Regulation of the cardioprotective adiponectin and its receptor AdipoR1 by salt
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Abstract

Both circulating adiponectin (APN) and cardiac APN exert cardioprotective effects, and improve insulin sensitivity and mitochondrial function. Low circulating APN serves as a biomarker for cardiovascular risk. Ablation of adiponectin receptor 1 (AdipoR1) causes myocardial mitochondrial dysfunction. Though high salt intake is a contributor to cardiovascular disease, how it modulates the expression of APN or AdipoR1 in cardiomyocytes is not known. We report that APN mRNA expression was attenuated in a dose dependent manner in mouse cardiomyocyte cell line HL-1 exposed to salt concentrations ranging from 0.75% to 1.5% for 12 hours. High salt exposure (0.88% and 1.25% for 12 hours) also suppressed APN and AdipoR1 protein expression significantly in rat cardiac muscle H9c2 cells. Co-immunostaining for AdipoR1 and Mitochondrial Complex 1 indicated that AdipoR1 may be co-localized with mitochondria. These data show for the first time that high salt is an important suppressor of cardiovascular protective APN and AdipoR1.

Key Words: adiponectin, adiponectin receptor type 1, salt, cardiovascular disease
Introduction

Adiponectin (APN) is an anti-inflammatory, anti-apoptotic and anti-atherogenic adipokine that improves insulin sensitivity and renders cardiovascular protection (Shibata et al. 2004; van Stijn et al. 2014.). Accumulating clinical evidence indicates that circulating APN levels are inversely correlated to obesity, diabetes and hypertension (Lee et al. 2016; Mantovani et al. 2016). Thus, low circulating APN levels are considered a risk factor for these metabolic diseases. Moreover, genetic polymorphisms in the APN gene modulate BP responses to dietary sodium and potassium intake (Chu et al. 2016). However, in conditions of chronic heart failure, APN resistance is observed since in such conditions circulating APN levels are increased with no effect on insulin resistance (Sente et al. 2016). It is unclear what sources contribute to this increase in circulating APN in chronic heart failure. Although, adipose tissue is the main source of APN, recent studies have identified localized production of APN in skeletal muscle cells and cardiomyocytes (Amin et al. 2010; Skurk et al. 2008). The exact roles of these local APN systems are yet to be elucidated.

APN receptors include AdipoR1 that is primarily expressed by skeletal muscle and AdipoR2 that is mainly expressed by liver (Silva et al. 2014; Singh et al. 2016). The predominant APN receptor expressed in cardiomyocytes is AdipoR1. AdipoR1 deficiency in the heart causes myocardial mitochondrial dysfunction in mice and highlights the critical role of APN and its receptors in mitochondrial function (Koentges et al. 2015). Previous studies have shown that APN and its receptors are expressed in adult ventricular cardiomyocytes (Ding et al. 2007). In diabetic rats, cardiac expression of APN and its receptors is suppressed. Angiotensin II type 1 receptor (AT1R) inhibitor telmisartan, and activation of peroxisome proliferator-
activated receptor gamma by rosiglitazone, elevate cardiac expression of APN and its receptors in diabetic rats (Guo Z et al. 2012 a,b).

Though Ang II infusion and activation of renin-angiotensin aldosterone system suppress circulating APN, Ang II has an opposing effect on cardiac APN. In neonatal rat ventricular myocytes Ang II induces elevation of APN mRNA and this effect is mediated via the angiotensin type-2 receptor (AT2R)/nitric oxide/cGMP/protein kinase G signaling pathway (Guo B et al. 2011). Since AT2R/nitric oxide/cGMP/protein kinase G signaling pathway attenuates Ang II-induced hypertension, increase in APN via this pathway in cardiomyocytes could be a protective mechanism. Both Ang II and high salt diet are major contributors to cardiovascular diseases including left ventricular hypertrophy and chronic heart failure (He et al. 2011). Interestingly, high salt induced dysregulation of glucose homeostasis could be reversed by activation of PPARδ/Adiponectin-Mediated inhibition of SGLT2 (Zhao et al. 2016). To date there are no studies that show the effect of high salt on the expression of cardiac APN or APNR1. This study was undertaken to examine the effect of high salt on the expression of APN and APNR1 under in vitro conditions using atrial and ventricular cardiac muscle cell lines of rat (H9c2) and mouse (HL-1).

