Effect of Irbesartan on Angiotensin II-Induced Adiponectin Expression in Human Cardiomyocytes

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Background: Excessive angiotensin II (AngII) can cause cardiac dysfunction and failure. Adiponectin is an abundant plasma protein secreted from adipocytes that elicits protective effects in the vasculature and myocardium. We therefore hypothesized that AngII may induce the expression of adiponectin in cultured human cardiomyocytes (HCM), and adiponectin expression could perhaps be modified by angiotensin type 1 receptor blocker (ARB) irbesartan.

Methods: The HCM were stimulated either with or without AngII, and the inhibitory effects of irbesartan on adiponectin expression were tested. The levels of phosphorylated extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) proteins induced by AngII, and the effects of specific inhibition on the ERK, p38, and JNK pathways on the AngII-induced adiponectin protein expression were also tested.

Results: AngII dose-dependently induced adiponectin expression in HCM. Pretreatment with irbesartan significantly blocked the increase of adiponectin protein by the AngII. AngII significantly increased phosphorylation of ERK, p38, and JNK, while PD98059, SB205380, and SP600125 attenuated the phosphorylation of the 3 signaling pathways, respectively, and adiponectin expression induced by AngII.

Conclusion: Our study revealed that AngII enhances adiponectin expression in cultured HCM, probably mediated through the ERK, p38, and JNK pathways. The use of ARB irbesartan can inhibit the AngII-induced adiponectin expression.

Key Words: Adiponectin • Angiotensin II • Angiotensin receptor blocker • Cardiomyocytes

INTRODUCTION

The rennin-angiotensin-aldosterone system (RAAS) is activated in patients with various hypertension and obesity-related conditions, including heart failure (HF).1-5 Angiotensin II (AngII), produced by the enzymatic cascade involved in RAAS, can cause cell proliferation and migration, vascular contraction, induce intracellular oxidative stress and promote tissue fibrosis.1,3,6 Moreover, RAAS exists not only in the circulatory system, but also can be activated inside many tissues and cells.1,2 Excessive myocardial AngII plays a critical role in cardiac remodeling, for it can promote cardiomyocyte hypertrophy, which leads to cardiac dysfunction and failure.1,2 Adiponectin is an abundant plasma adipokine derived from adipose tissue that elicits protective effects in the vasculature and myocardium.1 Adiponectin protects heart tissue from ischemia-reperfusion injury,7,8 inhibits hypertrophic signaling in the myocardium, and contributes to remodeling of the myocardial extracellular matrix.10-12 Circulating concentrations of adiponectin are
decreased in obese patients and inversely correlated with cardiovascular risk factors. Hypoadiponectinemia is an independent risk factor for the development of HF. On the other hand, plasma adiponectin concentrations have recently been shown to be increased in patients with overt HF, and high adiponectin levels were independently predictive of clinical outcomes in HF. These studies are difficult to interpret because systemic wasting, associated with high adiponectin levels, is a positive predictor of mortality in patients with HF.

Recent studies have demonstrated that adiponectin is synthesized and secreted by isolated human cardiomyocytes (HCM), and adiponectin is present in damaged cardiomyocytes. Skurk et al have also discovered the existence of a local cardiac adiponectin system, down-regulated in those patients with dilated cardiomyopathy. Although the amount of locally synthesized cardiac adiponectin is too small to contribute significantly to the circulating concentrations of adiponectin, as compared to that of adipose tissue, the adiponectin generated locally may still determine the biological effects on myocardial cells.

Recent data also demonstrated that AngII decreased plasma concentrations of adiponectin and adipose tissue levels of adiponectin mRNA through a reactive oxygen species-dependent mechanism. A RAAS blockade appears to increase plasma adiponectin, improve insulin resistance and metabolic disturbances, and reduce oxidative stress in adipose tissue in obese and/or hypertensive patients and animals with insulin resistance. However, the direct effect of AngII on cardiomyocyte adiponectin expression has not yet been investigated.

