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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). $LVFS=(LVEDd-LVESd)/LVEDd*100$, $LVEF=[(LVEDd)^3-(LVESd)^3]/(LVEDd)^3*100$.

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.

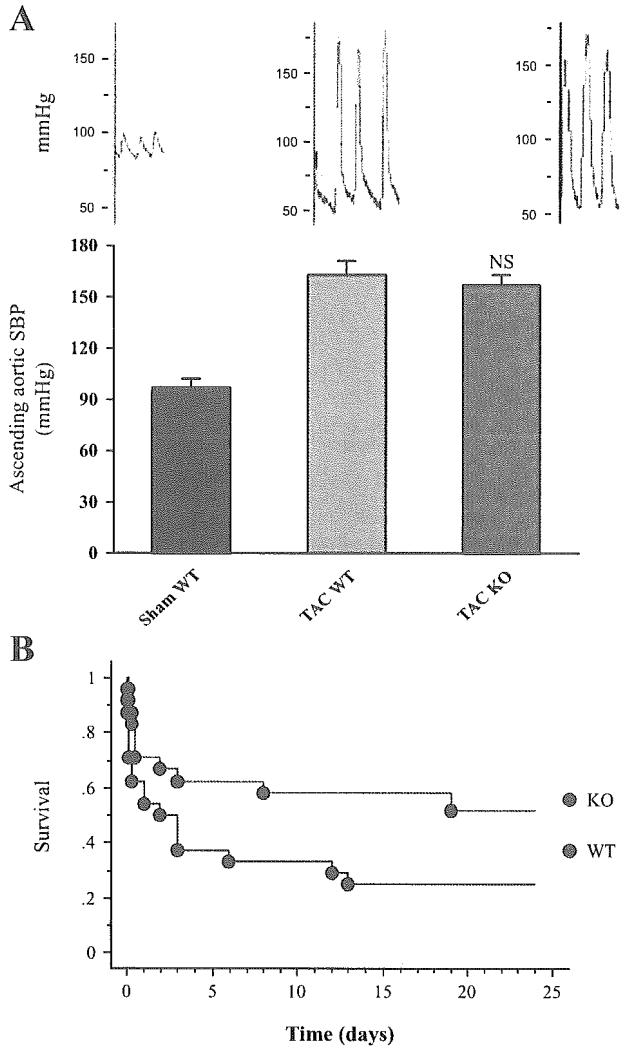

Fig. 1. Left ventricular pressure overload and survival. A) The ascending aortic systolic pressure measured with a 1.4 F catheter was similar in adiponectin KO and WT mice. NS: not significant vs. TAC WT. B) Kaplan–Meier survival analysis showed a significant higher mortality in adiponectin KO mice after 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

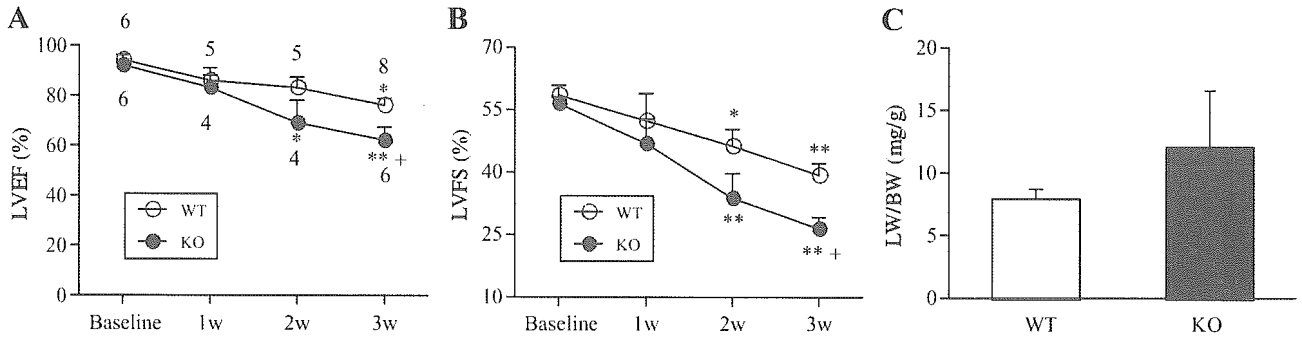

Fig. 2. The transition from hypertrophy to heart failure. A) Left ventricular ejection fraction (LVEF) and B) left ventricular fractional shortening (LVFS) were progressively depressed in adiponectin KO mice after 1 week of TAC, and the transition to heart failure occurred at 2 weeks after TAC in KO 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,

