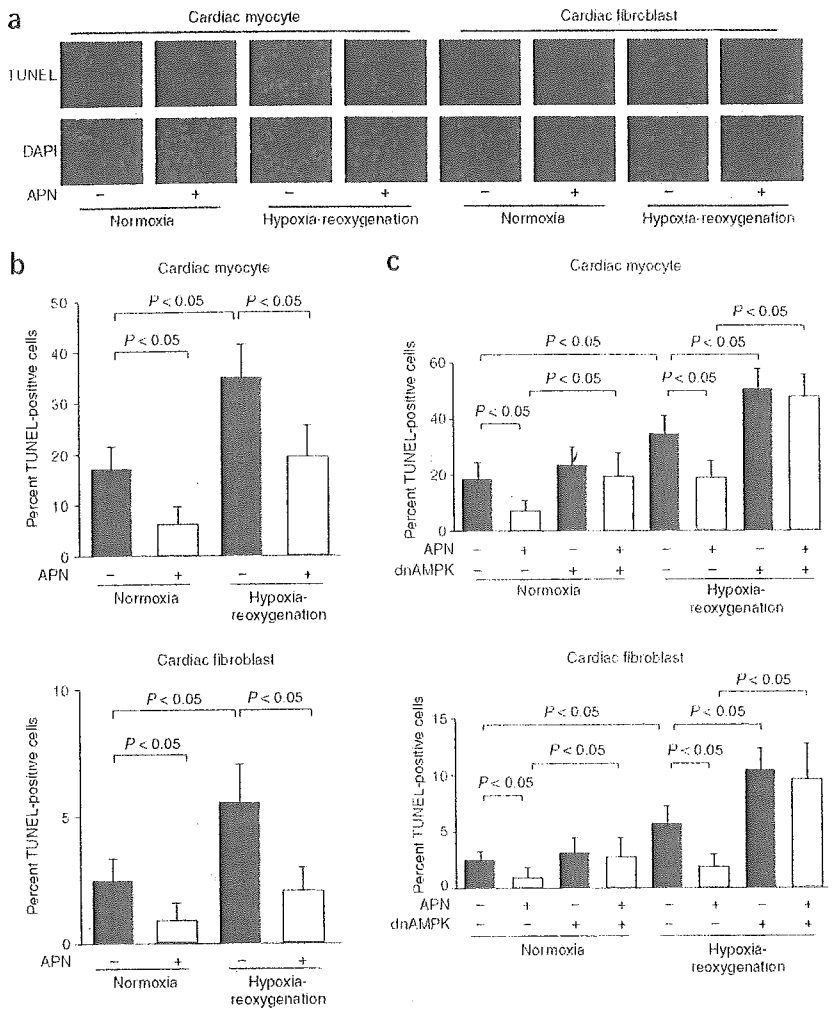

Figure 3 AMPK-dependent inhibition of cardiac myocyte and fibroblast apoptosis by adiponectin. Rat neonatal myocytes and fibroblasts were treated with adiponectin (30 μg/ml) or vehicle in serum-free media for 48 h under normoxic conditions or 12 h under hypoxic conditions followed by 24 h of reoxygenation. (a) Representative photomicrographs of TUNEL-positive cardiac myocytes and fibroblasts. Apoptotic nuclei were identified by TUNEL staining (green) and total nuclei by DAPI counterstaining (blue). (b) Quantitative analysis of TUNEL-positive cells under conditions of normoxia or hypoxia-reoxygenation with adiponectin (APN) or vehicle. TUNEL-positive nuclei were counted in several randomly selected fields and expressed as a percentage of the total number of nuclei. (c) Effect of Ad-dnAMPK on adiponectin inhibition of myocyte and fibroblast apoptosis under conditions of normoxia or hypoxia-reoxygenation. Cells were transduced with Ad-dnAMPK or Ad-βgal (control) for 24 h and then treated with adiponectin or vehicle under conditions of normoxia or hypoxia-reoxygenation. Results are presented as mean ± s.d. ($n = 3$).

