Introduction

There are a variety of experimental models of chronic heart failure (CHF). Isoproterenol (Iso)-induced CHF is a widely used model due to its simple methodology, low cost, and the clinically representative pathophysiological changes it produces. In this model, subcutaneous administration of the beta adrenoceptor agonist Iso produces patchy myocardial necrosis in a dose-related manner while retaining intact coronary vasculature. The pathophysiological and morphological changes observed in Iso-treated rats have been found to be similar to those observed following human myocardial infarction (MI)\(^1\). MI results in asymmetrical left ventricular remodeling, characterized by fibrosis at both the site of infarct and in the noninfarcted myocardium, in addition to hypertrophy of viable myocytes\(^2\). These cardiac structural adaptations facilitate compensated hemodynamic performance, but ultimately result in a high incidence of CHF and sudden cardiac death\(^3\).

Adiponectin acts as an anti-inflammatory and antiatherogenic cytokine and has been demonstrated to suppress cardiac hypertrophy in response to pressure overload\(^4, 5\). Adiponectin, a member of the adipokine superfamily, is expressed in adult ventricular cardiomyocytes and is upregulated by activation of peroxisome proliferator-activated receptor-gamma (PPAR-\(\gamma\))\(^6\). Adiponectin was recently shown to be suppressed by tumor necrosis factor-alpha (TNF-\(\alpha\))\(^7\), another cytokine that is expressed in and secreted from cardiomyocytes\(^8\). Locally generated TNF-\(\alpha\) plays a role in cardiac remodeling via its ability to stimulate apoptosis and inflammatory and fibrogenic responses in a pressure-overloaded heart, which in turn contribute to cardiac dysfunction\(^9, 10\).

Telmisartan attenuates isoproterenol-induced cardiac remodeling in rats via regulation of cardiac adiponectin expression

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Aim: To investigate whether telmisartan (Telm) pretreatment attenuates isoproterenol (Iso)-induced postinfarction remodeling (PIR) in rats, and whether the effect of Telm is associated with cardiac expression of adiponectin.

Methods: PIR was induced in male Wistar rats with two consecutive injections of Iso (80 mg/kg, sc) at an interval of 24 h. Primary culture of ventricular myocytes from neonatal rats was prepared. Iso-induced cardiomyocyte injury was assessed based on cell growth and lactate dehydrogenase (LDH) activity. Cardiac adiponectin expression was measured using qRT-PCR and immunoblot analysis.

Results: In the rats with PIR, Telm (10 mg·kg\(^{-1}\)·d\(^{-1}\), po for 65 d) suppressed Iso-induced increases in gravimetric parameters, cardiomyocyte diameter and collagen volume fraction, but had no effect on Iso-induced myocardial hypertrophy and interstitial fibrosis. The protective effect of Telm was associated with enhanced protein expression of cardiac adiponectin. In cultured cardiomyocytes, Telm (5–20 \(\mu\)mol/L) inhibited the cell death and LDH release induced by Iso (10 \(\mu\)mol/L), and reversed Iso-induced reduction in adiponectin protein expression. In cardiomyocytes exposed to Iso (20 \(\mu\)mol/L), GW9662 (30 \(\mu\)mol/L), a selective antagonist of PPAR-\(\gamma\), blocked the effects of Telm pretreatment on adiponectin protein expression, as well as the protective effects of Telm on Iso-induced cell injury.

Conclusion: Telm attenuates Iso-induced cardiac remodeling and cell injury, which is associated with induction of cardiac adiponectin expression.

