Role of adiponectin receptors in endothelin-induced cellular hypertrophy in cultured cardiomyocytes and their expression in infarcted heart

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Fujikura, Daisuke, Ken-ichi Kawabata, Yukio Saito, Tsuyoshi Kobayashi, Takamitsu Nakamura, Yasushi Kodama, Hajime Takano, Jyun-ee Obata, Yoshinobu Kitta, Ken Umetani, and Kiyotaka Kugiyama. Role of adiponectin receptors in endothelin-induced cellular hypertrophy in cultured cardiomyocytes and their expression in infarcted heart. Am J Physiol Heart Circ Physiol 290: H2409–H2416, 2006. First published January 13, 2006; doi:10.1152/ajpheart.00987.2005.—Adiponectin, an adipocyte-derived protein, plays a fundamental role in energy homeostasis of adipose tissue, plays a fundamental role in energy homeostasis and glucose and lipid metabolism in adipose tissue and has insulin-sensitizing effects on liver and skeletal muscle (3, 5, 9, 12). Shibata et al. (15, 16) recently demonstrated that adiponectin suppresses cardiac hypertrophy in response to pressure overload and protects the heart from ischemia-reperfusion injury. Recently, it has been shown that AMP-activated protein kinase (AMPK), an important regulator of the adiponectin signaling pathway (22), not only improves myocardial glucose and lipid metabolism but also prevents ventricular contractile dysfunction in the ischemic heart (14). It is also known that abnormalities in glucose and lipid metabolism in cardiac muscle are associated with heart failure (6, 13). Thus it is possible that adiponectin might exert cardioprotective properties in various heart diseases. Adiponectin exerts its action through two recently discovered receptors, AdipoR1 and AdipoR2 (21). Previous reports (2, 17) have shown that skeletal muscle produces adiponectin and expresses adiponectin receptors. However, the expression remains unclarified in cardiac muscle. Cardiac hypertrophy in the remote area of the infarcted heart is initially a compensatory response of myocardial tissue to increased mechanical load, but its early beneficial effects become maladaptive, leading to heart failure at a later phase of myocardial infarction (8, 19, 23). Among several neurohumoral factors activated after myocardial infarction, endothelin-1 (ET-1) plays an important role in the genesis of myocyte hypertrophy after myocardial infarction (8, 23). Thus this study examined the possible role of AdipoR1 and AdipoR2 in ET-1-induced cellular hypertrophy in cultured cardiomyocytes and AdipoR1 and AdipoR2 expression in infarcted hearts in animal models. The results demonstrate a potential role for the cardiac adiponectin system in the pathogenesis of cardiac hypertrophy.

MATERIALS AND METHODS

Materials. Rat recombinant full-length adiponectin was purchased from BioVision (Mountain View, CA) and globular adiponectin from Adipogen (Sungnam, Korea). Both adiponectins were derived from bacteria (Escherichia coli). The full-length adiponectin forms monomers, trimers, hexamers, and high-molecular-weight multimers, and the globular adiponectin forms monomers, dimers, and trimers. Anti-AdipoR1 and anti-AdipoR2 polyclonal antibodies were purchased from Alpha Diagnostic International

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Briefly, after trypsinization, the cells were collected by ultracentrifugation and diluted to 5 × 10^5 cells/ml in DMEM containing 10% FCS. Primary cultures of rat neonatal cardiomyocytes were prepared by trypsin-EDTA digestion from ventricles of 1- to 3-day-old Sprague-Dawley rats as described previously (15).

Preparation and culture of rat cardiomyocytes. The experimental protocol was approved by University of Yamanashi Animal Care and Use Committee, and procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996). Primary cultures of rat neonatal cardiomyocytes were prepared by trypsin-EDTA digestion from ventricles of 1- to 3-day-old Sprague-Dawley rats as described previously (15).

