Is the lack of adiponectin associated with increased ER/SR stress and inflammation in the heart?

Neeraja J Boddu1, Sue Theus1,2, Shoake Luo3, Jeanne Y Wei1,3,*, and Gouri Ranganathan1,3,*

1The Central Arkansas Veterans Healthcare System; Little Rock, AR USA; 2Department of Pathology; University of Arkansas for Medical Sciences; Little Rock, AR USA; 3Department of Geriatrics; University of Arkansas for Medical Sciences; Little Rock, AR USA

Keywords: adiponectin, ER/SR stress, heart, inflammation, H9C2 cells

Introduction

There is considerable clinical evidence linking diabetes and cardiovascular disease with ER/SR stress and tissue inflammation in cardiomyocytes. The adipose tissue is an endocrine organ that senses and responds to energy needs, metabolic changes, and inflammation. Among the endocrine functions of adipose tissue is the production of adipokines, such as adiponectin, leptin, and resistin. These adipokines regulate lipid synthesis and storage, gluconeogenesis, and glucose utilization in target organs, including muscle (skeletal and cardiac), adipose tissue, and liver. Adiponectin is the most abundant adipocyte-specific cytokine that has putative anti-diabetic and anti-atherogenic and cardioprotective properties.1,2 Adipogenesis activates adiponectin expression; however an inhibition occurs with the development of obesity, where the secretion of adiponectin is shown to be reduced by TNFα.3 ER stress downregulates adiponectin processing and consequently modifies adipokine secretion in human adipocytes.4 The levels of adiponectin are lower in diabetic patients as compared with non-diabetic subjects.3 Decreased circulating levels of plasma adiponectin may contribute to inflammation and insulin resistance leading to cardiac hypertrophy and heart failure.5 Adiponectin modulates cardiac remodeling in response to pressure overload through its ability to regulate adaptive angiogenesis.6 Adiponectin binds to and activates two trans-membrane receptors and both are expressed in cardiomyocytes. Adiponectin receptors are downregulated in obesity-linked insulin resistance and in metabolic syndrome.8 It has been shown that adiponectin activates AMPK in skeletal muscle, thereby directly regulating glucose metabolism and insulin sensitivity in vitro and in vivo.9 AMP activating protein kinase protects cardiomyocytes from endoplasmic reticulum (ER) stress by downregulating HSPA5, CHOP, and caspase.10 ER stress has been proposed as the immediate cause for chronic inflammation and reduced insulin action at the molecular, cellular, and organism levels.11 ER stress activates the unfolded protein response, which triggers both adaptive and apoptotic pathways.12 ER stress can also alter Ca2+ ATPase/Serca2A levels, thus regulating cardiac contractility.13,14

This study was designed to examine the cardioprotective role of adiponectin. We compared the adipose tissue and heart

*Correspondence to: Jeanne Y Wei; Email: weijeanne@uams.edu; Gouri Ranganathan; Email: ranganathangouri@uams.edu
Submitted: 08/21/2013; Revised: 10/01/2013; Accepted: 10/02/2013
http://dx.doi.org/10.4161/adip.26684
muscle of WT mice and ADKO mice in response to a high fat diet. Based on the changes in gene expression in the heart tissue, we hypothesized that the lack of adiponectin could trigger ER stress and increased inflammation. We therefore studied tunicamycin (an N-glycosylation inhibitor)- and palmitate (saturated fatty acid)-induced ER stress in triggering pro-inflammatory responses in cardiomyocytes. We examined the protective role of adiponectin in lowering inflammation, resulting in decreased ER stress and an increase in Serca2A, thus tilting the balance toward cardioprotective effects.

**Results**

**Effect of high fat diet on body weight and adiponectin**

High fat diet increased body weight in both the WT (30 ± 5%) and the ADKO (35 ± 8%) mice. There was no significant difference in weight gain between the WT and ADKO groups. High fat diet is known to decrease plasma adiponectin levels. High fat diet decreased adiponectin mRNA 3-folds in adipose tissue in WT mice, WT-CH: 2.8 ± 0.6, WT-HF*: 0.85 ± 0.29(* P < 0.05). Adiponectin mRNA was not detectable in adipose tissues of chow or high fat fed ADKO mice (data not shown).