NS398 but not by transduction with Ad-dnAMPK. LPS increased production of PGE_2 in cardiac neonatal myocytes and fibroblasts, consistent with observations in other cell types[25], and adiponectin further augmented the production of PGE_2 under these conditions in a COX-2–dependent, AMPK-independent manner (**Fig. 4b** and **Supplementary Fig. 4** online). In separate experiments, exogenous PGE_2 at a concentration of 350 pg/ml inhibited LPS-induced TNF-α production by 91% ± 4% in myocyte cultures (data not shown), indicating that functionally relevant levels of PGE_2 are produced in response to adiponectin stimulation *in vitro*. Adiponectin also increased the expression of COX-2, the rate-limiting step for PGE_2 synthesis, in both basal and LPS-stimulated myocytes and fibroblasts (**Fig. 4c** and **Supplementary Fig. 4** online). This induction was not suppressed by transduction with Ad-dnAMPK in either cell type. Finally, we also examined the effects of adiponectin on this regulatory system in cultures of rat cardiac myocytes prepared from adult left ventricle. Adiponectin inhibited LPS-induced production of TNF-α, enhanced production of PGE_2 in the presence or absence of LPS and increased basal and LPS-stimulated expression of COX-2 (**Supplementary Fig. 5** online).

The cardioprotective actions of PGE_2 are mediated, at least in part, by the EP_4 receptor that is highly expressed in heart[26]. Thus, to test whether EP_4 participates in the inhibitory effect of adiponectin on secretion of TNF-α, we treated neonatal cardiac myocytes or fibroblasts with the EP_4 receptor–selective antagonist AH23848 and then assessed LPS-induced production of TNF-α. AH23848 reversed the inhibitory actions of adiponectin on LPS-induced secretion of TNF-α from both myocytes and fibroblasts (**Fig. 4d** and **Supplementary Fig. 4** online). The COX-2 inhibitor NS398 also blocked the suppressive effect of adiponectin on LPS-induced secretion of TNF-α from myocytes and fibroblasts. Collectively, these data suggest that adiponectin suppresses LPS-induced secretion of TNF-α through a COX-2–PGE_2–EP_4–dependent pathway that is independent of AMPK signaling.

COX-2 contributes to the protective actions of adiponectin

To examine the role of COX-2 signaling in the cardioprotective action of adiponectin *in vivo*, we administered daily intraperitoneal injections of the COX-2 inhibitor NS398 (5 mg/kg) from 3 d before surgery until mice were killed. Although NS398 did not affect infarct size in Ad-βgal–treated wild-type and APN-KO mice, it abrogated the infarct-sparing actions of exogenous adiponectin by 53% in wild-type and 48% in APN-KO mice (**Fig. 5a**). Consistent with the results from LPS-stimulated cardiac myocyte and fibroblast cultures (**Fig. 4d** and **Supplementary Fig. 4** online), NS398 significantly reversed the suppressive effect of adiponectin on serum levels of TNF-α after infarction in both wild-type and APN-KO mice (**Fig. 5b**). Treatment with NS398 also abrogated the adiponectin-induced decrease in TUNEL-positive cells in wild-type and APN-KO mice (**Fig. 5c**). NS398 did not affect apoptosis or serum levels of TNF-α in Ad-βgal–treated wild-type or APN-KO mice. These data indicate that COX-2–dependent signaling contributes to the protective action of adiponectin in myocardial ischemia-reperfusion injury through the suppression of inflammatory cytokines and improved cell survival.

