Heart-bound adiponectin, not serum adiponectin, inversely correlates with cardiac hypertrophy in stroke-prone spontaneously hypertensive rats

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New Findings
- What is the central question of this study?
  An inverse correlation between circulating adiponectin and many diseases has been reported, but some studies have found no correlation. To evaluate this controversy, we investigated the relationship between heart-bound adiponectin and hypertension or cardiac hypertrophy, compared with serum adiponectin.
- What is the main finding and its importance?
  Using hypertensive and normotensive rats, we found that heart-bound adiponectin was inversely correlated with cardiac hypertrophy, suggesting that heart-bound adiponectin has a more important function in preventing cardiac hypertrophy than circulating adiponectin. Our study provides new insights regarding the role of adiponectin in diseases.

The inverse correlation between circulating adiponectin concentration and hypertension or cardiac hypertrophy is still controversial. In addition to circulating adiponectin, adiponectin is also bound to tissues such as the heart and skeletal muscle. In this study, we investigated the relationship of serum adiponectin and heart-bound adiponectin with hypertension and cardiac hypertrophy. Four types of hypertensive rats presenting different blood pressure levels were used at different ages, as follows: normotensive Wistar–Kyoto rats (WKYs); two sub-strains (strains C and B2, having low and high blood pressure, respectively) of spontaneously hypertensive rats (SHRs); and stroke-prone SHRs (SHRSPs). Blood pressure, heart-to-body weight ratio, serum adiponectin and heart-bound adiponectin were determined. Histopathological analysis of the heart was carried out to evaluate the relationship with heart-bound adiponectin. Serum adiponectin concentration was not inversely correlated with blood pressure or heart-to-body weight ratio. In contrast, heart-bound adiponectin levels were significantly lower in SHRSPs than in other strains at respective ages. This resulted from a decrease in T-cadherin expression, which induced adiponectin binding to tissues. No significant difference in heart-bound adiponectin among WKYs and SHRs (C and B2) was detected, indicating that heart-bound adiponectin is not related to hypertension. In addition, differences in heart-bound adiponectin did not affect AMP-activated protein kinase in the traditional adiponectin activation cascade.
Histopathological analysis revealed that heart-bound adiponectin was inversely correlated with cardiomyocyte hypertrophy and left ventricular wall thickness and, in part, with cardiac fibrosis. These results suggest that the decreased level of heart-bound adiponectin in SHRSPs is more related to their cardiac hypertrophy than circulating adiponectin.

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Introduction

Adiponectin, an adipocyte-derived hormone, plays an important role against metabolic syndrome (Kadowaki et al. 2006; Whitehead et al. 2006; Esfahani et al. 2015). Hypoadiponectinaemia is observed in obese subjects and in obesity-related diseases (Arita et al. 1999; Ryo et al. 2004; Okauchi et al. 2009). Thus, circulating adiponectin is considered a useful biomarker for evaluating disease risk. As adiponectin is an anti-metabolic syndrome hormone, there should be an inverse correlation between the adiponectin concentration and disease parameters. In fact, some studies have shown that the circulating adiponectin concentration is inversely correlated with hypertension (Adamczak et al. 2003; Hong et al. 2004; Iwashima et al. 2004; Shankar et al. 2008) and cardiac diseases (Hong et al. 2004; Pischon et al. 2004; Mitsuhashi et al. 2007). In contrast, other studies have found no correlation between the circulating adiponectin concentration and hypertension (Mallamaci et al. 2002; Chiba et al. 2010) or cardiac diseases (Kistorp et al. 2005; Tamura et al. 2007; Kalisz et al. 2015). At present, the relationship between circulating adiponectin concentration and hypertension or cardiac diseases is still controversial.