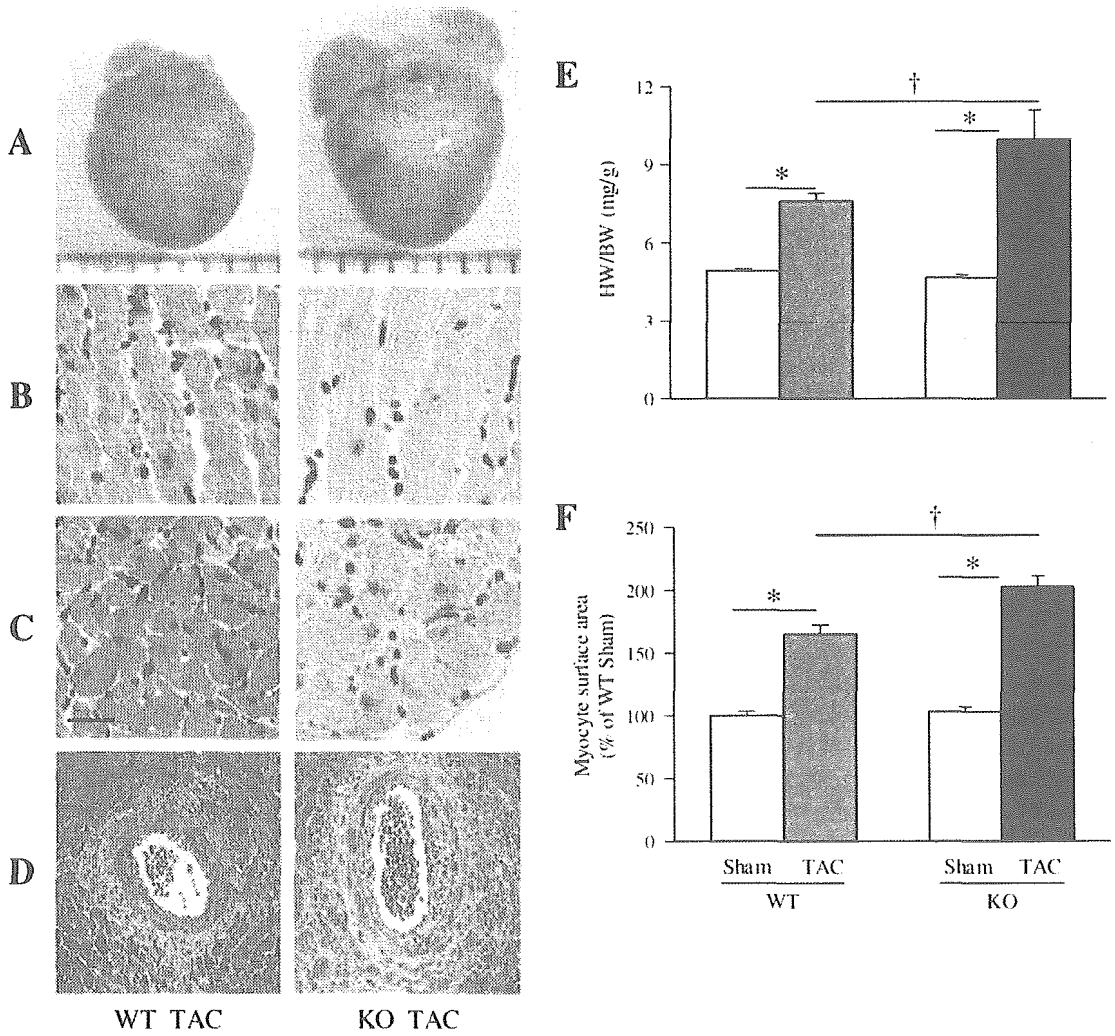

Fig. 3. Cardiac remodeling was more severe in KO mice. A) Representative pictures of cardiac hypertrophy in WT and KO mice at 3 weeks after TAC. B and C) Represent long-axis and cross-sectional views of cardiac myocytes with HE staining. D) Represents cardiac fibrosis with Azan staining (×100 magnification). 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. *$P<0.01$, †$P<0.05$. Bar=20 μm for B and C.