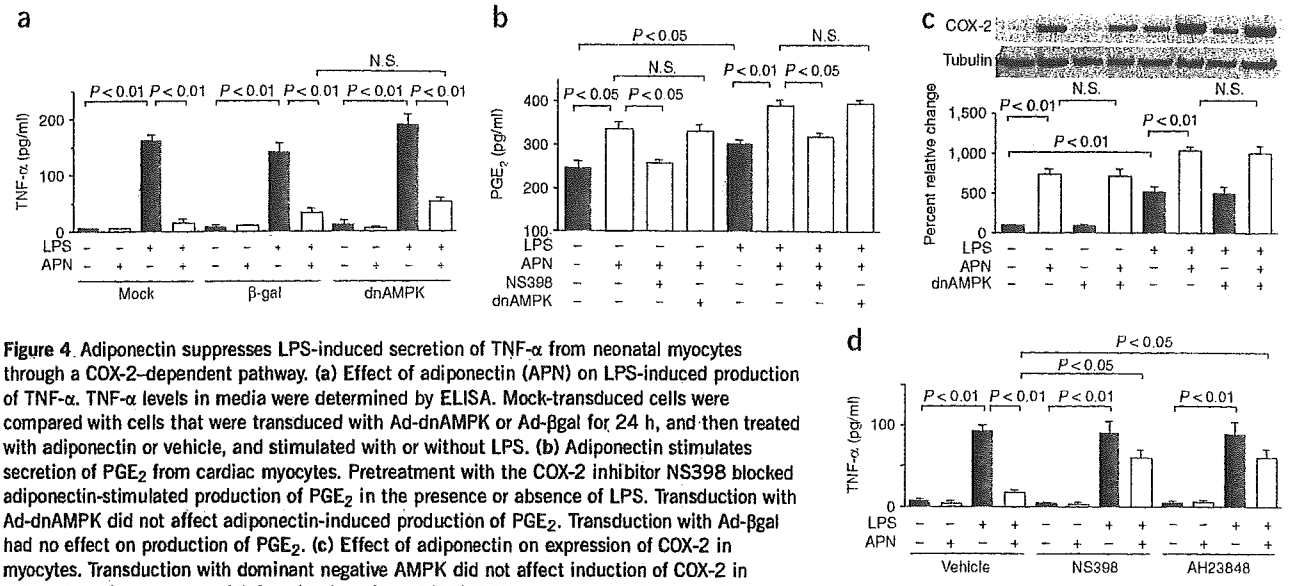

Figure 4 Adiponectin suppresses LPS-induced secretion of TNF-α from neonatal myocytes through a COX-2–dependent pathway. **(a)** Effect of adiponectin (APN) on LPS-induced production of TNF-α. TNF-α levels in media were determined by ELISA. Mock-transduced cells were compared with cells that were transduced with Ad-dnAMPK or Ad-βgal for 24 h, and then treated with adiponectin or vehicle, and stimulated with or without LPS. **(b)** Adiponectin stimulates secretion of PGE_2 from cardiac myocytes. Pretreatment with the COX-2 inhibitor NS398 blocked adiponectin-stimulated production of PGE_2 in the presence or absence of LPS. Transduction with Ad-dnAMPK did not affect adiponectin-induced production of PGE_2. Transduction with Ad-βgal had no effect on production of PGE_2. **(c)** Effect of adiponectin on expression of COX-2 in myocytes. Transduction with dominant negative AMPK did not affect induction of COX-2 in cultured cardiac myocytes. **(d)** Contribution of the COX-2–PGE_2 pathway to adiponectin inhibition of LPS-induced production of TNF-α from myocytes. Cells were pretreated with a EP_4-selective antagonist, AH23848, NS398 or vehicle, treated with adiponectin or vehicle and stimulated with or without LPS. Results are presented as mean ± s.d. (n = 3–5). N.S., not statistically significant.

Recombinant adiponectin protein minimizes infarct size

To test whether administration of adiponectin could minimize infarct area before or after ischemia-reperfusion, we administered recombinant adiponectin (1.0 μg/g) to wild-type mice either 30 min before the induction of ischemia, during ischemia or 15 min after reperfusion. The administration of adiponectin at any of these time points led to a reduction in infarct size relative to control mice (**Fig. 5d**). To examine the effect of adiponectin on hemodynamic properties, we measured left ventricular end-diastolic pressure (LVEDP) and the derivative of left ventricular pressure (dP/dt) using a micromanometer-tipped catheter at 24 h after ischemia-reperfusion surgery in wild-type mice that had received 1.0 μg/g adiponectin or vehicle 15 min before ischemia. Whereas control mice showed a marked elevation in LVEDP, the increase in LVEDP diminished in the adiponectin-treated animals (**Fig. 5e**). Furthermore, pretreatment with adiponectin increased dP/dt_{max} and decreased dP/dt_{min} at 24 h after ischemia-reperfusion (**Fig. 5f**). Finally, to test whether adiponectin affects echocardiographic parameters, we measured left ventricular fractional shortening by echocardiography on day 7 after operation in wild-type mice (**Fig. 5g**). Treatment with adiponectin before ischemia-reperfusion led to a statistically significant increase in fractional shortening, indicative of improved myocardial remodeling.