Measurements of mRNA and protein expression levels in myocardium and cultured cardiomyocytes. Total RNA was extracted from myocardial tissues, skeletal muscle (soleus muscle), intraperitoneal adipose tissue of rats and mice, and rat cultured cardiomyocytes. Expression levels of mRNA for adiponectin, AdipoR1, and AdipoR2 were quantified by a real-time two-step RT-PCR assay with use of SYBR green polymerase, and a sequence detection system (GeneAmp 5700, PE Applied Biosystems, Foster City, CA). The PCR primers are listed in Table 1. The GAPDH housekeeping gene was used for normalization of target gene expression.

For immunoblot analysis, the extracts of myocardial tissue, skeletal muscle, and intraperitoneal adipose tissue of rats and mice or the treated cells were matched for protein concentration (15 μg) with SDS-PAGE sample buffer and separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were incubated with the indicated primary antibodies overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibody at 1:20,000 dilution. The ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ) was used for detection. Intensity of the β-tubulin band was used as a loading control between samples.

Table 1. Sequences of sense siRNAs and PCR primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Real-time PCR</th>
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<tbody>
<tr>
<td>Mouse adiponectin</td>
<td>5′-GCAAGCTTCCCTGTGCTCTTAATC-3′</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-GGTACTGATGGTCGCCCTCC-3′</td>
<td></td>
</tr>
<tr>
<td>Mouse AdipoR1</td>
<td>5′-ACGGTTGAGAGTCATCCGGAT-3′</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5′-CTCTGGTCTGTAAGG-3′</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-GGCAGGATGTCACATTTGCCA-3′</td>
<td></td>
</tr>
<tr>
<td>Mouse AdipoR2</td>
<td>5′-GCCAGAAGGTAAAGGTTT-3′</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5′-AAGCATTTCGATTCTACAGAG-3′</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-GGAGCCATTCTCTGGCTT-3′</td>
<td></td>
</tr>
<tr>
<td>Rat AdipoR1</td>
<td>5′-TCTTCCTCAATGCGTGA-3′</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5′-GGCCTCTGGATGGCCACCTG-3′</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-TCCTGTTCCCTTGAGTAT-3′</td>
<td></td>
</tr>
<tr>
<td>Rat AdipoR2</td>
<td>5′-GAGGGATGTCACATTTGCCA-3′</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5′-ACGGATGTCACATTTGCCA-3′</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-GGCAGGATGTCACATTTGCCA-3′</td>
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siRNA, small interfering RNA; AMPK, AMP-activated protein kinase; AdipoR1 and AdipoR2, adiponectin receptors.
Measurement of protein synthesis and cell surface area in cultured cardiomyocytes. Protein synthesis in cultured cardiomyocytes was evaluated by incorporation of [3H]leucine into the cells as described in previous reports (11, 15). Briefly, cardiomyocytes on a 24-well plate were pretreated with or without adiponectin for 4 h. The cells were incubated for 42 h with or without ET-1 (100 nmol/l) and for an additional 6 h with 1 μCi/ml [3H]leucine (Amersham). The cultures were washed twice with ice-cold PBS and fixed with 10% TCA.

Fig. 2. Photomicrographs of cultured cardiomyocytes treated with PBS (A), ET-1 (100 nmol/l; B), full-length adiponectin (30 μg/ml) + ET-1 (100 nmol/l; C), or combination of small interfering RNAs (siRNAs) for AdipoR1 and AdipoR2 and adiponectin (30 μg/ml) + ET-1 (100 nmol/l; D).