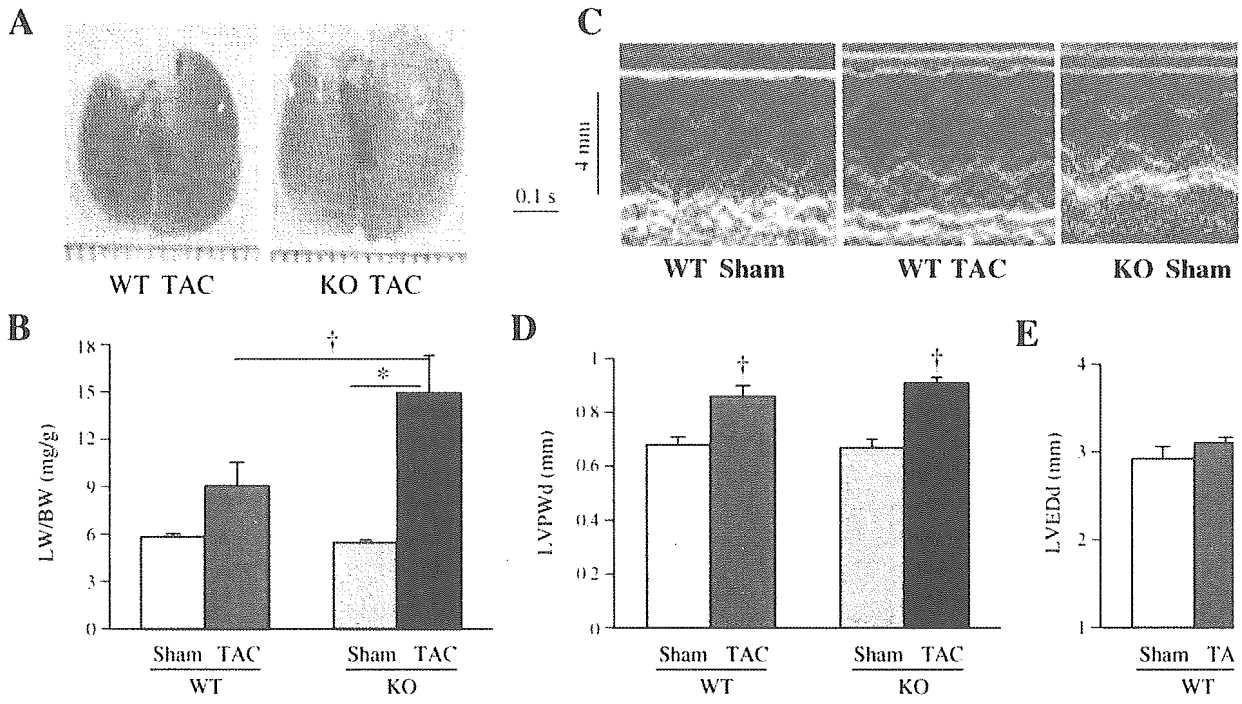

Fig. 4. Pulmonary congestion and echocardiographic findings at 3 weeks after TAC. The lungs of an adiponectin KO mouse were markedly enlarged compared with ratio (LW/BW) was markedly increased in KO mice compared with WT mice (B). *$P<0.01$, †$P<0.05$. Echocardiography (C) shows that the LV posterior wall di 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. †$P<$(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