**Effect of HF diet on ER stress and inflammation in ADKO adipose tissue**

To study the role of adiponectin in regulating ER/SR stress in the adipose tissue, we compared HSPa5, ATF6, and ERN1 in adipose tissues of WT and ADKO mice. HSPa5, ATF6, and ERN1 transcripts did not increase between chow fed WT and ADKO mice. HSPa5 increased by 30 ± 5% (* P < 0.05 vs. chow fed) in HF fed ADKO mice; however other markers of ER stress ATF6 and ERN1 were not altered significantly by HF diet (Fig. 1A).

TNFα transcript was increased by 20 ± 5% (* P < 0.05 vs. WT chow) but inflammatory marker CD68 was not increased in ADKO as compared with WT on a chow diet (Fig. 1B). HF diet increased TNFα by 20 ± 5% (* P < 0.05 vs. WT HF) and CD68 increased 3-fold in both WT and ADKO mice in response to HF diet († P < 0.05 vs. WT or ADKO chow) (Fig. 1B).

**Effect of high fat diet on ER/SR and inflammatory marker transcripts in ADKO heart left ventricle**

To examine whether ER/SR stress transcripts are regulated in the heart tissue by the lack of adiponectin, we compared HSPa5, ERN1, and GADD34 expression in WT and ADKO mice on chow or HF diets. There was no change in adiponectin mRNA in WT mice in response to HF (data not shown). As shown in Figure 2A, HSPa5 was increased by 30 ± 5% (* P < 0.05) in ADKO heart when compared with WT on a chow diet, but HSPa5 was not further increased by HF. ERN1, which encodes the ER stress response protein IRE1, increased 2-fold in ADKO heart as compared with WT on a chow (* P < 0.05); ERN1 was not further increased by HF. Growth arrest and DNA damage indicator transcript GADD34 increased 2-fold (* P < 0.05) in ADKO heart as compared with WT and HF increased GADD34 by 25 ± 5% (P < 0.05) in ADKO heart tissues. Thus, HSPa5, ERN1, and GADD34 are increased significantly in the ADKO heart.

![Figure 1. Effect of HF diet on ER stress and inflammation in ADKO adipose tissue. (A) Relative change in ER stress transcripts HSPa5, ATF6, and ERN1 in WT and ADKO mice fed chow or HF diet. Values shown are obtained from real time measurements of each transcript normalized to 18s RNA. Data are presented as means ± SD of 18s normalized expression. Mean ± SD, n = 5–6. *P < 0.05 vs. ADKO chow). (B) Change in inflammatory markers. Relative change in TNFα and CD68 transcripts in adipose tissues of WT and ADKO mice fed chow or HF diet. Values shown are obtained from real time measurements of each transcript normalized to 18s RNA. Mean ± SD, n = 5–6. *P < 0.05 vs. WT chow, ††P < 0.05 vs. chow groups in both WT and ADKO.](image)

To study the role of adiponectin in the induction of inflammation in the heart, we measured the change in CD68 and TNFα transcripts in WT and ADKO heart tissues. As shown in Figure 2B, both CD68 and TNFα increased in ADKO mice on a chow diet. The increase in CD68 was 80 ± 5% (* P < 0.05 vs. WT chow), and TNFα was increased by 30 ± 5% (* P < 0.05 vs. WT chow). High fat diet did not increase CD68 or TNFα significantly in the WT and ADKO mice. Thus, TNFα and CD68 are increased significantly in the ADKO heart.

**Effect of tunicamycin or palmitate on H9C2 cardiomyoblasts**

ER stress can be chemically induced in cultured cells using tunicamycin. To determine the effect of ER stress in cardiomyocytes, H9C2 cells were treated with increasing concentrations of tunicamycin for 6 h. The changes in ER stress indicators are shown in Figure 3A. HSPa5, GRP94, and ATF6 were upregulated 2-fold (* P < 0.05 vs. control) and adiponectin and adiponectin
receptor 1 were inhibited by 25 ± 5% (*P < 0.05 vs. control) in response to treatment with 0.1 μg/ml of tunicamycin or 200 μM palmitate conjugated with BSA were examined for changes in HSPa5 and Iκβα proteins using western blots (Fig. 3C). As shown in Figure 3D, following tunicamycin or palmitate treatments, HSPa5 normalized to β-actin was increased more than 5-fold (*P < 0.05 vs. control) and Iκβα normalized to β-actin decreased by 5-fold (*P < 0.05 vs. control). Iκβα protein expression was lower in DMSO control, when compared with BSA control (Fig. 3C); however, ER stress gene induction was comparable between DMSO and BSA controls (Fig. 3B). Thus ER/SR stress gene induction by tunicamycin or palmitate is accompanied a decrease in Iκβα.