Materials and Methods

Cell culture and treatments. The cardiac ventricular cell line H92C was grown in Dulbeco’s Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (both from ATCC) and 1% Penicillin/Streptomycin (Thermo Fisher). Mouse atrial cardiomyocyte HL-1 cells were a gift from Dr. William Claycomb at Louisiana State University Medical Center and cultured as described previously (Arnold et al. 2014; Mahmood and Pulakat 2015). For Ang II
and salt treatments (12 hours), cells were maintained in serum free medium supplemented with AngII (100nM), or salt (NaCl) concentrations adjusted to be between 0.75% to 1.5% of salt.

**RNA isolation and quantitative real-time RT-PCR.** Isolation of mRNA from HL-1 cells was performed using mirVana miRNA isolation kit (Ambion) following the manufacturer’s protocol. The mRNA was quantified using NanoDrop (Thermo Scientific) and stored at -80°C until further processing. c-DNA synthesis for mRNA and qRT-PCR was performed as described previously (Arnold et al. 2014). Taqman primers for mouse AdipoQ and 18S RNA (Gene Expression Assays) from Applied Biosystems Life Technologies were used. Experiments were performed in triplicates for each biological sample with Taqman Fast Universal PCR Master Mix 2X, (Applied Biosystems Life Technologies). 18S RNA was used as internal control. Relative quantification (RQ) values were obtained by determining ∆Ct values followed by determining ∆∆Ct values and then RQ values via the equation $2^{-\Delta\Delta C_t}$.

**Immunofluorescence.** Immunofluorescence analysis of the expression of APN1 and AdipoR1 in H9c2 cells in response to treatments with salt or Ang II or their combination was performed using anti-APN and anti-AdipoR1 antibodies (Abcam Inc, 1:50 dilution) and Alexa Fluor 488 or Alexa Fluor 594 goat anti-rabbit (Abcam Inc.) (1:200 dilution) as described previously (Arnold et al. 2014; Mahmood and Pulakat 2015). Analysis of co-localization of AdipoR1 with mitochondria was performed by double immunostaining using anti-AdipoR1 and anti-mitochondrial Complex 4 subunit 1 (MTCO1) antibodies. All experiments were done in at least triplicates.
Statistics. Statistical analysis was performed using the SPSS 20 software package. Results were expressed as mean ± SEM (standard error of mean). Differences among groups were tested by using One-Way ANOVA followed up with Tukey's test or t-test, as appropriate, and two-tailed $p$ values are reported. A $p$-value of 0.05 was considered statistically significant.

Results

Regulation of APN by salt and AngII in mouse atrial cardiac muscle cell line HL-1. As shown in Fig. 1A, exposure of HL-1 cells to salt (0.75% to 1.5%) significantly suppressed APN (Adipoq) mRNA expression in a dose dependent manner. In contrast, exposure to Ang II (100nM) increased APN (Adipoq) mRNA levels and this is consistent with previous reports (Guo B et al. 2011). To our knowledge, this is the first report that shows high salt suppresses endogenous expression of APN in cardiac cells.

Regulation of APN by salt in rat ventricular cardiac muscle cell line H9c2. To further validate that high salt suppresses APN expression in cardiac cells, we tested the effect of 1.25% salt on APN protein expression in rat cardiac muscle cell line H9c2. As shown in Fig. 1B, immunofluorescence analysis indicated that APN protein expression was suppressed by 33% in H9c2 cells in response to 1.25% salt.

AdipoR1 is also affected by high-salt conditions, while AngII can partially mitigate this effect. To further investigate the impact of high-salt exposure on the APN signaling in cardiac muscle, we investigated how it modulates AdipoR1 protein expression in H9c2 cells. As shown in Fig. 2, AdipoR1 protein expression was significantly suppressed in H9c2 cells exposed to
1.25% salt (50%) and 0.88% salt (21%). Ang II (100nM) did not significantly affect AdipoR1 protein expression under these experimental conditions. Addition of Ang II with 1.25% salt did not rescue salt-induced loss of AdipoR1 protein expression. However, addition of Ang II with 0.88% salt could restore AdipoR1 protein expression to levels comparable to control. Thus, Ang II could partially mitigate loss of AdipoR1 expression in response to salt when salt concentration was not very high. To our knowledge, this is the first report that shows high salt exposure suppresses AdipoR1 expression.

