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Deficiency in adiponectin exaggerates cigarette smoking exposure-induced cardiac contractile dysfunction: Role of autophagy

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ABSTRACT

Second hand smoke is an independent risk factor for cardiovascular disease. Adiponectin (APN), an adipose-derived adipokine, has been shown to offer cardioprotective effect through an AMPK-dependent manner. This study was designed to evaluate the impact of adiponectin deficiency on second hand smoke-induced cardiac pathology and underlying mechanisms using a mouse model of side-stream smoke exposure. Adult wild-type (WT) and adiponectin knockout (APNKO) mice were placed in a chamber exposed to cigarette smoke for 1 hour daily for 40 days. Echocardiographic, cardiomyocyte function, and intracellular Ca^{2+} handling were evaluated. Autophagy and apoptosis were examined using western blot. 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) staining was used to evaluate reactive oxygen species (ROS) generation. Masson trichrome staining was employed to measure interstitial fibrosis. Our data revealed that adiponectin deficiency provoked smoke exposure-induced cardiomyopathy (compromised fractional shortening, disrupted cardiomyocyte function and intracellular Ca^{2+} homeostasis, apoptosis and ROS generation). In addition, these detrimental effects of side-stream smoke were accompanied by defective autophagolysosome formation, the effect of which was exacerbated by adiponectin deficiency. Blocking autophagolysosome formation using bafilomycin A1 (BafA1) negated the cardioprotective effect of rapamycin against smoke extract. Induction of autophagy using rapamycin and AMPKα activation using AICAR rescued against smoke extract-induced myopathic anomalies in APNKO mice. Our data suggest that adiponectin serves as an indispensable cardioprotective factor against side-stream smoke exposure-induced myopathic changes possibly through facilitating autophagolysosome formation.

1. Introduction

Tobacco smoke exposure is a major modifiable risk factor for cardiovascular diseases and represents the leading preventable cause of death in US [1]. Epidemiologic and experimental animal studies have recently depicted that cigarette smoking may increase the incidence of myocardial infarction and inflammatory coronary artery diseases [2,3]. Given the close tie between cigarette smoking and global health problem, a thorough understanding of how cigarette and second-hand smoking contribute to the pathogenesis of cardiovascular diseases is pivotal to reduce smoking-related death [4,5]. Up-to-date, several mechanisms of action have been postulated for the deleterious effects of cigarette smoking including impaired mitochondrial function and energy metabolism caused by toxins in cigarette smoke, including oxidative radicals [2,6]. Recent evidence has suggested that long-term exposure to "second-hand" smoke decreases the levels of adiponectin in plasma and tissues [7,8].

Adiponectin, the most abundant adipocyte-derived cytokine in the plasma, modulates cardiac homeostasis via its interaction with a number of intracellular signaling pathways [9]. The plasma levels of adiponectin typically range from 3 to 30 μg/ml in healthy individuals [9,10]. However, decreased plasma adiponectin levels or hypoadiponectinemia is often observed in patients with increased cardiovascular risk and inflammation such as diabetes, hypertension, coronary artery disease, obesity and insulin resistance [10,11]. Adiponectin is believed to offer its beneficial cardiac effect

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through attenuation or mitigation of pro-inflammatory or impaired metabolic stresses, demonstrating a balance between protective and harmful factors [12]. For example, treatment with recombinant adiponectin inhibits reactive oxygen species (ROS)-induced cardiac remodeling through activating AMP-activated protein kinase (AMPKα) as well as inhibiting extracellular signal-regulated kinase (ERK) signaling and nuclear factor-κB (NF-κB) [13]. Recent evidence also indicated that adiponectin may modulate autophagy under various stress conditions both *in vitro* and *in vivo* [14,15].

Although the precise regulatory mechanisms for cell survival are complex, recent advances support the notion that a homeostatic balance of energy status and removal of damaged intracellular components through autophagic degradation are critical for prevention of cell death pathway (*e.g.,* apoptosis) induced by cigarette smoking [16,17]. Autophagy is an intracellular degradation (self-eating) machinery to engulf damaged or aged proteins and organelles to be removed in lysosomes and plays a major role in cell survival or death depending on the cellular context [18]. Normally, autophagy is present constitutively at a basal rate in most eukaryotic cells to maintain cardiomyocyte homeostasis [19]. However, recent evidence has demonstrated activation of autophagy in both physiological and pathological states [20]. Interestingly, dysregulation of autophagy is gaining increased attention in various heart diseases although the precise role of autophagy and autophagy flux remains elusive and may be model dependent [21].

Recent evidence has revealed that cigarette smoke exposure may initiate accumulation of ubiquitinated-proteins [22]. While the ubiquitin–proteasome system (UPS) is mainly responsible for the specific targeting proteins, autophagy-lysosome pathway can selectively remove misfolded or aggregated proteins or cell organelles [23]. Nonetheless, the effect of autophagy on side-stream smoke exposure-induced cardiac anomaly remains unknown. To this end, this study was designed to examine the effect of adiponectin on side-stream smoke exposure, which mimics second hand smoke exposure, -induced myocardial contractile dysfunction and autophagy dysregulation, if any. We hypothesized that facilitating cardiac autophagy may assist in the combat against smoke exposure-induced myopathic changes in the heart, especially in those with low circulating adiponectin levels.