The effect of adiponectin on ER stress and inflammation genes in H9C2 cardiomyoblast cells

Adiponectin in circulation has three multimeric forms. The function of the three forms is not clearly understood. However, low levels of total plasma adiponectin are associated with coronary artery disease and insulin resistance.17 The adiponectin-plus and adiponectin-minus medium was prepared as described in methods contained all three multimeric forms as shown by western blot in Figure 4. The isolates were also quantitated using ELISA (R&D Systems). Aliquots of adiponectin enriched and adiponectin depleted fractions were stored frozen at −80 °C until use.

To examine whether adiponectin can modulate ER/SR stress response genes in cardiomyocyte cultures, we examined the effect of adiponectin-enriched (plus) medium containing 100 ng/ml of adiponectin and adiponectin-depleted (minus) medium on changes in SR/ER stress response genes. H9C2 were treated with adiponectin-plus or adiponectin-minus medium for 0.5, 1, or 3 h, as indicated. We have examined SR/ER stress-response genes, HSPA5, GRP94, ATF6, and Serca2a (Ca2+-ATPase), and inflammation related genes TNFα and MCP1 (Fig. 5A). Each transcript is expressed relative to 18s mRNA expression. HSPA5 and GRP94 transcript levels decreased at 0.5 h and 1 h, and the effect was more pronounced at 3 h of adiponectin treatment (*P < 0.05). ATF6 decreased at 0.5 h and the decrease was sustained for 3 h. Thus all three key indicators of ER stress decreased significantly. Inflammation related gene, TNFα decreased by 60 ± 5% at 0.5 h and 1 h following adiponectin treatment (*P < 0.05), at 3 h, TNFα was comparable between the plus adiponectin and minus
adiponectin treatments. MCP1 which encodes the macrophage chemo attractant protein 1 was also significantly decreased by the addition of plus adiponectin medium at 0.5 h and 1.0 h (*P < 0.05 vs. minus medium). MCP1 mRNA was comparable between the plus adiponectin and minus adiponectin treatments at 3 h. Serca 2A transcript level did not change at 0.5 h but there was a significant increase at both 1 h and 3 h of plus adiponectin medium treatment as compared with minus adiponectin treated cells. It is likely that decrease of HSPA5/GRP78 and/or TNFα by adiponectin act as triggers in upregulating Serca 2A, thus altering Ca²⁺ homeostasis.

We examined the changes in HSPA5 and IκBα protein expression in H9C2 cardiomyoblast cell line treated with adiponectin minus and adiponectin plus medium for 1 h and 3 h (Fig. 5B). Cell lysates were examined by western blotting for HSPA5, IκBα, and β-actin expression. Data shown in Figure 5B represents one of three similar blots. Densitometric analysis of the HSPA5/IκBα and β-actin protein expression are normalized to β-actin. Data are plotted as arbitrary units relative to the mean ± SD of the control. n = 3 (*P < 0.05 vs. control DMSO, †P < 0.05 vs. control BSA).