Keywords: telmisartan; angiotensin II receptor blocker; congestive heart failure; cardiac remodeling; cardiac fibrosis; adiponectin; isoproterenol
Telmisartan (Telm) is a selective angiotensin II type 1 receptor antagonist that is widely used in the treatment of hypertension, CHF, and diabetic nephropathy\cite{11-13}. It has been shown to have beneficial effects on postinfarct ventricular remodeling\cite{14}. Recently, Telm was reported to have partial activity as a PPAR-γ agonist, enabling it to regulate metabolic and inflammatory pathways and improve left ventricular functions\cite{15,16}. Moreover, Telm treatment has been shown to reduce the expression of inflammatory cytokines, including TNF-α and its transcription factors\cite{17-19}. Angiotensin receptor blocker (ARB) treatment increases circulating adiponectin concentrations in humans and adiponectin mRNA levels in adipose tissue in hypertensive rats\cite{20-23}. ARB treatment also induces cardiac adiponectin expression in mice with viral myocarditis\cite{24}. In addition, Telm was recently shown to be present in plasma at higher levels than other ARBs following oral administration\cite{25}, suggesting that it is absorbed well when administered orally. To date, however, the effects of Telm on cardiac adiponectin have not been studied.

The present study was designed to investigate the cardioprotective effects of Telm in vitro and in vivo, specifically its effects on cardiac adiponectin and TNF-α. We also examined the necessity of PPAR-γ activity in the stimulatory effects of Telm on adiponectin expression in cardiomyocytes. Cardiomyocyte viability and lactate dehydrogenase (LDH) activation, which is released when cell injury occurs, were employed as indexes of injury severity in our in vitro experiments.

**Materials and methods**

**Animals**

Forty male Wistar rats (200–250 g body weight; 6–8 weeks of age) were obtained from the Experimental Animal Center of Hebei Medical University. This research was carried out in accordance with the National Research Council protocol for the care and use of laboratory animals. The animals were housed in the Laboratory Animal Center under conditions of a controlled ambient temperature of 22–24 °C with a 12-h light/dark cycle. Animals were provided with free access to standard rodent chow and tap water and were allowed to acclimate for a period of 7 d.

**Drugs and chemicals**

Telm was obtained from Boehringer Ingelheim International GmbH (Germany). Iso hemisulfate was dissolved in 0.9% saline and used within 10 min of preparation. All chemicals used in this study were of analytical grade and purchased from Sigma Chemicals (St Louis, MO, USA).

**Induction of cardiac remodeling**

To induce PIR, rats were administered two subcutaneous injections of 80 mg/kg homologous Iso (Sigma-Aldrich) or vehicle at 24 h intervals on 2 consecutive days, as described by PalfI A et al\cite{26}.

**Experimental groups**

After 7 d of acclimatization, the experimental animals were divided into the following treatment groups: 1) Iso, \( n=10; \) 2) Iso+Telm, \( n=10; \) 3) Telm, \( n=10; \) and 4) control (vehicle), \( n=10. \) Rats in the Telm and Iso+Telm groups were administered Telm orally (10 mg·kg\(^{-1}·d^{-1}\)) by intragastric intubation for a period of 65 d. All rats were fed a standard diet throughout this experimental period. Changes in body weight and food and water intake were noted for all animals at regular intervals throughout the experimental period.

**Tissue preparation**

Two months after the last Iso injection, each rat was weighed and sacrificed by intravenous injection of 2–3 mL of 10% KCl through the femoral vein. Immediately after the KCl injection, the thorax was opened and the heart was excised, rinsed with saline, blotted dry, and weighed. The left ventricle (LV) was dissected out by incising the right ventricle along the septum and then weighed. Heart weight to body weight and LV weight to body weight ratios were calculated. One segment of the LV was then fixed in 4% paraformaldehyde and embedded in paraffin. The other segment was rapidly frozen in liquid nitrogen and stored at -80 °C for further analysis.