Fig. 3. Effects of suppression of AdipoR1, AdipoR2, or AMP-activated protein kinase (AMPK)-α2 by siRNAs on inhibitory action of adiponectin on ET-1-induced hypertrophic responses in cultured cardiomyocytes. After transfection of small interfering RNA (siRNA), cardiomyocytes were pretreated with full-length (30 μg/ml) or globular (2.5 μg/ml) adiponectin and then incubated with ET-1 (100 nmol/l), and cell surface area and [3H]leucine incorporation were measured. Values are means ± SE (n = 6). *P < 0.05. A: effects of AdipoR1 siRNA, AdipoR2 siRNA, combining siRNAs, or unrelated siRNA on inhibitory action of full-length adiponectin on ET-1-induced increase in surface area. B: effects of siRNAs on inhibitory action of full-length adiponectin on ET-1-induced increase in [3H]leucine incorporation. C: effects of siRNAs on inhibitory action of globular (glb) adiponectin on ET-1-induced increase in [3H]leucine incorporation. D: effect of AMPKα2 siRNA on inhibitory action of full-length adiponectin on ET-1-induced increase in [3H]leucine incorporation.
After the cultures were washed, radioactivity in the TCA-precipitable materials was determined after solubilization in 0.25 N NaOH. NIH Image J analysis software was used to measure surface area of fixed cardiomyocytes. One hundred cells from randomly selected fields in three wells were examined for each condition. The cell surface area was determined in cells pretreated for 4 h with or without adiponectin or AICAR and then incubated with ET-1 (100 nmol/l), ANG II (100 nmol/l), or IGF-I (100 nmol/l) for 48 h.

RNA interference and transfection. Small interfering RNAs (siRNAs) were designed and synthesized by Invitrogen. The sequences of the sense siRNAs are listed in Table 1. The cultured cardiomyocytes were transfected with 270 nM siRNA with use of Lipofectamine 2000. After the cultures were washed, the medium was replaced with DMEM containing 0.5% FCS for 12 h. AdipoR1, AdipoR2, or AMPKα2 was suppressed with the appropriate siRNA for determination of the effects of adiponectin on AMPK and ACC phosphorylation and ET-1-induced cellular hypertrophy and ERK phosphorylation.

Animal models of myocardial infarction. Myocardial infarction was created in 12- to 16-wk-old male mice and rats by ligation of the left coronary artery under anesthesia with pentobarbital sodium (50 mg/kg ip) and ventilation with a respirator. The chest was closed with 7.0 polypropylene sutures, and the animals were killed and tissues were harvested 2 wk after the surgery.

Parts of the tissue samples from the left ventricle were quickly frozen and stored at −80°C until measurement of mRNA and protein expression levels. Other parts of the samples were fixed in 10% formalin solution and embedded in paraffin and then sliced into 5-μm-thick sections, which were stained by the immunoperoxidase method (Vectastain ABC Kit, Vector Laboratories) with use of the indicated primary antibodies. The samples were counterstained with hematoxylin.

Statistical analysis. Values are means ± SE. An unpaired t-test was used to compare the mean value between two groups. ANOVA with Scheffe’s F procedure for post hoc analysis was used for comparison among three or more groups. P < 0.05 was considered statistically significant.

RESULTS

Role of adiponectin receptors in inhibitory effects of adiponectin on ET-1-induced hypertrophy of cultured cardiomyocytes. Compared with cultured cardiomyocytes treated with ET-1 alone, recombinant full-length adiponectin dose dependently suppressed the ET-1-induced increase in cell surface area and the cellular incorporation of [3H]leucine (Figs. 1, A and B, and 2). Full-length adiponectin also inhibited the ANG II- or IGF-I-induced cellular hypertrophy (Fig. 1 C).

Fig. 4. Effects of siRNA transfection on protein expression levels of AdipoR1 (A), AdipoR2 (B), and AMPKα2 (C). Cultured cardiomyocytes were transfected with each siRNA, cultures were washed, and extracts of cells were used for immunoblot analysis. Intensity of β-tubulin band was used as a loading control between samples. Protein levels are expressed relative to nontreated control cells (n = 1). Values are means ± SE (n = 6). *P < 0.05 vs. control.
length adiponectin, and siRNA for AdipoR1, but not AdipoR2, reversed the actions of globular adiponectin (Fig. 3C). Neither siRNA for AdipoR1 nor siRNA for AdipoR2 in the absence of adiponectin changed ET-1-induced cellular hypertrophy (data not shown).