**Figure 3.** Effect of tunicamycin and palmitate on H9C2 cells. (A) Expression of ERSR transcripts, HSPA5, GRP94, ATF6, Serca2A, adiponectin (adip), adiponectin receptor1 (adpr1), and adiponectin receptor 2 (adpr2) in response to increasing concentration of tunicamycin for 6 h. H9C2 cells were treated with 0.1 μg/ml, 0.5 μg/ml, or 1.0 μg/ml of tunicamycin. Expression values shown were obtained from real time measurements of each transcript normalized to 18s RNA. Experiments were performed in triplicates. Data represent means of two independent experiments. Mean ± SD, n = 6, (*P < 0.05 vs. control). (B) Comparing changes in ER stress induction with 0.1 μg/ml of tunicamycin or 200 μM palmitate conjugated with BSA. ER stress transcripts HSPA5, GRP94, and ATF6 were upregulated by both treatments as shown in (B), (*P < 0.05 vs. respective controls). Expression values shown are obtained from real-time measurements of each transcript normalized to 18s RNA. Data represents mean of two independent experiments done in triplicate. Mean ± SD, n = 6. (C) Cytosolic extracts of H9C2 cells treated with 0.1 μg/ml of tunicamycin or 200 μM palmitate were analyzed by western blot. The same blot was analyzed for HSPA5, IκBα, and β-actin. Data shown represents one of three similar experiments. (D) Densitometric analysis of western blots. HSPA5 and IκBα protein expression are normalized to β-actin. Data are plotted as arbitrary units relative to the mean ± SD of the control. n = 3 (*P < 0.05 vs. control DMSO, †P < 0.05 vs. control BSA).
**Discussion**

The endoplasmic reticulum is contiguous with the nuclear membrane. Muscle cells have a smooth ER called the sarcoplasmic reticulum (SR) that regulates intracellular Ca^{2+} uptake and release, thus regulating relaxation and contraction respectively. In patients with heart failure, the chaperone glucose-responsive protein 78 (GRP78)/HSPA5 expression is increased. This changes the activation of sarcoplasmic reticulum (SR)/ER transmembrane sensors and initiates the UPR responses and ER/SR stress.\(^{18}\) The increase in ER stress genes can trigger inflammation or vice versa. Persistent ER stress activates the unfolded protein response that is associated with the pathophysiology of heart failure in humans.\(^{18}\) In mouse cardiomyocytes, inflammation resulting in NF\(_{\kappa}B\) activation exacerbates the ER stress response and triggers apoptotic effects.\(^{19}\) Chronic inflammation is a predictor of prognosis in heart failure. The failing myocardium has an augmented level of pro-inflammatory cytokines and NF\(_{\kappa}B\) activation.\(^{20}\)

Clinical investigations have demonstrated the association of hypoadohiponectinemia in patients with cardiovascular disease. Increased adiponectin levels correlate with a moderately decreased risk of coronary heart disease in men with diabetes.\(^{21,22}\) Therefore, it is relevant to examine the role of adiponectin in inducing ER/SR stress and inflammation in the heart tissue and heart cells in culture.

High fat diet increased body weight both in WT and ADKO mice, but we did not find a significantly higher weight gain in the ADKO. Other studies have shown increased weight gain in response to HF diet for 22 weeks,\(^{23}\) we think that the difference in results can be attributed to the shorter 12-week duration of our study. This duration of 12 weeks of high fat diet decreased adiponectin mRNA in adipose tissue of WT mice. The animals used in the earlier study were 4 mo of age;\(^{23}\) we have compared 11-mo-old WT and ADKO mice.

This study shows ER stress and inflammation are increased in heart tissues of adult ADKO mice. HSPA5, ERN1, and GADD34 are increased in the heart of ADKO mice. ER stress indicators HSPA5 and ERN1 are not further increased in response to HF diet. GADD34, which plays a protective role in restoring normal cell function by de-phosphorylation of eIF2\(_{\alpha}\),\(^{24}\) is increased in ADKO heart in response to HF diet. Markers of inflammation TNF\(_{\alpha}\) and CD68 are also increased in the heart of ADKO mice with no further increase in TNF\(_{\alpha}\) and CD68 in response to HF. Earlier studies have shown that ischemia–reperfusion increased TNF\(_{\alpha}\) expression in ADKO mice as compared with WT.\(^{25}\) Also adiponectin deficiency accelerated heart failure in ADKO mice during pressure overload through decrease in VEGF and AMPK-dependent regulatory mechanisms.\(^{7}\)