In the blood, adiponectin forms four homomultimer structures: three full-length types corresponding to a trimer, a hexamer and a polymer [high molecular weight (HMW)] consisting of at least 12-mers, and a truncated globular type (Kadowaki et al. 2006). The HMW and globular adiponectins have higher activity than the other forms. Three adiponectin receptors, AdipoR1, AdipoR2 and T-cadherin, have been reported. Globular and full-length adiponectins bind to AdipoR1 and AdipoR2, respectively. AdipoR1 is mainly expressed in the skeletal muscle and activates AMP-activated protein kinase (AMPK), whereas AdipoR2 is mainly expressed in the liver and activates peroxisome proliferator-activated receptor α (Kadowaki et al. 2006). T-cadherin binds only hexamer and HMW adiponectins (Hug et al. 2004). T-cadherin lacks a transmembrane region, being anchored by glycosylphosphatidylinositol to the membrane, and is structurally different from other traditional cadherins (Vestal & Ranscht, 1992). Therefore, little is known about the signalling pathways activated by adiponectin binding to T-cadherin. We (Inoue et al. 2010) and others (Denzel et al. 2010; Parker-Duffen et al. 2013) have reported that T-cadherin, but not AdipoR1 and AdipoR2, contributes to adiponectin continuous binding to specific tissues, such as skeletal muscle, heart and blood vessels. Thus, decreased T-cadherin expression causes an increase in the circulating adiponectin concentration. We showed that a decrease in adiponectin binding to skeletal muscle via T-cadherin causes severe hypercholesterolaemia and hyperinsulinaemia in rats fed a high-fat diet (Inoue et al. 2010). Denzel et al. (2010) reported that T-cadherin knockout mice showed cardiac hypertrophy in response to pressure overload stress and an increase of infarct size in response to ischaemia–reperfusion. Therefore, adiponectin–T-cadherin binding might be involved in the suppression of metabolic syndrome in stress conditions.

Our hypothesis is that heart-bound adiponectin is more important and more relevant to hypertension and cardiac diseases than circulating adiponectin. In the present study, we investigated the relationship between serum or heart-bound adiponectin with hypertension or cardiac hypertrophy in hypertensive rats.

Methods

Ethical approval

All experimental protocols conformed to the guidelines published in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (8th edition, revised 2011) and were approved by the Institutional Animal Experimentation Committee of Kindai University Faculty of Medicine (approval number KAME15-26).

Animals

Male Wistar–Kyoto rats/Kpo (WKYs), spontaneously hypertensive rats (SHRs)/Kpo (SHR C), SHR/Kpo2 (SHR B2) and stroke-prone SHR/Kpo (SHRSPs), maintained in the Kindai University Life Science Research Institution, were used at 6, 12 and 20 weeks of age. All animals (n = 8 per group in all experiments except for the determination of adiponectin mRNA expression) were housed in the animal centre with constant temperature
and humidity and were fed a Funabashi SP diet (Funabashi Farm, Japan) and tap water ad libitum. Rats were anaesthetized by inhalation of 4% isoflurane for induction and 1.5–3% isoflurane for maintenance and then killed by exsanguination.

Blood pressure was measured using the tail-cuff method. Rats were kept at 38°C for 10 min and then held without anaesthesia with a holding tool during measurement. The concentration of rat serum adiponectin was measured using an enzyme-linked immunosorbent assay (ELISA; Otsuka Pharmaceutical, Tokushima, Japan). For protein analysis, tissues were frozen in liquid nitrogen and then stored at −80°C until use. For pathological analysis, tissues were fixed in 10% buffered formalin (pH 7.4) and then embedded in paraffin.

Antibodies
Anti-rat adiponectin polyclonal antibody was purchased from Biovision (Milpitas, CA, USA), human cadherin-13 (T-cadherin) antibody from R&D Systems (Minneapolis, MN, USA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) conjugated with horseradish peroxidase from Medical & Biological Laboratories (Nagoya, Japan). The AMPK and acetyl-CoA carboxylase (ACC) antibody sampler kit was purchased from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies against rabbit and goat IgG conjugated with horseradish peroxidase from Abcam (Cambridge, MA, USA) were used for Western blotting. Adiponectin, T-cadherin, and GAPDH antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies for horseradish peroxidase were purchased from GE Healthcare Life Sciences (Uppsala, Sweden), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) conjugated with horseradish peroxidase from Medical & Biological Laboratories (Nagoya, Japan). The AMPK and acetyl-CoA carboxylase (ACC) antibody sampler kit was purchased from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies against rabbit and goat IgG conjugated with horseradish peroxidase were purchased from GE Healthcare Life Sciences (Uppsala, Sweden).