Since the mechanisms of adiponectin signaling are multiple and vary among their cellular sites of action, we hypothesize that, if adiponectin acts as a counter-regulatory factor for AngII, the expressions of adiponectin in cultured HCM should be increased under the stimulation of AngII, and the expressions of adiponectin could then be modified by angiotensin type 1 (AT1) receptor blocker (ARB) irbesartan.

Isolation of total RNA and real-time polymerase chain reaction (PCR)

Total RNA was isolated using a RNeasy Mini kit and a RNase-free DNase set (Qiagen, Valencia, CA, USA). RNA (2 μg) was reverse-transcribed and the relative content of mRNA was quantified by real-time TaqMan-PCR (LightCycler FastStart DNA Master SYBR Green I; Roche, Eppstein-Bremthal, Germany). The following primers for real-time PCR were designed in our laboratory using Real Quant (Roche, Eppstein-Bremthal, Germany) based on published sequences and were used in this study: human adiponectin sense primer: 5'–GGT GAG AAA GGA GAT CCA GGT–3'; antisense primer: 5’–TCC TTT CCT GCC TTG GAT T–3'; and human GAPDH sense primer: 5’–AGC CAC ATC GCT CAG ACA–3'; antisense primer: 5’–GCC CAA TAC GAC CAA ATC C–3'.

Western blot analysis

The cell lysate was prepared using cell lysis buffer (Cell Signaling, Beverly, MA, USA), and Western blot analyses were performed. The cell lysate (25 to 40 μg) were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes, and then underwent blotting. After being blocked with 5% skim milk in Tween-20/PBS, blots were incubated with various primary antibodies, including anti-human adiponectin, polyclonal anti-p38 mitogen-activated protein (MAP) kinase, monoclonal anti-phospho p38 MAP kinase antibodies (Cell Signaling, Beverly, MA, USA), extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK). Equal protein loading of the samples was verified by staining monoclonal α-tubulin antibody (Chemicon, Temecula, CA,
USA). Blots were then incubated with horseradish peroxidase-conjugated secondary antibodies. The signal was detected using Chemiluminescence Reagent Plus (NEN, Boston, MA, USA). The intensity of each band was scanned and quantified using a densitometer linked to computer software (ImageQuant, Amersham, UK).

The effect of AngII on cultured HCM and possible signal transduction pathways

To test the effect of AngII on adiponectin expression on HCM, the cultured HCM were pretreated with the growth medium supplemented with AngII at 50, 100, 150 and 200 ng/mL for 24 hours at 37 °C. To determine the roles of ERK, p38 MAP kinase, or JNK in the expression of AngII-induced adiponectin expression, HCM were pretreated with SP600125 (20 μM, Calbiochem, San Diego, CA, USA), SB203580 (10 μM, Calbiochem, San Diego, CA, USA), or PD98059 (10 μM, Calbiochem, San Diego, CA, USA) for 60 mins, respectively, and followed by the addition of AngII. The PD98059 is a specific and potent inhibitor of ERK. The SB203580 is a highly specific, cell permeable inhibitor of p38 kinase. The SP600125 is a potent, cell-permeable, selective, and reversible inhibitor of JNK. In experiments involving the AT1 receptor antagonist, irbesartan at 100 nM was added 30 mins before AII stimulation.

Statistics analyses

All values were expressed as mean ± SEM. Comparisons between multiple groups were determined by means of a one-way analysis of variance (ANOVA) followed by Dunnett’s test. A p value of less than 0.05 was considered statistically significant.

RESULTS

Induction of adiponectin on HCM by AngII

After HCM and AngII were incubated for 24 hours, the protein and mRNA expression of adiponectin were increased, as compared with those incubated with a control medium (Figure 1). The AngII-induced adiponectin protein and mRNA expression was dose-dependent. AngII at 100 ng/mL showed the maximal effect to enhance adiponectin protein and mRNA expression in HCM; hence, the concentration of AngII used for the following experiments was 100 ng/mL.