Based on these observations we hypothesized that the lack of adiponectin could be a likely trigger for the increased ER/SR stress and inflammation in the heart. We have conducted preliminary studies on rat cardiomyocytes and the addition of adiponectin for 1 h increased I\(_{\kappa}B\)\(\alpha\) protein expression. However the use of terminally differentiated primary cultures had several limitations including variation in cells number and viability in different isolation. We have examined this hypothesis in H9C2 rat cardiomyoblast cell line. We observed that the induction of SR/ER stress in H9C2 cells decreased adiponectin and adiponectin receptor mRNA expression and decreased in I\(_{\kappa}B\)\(\alpha\) protein expression. Also the induction of ER stress protein HSPA5 along with the decrease in I\(_{\kappa}B\)\(\alpha\) protein by palmitate treatment of H9C2 cells indicates ER stress is linked to inflammation in heart cells. Advanced age is associated with reduced palmitate oxidation and impaired diastolic function in the heart.\(^{26-28}\) It is known that the I\(_{\kappa}B\) family of proteins regulates activation of NF\(_{\kappa}B\) by sequestering NF\(_{\kappa}B\) in the cytoplasm. The degradation of I\(_{\kappa}B\) proteins results in the nuclear entry of NF\(_{\kappa}B\) dimmers. The levels of I\(_{\kappa}B\) protein expression is indicative of inflammatory status (reviewed in ref. 29). It is known that NF\(_{\kappa}B\) activation in heart failure promotes ER stress-mediated pro-inflammatory and pro-apoptotic changes.\(^{19}\)

Most importantly, our results show that adiponectin appears to shields H9C2 cells against ER stress induction. The addition of adiponectin decreased HSPA5, GRP94, and ATF6 transcripts within 30 min and the decrease persisted for 3 h following the addition of adiponectin. In addition, the inflammatory cytokines TNF\(_{\alpha}\) and MCP1 were also decreased by adiponectin, and this is accompanied by an increase Serca2a. This is particularly relevant because ER stress induction in H9C2 cells decreased Serca2a. Serca2a is known to control the contraction and relaxation of the heart muscle and impaired cardiac contractility and/or relaxation in heart failure is partially attributable to the reduced activity of the cardiac Serca2a pump.\(^{14,30}\) Myocardial relaxation is impaired with advancing age, and is associated with reduced Serca2a activity.\(^{26,31,32}\) It has been shown that increased saturated fatty acid levels impair cellular
Ca\textsuperscript{2+} handling and contraction in a ROS-dependent manner in normal rat cardiomyocytes.\textsuperscript{35} Hyperglycemia induced contractile dysfunction in rat cardiomyocytes is also likely to be due to oxidative stress induced decrease in the activity of Serca2a. Attenuation of oxidative stress normalized Ca\textsuperscript{2+} signaling and Serca2a activity.\textsuperscript{34}

It is likely that downregulation of ER stress transcript HSPA5/GRP78 by adiponectin acts as a trigger in upregulating Serca2a, thus altering Ca\textsuperscript{2+} homeostasis. Thapsigargin, an inducer of ER stress, abolished the calcium uptake and Serca2a activity of cardiac muscle.\textsuperscript{13} The immediate decrease in ER stress markers by adiponectin is accompanied by a decrease in the inflammatory

**Figure 5.** Change in ER stress and inflammation following adiponectin treatment. (A) H9C2 rat cardiomyoblasts were treated with adiponectin minus and adiponectin plus medium (100 ng/ml). RNA is extracted from cells at 0.5, 1, and 3 h of treatment. The transcripts for HSPA5, GRP94, ATF6, TNF\textsubscript{α}, and MCP1 were quantitated by real time PCR and normalized to 18s RNA. Data are presented as means ± SD of 18s normalized expression from of 2 independent experiments with 3 biological replicates (*\textit{P} < 0.05 vs minus medium). (B) H9C2 cardiomyocytes were treated as described above; cell lysates were analyzed by western blotting for the expression of HSPA5 and Iκβα. (C) Quantitation of HSPA5/β actin and Iκβα/β actin. Data are plotted as arbitrary units relative to the mean ± SD (*\textit{P} < 0.05 vs. minus medium).
cytokine TNFα, thus it is also likely that activation of Serca2a is linked to the anti-inflammatory role of adiponectin, which is shown to be partially mediated by reduced SRF activity in adipocytes. The ER/SR is recognized as an important organelle that controls the pathophysiology of heart failure. Therefore identifying molecular mechanisms which can regulate ER/SR genes and inflammation will provide future therapeutic targets.