DISCUSSION

Our data provide evidence that adiponectin confers resistance to acute myocardial damage. Adiponectin-deficient mice showed increased infarct size after ischemia-reperfusion, whereas exogenous adiponectin reduced infarct size in both adiponectin-deficient and wild-type mice. It has been shown in men that plasma adiponectin is a marker of risk for myocardial infarction[17]. The current study suggests that this reduction in adiponectin level is a causal factor that contributes to the severity of infarction. Adiponectin protects the heart from injury in response to ischemia-reperfusion through at least two mechanisms: improvements in viability of myocardial cells and suppression of cardiac production of TNF-α (**Fig. 5h**). The antiapoptotic action of adiponectin is likely to be mediated by the direct activation of AMPK signaling within cardiac myocytes. Consistent with this hypothesis, *ex vivo* experiments have shown that the expression of dominant negative AMPK in the heart from a cardiac myocyte–specific promoter leads to increased apoptosis and cardiac dysfunction after ischemia-reperfusion injury[19]. Furthermore, here we show that the level of activated AMPK was reduced in the hearts of APN-KO mice. Transduction with dominant negative AMPK abrogated the antiapoptotic activities of adiponectin in cardiac myocytes in response to serum deprivation and hypoxia-reoxygenation *in vitro*. Fibroblasts in the heart also participate in remodeling after ischemia[27], and here we show that adiponectin also protected cardiac fibroblasts from apoptosis through an AMPK-dependent mechanism. Adiponectin stimulation inhibits apoptosis of endothelial cells through an AMPK-dependent mechanism[20,28], and this feature could also contribute to the protective action of adiponectin. But we detected no significant difference in capillary density between wild-type and APN-KO mice 2 d after ischemia-reperfusion injury, suggesting that the effect of adiponectin on infarct size is not mediated by the angiogenic properties of this cytokine at this early time point. Finally, administration of adiponectin reduced CPK release into the circulation, indicative of diminished myocardial necrosis. This effect may be mediated by the ability of adiponectin to increase AMPK-induced glucose transport[29].

The mechanisms by which adiponectin suppress inflammatory reactions are poorly understood. Here we show that adiponectin functions to suppress myocardial production of TNF-α *in vitro* and *in vivo*. The increased production of proinflammatory cytokines is an important component of postischemic myocardial injury[30,31]. Studies have shown that TNF-α–deficient mice have decreased myocardial damage in response to ischemia-reperfusion injury and that treatment with TNF-α–specific antibody limits the damage caused by acute myocardial injury in rat hearts *ex vivo*[32,33]. Clinically, the reduction of plasma adiponectin levels after acute myocardial infarction is negatively correlated with plasma CRP levels[18], suggesting that