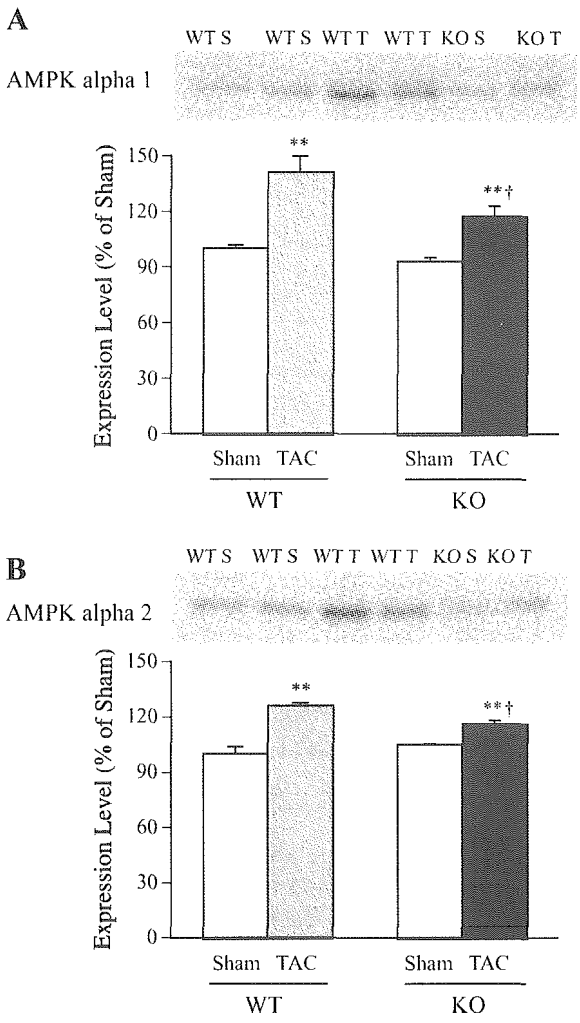

Fig. 5. Myocardial expression of AMPK. AMPKα_1 (A), α_2 (B) were increased in TAC mice, but the change was smaller in KO mice ($n=3$ in each group, $^{**}P<0.01$ vs. responding sham mice; $\dagger P<0.05$ vs. WT TAC). S: sham, T: TAC.

