AdipoR1/APPL1 Potentiates the Protective Effects of Globular Adiponectin on Angiotensin II-Induced Cardiac Hypertrophy and Fibrosis in Neonatal Rat Atrial Myocytes and Fibroblasts

Tengwei Cao¹, Zhen Gao¹, Lingyun Gu², Minglong Chen³, Bing Yang³, Kejiang Cao³, He Huang¹, Mingfang Li³*

¹College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing, P.R. China, ²Division of Cardiology, Jiangyin Hospital Affiliated to Southeast University, Jiangyin, P.R. China, ³Division of Cardiology, Department of Medicine, The First Affiliated Hospital of Nanjing Medical University, Nanjing, P.R. China, ⁴State Key Laboratory of Materials-Oriented Chemical Engineering, Nanjing, P.R. China

Abstract

Atrial hypertrophy and fibrosis are essential pathological features of atrial fibrillation. Recently, adiponectin has become a protein of interest due to its beneficial effects on cardiovascular diseases. However, the molecular mechanism of atrial structural remodeling and signaling pathways evoked by adiponectin remain unclear. In the present study, we investigated the cardioprotective effect of globular adiponectin (gAcrp) on angiotensin II-induced atrial hypertrophy and fibrosis in neonatal Sprague-Dawley rat. To further investigate the molecular mechanisms underlying the preventive effect of gAcrp, transfection of cells with siRNA was used to suppress the mRNA expression of adiponectin receptor 1 (AdipoR1) and its downstream adaptor protein APPL1. Non-silencing-Cy-3 labelled siRNA was used to determine transfection efficiency using fluorescence microscopy. The expression of atrial natriuretic peptide and procollagen type1 α-1, hypertrophy marker and fibrosis one, respectively, was detected by real-time PCR. Furthermore, the expression of adenosine monophosphate-activated protein kinase (AMPK), phosphatidylinositol 3-kinase 3 kinase (PI3K) and Akt was detected by western blotting. In addition, nuclear p65 translocation activity was analyzed by EMSA supershift assay. Our results showed that AdipoR1 and the adaptor protein APPL1 mediated the protective effects of gAcrp. In addition, the function of adiponectin and phosphorylation of AMPK were prominently diminished by inhibition of PI3K. Furthermore, nuclear factor-kB (NF-kB) transcription was diminished by the specific inhibition of AMPK. Taken together, AMPK pivotally interacts with NF-kB and PI3K, mediating the cardioprotective effect of adiponectin, and may serve as a therapeutic target for preventing atrial hypertrophy and fibrosis. Our present study suggests that gAcrp could ameliorate AngII-induced cardiac hypertrophy and fibrosis in rat atrial cells, which is mediated by the activation of AMPK signaling pathways. APPL1 and AdipoR1 are the key factors involved in the downstream of gAcrp approach.

Introduction

Atrial fibrillation (AF) is a common sustained arrhythmia in clinical practice [1]. AF is one of the leading causes of stroke among the elderly and accounts for one-third of strokes among patients over the age of 65 [2]. Atrial structural remodeling is one of the most pivotal substrates in AF and leads to progressive architectural aggravation of atria after continuous episodes of AF. The primary changes of structural remodeling involve myocytic hypertrophy, myolysis, and interstitial fibrosis [3]. It has been demonstrated that the activation of the renin-angiotensin system (RAS) plays a pivotal role in atrial structural remodeling of AF [4,5]. In addition, angiotensin II (AngII) has been shown to be a key trigger of atrial hypertrophy and fibrosis. However, the molecular mechanism of atrial structural remodeling still remains unclear.

At the cellular level, hypertrophy of cardiac ventricles is a result of increased cardiomyocyte cell volume, which is a procedure resulting from cellular signaling cascades and modulation of cellular energy mobilization [6]. One frequent observation of hypertrophy involves the fetal programming of gene expression with atrial natriuretic peptide (ANP) in ventricular myocytes and procollagen type1 α-1 (COL1A1) in ventricular fibroblasts.

Adiponectin (also known as Acrp30, AdipoQ, and GBP28), an adipocytokine secreted by adipocytes, has been the recent focus of intense research because of its insulin-sensitizing effect and possible therapeutic target for metabolic disorders [7,8]. There is mounting evidence supporting that exogenous adiponectin plays a central modulatory role in various pathophysiological conditions.

Citation: Cao T, Gao Z, Gu L, Chen M, Yang B, et al. (2014) AdipoR1/APPL1 Potentiates the Protective Effects of Globular Adiponectin on Angiotensin II-Induced Cardiac Hypertrophy and Fibrosis in Neonatal Rat Atrial Myocytes and Fibroblasts. PLoS ONE 9(8): e103793. doi:10.1371/journal.pone.0103793

