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Adiponectin Ameliorates Hyperglycemia-Induced Cardiac Hypertrophy and Dysfunction by Concomitantly Activating Nrf2 and Brg1

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Running head: Nrf2 and Brg1 ameliorate diabetic cardiomyopathy

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Abstract

Hyperglycemia-induced oxidative stress is implicated in the development of cardiomyopathy in diabetes that is associated with reduced adiponectin (APN) and heme oxygenase-1(HO-1). Brahma-related gene 1 (Brg1) assists nuclear factor-erythroid-2-related factor-2 (Nrf2) to activate HO-1 to increase myocardial antioxidant capacity in response to oxidative stress. We hypothesized that reduced adiponectin (APN) impairs HO-1 induction which contributes to the development of diabetic cardiomyopathy, and that supplementation of APN may ameliorate diabetic cardiomyopathy by activating HO-1 through Nrf2 and Brg1 in diabetes. Control (C) and streptozotocin-induced diabetic (D) rats were untreated or treated with APN adenovirus (1×10^9 pfu) three weeks after diabetes induction and examined and terminated one week afterward. Rat left ventricular functions were assessed by pressure-volume conductance system, before the rat hearts being removed to perform histological and biochemical assays. Four weeks after diabetes induction, D rats developed cardiac hypertrophy evidenced as increased ratio of heart weight to body weight, elevated myocardial collagen I content, and larger cardiomyocyte cross-sectional area (all $P < 0.05$ vs. C). Diabetes elevated cardiac oxidative stress (increased 15-F_{2t}-isoprostane, 4-hydroxynonenal generation, 8-Hydroxy-2'-deoxyguanosine, and superoxide anion generation), increased myocardial apoptosis, and impaired cardiac function (all $P < 0.05$ vs. C). In D rats, myocardial HO-1 mRNA and protein expression were reduced which was associated with reduced Brg1 and nuclear Nrf2 protein expression. All these changes were either attenuated or prevented by APN. In primarily cultured cardiomyocytes (CMs) isolated from D rats or in the embryonic rat cardiomyocytes cell line H9C2 cells incubated with high glucose (HG, 25mM), supplementation of recombinant globular APN (gAd, 2 μ g/mL) reversed HG-induced reductions of HO-1, Brg1, and nuclear Nrf2 protein expression and attenuated cellular oxidative stress, myocyte size, and apoptotic cells. Inhibition of HO-1 by ZnPP (10 μ M) or small interfering RNA (siRNA) cancelled all the above gAd beneficial effects. Moreover, inhibition of Nrf2 (either by the Nrf2 inhibitor, luteolin or siRNA) or Brg1 (by siRNA) cancelled gAd-induced HO-1

induction and cellular protection in CMs and in H9C2 cells incubated with HG. In summary, our present study demonstrated that APN reduced cardiac oxidative stress, ameliorated cardiomyocyte hypertrophy, and prevented left ventricular dysfunction in diabetes by concomitantly activating Nrf2 and Brg1 to facilitate HO-1 induction.

Keywords: Nuclear factor-erythroid-2-related factor-2, Adiponectin, Brahma-related gene 1, Cardiac hypertrophy, Diabetes

Introduction

Cardiovascular complications and myocardial hypertrophy are the leading causes of death in patients with diabetes mellitus. Clinically, this complication is characterized initially as myocardial hypertrophy and apoptosis, which then progresses to left ventricular diastolic dysfunction and eventually cardiac failure[1]. Hyperglycemia-induced oxidative stress has been considered as the main cause that triggers hypertrophic responses and apoptosis in the diabetic heart[2]. Therefore, therapies that can reduce oxidative stress may effectively attenuate the development and progression of diabetic cardiomyopathy.

Heme oxygenase-1 (HO-1), a stress-inducible enzyme with anti-apoptotic and anti-inflammatory properties, has been demonstrated as an important mediator in cytoprotective defense mechanism against oxidative insults and cardiac hypertrophy through the antioxidant capacity of its metabolite biliverdin and bilirubin, as well as the anti-inflammatory actions of its metabolite carbon monoxide[3]. Induction of HO-1 plays a key role in anesthetic propofol-mediated attenuation of hyperglycemia-induced cardiac hypertrophy[4]. Overexpression of HO-1 not only protects cardiomyocytes from angiotensin II-induced cardiac hypertrophy via suppressing reactive oxygen species (ROS) overproduction *in vivo* and *in vitro*[3] but also attenuates myocardial apoptosis and improves pathological left ventricular remodeling in the failing heart[5]. Thus, interventions that can enhance HO-1 induction may serve as a promising therapy against hyperglycemia-induced cardiac hypertrophy. However, the mechanism governing HO-1 induction in the heart of diabetic subjects and its importance in combating diabetic cardiomyopathy remain unclear.