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

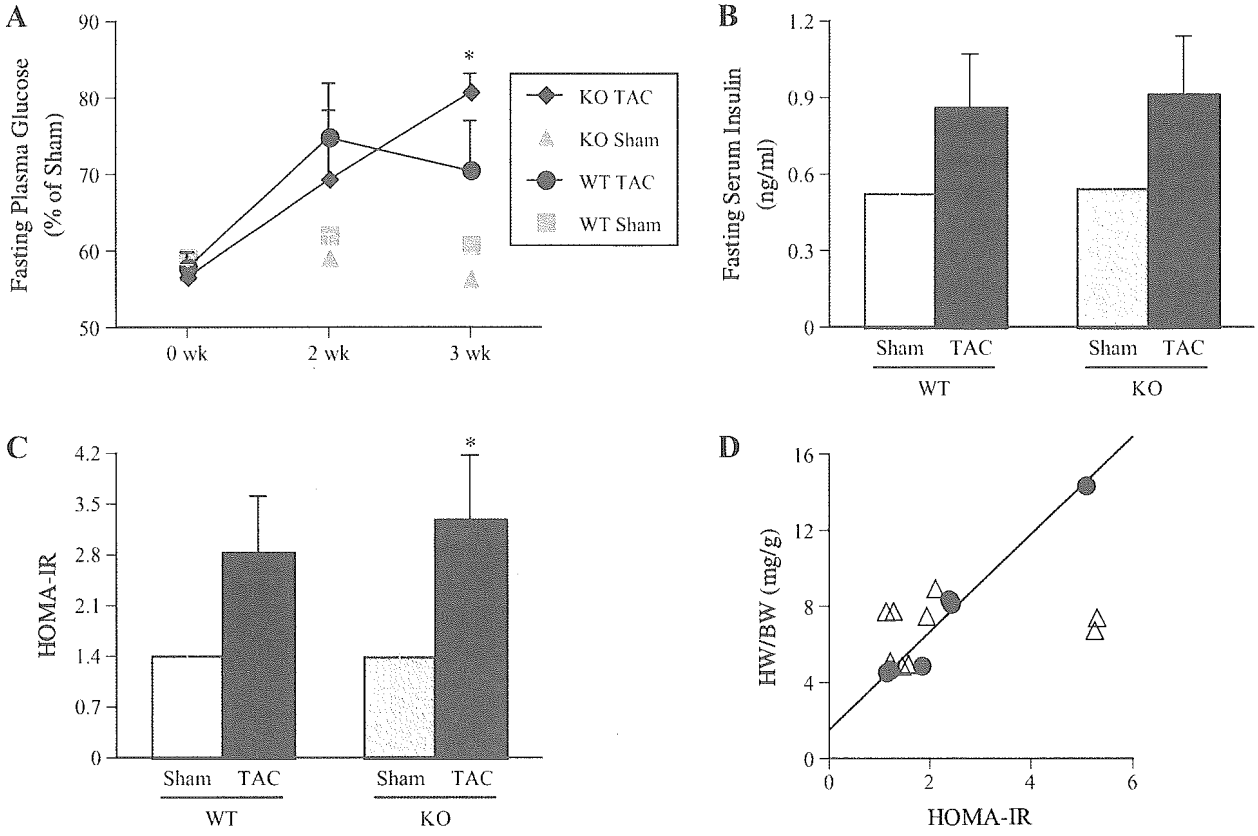

Fig. 6. Changes in glucose metabolism. Fasting glucose levels (A) were increased in adiponectin KO mice at 3 weeks after the onset of TAC, $*P<0.01$ vs. WT TAC ($n=5$ for all the groups at 0 week and for both sham groups at other two time points; $n=4$ for WT and KO TAC mice at 2 weeks, and $n=5$ and 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.

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Overexpression of Mitochondrial Transcription Factor A Ameliorates Mitochondrial Deficiencies and Cardiac Failure After Myocardial Infarction

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Background—Mitochondrial DNA (mtDNA) copy number is decreased not only in mtDNA-mutation diseases but also in a wide variety of acquired degenerative and ischemic diseases. Mitochondrial transcription factor A (TFAM) is essential for mtDNA transcription and replication. Myocardial mtDNA copy number and TFAM expression both decreased in cardiac failure. However, the functional significance of TFAM has not been established in this disease state.

Methods and Results—We have now addressed this question by creating transgenic (Tg) mice that overexpress human *TFAM* gene and examined whether TFAM could protect the heart from mtDNA deficiencies and attenuate left ventricular (LV) remodeling and failure after myocardial infarction (MI) created by ligating the left coronary artery. *TFAM* overexpression could ameliorate the decrease in mtDNA copy number and mitochondrial complex enzyme activities in post-MI hearts. Survival rate during 4 weeks of MI was significantly higher in Tg-MI than in wild-type (WT) littermates (WT-MI), although infarct size was comparable. LV cavity dilatation and dysfunction were significantly attenuated in Tg-MI. LV end-diastolic pressure was increased in WT-MI, and it was also reduced in Tg-MI. Improvement of LV function in Tg-MI was accompanied by a decrease in myocyte hypertrophy, apoptosis, and interstitial fibrosis as well as oxidative stress in the noninfarcted LV.

Conclusions—Overexpression of *TFAM* inhibited LV remodeling after MI. TFAM may provide a novel therapeutic strategy of cardiac failure. **(*Circulation*. 2005;112:683-690.)**

Key Words: free radicals ■ genes ■ heart failure ■ myocardial infarction ■ remodeling

Myocardial infarction (MI) leads to complex structural alterations (remodeling) involving both the infarcted and noninfarcted left ventricular (LV) myocardium. Early remodeling is LV cavity dilatation occurring during the early phase of MI, which is likely due to wall thinning of the infarct region. During the first several days, LV enlargement follows, and thereafter a progressive dilatation of the noninfarcted LV associated with myocyte hypertrophy and interstitial fibrosis occurs over weeks. These progressive changes in LV geometry contribute to the development of depressed cardiac function, clinical heart failure, and increased mortality. Accordingly, it is of critical importance to explore the mechanisms and to develop therapeutic strategies that will effectively inhibit this deleterious process.