Copyright: © 2014 Cao et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by grants from National Natural Science Foundation of China (Young Project, No. 81000083). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: huangh@njtech.edu.cn (HH); mingflee@hotmail.com (ML)
In addition to its well-characterized function in glucose and fatty acid metabolism, adiponectin has been extensively studied in recent years due to an apparent protective function in inflammation, metabolic syndrome, insulin resistance, atherosclerosis and cardiovascular disease [9]. The cardioprotective properties of adiponectin has been established recently in obesity-related diseases, including hypertrophic cardiomyopathy, myocardial ischemia–reperfusion injury, and heart failure [10]. Several studies have demonstrated an anti-hypertrophic effect of adiponectin on heart. Subsequently, these studies have also demonstrated that adiponectin influences cardiac remodeling in pathological conditions. Through stimulation of AMP-activated protein kinase signaling, adiponectin has been demonstrated to modulate the angiogenesis process in vivo in a mouse model of ischemia-induced angiogenesis [11]. It was previously reported that globular adiponectin inhibits AngII-induced cardiac ventricular hypertrophy [12]. Although many studies have attempted to elucidate the mechanisms responsible for cardiac ventricle remodeling, the various signaling pathways in atrial tissue remain unclear.

There are two putative distinct adiponectin receptors, AdipoR1 and AdipoR2, which are expressed at detectable levels in most mammalian tissues and cells. AdipoR1 is the preferentially abundant receptor expressed in skeletal muscle and heart tissues, and can be particularly detected in atrial myocytes [13]. The downstream adaptor protein, APPL1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif), interacts with AdipoR1. As such, both AdipoR1 and APPL1 participate in adiponectin-dependent activation of AMPK [14]. Despite detailed explanations in recent reports, both adiponectin and AMPK signaling pathways, and their regulation remain unclear in neonatal rat atrial myocytes and fibroblasts.

In the present study, we aimed to investigate the mechanism underlying the effect of globular adiponectin (gAcrp) on AngII-induced atrial impairment.

### Materials and Methods

#### Materials

Recombinant rat globular adiponectin (gAcrp) was purchased from BioVision (Mountain View, CA). Adiponectin was produced using bacteria (*Escherichia coli*). Dulbecco’s modified eagle medium (DMEM), Trypsin-EDTA, collagenase, penicillin/streptomycin, Opti-MEM Reduced-Serum Medium and fetal bovine serum (FBS) were all obtained from Gibco Laboratories (Grand Island, NY). Antibodies for phosphor-AMPK, whole AMPK, phosphor-Akt, whole Akt, phosphor-STAT3, whole STAT3, APPL1, and NF-kB p65 were acquired from Cell Signaling Technology (Beverly, MA). The antibody for AdipoR1 was from Abcam (Cambridge, UK). All target siRNAs and Cy3-labeled non-silencing siRNA (NS siRNA) were purchased from Ribobio Co. (Guangzhou, China), and TransIT-TKO transfection reagent was acquired from Mirus Bio Corporation (Madison, WI). AngII, compound C, BrdU and LY294002 were acquired from Sigma-Aldrich (St. Louis, MO). Finally, Ro31-8220 was obtained from Millipore (Billerica, MA). All reagents were of analytical grade.

#### Primary culture and identification of neonatal rat atrial myocytes and fibroblasts

All experimental procedures were approved by the Ethics Committee of Animal Research, Nanjing Medical University. Animals were used in agreement with the Animal Care and Experiment Committee of Nanjing Medical University guidelines. All primary cell extracts were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Neonatal rat atrial myocytes and fibroblasts were isolated as previously described [15]. In brief, the atria of 1 to 3-day-old Sprague-Dawley rats were minced under a dissecting microscope and dissociated in phosphate-buffered saline (PBS) containing 0.125% trypsin-EDTA and 0.05% type II collagenase for 6–8 cycles. Then, the cells were collected and incubated for 90 min at 37°C, 5% CO2 to allow the fibroblasts to adhere to tissue culture plates in high glucose (4.5 mg/l) DMEM with 10% FBS and 1% penicillin/streptomycin. The unattached myocytes were centrifuged and suspended in the same medium with 100 μM BrdU added. After 72 h, the atrial fibroblasts were removed using
AdipoR1/APPL1 Potentiates the Effects of gAcrp on Atrial Remodeling

A

actin

DAPI

Overlay

B

Atrial Myocyte

Relative expression of ANP

AngII

0 1 10 100 1000 (μM)

(μM)

C

Atrial Fibroblast

Relative expression of COL1A1

AngII

0 1 10 100 1000 (μM)

(μM)

D

Atrial Myocyte

Relative expression of ANP

AngII (10 μM)
gAcrp (2.5 μg/ml)

(μM)

E

Atrial Fibroblast

Relative expression of COL1A1

AngII (1 μM)
gAcrp (2.5 μg/ml)

(μM)

F

Atrial Myocyte

P-STAT3

STAT3

G

Atrial Fibroblast

P-STAT3

STAT3

F

Atrial Myocyte

Relative expression of STAT3 Phosphorylation

AngII (10 μM)
gAcrp (2.5 μg/ml)

(μM)

G

Atrial Fibroblast

Relative expression of STAT3 Phosphorylation

AngII (1 μM)
gAcrp (2.5 μg/ml)

(μM)
0.125% trypsin-EDTA solution and passaged at a 1:2 dilution. The second generation of fibroblasts were used in all subsequent experiments. The cells were serum starved for 12 h prior to commencing experiment. Of note, morphological examination and α-actin immunofluorescence were used to identify myocytes and ensure the positive rate of atrial myocytes approached 90%.