**Histological examination and Masson staining**

After being fixed for 4 h in 4% paraformaldehyde, the tissue blocks were dehydrated and embedded in paraffin; 5-μm-thick sections were then cut and stained with either hematoxylin-eosin or Masson’s trichrome stain. Eight light microscope visual fields were randomly selected for each Masson’s trichrome stained section and viewed using a computer-assisted image analysis system (Motic Med 6.0 CMIAS, China). To assess cardiomyocyte diameter, the cross-sectional margins of cardiomyocytes in the LV were marked with the cursor using the Image Pro Plus 4.1 software and the mean diameter was calculated; only cardiomyocytes that had complete cell boundaries and clear round intra- cytoplasmic nuclei were measured. Approximately 60–70 cardiomyocytes were randomly selected from five to seven images captured randomly at different sites in each specimen and the average cross-sectional diameter was calculated. The collagen volume fraction (CVF) was measured in five fields for each LV section. CVF values were determined by quantitative morphometry using an automatic image analyzer (IBAS 2.5, Kontron, Germany), such that CVF=fibrosis area/total area of myocardium. The CVF excluded scars and perivascular collagen areas. The pathologist performing the histopathological evaluations was blinded to the treatment group designations.

**Primary culture of neonatal rat ventricular myocytes (NRVMs)**

NRVMs were prepared as described previously\cite{27}. Briefly, ventricles from 1–3 d-old Wistar rats were minced and digested in phosphate-buffered saline (PBS) containing 0.1% trypsin and 0.04% type II collagenase for 8–10 cycles. The degraded tissue was then centrifuged and suspended in Dulbecco’s modified Eagle’s medium containing 15% fetal bovine serum. A single 2-h preplating step was performed to further increase the ratio of NRVMs to noncardiomyocytes. Noncar-
diomyocytes attached readily to the bottom of culture dishes, and the unattached myocytes were plated at $1\times10^5$ cells/cm$^2$ in the same medium as above and supplemented with 0.1 mmol/L BrdU. Cells were then held in serum-free medium for 24 h prior to being used in experiments. Morphological examination and staining with an anti-sarcomeric α-actin antibody were carried out to confirm the presence of NRVMs, with about 95% of the cells being identified as NRVMs.

Cell growth assay

The cytotoxic effect of Iso on cardiac myocytes was measured using the MTT assay as described elsewhere[28]. The cells were dispensed in 96-well flat bottomed microtiter plates (NUNC, Roskilde, Denmark) at a density of $5\times10^3$ cells/well. After 48 h of incubation, the cells were treated with Iso (10 μmol/L) and/or various concentrations of Telm for 48 h. Cell viability was calculated as follows:

$$\text{Viability} (\%) = \frac{(A_{490, \text{sample}} - A_{490, \text{blank}})}{(A_{490, \text{control}} - A_{490, \text{blank}})} \times 100$$

LDH activity-based cytotoxicity assay

LDH content released into the culture medium was measured as an index of LDH activity. The cells were incubated with various concentrations of Telm for 1 h prior to a 48 h incubation in the presence of isoproterenol (10 μmol/L). The substrate reaction buffer for LDH [0.5 mmol/L, with or without lactic acid, 0.66 mmol/L INT, 0.28 mmol/L PMS, and 1.3 mmol/L NAD$^+$ in Tris–HCl, pH 8.2] was added to collected samples of culture media. The absorbance value ($A_{490}$) at 490 nm for the LDH reaction was assayed after 1 and 5 min.

Measurements of protein expression levels in myocardium and cultured cardiomyocytes

For immunoblot analysis, extracts of myocardial tissue and treated cells were matched for protein concentration (15 μg) with SDS-PAGE sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. The membranes were incubated with the indicated primary antibodies overnight at 4 °C; 50 μg of denatured protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) for detection of adiponectin levels. Nonspecific binding was prevented by incubation with 5% nonfat dry milk for 1 h. The PVDF membranes were incubated overnight at 4 °C with anti-Acrp30 and anti-GAPDH primary antibodies diluted in TBS-T (1:1000) (Santa-Cruz Biotechnology, Santa Cruz, CA, USA). After washing in Tween-20 buffer, the membranes were incubated with fluorescent secondary antibodies. Labeling was quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, USA). Acrp30 expression was corrected by comparison to the endogenous control GAPDH. Band densities were scanned and quantified using a LEICA A550IW image analysis system.