Protein expression analysis by Western blotting
Adipose tissue and the left ventricular (LV) wall were homogenized with lysis buffer containing 150 mM Tris–HCl (pH 7.4), 1% Triton X, 1% sodium deoxycholate, 50 mM NaF, 1% Triton X and protease inhibitor cocktail (Sigma, St Louis, MO, USA). The homogenized samples were incubated at 4°C for 1 h and then centrifuged at 12,000 g at 4°C for 15 min. The aqueous phase between debris and fat was carefully collected and centrifuged again in the same conditions. The lysed material so obtained was mixed with 4 × sample buffer containing 0.25 M Tris–HCl (pH 6.8), 8% (v/v) SDS, 40% (v/v) glycerol, 8% (v/v) β-mercaptoethanol, 1 mM dithiothreitol and 0.04% (v/v) Bromophenol Blue and incubated at 95°C for 15 min. The samples were separated by SDS-PAGE using a 10% polyacrylamide gel and electro-transferred to a polyvinylidene fluoride membrane (Merck-Millipore, Billerica, MA, USA). Western blotting was then performed using antibodies against adiponectin, T-cadherin, AMPKα, phospho-AMPKα, ACC, phospho-ACC and GAPDH, and signals were detected using an LAS-4010 instrument (GE Healthcare Life Sciences). The signal intensity was analysed using ImageJ software (US National Institutes of Health, Bethesda, MD, USA) and normalized to that of GAPDH.

Adiponectin multimer analysis was carried out as previously described (Waki et al. 2003; Inoue et al. 2010). Briefly, heart lysates were mixed with 4 × sample buffer without reductants (β-mercaptoethanol and dithiothreitol) and incubated at room temperature for 1 h. The samples were separated by SDS-PAGE using a 5–20% gradient polyacrylamide gel (ATTO, Tokyo, Japan).

Real-time RT-PCR
RNA from the LV wall was purified using an RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany). Single-strand cDNA was synthesized from total RNA using a random hexamer primer with SuperScript IV (Invitrogen, Carlsbad, CA, USA). To analyse the expression levels of adiponectin and 18S rRNA, a 7900 HT real-time PCR System (Applied Biosystems, Foster city, CA, USA) was used. The primers for real-time PCR were as follows: adiponectin forward, 5′GGAAACCTTTGGTGAGGTGGATG-3′; reverse, 5′-GGTTCACTTATGACCAAGAA-3′; and 18S rRNA forward, 5′-ACTCAACACGGGAAACCTCA-3′; reverse, 5′-AACCAGACAAATCGCTCCAC-3′. The cDNA was amplified using the SYBR Premix Ex Taq (TaKaRa, Kyoto, Japan) following the manufacturer’s protocols. The results were normalized to the expression level of 18S rRNA.

Histopathological analysis
Heart tissue was stained with Haematoxylin and Eosin (H&E), Masson’s Trichrome (MT) and Sirius Red (SR). The LV wall thickness and heart muscle cross-sectional area were measured using H&E and MT staining, respectively, with the NIS-Elements D software (Nikon Instruments, Tokyo, Japan). Left ventricular wall thickness was measured at 10 points per rat. Heart muscle cross-sectional area was measured for 50 cardiomyocytes per rat. Cardiac fibrosis was measured using SR staining and ImageJ software.

Statistical analysis
Statistical analysis was carried out with R software (R Foundation for Statistical Computing, Vienna, Austria, https://www.r-project.org/). First, the data obtained were analysed using the Shapiro–Wilks normality test to determine whether to carry out parametric or non-parametric analyses. The Tukey and Steel–Dwass tests were used for parametric and non-parametric analysis, respectively. Correlation analyses were carried out with Pearson’s product–moment correlation coefficient and
Spearman’s rank correlation coefficient for parametric and non-parametric analyses, respectively.