mRNA expression analysis

To measure the mRNA levels of target genes, total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA samples (500 ng per sample) were reverse transcribed to cDNA with a Transcriptor First Stand cDNA Synthesis Kit (Roche, Mannheim, Germany). Two-step real-time quantitative PCR (qPCR) was used to detect the mRNA expression levels with Power SYBR Green (Applied Biosystems, Warrington, UK). The qPCR was performed using Mastercycler Realplex2 (Eppendorf, Germany) with the following conditions: 95°C for preheating for 10 min, and then 40 cycles of 95°C for 15 sec and 60°C for 1 min. Serial dilutions (10-fold) of an external standard with a known concentration were used to create a standard curve for each primer pair. The qPCR primers are listed in Table 1. To confirm amplification specificity, each qPCR product was tested by melting curve analysis. The qPCR products were electrophoresed on a 1.0% agarose gel and stained with Gold View. The bands were visualized with the Molecular Imager ChemiDoc™ XRS + Imaging System (Bio-Rad, Hercules, CA). The relative quantification of gene expression was used for the determination of the expression of mRNA of interest in comparison to housekeeping gene GAPDH transcripts by the 2−ΔΔCt method.

Western blot analysis

Atrial myocytes and fibroblasts grown on 6-well plates were harvested with 100 µl cell lysis buffer containing phosphatase inhibitor cocktail tablets (Roche, Mannheim, Germany) and phenylmethylsulfonyl fluoride (PMSF). Cells were scraped from the dish with a cell wiper to microfuge tubes. To maximize protein recovery, cells were also ultrasonicated (Sonics & Materials, Rockford, US). Protein concentrations in cell lysates were measured with a Pierce BCA protein assay kit (Thermo Scientific, Rockford, US). Protein samples (30 µg/lane) were separated by SDS-PAGE gel electrophoresis and transferred onto a 0.45-µm PVDF membranes (Millipore). The blots were blocked with 5% nonfat milk for 1 h at room temperature and then probed with rabbit anti-rat antibody overnight at 4°C followed by horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibodies at room temperature for 2 h. The immunoreactive proteins were rinsed 3 times in TBST and visualized by enhanced chemiluminescence detection (ECL, Thermo Scientific). The band density was scanned and quantified by Image Lab 2.0 software (Bio-Rad). β-actin was used as a loading control for all samples.
Statistical analysis

All data are presented as mean ± SD with the number of replicates (n) indicated in each case. Comparison of data between three or more groups was performed by one-way analysis of variance (ANOVA) with student-Newman-Keuls post hoc analysis using SPSS. Differences between groups were considered to be significant if the p value was <0.05.
Results

Globular adiponectin attenuates cardiac hypertrophy and fibrosis induced by AngII in myocytes and fibroblasts isolated from the atria of neonatal rats

To mimic atrial fibrillation, we constructed a model of atrial hypertrophy and fibrosis with myocytes and fibroblasts isolated from neonatal SD rats. After 72 h of culture, the isolated myocytes and fibroblasts were attached to plates at the appropriate density (Figure S1). The myocytes were identified using anti-α-actin antibody, which was recommended for the detection of rat α-cardiac actin [16]. As shown in Fig. 1A, atrial myocytes accounted for a large proportion of adherent cells. The results in Fig. 1B and C show that addition of AngII significantly increased ANP and COL1A1 expression in a dose-dependent manner. Nevertheless, concentration of AngII for the maximal effect varied in different cells, from 10 μM in atrial myocytes (p<0.01) down to 1 μM in atrial fibroblasts (p<0.05). All subsequent experiments were performed using AngII at these empirically determined concentrations. After 24 h of culture in serum-deprived medium, both atrial myocytes and fibroblasts were pretreated with gAcrp (2.5 μg/ml) for 1 h. (C) and (D) Quantitative analysis of AMPK phosphorylation shown in the upper panel was performed by densitometric analysis. Data were expressed as mean ± SD of three independent experiments. ***p<0.001 vs. NS siRNA control with gAcrp.

doi:10.1371/journal.pone.0103793.g003
STAT3 phosphorylation after incubation with AngII (Fig. 1F and G). Globular adiponectin induced 55% reduction in STAT3 phosphorylation in atrial myocytes and 40% reduction in atrial fibroblasts (both \( p < 0.001 \)).

Both APPL1 and AdipoR1 contribute to globular adiponectin-activated downstream signaling pathways

To further investigate the molecular mechanisms underlying the preventive effect of gAcrp on atrial hypertrophy and fibrosis, we examined whether APPL1 and AdipoR1 contributed to the

![Figure 4. ANP and COL1A1 expression in atrial cells after RNA interference of APPL1 and AdipoR1. Atrial myocytes (A) and fibroblasts (B) were transfected with siRNA targeting APPL1 or AdipoR1, or non-silencing (NS) siRNA, respectively. After 24 h incubation, cells were pretreated with gAcrp (2.5 \( \mu \text{g/ml} \)) before stimulation with indicated concentration of AngII for 24 h. Relative expression level of ANP and COL1A1 was then measured as described previously. Data shown were expressed as mean ± SD of three independent experiments as. *\( P < 0.05 \) vs. blank control, #\( P < 0.05 \) vs. AngII infusion, #\( P < 0.05 \) vs. AngII + gAcrp infusion. doi:10.1371/journal.pone.0103793.g004](image)