Angiotensin receptor blocker irbesartan inhibits AngII-induced adiponectin expression in HCM

HCM were pretreated with irbesartan (100 nM, an ARB) 30 mins before the addition of the control medium and 100 ng/mL AngII. The enhanced adiponectin protein expression in AngII-stimulated HCM was significantly reduced when the HCM were pretreated with the growth

Figure 1. Incubation for 24 hrs of human cardiomyocytes (HCM) with angiotensin II (AngII) increased the protein and mRNA expression of adiponectin as compared with those incubated with control medium. The AngII-induced adiponectin protein and mRNA expression was dose-dependent. AngII at 100 ng/mL showed the maximal effect to enhance adiponectin protein and mRNA expression in HCM. *p < 0.05, compared to baseline condition.
medium supplemented with irbesartan (89% reduction, p < 0.0001, Figure 2).

Possible signal transduction pathways in AngII-induced adiponectin expression

AngII at 100 ng/mL significantly increased the adiponectin protein expression while compared with the control medium. As shown in Figure 3, phosphorylated ERK, p38, and JNK proteins were induced by AngII stimulation in time-dependent manners. In Figure 4, the western blot also demonstrated that the increase of AngII-induced adiponectin protein expression was significantly, while partially, attenuated after the addition of PD98059, SB203580 and SP600125, 60 mins before AngII stimulation. The results suggest that AngII increases adiponectin protein expression probably through all the three signal transduction pathways.

DISCUSSION

The main findings of this study are: (1) the increased adiponectin expression to AngII-stimulated HCM was reduced when the HCM were pretreated with ARB irbesartan; (2) stimulation of cultured HCM with AngII significantly increased phosphorylated ERK, p38, and JNK protein expressions; and (3) the increased expression of adiponectin was inhibited by specific inhibitors of the 3 signaling pathways. To the best of our knowledge, these findings have never been reported previously.

In pathological state, AngII functions as a local biologically active mediator in the progression of cardiovascular remodeling. Excessive production of local

![Figure 2. Human cardiomyocytes (HCM) were pretreated with irbesartan (100 nM, an antagonist of the angiotensin type 1 receptor), 30 mins before the addition of control medium and 100 ng/mL angiotensin II (AngII). The enhanced adiponectin protein expression in AngII-stimulated HCM was significantly reduced when the HCM was pretreated with the growth medium supplemented with irbesartan (89% reduction, p < 0.0001). One representative example of 3 experiments is shown here. *p < 0.05, compared to baseline condition; $ p < 0.05, compared to AngII-induced adiponectin protein expression.](image)

![Figure 3. Angiotensin II (AngII) at 100 ng/mL significantly increased the adiponectin protein expression, compared with the control medium. Phosphorylated ERK, p38, and JNK proteins were induced by AngII stimulation in time-dependent manners. *p < 0.05, compared to baseline condition.](image)
AngII in the myocardium promotes cardiac myocyte hypertrophy,\(^1,3\) and enhances synthesis of extracellular matrix.\(^1,6\) AngII is also an important stimulus to trigger the initial steps toward myocardial cell degeneration and death, and thus plays a critical role in the maladaptive myocardial remodeling and HF.\(^2\) In this context, RAAS blockade is a proven effective therapeutic approach to the treatment of chronic HF.\(^4,5\) The underlying mechanisms may include the reduction of circulating AngII and aldosterone levels, or a decrease in AngII binding to AT1 receptor.\(^4,5\)