Mitochondria have their own genomic system, mitochondrial DNA (mtDNA), a closed-circular double-stranded DNA molecule. MtDNA contains 2 promoters, the light-strand and heavy-strand promoters (LSP and HSP, respectively), from which transcripts are produced and then processed to yield the individual mRNAs encoding 13 subunits of the oxidative phosphorylation system, ribosomal and transfer RNAs.[1,2] Transcription from the LSP also produces RNA primer, which is necessary for initiating mtDNA replication. Mitochondrial function is controlled by the mtDNA as well as factors that regulate mtDNA transcription and/or replication.[3] This raises the possibility that mitochondrial gene replication and thus the mitochondrial DNA copy number and/or mitochondrial gene transcription are impaired in heart failure. Indeed, heart failure is frequently associated with qualitative and quantitative defects in mtDNA.[4–7] Recently, we demonstrated that the decline in mitochondrial function and mtDNA copy number plays a major role in the development of heart failure that occurs after MI.[8,9]

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Mitochondrial transcription factor A (TFAM) is a nucleus-encoded protein that binds upstream of the LSP and HSP of mtDNA and promotes transcription of mtDNA. It also plays an important role in regulating mtDNA copy number.[10] In fact, disruption of the *Tfam* gene in mice causes depletion of mtDNA, loss of mitochondrial transcripts, loss of mtDNA-encoded polypeptides, and severe respiratory chain deficiency.[11] Moreover, targeted disruption of *Tfam* in cardiac myocytes induced deletion of mtDNA and dilated cardiomyopathy.[12,13] These lines of evidence obtained from knockout mice have established a critical role for TFAM in regulation of mtDNA copy number and mitochondrial function as well as maintenance of the physiological function of the heart in vivo. In addition, a reduction in TFAM expression has been demonstrated in several forms of cardiac failure.[7,9,14,15] Therefore, an increase in *TFAM* expression may exert beneficial effects on cardiac remodeling after MI. However, it has not yet been analyzed whether an increase in *TFAM* expression can ameliorate mitochondrial dysfunction in heart failure and whether this protein may have therapeutic potential. To address these questions, we created transgenic (Tg) mice containing human *TFAM* gene. Accordingly, human *TFAM* Tg mice and their wild-type (WT) littermates were randomized to have either a large transmural MI induced by coronary artery ligation or sham operation.

Methods

Generation of Tg Mice

Human *TFAM* cDNA was inserted into the unique *Eco*RI site between the CAG (modified chicken β-actin promoter with CMV-IE enhancer) promoter and 3′-flanking sequence of the rabbit β-globin gene of the pCAGGS expression vector[16] and used to generate Tg mice (Figure 1A). The pronuclei of fertilized eggs from hyperovulated C57BL/6 mice were microinjected with this DNA construct. The presence of the *TFAM* transgene was confirmed by polymerase chain reaction (PCR) before the experiments. Four independent founder lines were identified and mated to C57BL/6 WT mice to generate pure C57BL/6 genetic background WT and Tg offspring. Heterozygous Tg mice were used at 10 to 13 weeks of age. The study was approved by our Institutional Animal Research Committee and conformed to the animal care guidelines of the American Physiological Society.

Western Blotting

The protein levels human TFAM and mouse Tfam were analyzed in cardiac tissue homogenates by Western blot analysis with a polyclonal antiserum against human TFAM and mouse Tfam, respectively. In brief, the LV tissues were homogenized with the lysis buffer (1% SDS, 1.0 mmol/L sodium orthovanadate, 10 mmol/L Tris; pH 7.4). After centrifugation, equal amounts of protein (5 μg protein per lane), estimated by the Bradford method with the use of a protein assay (Bio-Rad), were electrophoresed on a 12.5% SDS–polyacrylamide gel and then electrophoretically transferred to a nitrocellulose membrane (Millipore). After blocking with 5% nonfat milk in PBS containing 0.05% Tween-20 at 4°C overnight, the membrane was incubated with the first antibody and then with the peroxidase-linked second antibody (Amersham Pharmacia). Chemiluminescence was detected with an ECL Western blot detection kit (Amersham Pharmacia) according to the manufacturer's recommendation.