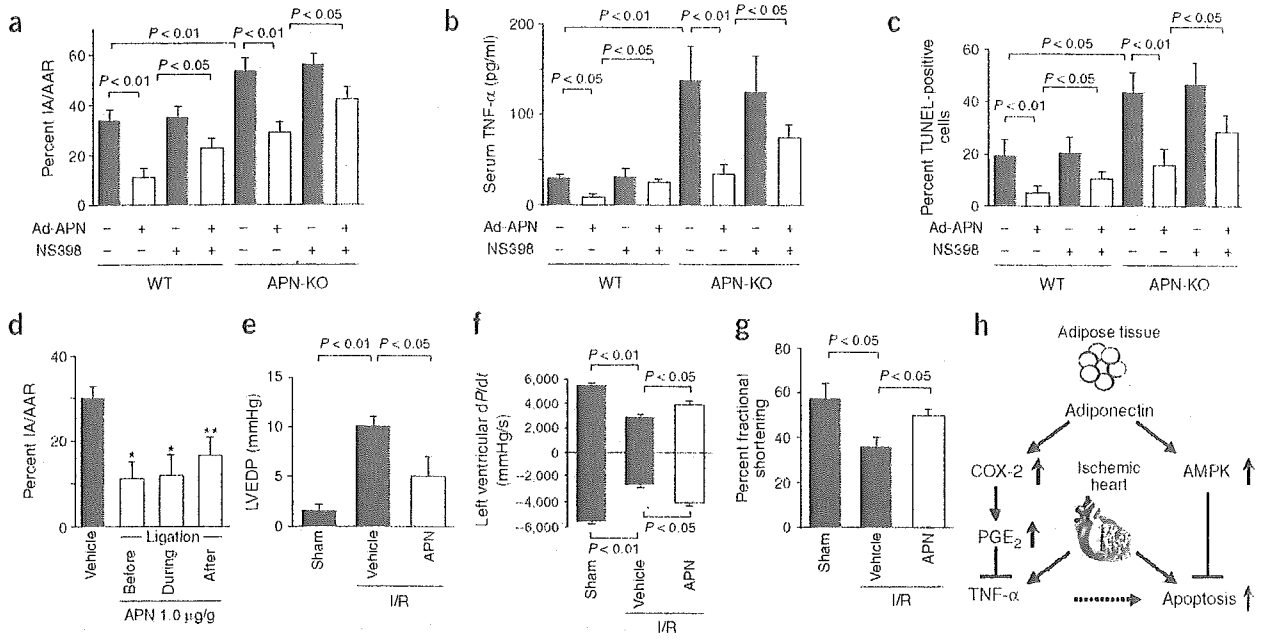

Figure 5 Inhibition of COX-2 partially prevents the protective actions of adiponectin on myocardial infarct size after ischemia-reperfusion injury in wild-type (WT) and APN-KO mice. **(a)** The COX-2 inhibitor NS398 was injected intraperitoneally from 3 d before ischemia-reperfusion injury until mice were killed, and Ad-APN or Ad-βgal was injected into the jugular vein 3 d before surgery. Infarct size in the heart tissue was quantified ($n = 5$ mice per experimental group). IA/AAR, ratio of infarct area to AAR. **(b)** NS398 reversed the suppressive effect of adiponectin on serum levels of TNF-α, after infarction in both wild-type (WT) and APN-KO mice. **(c)** NS398 increased the frequencies of TUNEL-positive cells after ischemia-reperfusion in Ad-APN–treated wild-type and APN-KO mice. **(d)** Administration of recombinant adiponectin minimized the effects of ischemia-reperfusion on infarct size and heart function. Quantification of infarct size in wild-type mice treated with recombinant adiponectin before, during and after ischemic injury ($n = 5$ per group). $^{*}P < 0.01$, $^{**}P < 0.05$ versus vehicle. **(e)** Effect of recombinant adiponectin on left ventricular end-diastolic pressure (LVEDP) in wild-type mice at 24 h after sham operation or ischemia-reperfusion. Recombinant adiponectin (1.0 μg/g) or vehicle was injected in wild-type mice before ischemia-reperfusion ($n = 5$). **(f)** Left ventricular dP/dt in wild-type mice ($n = 5$) treated with adiponectin (1.0 μg/g) or vehicle at 24 h after sham operation or ischemia-reperfusion. **(g)** Left ventricular fractional shortening assessed by echocardiography in wild-type ($n = 4$) treated with recombinant adiponectin (1.0 μg/g) at 7 d after ischemia-reperfusion. **(h)** Adiponectin protects the myocardium from cardiac injury in response to ischemia by protecting cardiac cells from apoptosis through activation of AMPK signaling and by the suppression of cardiac production of TNF-α by the activation of the COX-2–PGE_2 pathway.

hypoadiponectinemia is associated with an increased inflammatory response to acute myocardial ischemia. We found that adiponectin deficiency resulted in markedly higher TNF-α levels in the serum and heart tissue after ischemia-reperfusion injury, whereas elevated adiponectin expression reduced serum and myocardial TNF-α levels in both APN-KO and wild-type mice. Adiponectin also suppressed LPS-induced production of TNF-α in cultured cardiac myocytes and fibroblasts. This anti-inflammatory action was independent of AMPK signaling.

Our results indicate that the inhibitory action of adiponectin on myocardial production of TNF-α results from an activation of the COX-2–PGE_2–EP_4 pathway. COX-2 has important protective roles in the regulation of myocardial damage after ischemia-reperfusion injury[34,35], and it could function to limit oxidative damage in the heart[36]. Furthermore, the COX-2 metabolite PGE_2 protects hearts from ischemia-reperfusion injury, an effect that is mediated by the EP_3 and EP_4 receptor subtypes[26,37]. We found that adiponectin increased expression of COX-2 and release of PGE_2 from myocytes and fibroblasts in an AMPK-independent manner. COX-2 also produces PGI_2, which is reported to have cardioprotective effects against ischemia-reperfusion injury[38].