![Figure 5. Role of PI3K in the activation of AMPK/Akt signaling pathway induced by gAcrp in atrial myocytes and fibroblasts. Cells were pretreated with 20 \( \mu \text{M} \) LY294002 for 1 h and then incubated with gAcrp (2.5 \( \mu \text{g/ml} \)) for another 1 h. Data were expressed as mean ± SD of three independent experiments. *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \) vs. blank control, #\( P < 0.05 \); ###\( P < 0.001 \) vs. gAcrp only. doi:10.1371/journal.pone.0103793.g005](image)
downstream signaling pathways activated by gAcrp. We used siRNA transfection to suppress the mRNA expression of APPL1 and AdipoR1. As primary cultured cells can be difficult to be transfected with siRNA, we used Cy3-labeled NS siRNA to determine the transfection efficiency (Fig. 2A and B). The specific knockdown of APPL1 and AdipoR1 was verified by qPCR analysis (Fig. 2C, D, E and F). As shown in Fig. 2C and E, treatment with targeted siRNA dramatically reduced APPL1 mRNA expression by 70% \( (p, 0.01) \) and AdipoR1 by 75% \( (p, 0.001) \) in atrial myocytes. Similar to atrial myocytes, APPL1 and AdipoR1 expression were significantly decreased by treatment with targeted siRNA as compared with controls in atrial fibroblasts (Fig. 2D and F). As shown in Fig. 2C and E, treatment with targeted siRNA dramatically reduced APPL1 mRNA expression by 70% \( (p<0.01) \) and AdipoR1 by 75% \( (p<0.001) \) in atrial myocytes. Similar to atrial myocytes, APPL1 and AdipoR1 expression were significantly decreased by treatment with targeted siRNA as compared with controls in atrial fibroblasts (Fig. 2D and F).

We further examined the functional significance of AdipoR1 and APPL1 interaction with gAcrp in AngII-treated atrial myocytes and fibroblasts. As shown in Fig. 4, real-time qPCR analysis indicated that ANP and COL1A1 mRNA expression increased after silencing of APPL1 or AdipoR1. The silencing of APPL1 and AdipoR1 significantly abrogated the protective effect of gAcrp, which suggests that APPL1 and AdipoR1 may play an important role in the interaction of downstream signaling pathways.

Globular adiponectin mediates AMPK and PI3K/Akt signaling axis to protect atrial cells from hypertrophy and fibrosis

AMPK is a critical metabolic regulator of adiponectin downstream signaling action. We previously demonstrated that activation of AMPK though adiponectin could protect atrial myocytes (data not shown). Moreover, the AMPK activator 5-aminimidazole-4-carboxamide-1-b-d-ribofuranoside (AICAR, 1 nM) could simulate this effect (Figure. S2). In contrast, an AMPK inhibitor, compound C (10 \( \mu \)M), markedly inhibited this effect (Figure. S3). Thus, we investigated the underlying mechanism of how AMPK stimulates a series of metabolic pathways to ameliorate AngII-induced atrial hypertrophy and fibrosis. Western blot analysis detected AMPK and Akt activity after pretreatment with LY294002 (30 \( \mu \)M) for 1 hour. As illustrated in Fig. 5A, treatment with gAcrp significantly increased AMPK phosphorylation following AngII treatment.

![Figure 6. ANP and COL1A1 expression in atrial cells pretreated with 20 \( \mu \)M LY294002 or Ro31-8220. Atrial myocytes (A) and fibroblasts (B) were incubated with gAcrp (2.5 \( \mu \)g/ml), or pretreated with 20 \( \mu \)M LY294002 for 1 hour and then incubated with AngII for 24 hours. Data were expressed as mean ± SD of three independent experiments. *\( p<0.05 \) vs. blank control, \#\( p<0.05 \) vs AngII infusion, \$\( p<0.05 \) vs. AngII + gAcrp infusion. Atrial myocytes (C) and fibroblasts (D) were incubated with gAcrp (2.5 \( \mu \)g/ml), or pretreated with 2 \( \mu \)M Ro31-8220 for 1 hour, and then incubated with AngII for 24 hours. Data were expressed as mean ± SD of three independent experiments. \*\( p<0.05 \), \#\( p<0.05 \) vs. blank control, \$\( p<0.05 \) vs. AngII + Ro31-8220. doi:10.1371/journal.pone.0103793.g006

AdipoR1/APPL1 Potentiates the Effects of gAcrp on Atrial Remodeling
with the addition of LY294002 (lane 3 and 4). However, pretreatment of cells with LY294002 prominently reduced the phosphorylation of Akt (lane 4). Atrial fibroblasts displayed the same phenomena (Fig. 5B). Together, these data indicated that the activation of Akt by gAcrp occurred through a pathway that required the activation of PI3K in atrial cells. Based on this signaling axis, atrial myocytes and fibroblasts were pretreated with LY294002 for 60 min and then treated with gAcrp (2 μg/ml). After incubation with AngII for 24 h, ANP and COL1A1 mRNA expression was analyzed by qPCR. As seen in Fig. 6A and 6B, acute treatment with LY294002 (lane 5, p<0.05) attenuated gAcrp protection against atrial hypertrophy and fibrosis. However, Ro31-8220, an Akt activator, exerted its function by inhibiting AngII-induced atrial hypertrophy and fibrosis (Fig. 6C and 6D, lane 4, p<0.05).