Results

Basic characteristics of WKYs, SHRs and SHRSPs

To investigate the relationship between adiponectin and hypertension or cardiac hypertrophy, the basic characteristics of WKYs, SHR C, SHR B2 and SHRSPs were determined (Table 1). Systolic blood pressure among age-matched strains was significantly different except between 6-week-old SHR C and B2. Body weight and the weight of visceral adipose tissues, including epididymal and retroperitoneal adipose tissues, was inversely correlated with blood pressure among age-matched strains at all ages (data not shown). Heart weight showed a significant inverse correlation with blood pressure in 6- and 12-week-old rats ($R^2 = 0.364$, $P < 0.001$ and $R^2 = 0.235$, $P = 0.005$, respectively) and positive correlation in 20-week-old rats ($R^2 = 0.264$, $P = 0.002$). To evaluate cardiac hypertrophy more accurately, the heart-to-body weight ratio (in milligrams per gram) of all rats was calculated. The heart-to-body weight ratios showed a weak positive correlation with blood pressure in 6-week-old rats ($R^2 = 0.128$, $P = 0.044$) and a strong positive correlation in 12- and 20-week-old animals ($R^2 = 0.826$, $P < 0.001$ and $R^2 = 0.668$, $P < 0.001$, respectively), indicating that heart weight and heart-to-body weight ratio increase with the development of hypertension. No significant difference was observed in serum adiponectin concentrations in 6-week-old rats from all strains. For 12-week-old rats, serum adiponectin concentrations in SHR B2 and SHRSP strains were significantly higher than those in WKY and SHR C strains. Serum adiponectin concentrations of 20-week-old SHRSP rats were significantly higher than those of age-matched animals from other strains. The serum adiponectin concentration in SHR B2 was significantly higher than those in WKY and SHR C strains. Inverse correlations between serum adiponectin and blood pressure or heart-to-body weight ratio were not observed at the respective ages (Table 1). Therefore, these results indicate that serum adiponectin concentration is not related to hypertension and cardiac hypertrophy among these rats.

Adiponectin production in adipose tissue

Western blotting analysis of lysates from retroperitoneal adipose tissues showed that adiponectin monomer was detected as a doublet in reducing and heating conditions (Fig. 1A), as previously described (Inoue et al. 2010). The upper band was similar to that of circulating

| Parameter | WKY | SHR C | SHR B2 | SHRSP |
|-----------|-----|-------|--------|-------|
| 6 weeks   |     |       |        |       |
| Blood pressure (mmHg) | 121.4 ± 3.8 | 139.4 ± 8.0*** | 139.8 ± 5.0*** | 160.3 ± 4.7***,†††,‡‡‡ |
| Body weight (g) | 195.4 ± 8.1 | 176.3 ± 8.8*** | 172.4 ± 7.7*** | 155.0 ± 8.2***,†††,‡‡‡ |
| Adipose tissue weight (g) | 2.619 ± 0.283 | 1.448 ± 0.192** | 1.584 ± 0.177** | 1.445 ± 0.143* |
| Heart weight (g) | 0.784 ± 0.027 | 0.762 ± 0.034 | 0.748 ± 0.034 | 0.670 ± 0.053***,†††,‡‡‡ |
| Heart weight/body weight (mg g⁻¹) | 4.019 ± 0.196 | 4.329 ± 0.186** | 4.340 ± 0.173** | 4.308 ± 0.167* |
| Serum adiponectin (μg ml⁻¹) | 2.71 ± 0.30 | 2.77 ± 0.37 | 3.30 ± 0.56 | 3.06 ± 0.55 |
| 12 weeks  |     |       |        |       |
| Blood pressure (mmHg) | 148.1 ± 2.7 | 171.5 ± 11.6*** | 186.6 ± 9.5***,‡‡‡ | 235.6 ± 3.7***,†††,‡‡‡ |
| Body weight (g) | 373.6 ± 12.0 | 341.4 ± 9.1*** | 317.4 ± 11.6***,††† | 270.9 ± 13.3***,†††,‡‡‡ |
| Adipose tissue weight (g) | 11.370 ± 1.263 | 7.349 ± 1.275** | 6.734 ± 0.617** | 6.730 ± 0.758** |
| Heart weight (g) | 1.257 ± 0.055 | 1.230 ± 0.049 | 1.220 ± 0.042 | 1.165 ± 0.073** |
| Heart weight/body weight (mg g⁻¹) | 3.364 ± 0.126 | 3.606 ± 0.157* | 3.813 ± 0.145** | 4.299 ± 0.145*,†††,‡‡‡ |
| Serum adiponectin (μg ml⁻¹) | 2.32 ± 0.36 | 2.37 ± 0.22 | 3.16 ± 0.69* | 2.97 ± 0.28† |
| 20 weeks  |     |       |        |       |
| Blood pressure (mmHg) | 153.5 ± 8.9 | 172.4 ± 3.9*** | 209.4 ± 5.6***,††† | 270.0 ± 8.8***,†††,‡‡‡ |
| Body weight (g) | 427.3 ± 21.1 | 423.5 ± 10.7 | 391.1 ± 13.3††† | 349.5 ± 14.5**,†††,‡‡‡ |
| Adipose tissue weight (g) | 13.068 ± 1.564 | 10.710 ± 2.364 | 10.272 ± 1.721*** | 8.899 ± 1.743***,†††,‡‡‡ |
| Heart weight (g) | 1.424 ± 0.076 | 1.443 ± 0.084 | 1.481 ± 0.065 | 1.519 ± 0.059 |
| Heart weight/body weight (mg g⁻¹) | 3.333 ± 0.119 | 3.410 ± 0.247 | 3.790 ± 0.202*** | 4.354 ± 0.251**,†††,‡‡‡ |
| Serum adiponectin (μg ml⁻¹) | 1.55 ± 0.28 | 2.21 ± 0.81 | 2.37 ± 0.31* | 3.60 ± 0.67***,†††,‡‡‡ |

Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 versus age-matched WKY; †P < 0.05, ††P < 0.01, †††P < 0.001 versus age-matched SHR C; and ‡P < 0.05, ‡‡P < 0.01, ‡‡‡P < 0.001 versus age-matched SHR B2. §Adipose tissue weight represents the weight of both epididymal and retroperitoneal adipose tissues.
Heart-bound adiponectin in cardiac hypertrophy

Heart-bound adiponectin and T-cadherin expression in the heart

Based on the results of adiponectin concentrations in sera and adipose tissues from all rats tested, we speculated that higher concentrations of serum adiponectin in hypertensive rats, especially in SHRSPs, result from a decrease in heart-bound adiponectin. Western blotting analysis of LV wall lysate (Fig. 2A) showed a polypeptide corresponding to the single monomer migrating as serum adiponectin (data not shown), a pattern different from

![Image of Western blot analysis](image)

**Figure 1. Adiponectin production in adipose tissue**

*Panel A* shows Western blot of adipose tissue lysates in reducing and heating conditions. *Panel B* displays densitometric analysis of *Panel A*. Doublet adiponectin detection (upper band, lower band, and a combination of both) was normalized with GAPDH. Data are presented as means ± SD, and the mean value of WKYs was set as 1.0.

*adiponectin (Inoue et al. 2010). The difference between the monomer doublet adiponectin is attributed to hydroxylation or glycosylation (Wang et al. 2006). The signal intensities corresponding to the upper and lower bands individually and in combination (total) from all rats were compared. No significant difference was found among all age-matched rats in the three comparisons above (Fig. 1B), suggesting that hypertensive rats, which present higher concentrations of circulating adiponectin (Table 1), did not increase adiponectin production in adipose tissue.*
Figure 2. Heart-bound adiponectin and T-cadherin expression in the heart
A, Western blot of left ventricular lysates in reducing and heating conditions. B and C, densitometric analysis of heart-bound adiponectin (B) and T-cadherin (C). Adiponectin and T-cadherin levels were normalized to those of GAPDH. D, Western blot of left ventricular lysates in non-reducing and
that observed in adipose tissue (Fig. 1A). Heart-bound adiponectin decreased significantly in SHRSps when compared with age-matched animals from other strains at all ages (Fig. 2B). Heart-bound adiponectin levels among WKY and SHR strains (C and B2) were not significantly different, indicating that heart-bound adiponectin is not related to hypertension in hypertensive rats.

To exclude the possibility that the observed heart-bound adiponectin is a product of the heart adiponectin gene, adiponectin mRNA expression in the LV wall was determined. The results show that adiponectin transcript levels were not significantly different among age-matched rats (Table 2), and no correlation with heart-bound adiponectin was observed (data not shown). In addition, adiponectin expression in the heart was detected at very low levels compared with that in retroperitoneal adipose tissue (10^3- to 10^4-fold difference, depending on age; data not shown), indicating that adiponectin expressed in the heart represents a very small fraction of total adiponectin in heart lysates as well as in the circulation.