Adiponectin induced AMPK phosphorylation and inhibited ET-1-induced ERK1/2 phosphorylation, which was also reversible by transfection of siRNA for AdipoR1 or AdipoR2 in cultured cardiomyocytes (Fig. 5, A and B). Transfection of siRNA for AMPKα2 reduced the inhibitory effect of adiponectin on ET-1-induced cellular incorporation of [3H]leucine (Fig. 3D) and ERK phosphorylation (Fig. 5B), in parallel with suppression of AMPKα2 protein expression levels (Fig. 4C). Adiponectin induced ACC phosphorylation, which was also reversed by AMPKα2 siRNA (Fig. 5C).

Effects of AICAR on ET-1-induced cellular hypertrophy and ERK phosphorylation. AICAR dose dependently inhibited the ET-1-induced increase in cell surface area of the cultured cardiomyocytes (Fig. 1D). AICAR inhibited ERK1/2 phosphorylation induced by ET-1 treatment but did not affect ERK1/2 phosphorylation at baseline (Fig. 1E).

Expression of adiponectin and its receptors in normal and infarcted hearts in animal models. Protein expression levels of AdipoR1, AdipoR2, and adiponectin in the left ventricle were similar in magnitude to those in skeletal muscle in mice (Fig. 6, E–H); however, mRNA levels of AdipoR1 and AdipoR2 were higher in the ventricle than in skeletal muscle (Fig. 6, A and B). The mRNA expression level of AdipoR1 was higher than that of AdipoR2 in the left ventricle (Fig. 6D). Compared with the normal left ventricle, expression levels of AdipoR1 mRNA and protein were decreased in the remote, as well as the infarcted, area 2 wk after myocardial infarction in mice (Fig. 7). Expression levels of AdipoR2 mRNA and protein were decreased in the infarcted area. AdipoR2 expression levels had a tendency to decrease in the remote area, but the change was not significant (Fig. 7).
Levels of AdipoR1 and AdipoR2 in cultured cardiomyocytes were performed to determine the effect of these neurohumoral factors on mRNA expression levels of adiponectin receptors in cultured cardiomyocytes. Because TNF-α, ANG II, and norepinephrine, as well as ET-1, have been reported to play a possible role in the pathogenesis of postinfarct ventricular remodeling (4, 8, 18, 19), experiments were performed to determine the effect of these neurohumoral factors on mRNA expression levels of AdipoR1 and AdipoR2 in cultured cardiomyocytes. TNF-α and norepinephrine significantly inhibited mRNA expression levels of AdipoR1 and AdipoR2 in cultured cardiomyocytes (Fig. 9).

**Discussion**

Using siRNAs specific for AdipoR1 and AdipoR2, we have shown that AdipoR1 and AdipoR2 mediated the suppressive effects of full-length adiponectin on ET-1-induced cardiomyocyte hypertrophy. AdipoR1 and AdipoR2 were expressed in the left ventricle and skeletal muscle to a similar extent. Furthermore, AdipoR1 and AdipoR2 expression levels were decreased in the infarcted area of the left ventricle. Also, AdipoR1 expression levels were significantly decreased in the remote area of the left ventricle. AdipoR2 expression levels had a tendency to decrease in the remote area, but the change was not significant. ET-1 has previously been shown to contribute to cardiomyocyte hypertrophy, leading to heart failure after myocardial infarction (8, 23). Therefore, the present results indicate that the myocardial expression of AdipoR1 and AdipoR2 might play a role in the regulation of cardiomyocyte hypertrophy after myocardial infarction in the remote, as well as the infarcted, area.