**Globular adiponectin attenuated AngII-induced NF-κB activation and translocation in atrial myocytes via AMPK activation**

NF-κB activation has been reported to be essential for hypertrophic growth of atrial myocytes during translocation stimulated by the hypertrophic agonist AngII. Nuclear localization of NF-κB response to AngII was observed (Fig. 7) but globular

---

**Figure 7. Effect of gAcrp on NF-κB translocation in atrial myocytes.** (A) Atrial myocytes were pretreated with or without gAcrp (2.5 μg/ml) for 1 h and then stimulated AngII (10 μM) for 1 h. Cytosol proteins were extracted and immunblotted with anti-p65 antibody. Anti-β-actin antibody was used as control. Data were expressed as mean ± SD of three independent experiments. *P<0.05 vs. blank control, †P<0.05 vs. AngII infusion. (B) Atrial myocytes were pretreated with compound C (10 μM, lane 7) or gAcrp (2.5 μg/ml, lane 6), and then incubated with AngII for 60 min (lanes 4, 6 and 7). Lanes 1 and 2 indicated that nonspecific probes and control IgG were regarded as negative controls. Lanes 3–7 used anti-p65 antibody to certify that all samples in the membrane are significant.

doi:10.1371/journal.pone.0103793.g007

**Figure 8. Schematic model of the proposed effects of gAcrp on Ang II-induced atrial remodeling.** Treatment of AngII induces hypertrophy and fibrosis in atrial cells. gAcrp suppresses AngII-induced hypertrophy and fibrosis in atrial cells through activation of AMPK and downstream signaling pathways. The inhibitory effect of gAcrp on AngII-induced atrial hypertrophy and fibrosis is mediated through AdipoR1- and APPL1-dependent mechanisms.

doi:10.1371/journal.pone.0103793.g008
adiponectin was found to abrogate this response. As shown in Fig. 7A, NF-κB p65 expression decreased in the cytosol (lane 2, p<0.05) as mediated by AngII. Notably, the decrease in cytosol was significantly reversed by the stimulation of gAcrp, whereas gAcrp alone had no significant effect on atrial myocyte basal NF-κB p65 expression (lane 4, p>0.05 and lane 3, p>0.05).

Subsequently, we investigated the DNA p65 interaction to confirm that gAcrp inhibited NF-κB translocation in hypertrophic atrial myocytes induced by AngII. The EMSA results shown in Fig. 7B indicate that the DNA binding ability of NF-κB p65 was prominently increased (lane 4) by AngII but was suppressed by pretreatment with gAcrp (lane 6). Moreover, with pretreatment with compound C (10 μM, an AMPK inhibitor), a larger band shift was observed (lane 7). Anti-NF-κB p65 antibody supershift assays demonstrate that all nuclear extracts specifically contained this transcription factor (lanes 3–7).

Discussion

Here we present new data showing that incubation of gAcrp disrupts AngII-induced atrial hypertrophy and fibrosis in the neonatal rat atrial cells via multiple signaling pathways (see schema in Fig. 8). We investigated the roles of AdipoR1 and APPL1 in the gAcrp-induced signaling pathway that led to protective effects against atrial hypertrophy and fibrosis in neonatal rat atrial myocytes and fibroblasts. Our results demonstrated that downregulation of AdipoR1 or APPL1 caused a prominent attenuation of gAcrp-induced AMPK activation and related downstream signaling pathways. Furthermore, we found that gAcrp, along with AMPK/P38K/Akt, activated signaling, and accelerated NF-κB p65 translocation from the cytosol to the nucleus.

It is well known that plasma adiponectin is negatively regulated by obesity [17]. Adiponectin is the most abundant adipose tissue-derived hormone. Adiponectin exists in two multimers, full-length adiponectin and globular adiponectin [8]. In the present study, we concentrated on globular adiponectin, which has been demonstrated to produce multiple cardioprotective effects, including anti-hypertrophic, anti-fibrotic and anti-ischemia-reperfusion injury effects [10] because this form has a higher affinity for myocytes than full-length adiponectin [18]. Cloning of adiponectin receptors has facilitated the understanding of the molecular mechanisms of adiponectin action [13]. The suppression of AdipoR1 expression largely reduced gAcrp binding with it in myocytes [13].

It has been demonstrated that binding of the docking protein APPL1 with AdipoR1 interacts with the adiponectin signaling axis, and mediates metabolic effects in a variety of cell types [19,20]. APPL1 also acts as a critical regulator of the crosstalk between adiponectin signaling and insulin signaling pathways [21]. Here, we demonstrated, for the first time, in primary neonatal rat atrial myocytes and fibroblasts that APPL1 regulates gAcrp action, promoting the expression of ANP and COL1A1 in hypertrophic atria. APPL1 acts as a vital mediator involved in adiponectin-induced AMPK, P38K activation, phosphorylation of p38 MAPK and Akt [19,21,22]. In accordance with this finding, our proteomics-based analysis also clarified the interaction between APPL1 and the aforementioned signaling pathways. On the basis of these results, we can affirm that AMPK activation by gAcrp prevented AngII-induced hypertrophy and fibrillation.