Statistical analysis

All data are expressed as mean±SD. Group mean values were compared using one-way analysis of variance (ANOVA) followed by a Tukey’s multiple comparison test where appropriate. For all comparisons, $P<0.05$ was used to establish statistically significant results.

Results

Influence of Telm on Iso-induced effects on gravimetric parameters

The gravimetric parameters in hearts of intact and PIR animals are presented in Figure 1. Rats treated with Iso alone showed significantly elevated heart mass normalized to body mass, while Iso-treated rats that had been pretreated with Telm did not show these unfavorable changes (Figure 1A). Telm pre-treatment also reduced the gain in Iso-induced ventricular mass (Figure 1B). Heart mass and ventricle/body mass ratio did not differ between rats treated with Telm alone and control (no drug) rats (Figure 1).

Impact of Telm on Iso-induced myocardial hypertrophy and interstitial fibrosis

Compared to untreated PIR (isoproterenol alone) animals, PIR animals given Telm showed significantly attenuated Iso-induced increases in mean myocyte diameter (Figure 2) and collagen volume fraction (Figure 3). Treatment with Telm alone had no effect on these values ($P>0.05$ vs control) (Figure 2, 3).

Protective effects of Telm against PIR are associated with enhanced protein expression of adiponectin

Relative to intact hearts, TNF-α expression in hearts with PIR was increased by about three-fold ($P<0.01$) while adiponectin expression in hearts with PIR was decreased by about half.
Oral administration of Telm significantly attenuated these PIR-related changes in TNF-α and adiponectin expression (Figure 4).

**Telm inhibits Iso-induced cell death in cardiomyocytes**

Cardiomyocytes were exposed to Iso (10 μmol/L) alone, Iso (10 μmol/L) with Telm (5–20 μmol/L), or vehicle for 48 h. The cell death inhibition ratio of Telm-treated cells relative to Iso only-treated cells ranged from 40.2±4.5% (5 μmol/L Telm) to 92.6±7.1% (20 μmol/L Telm) (Figure 5A). Telm protected Iso-treated cardiomyocytes from cell death in a dose-dependent manner and had no cytotoxic effects on the cells.

It is worth noting that we observed a marked increase in the cardiomyocyte beating rate 6 h after Iso treatment. The cardiomyocyte beating rate was then weakened in a time-dependent manner such that by 48 h following Iso treatment, no cell beating was observed. On the contrary, cells that were exposed to Telm and Iso simultaneously continued to exhibit steady beating throughout the 48 h experimental period.

**Telm inhibited Iso-induced LDH release in cardiomyocytes**

Iso (10 μmol/L) nearly doubled LDH release in cardiomyocytes relative to cells not exposed to any drug (P<0.01). Pretreatment with Telm for 48 h reduced Iso-induced LDH release in a dose-dependent manner; at 20 μmol/L, Telm-pretreated, Iso-exposed cells had LDH release levels that were similar to those of untreated control cells (Figure 5B). Telm alone (5–20 μmol/L) did not affect LDH release (data not shown).

**Telm treatment blocked Iso effects on adiponectin protein expression in cardiomyocytes via a mechanism involving PPAR-γ activation**

After incubation with 10 μmol/L Iso, adiponectin protein expression in cardiomyocytes was decreased relative to cells not exposed to any drug. However, in cells that were pretreated with Telm (20 μmol/L), this decrease did not occur. Treating Iso-exposed cells with the selective PPAR-γ antagonist GW9662 (30 μmol/L) potently blocked the effects of Telm.
pretreatment on adiponectin expression (Figure 6). GW9662 in the absence of Telm did not change the Iso-induced decrease of adiponectin expression (data not shown).

Inhibiting PPAR-γ activity with the selective antagonist GW9662 blocked the protective effects of Telm against Iso-induced cell injury

Inhibition of PPAR-γ activity with the selective antagonist GW9662 (30 μmol/L) potently blocked the protective effects of Telm against Iso-induced cell injury (Figure 7A and 7B). GW9662 alone (in the absence of Telm) had no observable effects on Iso-induced cell injury (data not shown).