It has been previously shown that AMPK is involved in the signaling pathway for the metabolic effects of adiponectin (22). Furthermore, activation of AMPK was shown to suppress ERK phosphorylation, which leads to cardiac hypertrophy under pressure loading (15). The present study showed that adiponectin induced AMPK phosphorylation in association with suppression of ET-1-induced ERK phosphorylation in cultured cardiomyocytes. Furthermore, siRNA for AMPK also suppressed the inhibitory effects of adiponectin on ET-1-induced cellular hypertrophy. Taken together, the inhibitory effects of adiponectin on ET-1-induced cellular hypertrophy may be at least partly mediated via the AMPK-ERK pathway in cultured cardiomyocytes. In support of this notion, the present study also showed that AICAR, a specific stimulator of AMPK, mimicked the results obtained with adiponectin. Furthermore, AMPK activation is known to stimulate fatty acid oxidation, which may lead to inhibition of cardiomyocyte hypertrophy (1, 6, 14, 15, 22). The present study also showed that adiponectin induced phosphorylation of ACC, an important regulator of fatty acid oxidation, through AMPK. Thus it is also possible that adiponectin might influence myocardial energy substrate utilization, including glucose uptake and fatty acid oxidation through AMPK, and, thereby, block hypertrophic growth and, in addition, the suppressive effect of AMPK on ERK phosphorylation. The present study also showed that adiponectin suppressed the hypertrophic response of cultured cardiomyocytes to IGF-I, which stimulates the IGF-I receptor, a tyrosine kinase receptor distinct from G protein-coupled receptors. It has been reported that postreceptor signaling cascades of IGF-I share the ERK pathway to cardiac hypertrophy (7). It remains to be determined whether adiponectin may uniformly suppress cardiac hypertrophy induced by stimulations converging to a common pathway with ERK.

Although adiponectin is produced in the heart, its expression in the myocardium was extremely low compared with that in adipose tissue. Therefore, circulating adiponectin, rather than adiponectin produced locally in the myocardium, seems to act as the predominant ligand for myocardial adiponectin receptors; however, adiponectin produced in the myocardium may function in an autocrine or paracrine manner. The biological activities of adiponectin have been shown to depend on the structure and the oligomeric state (10, 20). Adiponectin in human or mouse serum formed trimers, hexamers, and high-molecular-weight species (10, 20). It is not clear whether these...
oligomers may have different affinities for AdipoR1 and AdipoR2, leading to different biological actions of adiponectin in assays in vitro. The present study using siRNA for AdipoR1 and AdipoR2 showed that both receptors were involved in the effects of full-length adiponectin, whereas AdipoR1, but not AdipoR2, mediated the effects of globular adiponectin. The different roles of AdipoR1 and AdipoR2 may be explained by the different affinity of these receptors for full-length and globular adiponectin, as previously reported (9, 21).

The precise regulatory mechanisms for the myocardial expression of AdipoR1 and AdipoR2 remain undetermined, but the present study showed that AdipoR1 and AdipoR2 expression was suppressed by TNF-α and norepinephrine, which importantly participate in the pathogenesis of myocardial remodeling (4, 18). This finding is reminiscent of counteractions between adiponectin and TNF-α on insulin sensitivity in adipocytes (5, 9, 12). The present immunohistochemical study showed that the adiponectin receptors were expressed mainly in myocytes and that they were weakly expressed in fibrous tissue of the infarcted myocardium. Therefore, a loss of cardiomyocytes may contribute to a decrease in mRNA and protein expression levels of the adiponectin receptors in the infarcted myocardium. However, it is possible that TNF-α and norepinephrine may participate in the decrease in expression of the adiponectin receptors, especially AdipoR1, in the remote myocardium of the infarcted heart. It remains to be determined whether expression of adiponectin receptors per cardiomyocyte is decreased in the surviving myocardium in the infarcted area.

In conclusion, AdipoR1 and AdipoR2 mediate the inhibitory effects of adiponectin on ET-1-induced cardiomyocyte hypertrophy, and AMPK is involved in signal transduction through these receptors. The present study suggests that the myocardial expression of AdipoR1 and AdipoR2 might play a role in the pathogenesis of ET-1-related cardiomyocyte hypertrophy and subsequent heart failure after myocardial infarction. Furthermore, this study may provide a clue regarding mechanisms of cardiac metabolic disorder in ischemic heart disease.
ADIPONECTIN RECEPTORS IN CARDIOMYOCUTES

This study was supported by Grants-in-Aid B2-15390244, for Priority Areas C and 15012222 for Medical Genome Science from the Ministry of Health, Labor, and Welfare, and for Comprehensive Research on Aging and Health (n = 6). *P < 0.05 vs. PBS.

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