Adipose tissue is an important source of adipokines, which have a broad array of physiologic effects, including their important regulatory role in myocardial function.\(^12\) The events which these adipokines can regulate include alterations in myocardial metabolism, cardiomyocyte hypertrophy, cell death, and structure and composition of the extracellular matrix.\(^12\) Among the various adipokines, adiponectin has been reported to play an important role in the regulation of cardiac remodeling. Adiponectin inhibits hypertrophic signaling in the myocardium through activation of AMP-activated protein kinase (AMPK) signaling.\(^7,10\) In addition to its vascular effects that indirectly protect ischemic-reperfused cardiomyocytes, in vitro studies have demonstrated that adiponectin promotes cell survival and inhibits cell death.\(^7,9\) Moreover, adiponectin protects against cardiac fibrosis and dysfunction.\(^7,11,12\) Furthermore, the enhanced cardiac hypertrophy to pressure overload, and the worsening of myocardial ischemia-reperfusion injury in adiponectin-deficient animals was ameliorated by supplementation of exogenous adiponectin, which suggests that adiponectin may directly protect cardiomyocytes.\(^7,10\) The severe cardiac fibrosis and the dysregulation of reactive oxygen species-related mRNAs and left ventricular dysfunction observed in AngII-infused adiponectin knockout mice was also improved by adenovirus-mediated adiponectin treatment.\(^11\)

Initially, adiponectin was considered to be exclusively synthesized and secreted by adipocytes. Adiponectin is abundantly present in serum exerting its effects in distant target tissues through the circulation. As mentioned earlier, circulating adiponectin levels are decreased in obese patients and inversely correlated with cardiovascular risk factors.\(^13\) Hypoadiponectinemia is an independent risk factor for the development of HF.\(^14\) However, plasma adiponectin concentrations are increased in patients with overt HF, and high adiponectin levels are independently predictive of clinical outcomes in HF.\(^15-17\) The increase in plasma adiponectin in HF patients might represent preservation of the physiologic response to the change in body fat and may play a pathophysiologic role in the wasting process of cardiac cachexia.\(^30\) But it might also represent a compensatory response to prevent the progression of HF, much like the counter-regulating hormone natriuretic peptides.\(^31\) If so, with successful anti-failure therapy, the plasma adiponectin concentrations should return to basal conditions with improvement of HF. In support of this hypothesis, a recently published report demonstrated that the treatment with beta-blocker carvedilol was associated with reduced plasma adiponectin concentration; while the decrease in plasma adiponectin levels was associated with the improvement of left ventricular ejection fraction in HF patients.\(^31\) However, RAAS blockade with angiotensin converting enzyme inhibitors or ARB has been reported as being able to increase the plasma adiponectin concentration in patients with hypertension.

**Figure 4.** The Western blot demonstrated that the increase of angiotensin II (AngII)-induced adiponectin protein expression was significantly, but partially, attenuated after the addition of PD98059, SB203580, and SP600125 60 mins before AngII stimulation. One representative example of 3 experiments is shown here. * p < 0.05, compared to baseline condition; $ p < 0.05, compared to AngII-induced adiponectin protein expression.
and insulin resistance, and in mice with viral myocarditis.\textsuperscript{33-35} Moreover, the reports on the effects of RAAS blockade on circulating levels of adiponectin, specific in patients with overt HF, are scarce and inconsistent.\textsuperscript{31,34} Therefore, there could be no consistent results of anti-failure drugs on the effects of plasma adiponectin. It is only fair to emphasize here that the present study was designed to merely study the possible physiologic protective result of higher adiponectin under AngII stimulation at cardiomyocytes. Through our test results, we cannot infer how the long-term use of ARB affects circulating levels of adiponectin in HF patients, since the response of cardiomyocytes to AngII stimulation, while belonging to “rescue” or “response to injury”, last but a relatively short period of time. Hence, the effects of anti-failure drugs on adipokines and body wasting in patients with HF and the prevention and treatment of cachexia and cachexia in HF are topics deserving further research. Moreover, whether or not adipokines may become potential targets for the treatment of HF also calls for further investigation.