2. Materials and methods

2.1. Experimental animals and side-stream smoke exposure

The animal procedures described in this study were approved by the University of Wyoming Institutional Animal Use and Care Committee (Laramie, WY, USA). In brief, adult C57 wild-type mice and adiponectin knockout (APNKO) mice were placed in an exposure box with 1 cigarette smoke for 1 h daily for 40 days (Golden Monkey-brand of cigarette; TAR: 13 mg; Nicotine: 1.1 mg; CO: 15 mg) [24–26]. The cigarette smoke concentration used in our study was $125.5 \pm 8.4\,mg/m^3$ ($n = 8$) total suspended particulates (TSP), similar to the levels reported previously [27]. Adiponectin knockout mice were genotyped by PCR using the following primers: TGG ATG CTG CCA TGT TCC CAT (WT forward), CTT GTG TCT GTG TCT AGG CCT T (WT reverse) and CTC CAG ACT GCC TTG GGA (mutant reverse). All mice were maintained at 22 °C with a 12/12-light/dark cycle and received lab chow and water *ad libitum*.

2.2. Serum adiponectin level assay

Serum adiponectin levels were detected using an adiponectin ELISA kit according to the manufacturer's instructions (Abcam). Briefly, serum samples were diluted at 1:400 into a 1X Diluent N solution. Fifty μl of adiponectin standards or serum samples were added onto the 96-well plates and were incubated for two hours prior to a rinse with 1X wash buffer included in the kit. Then 50 μl of 1X biotinylated adiponectin antibody was added and was incubated for another hour prior to a rinse with wash buffer. Fifty μl of 1X SP conjugate and 50 μl of chromogen substrate were added in sequence and were incubated for 30 min and 10 min, respectively. Fifty μl Stop Solution was added to terminate the reaction before absorbance measurement using a microplate reader at the wavelength of 450 nm.

2.3. Echocardiographic assessment

Cardiac geometry and function were evaluated in anesthetized (ketamine 80 mg/kg and xylazine 12 mg/kg, i.p.) mice using 2-D guided M-mode echocardiography (Sonos 5500) equipped with a 15–6 MHz linear transducer. Left ventricular anterior and posterior wall dimensions during diastole and systole were recorded from three consecutive cycles in M mode using methods adopted by the American Society of Echocardiography. Fractional shortening was calculated from LV end-diastolic (EDD) and end-systolic (ESD) diameters using the equation $(EDD - ESD)/EDD \times 100$ [28].

2.4. Isolation of cardiomyocytes

Murine cardiomyocytes were isolated as previously described [29]. After ketamine/xylazine sedation, hearts were removed and perfused with Ca^{2+}-free Tyrode's solution containing (in mM): NaCl 135, KCl 4.0, $MgCl_2$ 1.0, HEPES 10, NaH_2PO_4 0.33, glucose 10, butanedione monoxime 10, and the solution was gassed with 5% CO_2/95% O_2. Hearts were digested with Liberase Blendzyme 4 (Hoffmann-La Roche Inc., Indianapolis, IN, USA) for 20 min. Left ventricles were removed and minced before being filtered. Tissue pieces were gently agitated and pellet of cells was resuspended. Extracellular Ca^{2+} was added incrementally back to 1.20 mM over a period of 30 min. Isolated myocytes were used within 8 hrs of isolation. Normally, a yield of 50–60% viable rod-shaped cardiomyocytes with clear sarcomere striations was achieved. Only rod-shaped myocytes with clear edges were selected for mechanical study.

2.5. Cell shortening/relengthening

Mechanical properties of cardiomyocytes were assessed using a SoftEdge MyoCam system (IonOptix, Milton, MA, USA). In brief, cells were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus IX-70) and superfused (~1 ml/min at 25 °C) with a buffer containing (in mM) 131 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES at pH 7.4. The cells were field stimulated with suprathreshold voltage at a frequency of 0.5 Hz using a pair of platinum wires placed on opposite sides of the chamber connected to a FHC stimulator (Brunswick, NE, USA). The myocyte being studied was displayed on the computer monitor using an IonOptix MyoCam camera. An IonOptix SoftEdge software was used to capture changes in cell length during shortening and relengthening. Cell shortening and relengthening were assessed using the following indices: resting cell length, peak shortening (PS), time-to-PS (TPS), time-to-90% relengthening (TR_{90}), and maximal velocity of shortening/relengthening (± dL/dt) [30].

2.6. Intracellular Ca^{2+} transients

A cohort of myocytes was loaded with fura-2/AM (0.5 μM) for 10 min, and fluorescence intensity was recorded with a dual-excitation fluorescence photomultiplier system (IonOptix). Myocytes were placed onto an Olympus IX-70 inverted microscope and imaged through a Fluor 40 oil objective. Cells were exposed to light emitted by a 75W lamp and passed through either a 360