Immunohistochemistry

Frozen sections of cardiac tissues were incubated in the presence of 100 nmol/L Mitotracker Red CMXRos (Molecular Probes) at 37°C for 20 minutes. We did not repeat freezing-thawing to avoid the loss of mitochondrial integrity. After they were washed with PBS (10 mmol/L sodium phosphate, pH 7.4, and 150 mmol/L NaCl), the sections were fixed with 3.7% formaldehyde for 5 minutes. After they were washed, the fixed sections were incubated with 100-fold diluted anti-TFAM affinity purified antibodies (10 μg/mL) in PBS at 4°C overnight. Fluorescence images were taken with a confocal laser scanning microscope (Bio-Rad MRC 1000) with laser beams of 488 and 568 nm for excitation.

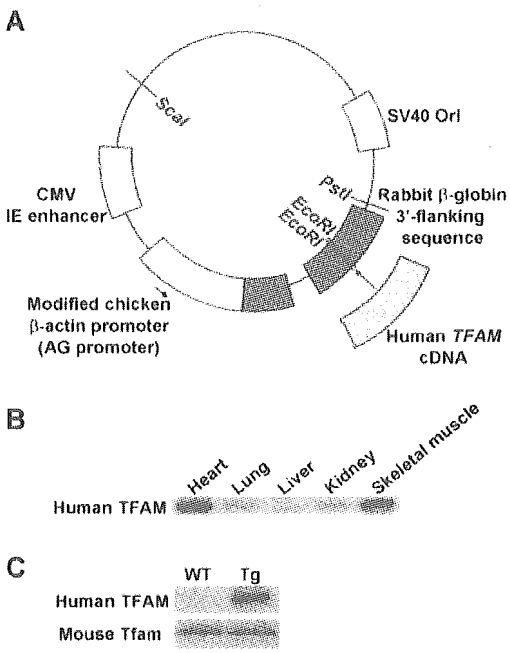

Figure 1. Characterization of human *TFAM* Tg mice. A, Diagram of the human *TFAM* transgenic construct. Plasmid was constructed by inserting a human *TFAM* cDNA (0.74 kb) into the unique *Eco*RI site between the CAG promoter and 3′-flanking sequence of the rabbit β-globin gene of the pCAGGS expression vector. Tg mice harboring human *TFAM* cDNA were identified by PCR with genomic DNA prepared from tail biopsies. CMV indicates cytomegalovirus; IE, immediate early; SV40, simian virus; and Ori, origin of DNA replication. B, Western blot analysis of human TFAM protein in various tissues from Tg mice. Total protein extracts from heart, lung, liver, kidney, and skeletal muscle were probed with a polyclonal antiserum against human TFAM. The antibody recognized TFAM as a single band of 24 kDa. C, Western blot analysis of human TFAM and mouse Tfam protein levels in the heart from Tg and WT mice.

Creation of MI

We created MI in mice by ligating the left coronary artery. Sham operation without coronary artery ligation was also performed.[9] Tail clips were applied, and a PCR protocol was performed to confirm the genotype by a group of investigators. Next, MI was induced in these mice by another subset of investigators, who were not informed of the genotyping results. This assignment procedure was performed with numeric codes to identify the animals.

Survival

To perform the survival analysis, cages were inspected for deceased animals during the study period of 4 weeks. All deceased mice were examined for the presence of MI as well as pleural effusion and cardiac rupture.

We performed the subsequent molecular (mtDNA copy number and mtRNA), biochemical (mitochondrial enzyme activity and apoptosis), and histopathological (myocyte cross-sectional area, collagen volume fraction, and mitochondrial ultrastructure) analysis by using the LV from sham-operated mice and the noninfarcted LV from MI mice.