Materials and Methods

Experimental reagents

Stock solutions for tunicamycin (1 mg/ml) and palmitate (100 mM) (Sigma) were dissolved in dimethyl sulfoxide. Palmitate was conjugated to fatty acid free BSA (dissolved in DMEM cell culture medium) at a 2.5:1 molar ratio by incubating the mixture at 45 °C for 20 min and filter sterilized before adding to cell cultures. H9C2 cells were treated with DMSO (vehicle control), tunicamycin, palmitate–BSA conjugate (200 μM) or the same concentration of BSA in serum free DMEM medium (GIBCO) at a 2.5:1 molar ratio by incubation (100 mM) (Sigma) were dissolved in dimethyl sulfoxide.

Experimental reagents

Total RNA was isolated from H9C2 cardiomyoblasts using RNeasy kit (Quigen Sciences), and performed according to the manufacturer’s instructions. Adipose or heart tissue samples were extracted using Quizol (Quigen) followed by purification using Quigen RNeasy kit extraction kit. RNA was analyzed for integrity using agarose gels and quantitated using Nanodrop1000 spectrophotometer. Total RNA (500 ng) was converted to cDNA using high capacity RNA to cDNA transcription kit (Applied Biosystems). Reverse transcribed RNA (cDNA) was amplified with 1× SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers using an ABI 7900 RT PCR system. Primers sequences designed to span an intron are described below. In a few instances when the primers did not span an intron, controls using the same concentration of RNA were also amplified with gene-specific primers. Samples were normalized to 18S RNA. Standard curves were generated using pooled cDNA from samples being compared with determine the relative change in gene expression. Duplicate measurements were performed for analysis.

The following primers were used to analyze gene expression in mouse adipose and cardiac tissues. 18S F 5′-ATCAACTTTTC GTGGTATGC-3′, 18S R 5′-TCTTTGGAGTG TGTTAGCCG-3′; Adiponectin F 5′-GTGGCAAGCT CTCTTGTCC-3′; Adiponectin R 5′-ATCACCAGCT CAAAGTTC, HSPa5/GRP78 F 5′-AGATGGATG TGGAAAGC-3′, R 5′-AGTTCGTCA GGCCCTTGTT-3′; GADD34 F 5′- AGCCTAAGCG GAGGGAGAA-3′, R 5′-ATCCCAACAG CAGTGGAAGAG-3′, 18S R 5′-CTCAGGATTC CGCAAGGATT-3′. CD 68 F 5′-CAGGTGGGGA TGGTATACGGTC-3′, R 5′-GGTGAAGAAAGCTTGGGG CAT-3′. TNF F 5′-CAGGCAAGGA ACCCTTGAGA-3′, TNF R 5′-AGCCTCCAAG CCGAGAATCT-3′.

The following primers were used to analyze gene expression in H9C2 RNA: 18S F 5′-ATCAACTTTTC GTGGTATGC-3′, 18S R 5′-TCTTTGGAGTG TGTTAGCCG-3′, HSPa5 F 5′-CTCCTGCGTTT CGGGCTACA-3′, HSPa5 R 5′-TCTTTGGAGTG CGCCTTGAGAC-3′, GRP94 F 5′-GCCGCCACGC CATGAGGGTC-3′, R 5′-CCGGAAAGGC AGCAGGAGGC-3′, ATF6 F 5′-GGCCTCCAAG TGGCTGAC-3′, ATF6 R 5′-ACCTTCCAGT CCATCAGA-3′, Serca 2a F 5′-GGAATACATC ACCCTTGAGC-3′, Serca 2a R 5′-CTCCTCAGCC AAAATAGGA C-3′, Adiponectin F 5′-AATCCCTGCC AGTCAAGA-3′, Adiponectin R 5′-TCTCAGGAG TGCCATCTCT-3′, Adiponectin receptor 1 F 5′-GCAGCAAGGA GCAGGAGTGT-3′, Adiponectin receptor 1 R 5′-ACTGTTGGTG CCTTGACAAA-3′, Adiponectin

Cell culture

H9C2 rat cardiomyoblast cell line (ATCC) was maintained in high glucose (4500 mg/l) Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. For experiments cells were grown to 80–90% confluence. Prior to treatment cells were washed with PBS and placed in serum free DMEM medium for 4 h.