The protective action of adiponectin overexpression on myocardial infarct size was inhibited when we administered a COX-2 inhibitor to either wild-type or APN-KO mice. Abrogation of adiponectin-protective actions by inhibition of COX-2 was associated with increases in apoptosis and production of TNF-α. In contrast, *in vitro* studies showed that the antiapoptotic actions of adiponectin were not reversed by inhibition of COX-2. These data suggest a complex interplay between COX-2–dependent production of TNF-α and cell-death pathways in the heart that are not reflected by experiments with cultured cardiac cells (**Fig. 5h**). In this regard, production of TNF-α after ischemia-reperfusion injury has been shown to have a major role in apoptosis and myocardial damage[39,40]. Presumably, the proapoptotic actions of COX-2 inhibition are only observed *in vivo* because cardiac levels of TNF-α are higher than those produced in the cell-culture experiments or because a large portion of the apoptosis observed *in vivo* is the consequence of inflammatory cell infiltration in response to this cytokine.

Notably, inhibition of COX-2 had no detectable effect on infarct size in untreated mice. These data are consistent with reports showing that although COX-2 is required for late preconditioning, treatment with COX-2 inhibitors does not result in a statistically significant increase in infarct size[34,41,42]. The inability of COX-2 inhibitors to influence infarct size in untreated mice could result from the decline in adiponectin levels that occurs in response to acute myocardial injury. In our studies, adiponectin levels declined 32% after ischemia-reperfusion injury (data not shown), which is comparable to the 29% decrease found in men 72 h after a myocardial infarction[18].

Recent clinical trials indicate that treatment with selective COX-2 inhibitors results in an increased risk for myocardial infarction and other serious cardiovascular events[43,44]. The adverse effects of COX-2 inhibitors on cardiovascular events are thought to result from a perturbation of vascular homeostasis due to reduced synthesis of prostacyclin[45]. Our data suggest that inhibition of COX-2 could also contribute to the severity of myocardial infarction by interfering with the protective actions of adiponectin on cardiac myocytes.

Previous studies have shown that adiponectin might have potential utility for the treatment of a variety of chronic diseases including obesity[46], insulin resistance[47,48] and hypertrophic cardiomyopathy[16]. But adiponectin is an abundant serum protein and its long-term administration could be problematic. Here, it is shown that adiponectin can limit the damage from an acute myocardial infarction, suggesting that the short-term administration of this factor may have practical clinical utility.

METHODS

Materials. We purchased phosphorylated AMPK (Thr172) and pan-α-AMPK from Cell Signaling Technology. We purchased COX-2–specific antibody and NS398 from Cayman Chemical Co; tubulin-specific antibody from Oncogene; phosphorylated ACC (Ser79), ACC and c-Myc–specific tag antibody from Upstate Biotechnology. We purchased LPS of *E. coli* 0127 and AH23848 from Sigma Chemical Co. We prepared recombinant mouse adiponectin in *E. coli* as described previously[20]. Adenovirus vectors containing the gene for β-galactosidase (Ad-βgal), full-length mouse adiponectin (Ad-APN) and dominant negative AMPKα2 (Ad-dnAMPK) were described previously[14,49].

Mouse model of myocardial ischemia-reperfusion. Studies using APN-KO and wild-type mice in a C57BL/6 background were approved by the Institutional Animal Care and Use Committee at Boston University[14]. We anesthetized 10–12-week-old mice with sodium pentobarbital. We cannulated the trachea with a polyethylene tube connected to a respirator with a tidal volume of 0.6 ml (110 breaths/min). We performed left thoracotomy between the fourth and fifth ribs. We removed the pericardial tissue and visualized the LAD artery under a microscope and ligated it with 8-0 silk suture using a snare occluder. We subjected mice to 30 min of LAD ligation followed by 48 h of reperfusion. In some experiments, we injected 2×10^8 plaque-forming units of Ad-APN or Ad-βgal into the jugular vein of mice 3 d before the ischemia-reperfusion injury. We determined mouse adiponectin levels by ELISA kit (Otsuka Pharmaceutical Co. Ltd.)[16]. In some experiments, we intraperitoneally injected the COX-2 inhibitor NS398 (5 mg/kg/d) or vehicle (dimethylsulfoxide) into the abdomen of the APN-KO and wild-type mice from 3 d before ischemia-reperfusion injury and until the mice were killed. In other experiments, we injected recombinant adiponectin or PBS vehicle into the jugular vein of mice at 30 min before LAD ligation, after 15 min of LAD ligation or 15 min after reperfusion. We performed hemodynamic measurements after 24 h of reperfusion using a 1.4F catheter tip micromanometer (ARIA, Millar Instruments) inserted through the right carotid artery into the left ventricular cavity. We analyzed the first derivative of left ventricular pressure (dP/dt) using Power Lab SP Software (Ad Instruments). We performed echocardiography with an Acuson Sequoia C-256 machine using a 15-MHz probe. We calculated fractional shortening as (LVEDD – LVESD)/LVEDD × 100 and expressed the result as a percentage. LVEDD is left ventricular end diastolic dimension and LVESD is left ventricular end systolic dimension.