Several pieces of evidence suggest that exogenous norepinephrine, endothelin-1 (ET-1), and AngII can trigger the pathogenesis of hypertrophic cultured cardiomyocytes and ventricular remodeling [23,24]. Angiotensin has 7 subtypes with vasodilator property. AngII produces important cell signaling cascades and related effects on physiology and pathology in the cardiovascular system [25]. For example, through the G protein-dependent pathway, AngII activates serine/threonine kinases including ERK1/2, p38 MAPK, and JNK, which are responsible for cell growth and hypertrophy. Moreover, activation of JAK/STAT pathway is involved with non-receptor tyrosine kinases by AngII [25]. A previous study demonstrated tyrosine phosphorylation of STAT3 by AngII in a dose-dependent and time-dependent manner in hypertrophic atrial myocytes and fibroblasts [5]. It is reasonable to speculate that ANP and COL1A1 could be regulated by AngII in a dose-dependent manner. Our results indicate significant increase of ANP and COL1A1 expression in the middle of the AngII concentration gradient (10 μM for ANP, and 1 μM for COL1A1) in neonatal rat atrial tissue.

Until recently, multiple gene regulating methods were used to modulate the expression of APPL1 and AdipoR1, the key adaptor protein of adiponectin and its downstream signaling pathway in cell metabolism [26]. Using transient transfection with APPL1 and AdipoR1 target siRNA, we found that both ANP and COL1A1 expressions were significantly inhibited, whereas the non-silencing siRNA did not produce similar effect. Our finding suggests that the inhibition of APPL1 and AdipoR1 expression could attenuate AMPK phosphorylation activated by gAcrp.

To further explore another possible mechanism of cardioprotection mediated by adiponectin, we attempted to investigate Akt and the major, but not only, upstream kinase P3K in atrial cells. Adiponectin induced P3K activation and adenosinergic transduction of dominant-negative P3K, and previous research verified that P3K recovered Akt levels increased by adiponectin in endothelial cells [27]. The selective P3K inhibitor LY294002 could blunt adiponectin-induced AMPK activation in endothelial cells [28]. Conversely, our results indicated that LY294002 alone activated AMPK instead of abrogating phosphorylation of AMPK. This discrepancy may be due to the different experimental methods, cell culture conditions in vitro, and animal species used. The decreased phosphorylation level of Akt could not be recovered with LY294002. These results again emphasize that the signaling axis P3K/Akt was independent of AMPK in the atrial cells.

A number of downstream signaling pathways involved in adiponectin action have been revealed in recent years. AMPK is a serine/threonine protein kinase. Activation of AMPK by adiponectin can stimulate extracellular signal-regulated kinase (ERK) activity and a-adrenergic receptor norepinephrine-induced hypertrophy in neonatal cardiac ventricular myocytes [29]. Another pathway of Akt (protein kinase B) exerts a central role, involving the phosphatidylinositol 3-kinase (PI3K) pathway, in insulin-mediated glucose uptake. The process of the enhancement of insulin action on glucose uptake and phosphorylation of Akt was sensitized by adiponectin in the rat skeletal muscle L6 cell line [30]. Nuclear factor κB (NF-κB), is a key transcription factor that modulates the processes of inflammation, immune response and cell proliferation. Many studies have recently demonstrated that activation of NF-κB may be involved in cardiac structural remodeling [31]. In addition, the activation of NF-κB by AngII-induced cardiac hypertrophy was attenuated by adiponectin via a signaling transduction pathway that involves AMPK [12].

Akt plays a central role in cell signaling pathways of metabolism, proliferation and apoptosis. By binding to its receptors, gAcrp facilitates Akt phosphorylation in 3 myocytes cell lines [30]. Based on this finding, we hypothesize the selective Akt activator Ro31-8220 may exert the same effect as gAcrp in atrial myocytes and fibroblasts. Therefore, our data raises the intriguing possibility that the activation of Akt can mimic gAcrp action in playing a protective role in atrial cells while maintaining normal cardiac

PLOS ONE | www.plosone.org 10 August 2014 | Volume 9 | Issue 8 | e103793

AdipoR1/APPL1 Potentiates the Effects of gAcrp on Atrial Remodeling
efficiency. Conversely, the simple inhibition of Akt cannot prevent the cardioprotective effect of gAcrp. Therefore, the PI3K/Akt signaling pathway is not the only pathway that can account for the protective role of gAcrp in hypertrophic atrial cells. The PI3K/Akt signaling pathway could be one of the most important signal transduction pathways that regulate atrium hypertrophy and fibrosis.

Previous studies have shown that adiponectin protects against myocardial hypertrophy via inhibition of NF-kB translocation, which is induced by AngII in ventricular cardiomyocytes [12]. In addition, the attenuation of NF-kB activation through the phosphorylation of AMPK plays a promotional role in vascular endothelial cells [32]. Our data clearly suggest that AngII can potentially increase the protein expression of NF-kB p65 in nuclear extracts but can only slightly increase NF-kB p65 expression in cytoplasmic extracts. Consistent with the finding, strongly restrained AMPK activation can promote NF-kB p65 nuclear transfer, which reverses the signaling cascade activated by gAcrp.