**AdipoR1 may be co-localized with mitochondria.** AdipoR1 is a seven transmembrane domain receptor that is structurally and functionally distinct from classical G-protein coupled receptors, since it has an inverted membrane topology with a cytoplasmic amino terminus and a short extracellular carboxyl terminus. AdipoR1 deficiency in myocardium is reported to induce mitochondrial dysfunction (Koentges et al. 2015). Since this observation highlights the crucial role of AdipoR1 in mitochondrial structure and function, we investigated whether AdipoR1 is present on mitochondria. As shown in Fig. 3, co-immunostaining with anti-AdipoR1 and anti-MTCO1 (mitochondria marker) showed that AdipoR1 seems to be present on mitochondria, in addition to the cell membrane.

**Discussion**

Accumulating clinical evidence show that a low circulating level of APN is an independent risk factor for cardiovascular disease, whereas patients with high circulating APN have a significantly reduced risk of myocardial infarction even after adjustment for body mass index, history of diabetes and hypertension (Koentges et al. 2015; Lee et al. 2016; Mantovani et
Mice lacking adiponectin exhibit enhanced concentric cardiac hypertrophy following pressure overload and increased myocardial infarct size following ligation of the left anterior descending (LAD) coronary artery, and these pathologies could be ameliorated by administration of recombinant adiponectin (Shibata et al. 2004, 2005). APN is synthesized and secreted by human and murine cardiomyocytes and endogenously produced adiponectin protects cardiomyocytes from hypertrophy induced by high fat diet (Amin et al. 2010). Additionally, loss of AdipoR1 induced myocardial mitochondrial dysfunction whereas loss of AdipoR2 did not have such effect. Thus APN-AdipoR1 signaling has an important role in cardiomyocyte survival and mitochondrial function.

Despite extensive epidemiological evidence that suggests a high-salt diet is an independent predictor of heart disease along with heart failure, the exact mechanisms by which high-salt increases this susceptibility is not clear. Given the role of APN-AdipoR1 signaling in cardiomyocyte survival, we hypothesized that high salt may suppress this cardioprotective signaling. Data presented here show that APN expression can be suppressed in cardiac muscle cell lines derived from mouse atrium (HL-1) and rat ventricle (H9c2) by high salt conditions. Though these cell lines do not exhibit all properties of primary cardiomyocytes, they are widely used as surrogates to study cardiac signaling mechanisms. We found that APN (AdipoQ) mRNA expression was suppressed in a dose dependent manner by high salt conditions, and at a salt concentration of 1.5%, there was almost no expression of AdipoQ mRNA in HL-1 cells as detected by quantitative RT-PCR (Fig. 1A). The fact that 18S mRNA expression in the HL-1 cells treated with 1.5% salt was similar to that of untreated HL-1 cells validated the severe suppression of AdipoQ mRNA expression by 1.5% salt. Previous studies have shown that Ang II
elevates expression of APN in neonatal cardiomyocytes (Guo B. et al. 2011). Consistent with this, we also observed an elevation of AdipoQ mRNA in HL-1 cells in response to Ang II treatment.

The suppression of APN protein by high salt conditions in H9c2 cells further confirmed the fact that salt is a negative regulator of APN expression (Fig. 1B). In H9c2 cells, Ang II treatment did not significantly affect AdipoR1 expression. However, Ang II treatment could rescue 0.88% salt-induced suppression of AdipoR1 protein expression (Fig. 2). These observations indicate a novel cross talk between salt-induced signaling and Ang II that is mediated via APN. However, Ang II treatment was ineffective in restoring the AdipoR1 protein expression in the presence of 1.25% salt (Supplemental Fig. S1). Therefore, the protective effect of Ang II in improving AdipoR1 expression in the presence of high salt is dependent on the extent of salt in the medium.

The heart tissues of mice lacking AdipoR1 gene expression showed significant mitochondrial structural and functional damage (Koentges et al. 2015). Thus, AdipoR1 has a critical role in mitochondrial survival. However, it is not known whether AdipoR1 is present on the mitochondrial membranes. Data presented here suggest that mitochondrial complex 4 subunit 1 (MTCO1) seems to co-localize with AdipoR1. Therefore, it is tempting to speculate that endogenous APN in cardiac muscle cells may directly regulate AdipoR1 signaling in an intracrine manner and protect mitochondrial structure and function.
Collectively, our data shows for the first time, to our knowledge, a novel pathway by which high-salt conditions can lead to suppression of cardioprotective APN and its receptor AdipoR1 directly within cardiomyocytes (Fig. 4). Considering that high salt diet is associated with cardiac hypertrophy, diastolic dysfunction and heart failure, the role of suppression of cardioprotective APN and AdipoR1 by high salt diet warrants further investigation.