Determination of area at risk and infarct size. We reoccluded the LAD artery, and injected 1 ml of 1.0% Evans blue (Sigma Chemical Co.) through the jugular vein to delineate the nonischemic tissue. We then excised the heart, washed it with PBS and cut it into four transverse slices. We stained slices for 5 min at 23 °C with 1.0 ml of 1.5% 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co.) to determine infarct area. We weighed sections and photographed them under a microscope. We determined left ventricular area, AAR and infarct area by computerized planimetry using Image J software. We expressed infarct area as a percentage of the AAR and left ventricular area.

Analysis of myocardial injury and apoptosis. We assessed an index of myocyte injury by determining the release of CPK (Catachem Inc.). We collected blood from tail veins at 6 h after operation. We qualitatively analyzed myocardial apoptosis by TUNEL staining as previously described[50]. We examined five randomly chosen microscopic fields from four different sections in each tissue block for each mouse specimen.

Measurement of TNF-α. We quantified plasma TNF-α with the use of ELISA kits (R&D Systems). We quantified *Tnf* mRNA in myocardium by real-time PCR. We prepared total RNA using a Qiagen kit. We produced cDNA using ThermoScript RT-PCR Systems (Invitrogen). We performed PCR on iCycler iQ Real-Time PCR Detection System (BIO-RAD) using SYBR Green I as a double-standard DNA-specific dye (Applied Biosystems)[9]. We used the following primers: 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, and 5′-TGGGAGTAGA CAAGGTACAACCC-3′ for *Tnf* and 5′-TCACCACCATGGAGAAGGC-3′ and 5′-GCTAAGCAGTTGGTGGTGCA-3′ for *Gapdh*.

Cell culture and adiponectin treatment. We prepared primary cultures of neonatal and adult rat ventricular myocytes as described previously. We cultured neonatal myocytes in DMEM containing 7% FCS[16]. We cultured adult myocytes in ACTT medium. We cultured neonatal rat ventricular nonmyocytes, which are predominantly fibroblasts, in DMEM containing 1% FCS and passaged them with trypsin-EDTA. We examined cell number and TUNEL-positive cells after serum deprivation for 48 h under normoxic conditions or 12 h of hypoxia (<1% O_2 and 5% CO_2, 37 °C) followed by 24 h of reoxygenation (21% O_2 and 5% CO_2, 37 °C) in the presence or absence of recombinant adiponectin (30 μg/ml). We generated hypoxia by using a GasPak Plus system (Becton Dickinson). We pretreated myocyte cultures for 18 h in the presence or absence of bacterially produced adiponectin[20] (30 μg/ml) and subjected them to LPS stimulation (1 μg/ml) for 6 h. In other experiments, we preincubated the cells with AH23848 (100 μM), NS398 (2 μM) or vehicle for 30 min before adiponectin treatment. We infected cells with Ad-βgal and Ad-dnAMPK at a multiplicity of infection of 50 for 24 h before treatments. We measured levels of TNF-α, IL-1β and IL-6 using ELISA (R&D Systems). We also determined PGE_2 concentrations by ELISA (Cayman Chemical Co.).

Western blot analysis. We homogenized tissue samples obtained at 48 h after surgery and separated proteins (30 μg) with denaturing SDS 10% polyacrylamide gels. After transfer to membranes, we performed immunoblot analysis with the indicated antibodies at a 1:1,000 dilution. This was followed by incubation with secondary antibody conjugated with horseradish peroxidase at a 1:5,000 dilution. We used the ECL-PLUS Western Blotting Detection kit (Amersham Pharmacia Biotech) for detection. We normalized the relative changes to the tubulin signal and expressed them as percent relative to control.

Statistical analysis. Data are presented as mean ± s.d. We performed statistical analysis using Scheffe F test or analysis of variance to determine group differences. A value of $P < 0.05$ was accepted as statistically significant.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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