As we and others have shown, T-cadherin, but not AdipoR1 and AdipoR2, contributes to continuous adiponectin binding to skeletal muscle, heart and smooth muscle (Denzel et al. 2010; Inoue et al. 2010; Parker–Duffen et al. 2013), and T-cadherin expression in the heart was also analysed (Fig. 2A). The results showed that T-cadherin expression in the heart decreased significantly in SHRSps compared with age-matched rats from other strains at all ages, indicating that the decrease in heart-bound adiponectin in SHRSps results from reduced T-cadherin expression in the heart (Fig. 2A and C). Furthermore, multimer structure analysis of the heart-bound adiponectin showed that HMW adiponectin decreased significantly in SHRSps, and no significant difference was observed between WKY and SHR strains (Fig. 2D and E), consistent with results in the monomer analysis (Fig. 2B). However, the levels of hexamer adiponectin in SHRSps were significantly different from those of all other rats at 12 weeks of age and from SHR C at 6 and 20 weeks of age. Trimers were hardly detected, because T-cadherin can bind hexamer and HMW adiponectin.

Activation of AMPK and ACC by heart-bound adiponectin

The AMPK activation cascade induced by adiponectin is reported to participate in the prevention of cardiac hypertrophy (Shibata et al. 2004). To analyse whether activation of the AMPK cascade is affected by adiponectin binding to the heart, phosphorylation of AMPK and ACC was measured by Western blotting (Fig. 3A). The results showed that there was no significant difference in phosphorylation of AMPK and ACC among all age-matched rats (Fig. 3B). In addition, correlation analysis showed that heart-bound adiponectin and circulating adiponectin were not related to phosphorylation of AMPK or ACC at any age (data not shown). These results indicate that the differences in heart-bound adiponectin through T-cadherin do not affect the traditional adiponectin cascade through AdipoR1 and AdipoR2.

Determination of cardiomyocyte hypertrophy and fibrosis

To analyse the relationship between heart-bound adiponectin and cardiac hypertrophy in detail, histopathological analysis of the heart was carried out. Representative images of the heart from rats at all ages are shown in Fig. 4. No significant difference was found in LV wall thickness among 6-week-old rats (Fig. 5A). The WKY and SHRSps strains had significantly thicker LV walls than the SHR C and B2 strains at 12 weeks of age. In 20-week-old rats, a significant difference was observed only in SHRSps compared with all other strains. The heart muscle cross-sectional area of SHRSps was significantly larger than those of other rats at the corresponding ages, but no significant difference was observed between WKY and SHR strains (Fig. 5B). These results were consistent with the analysis of heart-bound adiponectin (Fig. 2B). The area of fibrosis in 6-week-old WKYSs was significantly smaller than that in other age-matched rats (Fig. 5C). In addition, 20-week-old SHRSps showed a significantly larger fibrotic area compared with that of age-matched SHR C.

Correlation between heart-bound adiponectins and histopathological data

Western blotting analysis indicated the possibility of a close relationship between heart-bound adiponectin and heart muscle cross-sectional area (Figs 2B and 5B). Correlation analysis showed a moderate inverse correlation at 12 and 20 weeks of age (Table 3). Correlations with LV wall thickness and cardiac fibrosis were also analysed. Moderate inverse correlations were observed with LV wall thickness at 12 and 20 weeks of age (Table 3),
indicating that the thickness results from cardiomyocyte hypertrophy. A weak correlation was also observed with cardiac fibrosis at 20 weeks of age (Table 3). Furthermore, the presence of multimer adiponectins (HMW and hexamer) bound to the heart was determined. In contrast to monomer analyses, HMW adiponectin levels presented a moderate and inverse correlation with heart muscle cross-sectional area in all ages tested (Table 3). However, no correlation with cardiac fibrosis was found. A moderate inverse correlation was also detected between hexamer adiponectin and heart muscle cross-sectional area at all ages (Table 3). In contrast to HMW adiponectin, a weak inverse correlation was observed in cardiac fibrosis at 20 weeks of age. The different correlations obtained between HMW and hexamer adiponectins suggest that these two types of adiponectins might have different biological activities.