Sources of Funding

This work was supported, in part, by the Life Science Mission Enhancement Fund from UM-Columbia (LP), as well as research grant, NIH NHLBI 1R01HL118376-01 (LP). This work was also supported by resources, including the use of facilities, from Research Services, Harry S. Truman Memorial Veterans Hospital.

References


Figure legends:

**Fig. 1.** APN (AdipoQ) mRNA levels are suppressed by high salt conditions in a dose-dependent manner in mouse atrial HL-1 cells and APN protein expression is suppressed by high salt conditions in rat ventricular cardiac muscle cell line H9c2. A) Graph shows qRT-PCR data on the expression of APN (AdipoQ) mRNA in response to high salt conditions in mouse HL-1 cells. APN mRNA expression was suppressed in a dose dependent manner by high salt conditions (n=3 biological replicates). B) Graph shows immunofluorescent intensity quantification of Rat H9c2 probed for APN. Expression of APN was significantly suppressed in cells treated with 1.25% NaCl containing medium C) Representative immunofluorescent images showing APN expression in H9c2 cells (n > 15). Values are means ± SEM. * = p < 0.05 vs Control.

**Fig. 2.** AdipoR1 protein expression is suppressed by high salt conditions in H9c2 cells and Ang II could partially restore AdipoR1 protein expression. A) Graph shows immunofluorescence intensity quantification for Rat H9c2 probed for AdipoR1. Suppression of AdipoR1 was observed in response to 0.88% and 1.25% salt treatment and this effect was reversed by 0.88% Salt+AngII (n=3 for each group, average of 40 cells measured per group). B) Representative images of H9c2 cells stained with anti-AdipoR1 (scale bars = 50µm). Values are means ± SEM. * = p < 0.05 vs. Control, † = p < 0.05 vs. 0.88% Salt.

**Fig. 3.** AdipoR1 may be present on mitochondria. Representative images generated by Co-staining of H9c2 cells for Adiponectin Receptor (AdipoR1) and Mitochondrial Complex 4 Subunit 1 (MTCO1: as a mitochondrial marker) shows a pattern of co-localization between the
two proteins suggesting the presence of the AdipoR1 on the mitochondrial surface. Orange color represents overlap of the fluorescent signals. Areas of significant overlap are indicated by white arrows.

**Fig. 4. Schematic representation of the effect of high salt and Angiotensin II (Ang II) on cardiac adiponectin signaling.** A) In normal conditions, it is known that adiponectin acts through the Adiponectin receptor (AdipoR1) present on the cell surface to improve cardiac muscle survival, insulin sensitivity and mitochondrial function. Our data suggest presence of AdipoR1 on the mitochondrial surface and imply a potential intracrine signaling for adiponectin via mitochondrial AdipoR1. B) In conditions of high salt, both adiponectin and AdipoR1 expression are suppressed which decreases cell survival, insulin sensitivity and mitochondrial function, ultimately leading to cardiac dysfunction. C) Treatment of cardiac cells with AngII increases adiponectin expression via Angiotensin II Type 2 (AT2R) mediated activation of the nitric oxide/cGMP/Protein Kinase G pathway (as shown in literature; Guo B et al. 2011). D) Co-treatment of cardiac cells in high salt with AngII abolishes the suppressive effects of high salt on AdipoR1 expression (returning it to normal levels) while their effect on cardiac adiponec
APN (AdipoQ) mRNA levels are suppressed by high salt conditions in a dose-dependent manner in mouse atrial HL-1 cells and APN protein expression is suppressed by high salt conditions in rat ventricular cardiac muscle cell line H9c2.

Fig. 1

265x186mm (300 x 300 DPI)
AdipoR1 protein expression is suppressed by high salt conditions in H9c2 cells and Ang II could partially restore AdipoR1 protein expression.

Fig. 2.

AdipoR1 protein expression is suppressed by high salt conditions in H9c2 cells and Ang II could partially restore AdipoR1 protein expression.
AdipoR1 may be present on mitochondria
Fig. 3.
237x88mm (300 x 300 DPI)
Schematic representation of the effect of high salt and Angiotensin II (Ang II) on cardiac adiponectin signaling. Fig. 4

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