RNA isolation and gene expression

Animals

Male Sprague–Dawley rats (200–225 g) were purchased from Harlan Sprague–Dawley. Animal care was performed in accordance with IACUC guidelines. Adipocytes were prepared from epididymal fat pads, as described previously. Adiponectin knockout mice (ADKO) as well as C57 Bl6 mice (WT) mice were purchased from Jackson Labs. Animals were bred using the breeding protocol at the CAVHS animal facility. Eleven- to 12-mo-old male AD-KO and WT mice weighing 20–25 g were purchased from Harlan Sprague–Dawley. Animal care was performed in accordance with IACUC guidelines. Adipocytes were prepared from epididymal fat pads, as described previously.

Adiponectin isolation

Commercial sources of adiponectin were of mouse and human origin. We have isolated rat adiponectin from primary cultures of rat adipocytes maintained for 18 h in serum free DMEM culture medium (GIBCO). Adiponectin-enriched fractions were prepared by purification of adipocyte conditioned medium on sucrose density gradients as described earlier. The fractions were examined for adiponectin by western blotting and the two enriched fractions were pooled as adiponectin-containing (plus) medium. Adiponectin-depleted (minus) fractions were prepared by immunoprecipitation of adiponectin using a specific antibody followed by removal of the complex using protein-G beads. The adiponectin containing and adiponectin depleted fractions were examined by western blotting using rat adiponectin antibody (R&D Systems) and quantified using ELISA (R&D Systems).

Materials and Methods

Adipocyte Volume 3 Issue 1

16
Western blot analysis
Following the treatment, total protein extracts were prepared using protein lysis buffer (Cell Signaling) as described previously. Briefly, equal amounts of protein (50 µg) were separated on 10% SDS-polyacrylamide gels (Mini-PROTEAN 11, Bio-Rad) and transferred to nitrocellulose membranes (0.45 µm pore size, Amersham Hybond-ECL, GE Healthcare, Inc.). Membranes were incubated overnight at 4 °C in anti-HSP65 (1:1000), anti-IκBα (1:1000). After washing the excess of primary antibody; membranes are probed with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000). Antigens were detected using chemiluminescence reagents (Santa Cruz Biotechnology, Inc.). To ensure equal loading of protein, the blots were normalized to β-actin by probing each blot subsequently with β-actin antibody. The blots were scanned using ChemiDoc XRS imaging and analysis software and densitometric analysis was performed Quantity One Image Analysis software (v4.6.3; BioRad).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
This research was supported by REAP Grant funds from the Veterans Administration to G.R. and from the National Institutes of Health AG 028718 to J.Y.W.

References
1. Berg AH, Combs TP, Scherer PE. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. Trends Endocrinol Metab 2002; 13:84-9; PMID:11854024; http://dx.doi.org/10.1016/S1043-2701(01)00524-0
2. Hopkin TA, Ouchi N, Shibata R, Walsh K. Adiponectin actions in the cardiovascular system. Cardiovasc Res 2007; 74:11-8; PMID:17140553; http://dx.doi.org/10.1016/j.cardiores.2006.10.009
3. Berg AH, Scherer PE. Adipose tissue, inflammation, and cardiovascular disease. Circ Res 2005; 96:939-49; PMID:15899081; http://dx.doi.org/10.1161/01..RES.000016356.62972.34
4. Mondal AK, Das SK, Varma V, Nolen GT, McGehee DB, Ory DS, Muller DL. Adiponectin regulates the development and function of pulmonary endothelial cells. J Mol Cell Cardiol 2008; 49:210-20; PMID:18576634; http://dx.doi.org/10.1016/j.yjmcc.2007.11.010
5. Dutta S, Belkowska A, Widomska A, Nolen GT, McGehee DB, Ory DS. Adiponectin regulates the development and function of pulmonary endothelial cells. J Mol Cell Cardiol 2008; 49:210-20; PMID:18576634; http://dx.doi.org/10.1016/j.yjmcc.2007.11.010
6. Bełtowski J, Jamroz-Wiśniewska A, Widomska A, and cardiovascular disease. Trends Endocrinol Metab 2002; 13:84-9; PMID:11854024; http://dx.doi.org/10.1016/S1043-2701(01)00524-0
7. Shimano M, Ouchi N, Shibata R, Walsh K. Adiponectin deficiency exacerbates cardiac dysfunction following pressure overload through disruption of an AMPK-dependent angiogenic response. J Mol Cell Cardiol 2010; 49:210-20; PMID:20206634; http://dx.doi.org/10.1016/j.yjmcc.2010.02.021
8. Kawabata T, Kamon J, Minokoshi Y, Ito Y, Wang H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 2002; 8:1288-95; PMID:12368907; http://dx.doi.org/10.1038/nm788
9. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Wang H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 2002; 8:1288-95; PMID:12368907; http://dx.doi.org/10.1038/nm788
30. Raeymaekers L, Vandecaetsbeek I, Wayzack F, Vangheluwe P. Modeling Ca2+ dynamics of mouse cardiac cells points to a critical role of SERCA's affinity for Ca2+. Biophys J 2011; 100:1216-25; PMID:21354994; http://dx.doi.org/10.1016/j.bpj.2011.01.024