**Discussion**

In the present study, we evaluated the effects of circulating and heart-bound adiponectins on blood pressure and

| Age     | WKY      | SHR C   | SHR B2  | SHRSP   |
|---------|----------|---------|---------|---------|
| 6 weeks | 1.000 ± 0.537 | 0.630 ± 0.308 | 0.643 ± 0.313 | 0.462 ± 0.187* |
| 12 weeks| 0.214 ± 0.175  | 0.335 ± 0.281  | 0.193 ± 0.091  | 0.129 ± 0.139  |
| 20 weeks| 0.290 ± 0.107  | 0.167 ± 0.071  | 0.139 ± 0.071  | 0.165 ± 0.087  |

*Messenger RNA expression levels (n = 5 per group) were normalized using 18S rRNA levels. Data are presented as means ± SD, and the mean value of WKY was set as 1.0. *n = 4.*
Figure 4. Representative images of heart tissue
Haematoxylin and Eosin (H&E), Masson’s Trichrome (MT) and Sirius Red (SR) staining of tissues from 6-, 12- and 20-week-old rats. Scale bars represent 50 μm.
cardiac hypertrophy by using normotensive (WKY) and hypertensive (SHR and SHRSP) rats. Our results showed that: (i) circulating adiponectin concentrations were not inversely correlated with any of the hypertension-related parameters tested in this study; (ii) heart-bound adiponectin did not relate to hypertension; and (iii) heart-bound adiponectin was inversely correlated with cardiomyocyte hypertrophy, LV wall thickness and, in part, with cardiac fibrosis.

The relationship between circulating adiponectin and cardiac hypertrophy is still controversial. In studies showing correlations between circulating adiponectin and cardiac diseases, some subjects with high concentrations of circulating adiponectin developed cardiac diseases (Pischon et al. 2004; Mitsuhashi et al. 2007). The present study proposes an explanation for such subjects, who might present a decrease in heart-bound adiponectin. In contrast, several groups reported that subjects with cardiac diseases present higher adiponectin concentrations and HMW adiponectin in the blood (Kistorp et al. 2005; Karas et al. 2014). These observations might result from cardiomyocyte necrosis, which leads to the release of heart-bound adiponectin, resulting in higher adiponectin concentrations in the blood. T-cadherin disorder and T-cadherin knockout in rodents are strongly related to high adiponectin concentrations in the blood (Denzel et al. 2010; Inoue et al. 2010). Therefore, heart-bound adiponectin and T-cadherin expression are also considered important factors to determine circulating adiponectin concentrations.

It has been reported that other tissues besides adipose tissue express adiponectin (Delaigle et al. 2004, 2006; Pineiro et al. 2005; Guo et al. 2007; Krause et al. 2008). Pineiro et al. (2005) and Guo et al. (2007) reported that the heart expresses and secretes adiponectin. However, adiponectin detected in heart lysates was considered in the present study as circulating adiponectin rather than from the heart itself. This conclusion is based on the following observations: (i) when adiponectin is expressed in tissues and cells, endogenous and exogenous

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**Figure 5. Histopathological analysis of the heart**

A, left ventricular (LV) wall thickness. B, heart muscle cross-sectional area. C, cardiac fibrosis area. Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 versus age-matched WKY; †P < 0.05, ††P < 0.01, †††P < 0.001 versus age-matched SHR C; and ‡P < 0.05, ‡‡P < 0.01, ‡‡‡P < 0.001 versus age-matched SHR B2.
Heart-bound adiponectin in cardiac hypertrophy

Table 3. Correlation analysis between heart-bound adiponectins and histopathological data

| Parameter            | 6 weeks old | 12 weeks old | 20 weeks old |
|----------------------|-------------|--------------|--------------|
| Cross-sectional area |             |              |              |
| Monomer              | —           | Negative     | Negative     |
|                      |             | $R^2 = 0.268, P = 0.003$ | $R^2 = 0.379, P < 0.001$ |
| Hexamer              | Negative    | Negative     | Negative     |
|                      | $R^2 = 0.241, P = 0.004$ | $R^2 = 0.357, P < 0.001$ | $R^2 = 0.168, P = 0.020$ |
| HMW                  | Negative    | Negative     | Negative     |
|                      | $R^2 = 0.314, P < 0.001$ | $R^2 = 0.273, P = 0.002$ | $R^2 = 0.352, P < 0.001$ |
| LV wall thickness    |             |              |              |
| Monomer              | —           | Negative     | Negative     |
|                      |             | $R^2 = 0.251, P = 0.003$ | $R^2 = 0.332, P < 0.001$ |
| Hexamer              | —           | Negative     | Negative     |
|                      |             | $R^2 = 0.266, P = 0.002$ | $R^2 = 0.335, P < 0.001$ |
| HMW                  | —           | Negative     | Negative     |
|                      |             | $R^2 = 0.418, P < 0.001$ | $R^2 = 0.284, P = 0.002$ |
| Area of fibrosis     |             |              |              |
| Monomer              | —           | —            | Negative     |
|                      |             |              | $R^2 = 0.155, P = 0.026$ |
| Hexamer              | —           | —            | Negative     |
|                      |             |              | $R^2 = 0.208, P = 0.009$ |
| HMW                  | —           | —            |              |

Abbreviations: HMW, high molecular weight; LV, left ventricular; Negative, negative correlation; and —, no correlation.