Recent studies have demonstrated that adiponectin is present in damaged myocytes, and adiponectin is synthesized and secreted by isolated HCM. These findings suggest that the damaged cardiac cells may possess an adiponectin autocrine/paracrine system.\textsuperscript{19-21} Skurk et al. have demonstrated the existence of such a local cardiac adiponectin system, which is dysregulated in dilated cardiomyopathy, and suggested that adiponectin has a part to play in the pathogenesis of dilated cardiomyopathy.\textsuperscript{22} In their report, they showed, for the first time, that adiponectin and adiponectin receptor expression in healthy human hearts and down-regulation of intramyocardial adiponectin mRNA and protein in patients with moderate and severe dilated cardiomyopathy were independent of circulating adiponectin concentrations.\textsuperscript{22} The mechanisms underlying this down-regulation remain unknown. However, given the fact that, at the organ level, microvascular endothelium modulate local levels of adiponectin, the inflamed microvascular networks in HF may contribute to the generalized loss of physiological cardiomyocyte adiponectin levels in the diseased states,\textsuperscript{33,36} where the increased oxidative stress and the cross-talk between inflammatory cells and cardiomyocytes are also potential mechanisms.\textsuperscript{37,38}

Our finding that treatment with an ARB irbesartan can decrease AngII-induced adiponectin release from cultured HCM supports of the hypothesis that, in physiologic conditions, endogenously produced local AngII stimulates transcription and expression of adiponectin in cardiomyocytes, which elicit protective effects in the myocardium. In another of our studies, we have also demonstrated that endothelin-1, one of the neurohumoral factors activated in HF, which plays an important role in the genesis of myocyte hypertrophy, significantly increased cell size and adiponectin expression in HCM.\textsuperscript{39} However, in failing hearts and injured heart tissues, this autocrine system may be down-regulated, partially through the aforementioned mechanisms.\textsuperscript{22,35,36} Actually, in an animal model with acute myocarditis, the expression of adiponectin can be induced by using ARB can-desartan, probably to provide cardioprotective effects against HF.\textsuperscript{32,33}

Our study also revealed that AngII enhances adiponectin expression in cultured HCM, probably mediated through the ERK, p38, and JNK pathways. As much as we know, each MAP kinase (MAPK) signaling cascade is involved in the mediation and regulation of a large number of distinct and even opposing cellular processes. This diversity raises the question as to how the specificity of each signaling cascade is regulated. Several mechanisms have been proposed to explain the signaling specificity and time-dependent effects of various MAPKs: the duration and strength of the signal, the interaction with various scaffold proteins, the cross-talk with other signaling pathways, activated or inhibited simultaneously with the MAPK cascades, the distinct sub-cellular localization of components of the cascades, and so forth.\textsuperscript{40} Furthermore, the mechanisms of adiponectin signaling are multiple and vary in terms of their cellular sites of action. For example, peroxisome proliferator-activated receptor-γ (PPARγ) stimulates adiponectin transcription in adipocytes; whereas, AngII inhibits it through a reactive oxygen species-dependent mechanism. In the presence of an ARB, AngII is bound to the angiotensin type 2 (AT2) receptor. AT2 receptor activation stimulates PPARγ, leading to adiponectin production.\textsuperscript{24,25} Therefore, adiponectin may respond differentially to different pathologic stimuli in different cell types in different physiologic and pathologic conditions. Further investigation into the pathophysiologic interaction among adiponectin, adiponectin receptors, together
with their interplay in signal transduction, will be most fascinating, and has the potential to lead to the discovery of novel treatment strategies for cardiovascular disease and HF.

Certain limitations of the present study are noted as follows: (1) we did not investigate whether exogenous adiponectin suppresses AngII expression; (2) the present study did not exclude a role for the effects of AngII on the expression of other factors involved in adiponectin expression; (3) nor did it rule out the possibility that the molecules tested could act through unrelated mechanisms in addition to AT1 receptor activation.

CONCLUSION

The test results discovered in our study clearly indicate that AngII increases adiponectin expression in cultured cardiomyocytes through activation of AT1 receptor and the ERK, p38, and JNK signaling pathways. The AngII-induced adiponectin protein synthesis can be inhibited by the ARB irbesartan. This study may proffer some clue to research into mechanisms of adiponectin derangement in the pathophysiology of HF.

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