Cardiomyocyte dysfunction leads to AF, which is a pathological condition closely related to obesity and diabetes. As a major adipocyte-secreted adipokine that is present abundantly in circulation, adiponectin has an important role in maintaining vascular homeostasis, sustaining myocardial diastolic normalization [33] and suppressing atherosclerosis [20]. Nevertheless, the cellular mechanism underlying these protective effects of adiponectin does not account for its systemic effect. In this study, we further investigated the signaling cascade with adiponectin-triggered AMPK and PI3K pathways in atrial myocytes and fibroblasts. The present study focused on the in vitro model of hypertrophy and fibrosis using cultured cells. The in vivo effect of adiponectin and analysis of dysfunctional atrial tissue need to be further investigated.

Conclusions

In conclusion, the current study provides novel supporting evidence that multiple signaling pathways may involve in the cardioprotective effect of adiponectin in neonatal rat hypertrophic atrial myocytes or fibrotic atrial fibroblasts, as induced by AngII. AMPK and PI3K participate the signal transduction through AdipoR1, and the anchoring protein APPL1 is involved in both the gAcrp/AMPK/NF-kB and gAcrp/AMPK/PI3K/Akt signaling axis as part of effective pathways for protection against atrium hypertrophy and fibrillation. Moreover, this report may provide preliminary evidence on the mechanisms of AngII-induced atrial structural remodeling in AF and atrial metabolic disorder.

Supporting Information

Figure S1 Compound C partially inhibits the cardioprotection mediated by gAcrp. Atrial myocytes were incubated with gAcrp (2.5 μg/ml), or pretreated with compound C (AMPK inhibitor, 10 μM) for 1 h and then incubated with AngII for 24 h. Data were expressed as mean ± SD of three independent experiments. **P<0.01 vs. blank control. ##P<0.01 vs AngII infusion. ***P<0.01 vs AngII+ gAcrp infusion. (DOC)

Figure S2 AICAR inhibits hypertrophy of atrial myocytes induced by AngII. Atrial myocytes were pretreated with specific activator of AMPK (AICAR, 1 nM) for 1 h, and then incubated with AngII (10 μM) in the presence of AICAR for 24 h. Data were expressed as mean ± SD of three independent experiments. **P<0.01 vs. blank control, ***P<0.01 vs AngII infusion. (DOC)

Figure S3 Photomicrographs (X100) of cultured atrial myocytes and atrial fibroblasts incubated 72 h after seeding on 6-wells plate. (A) Atrial myocytes beating rate approaching 110 times per minute. (B) Atrial fibroblasts. (DOC)

Acknowledgments

We thank Dr. Fang Wang for her technical assistance. Dr. Mingfang Li is an Assistant Fellow at the Collaborative Innovation Center For Cardiovascular Disease Translational Medicine of Nanjing Medical University.

Author Contributions

Conceived and designed the experiments: ML TC HH. Performed the experiments: TC LG. Analyzed the data: TC ZG. Contributed reagents/materials/analysis tools: MC BY KC. Wrote the paper: TC ML HH.