Adiponectin monomers are detected as a doublet (Pajvani et al. 2003; Waki et al. 2003; Inoue et al. 2010), and heart lysates from all rats in the present study showed a single polypeptide that migrated as serum adiponectin; (ii) adiponectin gene expression in the LV wall was much lower than that in adipose tissue ($10^4$- to $10^5$-fold in adipose tissue), which had a much heavier weight than the heart (Table 1); and (iii) no adiponectin is detected in heart lysates from T-cadherin knockout mice (Denzel et al. 2010).

A difference was observed in heart-bound adiponectin between SHRSP and other rats, but no difference was detected between WKY, SHR C and SHR B2 strains. These results suggest that heart-bound adiponectin as well as circulating adiponectin concentrations are not related to hypertension in hypertensive rats. However, heart-bound adiponectin, especially HMW and hexamer adiponectin, were correlated with cardiomyocyte hypertrophy. These results are in agreement with those obtained by Shibata et al. (2004) and Fujita et al. (2008), showing that: (i) angiotensin II infusion does not result in blood pressure differences between adiponectin knockout and wild-type mice; and (ii) angiotensin II induces cardiac hypertrophy and fibrosis in adiponectin knockout mice. Our data showed a weak correlation between heart-bound adiponectin (monomer and hexamer, but not HMW) and cardiac fibrosis only in 20-week-old rats. Therefore, hexamer adiponectin may partly correlate with cardiac fibrosis, although fibrosis might be more related to other factor(s). Moreover, we need to evaluate cardiac fibrosis in rats at older ages to confirm this observation.

The mechanisms by which adiponectin protects against cardiac hypertrophy remain unclear. We analysed phosphorylation of AMPK and ACC because AMPK activation is reported to suppress cardiac hypertrophy (Shibata et al. 2004). Our results show that heart-bound adiponectin is not related to AMPK–ACC cascade activation. Shibata et al. (2004) showed that addition of adiponectin has a protective effect against cardiomyocyte hypertrophy. They concluded that AMPK activation by trimer adiponectin, but not hexamer or HMW, is important for the protective effect of adiponectin. In our study, similar to what was described by Shibata et al. (2004), no AMPK activation by hexamer or HMW adiponectins was observed.

In contrast, Denzel et al. (2010) reported that other adiponectins besides trimer adiponectin are necessary for the protective effects induced by activating AMPK from pressure overload and ischaemia–reperfusion by using T-cadherin knockout mice. We cannot discuss the effects of trimer on cardiac hypertrophy, because we could not detect trimer adiponectin in heart lysates. However, prevention or development of cardiac hypertrophy by AMPK activation is still controversial (Byrne et al. 2014). Thus, it is possible that another pathway might be involved in suppression of cardiac hypertrophy by heart-bound adiponectin through T-cadherin. When compared with monomer adiponectin, HMW and hexamer adiponectins were more protective against cardiac hypertrophy, because
both these adiponectins were inversely correlated with heart muscle cross-sectional area in all ages tested, whereas monomer adiponectin at 6-week-old rats (Table 3) was not. The different correlations between heart muscle cross-sectional area and monomer and multimer adiponectins remain unclear. Given that adiponectin forms hybrid multimers with Cq1/tumour necrosis factor-related protein (Wong et al. 2009), it is possible that adiponectin from adiponectin−Cq1/tumour necrosis factor-related protein hybrid multimers might be detected in monomer analysis, but hardly detected in multimer analysis with an anti-adiponectin antibody.

The present study suggests the importance of heart-bound adiponectin for cardiac hypertrophy in hypertensive rats. However, we were unable to determine how heart-bound adiponectin protects against cardiac hypertrophy. Further studies are needed to clarify the specific mechanisms underlying the relationship between cardiac hypertrophy and heart-bound adiponectin.

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Additional information

Competing interests

None declared.

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