**Discussion**

In the present study, oral Telm pretreatment prevented changes in gravimetric parameters and reduced myocardial hypertrophy and interstitial fibrosis in Iso-induced PIR hearts. Oral Telm pretreatment also reversed MI-induced decreases in adiponectin expression as well as MI-induced increases in TNF-α expression. Cardiac expression of adiponectin mRNA negatively correlated with expression of cardiac TNF-α mRNA. Importantly, heart size and interstitial fibrosis were not influenced by Telm in non-PIR hearts, and Telm led to an increased performance of the postinfarct heart. Cardiomyocytes treated *in vitro* with Iso alone showed elevated LDH activity accompanied by markedly inhibited cell growth; Telm inhibited Iso-induced cell death and LDH release in cardiomyocytes. Furthermore, we demonstrated that administration of the PPAR-γ antagonist GW9662 blocked the stimulatory effects of Telm on adiponectin expression in cardiomyocytes. The ability of Telm to promote preservation of myocardial adiponectin expression may represent an important mechanism for the beneficial effects of Telm in PIR.

Our demonstration that PPAR-γ activity was necessary for Telm-induced reversal of Iso-induced decreases in adiponectin expression...
in their number, which together with the increased interstitial fibrosis ultimately impairs heart performance and decreases its elasticity. Recently, inflammatory pathways and a pro-inflammatory cytokine mechanism have been invoked to explain Iso-induced PIR through the promotion of matrix metalloproteinase expression in cardiac fibroblasts[38]. These post-MI changes contribute to the progression toward CHF, characterized by a progressive deterioration in heart function. Autopsies have revealed the presence of adiponectin in the interstitium of early stage MI lesions, and the presence of adiponectin was further found to lie linearly along the border of vital myocardium and at the periphery of surviving cardiomyocytes around lesions in granulation stage MI lesions[37]. Expression of adiponectin has also been reported in the injured myocardium of patients with cardiomyopathy[39]. Our study reveals for the first time that the protective effects of Telm in vivo and in vitro are associated with enhanced expression of cardiac adiponectin. Thus we posit that Telm may induce cardiac adiponectin expression in the remodeling of hearts and in injured cardiomyocytes.

In summary, we have demonstrated that Telm treatment attenuates Iso-induced PIR in rats and exerts protective effects on Iso-treated rat cardiomyocytes. The cardioprotective actions of Telm may be mediated by the activation of ARB/PPAR-γ/adiponectin pathways and appear to involve cardiac expression of the anti-inflammatory cytokine, adiponectin, together with suppression of the inflammatory cytokine, TNF-α. The precise cardiac adiponectin-regulated signaling that occurs during Iso-induced PIR has yet to be delineated.

Author contribution
Yong-jun LI and Bing-yan GUO designed the study; Bing-yan GUO, Rui HAN, Shao-ling YANG, Hong ZHOU, and Mei WANG performed the research; Yong-jun LI, Ying-hui SHI, and De-rong HAN contributed new analytical tools and reagents; Bing-yan GUO analyzed the data; and Bing-yan GUO wrote the paper.

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Our findings showing increased expression of cardiac TNF-α protein accompanied by decreased expression of adiponectin in the presence of myocardial injury are consistent with prior studies suggesting that the two factors may work in opposition to each other. For example, adiponectin has been shown to provide protection against TNF-α-mediated myocardial injury and apoptosis in animal models of MI[33]. Furthermore, adiponectin knockout mice show elevated TNF-α expression, which can be attenuated by adiponectin[34]. Thus, TNF-α and adiponectin appear to have an antagonistic relationship, with one cytokine negatively regulating the expression of the other[35].

After MI, initial loss of the myocardium can induce progressive ventricular enlargement and deposition of interstitial collagen, changes which are intended to adapt the heart to its altered situation. However, increased muscle mass is achieved by the enlarging of cardiomyocytes rather than an
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