References

1.  Kannel WB, Abbott RD, Savage DD, McNamara PM (1982) Epidemiologic features of chronic atrial fibrillation: the Framingham study. New Engl J Med 306: 1018–1022.
2.  Benjamin EJ, Wolf PA, D’Agostino RB, Silbershatz H, Kannel WB, et al. (1998) Impact of atrial fibrillation on the risk of death the Framingham Heart Study. Circulation 98: 946–952.
3.  Allessie M, Asuma J, Schotten U (2002) Electrical, contractile and structural remodeling during atrial fibrillation. Cardiovasc Res 54: 230–246.
4.  Li D, Shinagawa K, Pang L, Leung TK, Cardin S, et al. (2001) Effects of angiotensin-converting enzyme inhibition on the development of the atrial fibrillation substrate in dogs with ventricular tachypacing–induced congestive heart failure. Circulation 104: 2608–2614.
5.  Tsai C-T, Lai L-P, Kuo K-T, Hwang J-J, Hsieh C-S, et al. (2008) Angiotensin II activates Signal Transducer and Activators of Transcription 3 via Rac1 in Atrial Myocytes and Fibroblasts Implication for the Therapeutic Effect of Statin in Atrial Structural Remodeling. Circulation 117: 344–355.
6.  Sugden PH, Clerk A (1998) Cellular mechanisms of cardiac hypertrophy. Journal of molecular medicine 76: 725–746.
7.  Hu E, Liang P, Spiegelman BM (1996) AdipoQ is a novel adipose-specific gene dysregulated in obesity. J Biol Chem 271: 10670–10673.
8.  Kadowaki T, Yamauchi T (2005) Adiponectin and adiponectin receptors. Endocr Rev 26: 493–491.
9.  Diczfalusy E (2003) The role of the novel adipocyte-derived hormone adiponectin in human disease. Eur J Endocrinol 148: 293–300.
10. Ouchi N, Shibata R, Walsh K (2006) Cardioprotection by adiponectin. Trends Cardiovasc Med 16: 141–146.
11. Shiibata R, Ouchi N, Kihara S, Sato K, Funahashi T, et al. (2004) Adiponectin stimulates angiogenesis in response to tissue ischemia through stimulation of an-activated protein kinase signaling. J Biol Chem 279: 29870–29874.
12. Wang C, Li L, Zhang ZG, Fan D, Zhu Y, et al. (2010) Globular adiponectin inhibits angiotensin II-induced nuclear factor kB activation through AMP-activated protein kinase in cardiac hypertrophy. J Cell Physiol 222: 149–155.
13. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, et al. (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423: 762–769.
14. Zhou L, Deepa SS, Etzler JC, Ryu J, Mao X, et al. (2009) Adiponectin activates AMP-activated protein kinase in muscle cells via AMPK/LKB1-dependent and phospholipase C/Ca2+/Ca2+/calmodulin-dependent protein kinase kinase-dependent pathways. J Biol Chem 284: 22426–22435.
15. Chlopickova S, Pietrowska J, Miketova P (2001) Neonatal rat cardiomyocytes-a model for the study of morphological, biochemical and electrophysiological characteristics of the heart. Biomedicinal Papers-Palacky University in Olomouc 145: 49–55.
16. Barkawol K, Hartwig J (1995) Actin cytoskeleton: setting the pace of cell movement. Curr Biol 5: 1000–1002.
17. Caup M, Havel P, Utschneider K, Carr D, Sinha M, et al. (2003) Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. Diabetologia 46: 459–469.
18. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, et al. (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 8: 1208–1209.
19. Fang X, Palanivel R, Cresser J, Schram K, Ganguly R, et al. (2010) An APPL1-AMPK signaling axis mediates beneficial metabolic effects of adiponectin in the heart. Am J Physiol-endoc M 299: E721–E729.

20. Tian L, Luo N, Zhu X, Chung B-H, Garvey WT, et al. (2012) Adiponectin-AdipoR1/2-APPL1 signaling axis suppresses human foam cell formation: differential ability of AdipoR1 and AdipoR2 to regulate inflammatory cytokine responses. Atherosclerosis 221: 66–75.

21. Mao X, Kikani CK, Raojas RA, Langlais P, Wang L, et al. (2006) APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. Nat Cell Biol 8: 516–523.

22. Yang L, Lin H-K, Ahluwaijri S, Xie S, Wang L, et al. (2003) APPL suppresses androgen receptor transactivation via potentiating Akt activity. J Biol Chem 278: 16520–16527.

23. Dougherty RN, Whalley GA, Walsh HA, Gamble GD, Lopez-Sendon J, et al. (2004) Effects of Carvedilol on Left Ventricular Remodeling After Acute Myocardial Infarction The CAPRICORN Echo Substudy. Circulation 109: 201–206.

24. Fujikawa D, Kawahata K-i, Saito Y, Kobayashi T, Nakamura T, et al. (2006) Role of adiponectin receptors in endothelin-induced cellular hypertrophy in cultured cardiomyocytes and their expression in infarcted heart. Am J Physiol-heart C 290: H2409–H2416.

25. Mehta PK, Griendling KK (2007) Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. Am J Physiol-cell Ph 292: C82–C97.

26. Park M, Youn B, Zheng X-I, Wu D, Xu A, et al. (2011) Globular adiponectin, acting via AdipoR1/ADPL1, protects H9c2 cells from hypoxia/reoxygenation-induced apoptosis. PloS one 6: e19143.

27. Chandrasekar B, Boylston WH, Venkatachalam K, Webster NJ, Prabhu SD, et al. (2008) Adiponectin blocks interleukin-1β-mediated endothelial cell death via APPL1-dependent AMP-activated protein kinase (AMPK) activation and IKK/NF-κB/PTEN suppression. J Biol Chem 283: 24089–24098.

28. Chen H, Montagnani M, Funahashi T, Shimomura I, Quon MJ (2003) Adiponectin stimulates production of nitric oxide in vascular endothelial cells. J Biol Chem 278: 45021–45026.

29. Shibata R, Ouchi N, Ito M, Kihara S, Shiojima I, et al. (2004) Adiponectin-mediated modulation of hypertrophic signals in the heart. Nat Med 10: 1304–1309.

30. Sattar AA, Sattar R (2012) Globular adiponectin activates Akt in cultured myocytes. Biochem Bioph Res Co 424: 753–757.

31. Purcell NH, Tang G, Yu C, Mercario F, DiDonato JA, et al. (2001) Activation of NF-κB is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes. Proceedings of the National Academy of Sciences 98: 6668–6673.

32. Hattori Y, Suzuki K, Hattori S, Kasai K (2006) Metformin inhibits cytokine-induced nuclear factor κB activation via AMP-activated protein kinase activation in vascular endothelial cells. Hypertension 47: 1183–1188.

33. Zhu W, Cheng K, Vanhouette P, Lam K, Xu A (2008) Vascular effects of adiponectin: molecular mechanisms and potential therapeutic intervention. Clin Sci 114: 361–374.