31. Zhang X, Azhar G, Furr MC, Zhong Y, Wei JY. Model of functional cardiac aging: young adult mice with mild overexpression of serum response factor. Am J Physiol Regul Integr Comp Physiol 2003; 285:R552-60; PMID:12990581

32. Azhar G, Zhang X, Wang S, Zhong Y, Quick CM, Wei JY. Maintaining serum response factor activity in the older heart equal to that of the young adult is associated with better cardiac response to isoproterenol stress. Basic Res Cardiol 2007; 102:233-44; PMID:17122890; http://dx.doi.org/10.1007/s00395-006-0634-z

33. Fauchon J, Andersson DC, Zhang SJ, Lanner JT, Wibom R, Karz A, Brutor JD, Westerblad H. Effects of palmitate on Ca(2+) handling in adult control and ob/ob cardiomyocytes: impact of mitochondrial reactive oxygen species. Diabetes 2007; 56:1136-42; PMID:17229941; http://dx.doi.org/10.2337/db06-0739

34. Tang WH, Cheng WT, Krawtsov GM, Tong XY, Hou XY, Chung SK, Chung SS. Cardiac contractile dysfunction during acute hyperglycemia due to impairment of SERCA by polyol pathway-mediated oxidative stress. Am J Physiol Cell Physiol 2010; 299:C643-53; PMID:20573996; http://dx.doi.org/10.1152/ajpcell.00137.2010

35. Johnson C, Williams R, Wei JY, Ranganathan G. Regulation of Serum Response Factor and Adiponectin by PPARγ Agonist Docosahexaenoic Acid. J Lipids 2011; 2011:670479; PMID:21792203; http://dx.doi.org/10.1155/2011/670479

36. Zhang X, Azhar G, Helms S, Burton B, Huang C, Zhong Y, Gu X, Fang H, Tong W, Wei JY. Identification of New SRF Binding Sites in Genes Modulated by SRF Over-Expression in Mouse Hearts. Gene Regul Syst Bio 2011; 5:41-59; PMID:21792203

37. Rodbell M. Metabolism of isolated fat cells. 1. Effects of hormone on glucose metabolism and lipolysis. J Biol Chem 1964; 239:375-80; PMID:14160133

38. Saffari B, Ong JM, Kern PA. Regulation of adipose tissue lipoprotein lipase gene expression by thyroid hormone in rats. J Lipid Res 1992; 33:241-9; PMID:1569376

39. Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schultheiss T, Engel J, Brownlee M, Scherer PE. Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications for metabolic regulation and bioactivity. J Biol Chem 2003; 278:9073-85; PMID:12496257; http://dx.doi.org/10.1074/jbc.M207198200

40. Wang X, McLennan SV, Allen TJ, Twigg, SM. Regulation of pro-inflammatory and pro-fibrotic factors by CCN2/CTGF in H9c2 cardiomyocytes. J Cell Commun Signal 2010; 4:15-23; PMID:20195389; http://dx.doi.org/10.1007/s12079-009-0083-1