Recent studies have demonstrated the importance of nuclear factor-erythroid-2-related factor-2 (Nrf2) as an upstream stimulator of HO-1[6, 7]. Deficiency of Nrf2 impairs HO-1 induction and exaggerates oxidative stress both in angiotensin-II induced myocardial hypertrophy[8] and in hearts under pressure overload[9]. While

enhancement of Nrf2 attenuates the development of diabetic cardiomyopathy through increasing HO-1 in type 1 diabetic mouse[10]. Of note, Nrf2-mediated HO-1 induction needs the participation of brahma-related gene 1 (Brg1), a key subunit of ATP-dependent remodeling complexes. In response to oxidative stress, Nrf2 recruits Brg1 for Z-DNA formation and RNA polymerase II recruitment, which is critical in Nrf2-mediated inducible expression of HO-1[11, 12]. Although over-expression of Brg1 has been shown to mediate inflammation-induced endothelial injury in the pathogenesis of atherosclerosis[13], our previous finding showed that STZ-induced diabetic rats exhibited cardiomyopathy and dysfunction that was associated with reduced myocardial Brg1. Antioxidant treatment with N-acetylcysteine (NAC)[14] not only restored cardiac Brg1 expression but also attenuated diabetic cardiac hypertrophy and dysfunction[1]. More importantly, NAC significantly attenuated the reductions in cardiac and systemic levels of adiponectin (APN), an adipocyte-derived protein with anti-diabetic and anti-inflammatory properties that has been shown to ameliorate pressure-induced[15] and angiotensin II-stimulated[16] cardiac hypertrophy. NAC mediated attenuation of diabetic cardiomyopathy is also associated with restoration of cardiac HO-1 expression[17]. However, the relative importance and especially the potential interplay among APN, Nrf2 and Brg1 in the context of hyperglycemia induced oxidative stress and diabetic cardiomyopathy has not been elucidated.

APN deficiency has been shown to exacerbate concentric cardiac hypertrophy and increase mortality in response to pressure overload[15]. However, plasma and cardiac APN levels are reduced in diabetic patients[18] and STZ-induced diabetic rats[19, 20]. Moreover, oxidative stress can down-regulate APN level and impair APN cardioprotective signaling pathways[20, 21]. All these suggest that reduction of APN may be the major factor that rendered the diabetic hearts more sensitive to hyperglycemia-induced hypertrophy and cardiac dysfunction. Given that APN and HO-1 pathways are interactive and HO-1 induction mediated cardioprotection in the diabetic rats involves the enhancement of APN production[22], it is plausible that

APN supplementation may attenuate cardiac hypertrophy and cardiac dysfunction via increasing HO-1 induction in diabetes which needs the participation of Nrf2 and Brg1.

We, therefore, hypothesized that APN may inhibit hyperglycemia-induced cardiomyocyte hypertrophy and apoptosis and improve cardiac function *in vivo* by a mechanism involving the activation of Nrf2 and the subsequent HO-1 induction, and this process needs the participation of Brg1.

Research Design and Methods

Animals and Induction of Diabetes

Male Sprague-Dawley rats (250 ± 8 g, 6-8 weeks) obtained from the Laboratory Animal Unit (The University of Hong Kong) were used in this study. All rats were housed in a room at controlled temperature (23 ± 1 °C) with a 12 hours light-dark cycle, and given free access to standard chow and water. Type 1 diabetes was induced as described[23]. Briefly, STZ was injected via tail vein at a dose of 65 mg/kg body weight (Sigma-Aldrich, St. Louis, MO) in 0.1 M citrate buffer (pH 4.5) or citrate buffer alone as control under anesthesia. One week after STZ injection, rats exhibiting hyperglycemia (blood glucose 16.7 mmol/L) were considered diabetic and subjected to outlined experiments. At termination (4 weeks after diabetes induction), cardiac functions were determined, rats were then deeply anesthetized with sodium pentobarbital (65 mg/kg), and hearts were either rapidly excised for cardiomyocyte isolation or frozen in liquid nitrogen for later analysis. All experimental protocols used in this study were approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong (reference number: 2684-12).

Animal Experimental Protocol

Rats were randomly divided into four groups (n=8 per group): control (C), diabetes (D), diabetes treated with adiponectin adenovirus (D + APN, 1×10^9 pfu) or luciferase as control (D + LacZ, 1×10^9 pfu). Recombinant adenovirus expressing adiponectin or luciferase was tail-vein-injected into rats 1 week prior to tissue collection[20]. The increased expression level of adiponectin was confirmed by enzyme-linked immunosorbent assay kit (AdipoGen, Inc., Incheon, South Korea). The lowest level of adiponectin that can be detected by this assay is 50 pg/mL as described in the assay kit's instruction with the most reliable detection range between 1.5-12 ng/ml, and as such our samples were diluted accordingly before performing the assay, and the intra-assay and inter-assay variances (%CV) range were 9.4% and 10.5%, respectively.

Measurement of Left Ventricular Function

The global cardiac functions were monitored by using a pressure-volume (PV) conductance catheter (AD Instruments, Colorado Springs, CO, USA) placed into the left ventricle through right carotid artery and connected to a computer equipped with an advantage PV control box software (AD Instruments, Colorado Springs, CO, USA) as previously described[23]. The cardiac functional parameters were recorded, including heart rate (HR); left ventricular end-systolic pressure (Pes); left ventricular end-diastolic pressure (Ped); stroke volume (SV); left ventricular ejection fraction (EF); stroke work (SW); cardiac output (CO). The load-independent contractility parameters including the maximal slope of systolic pressure increment (dP/dt_{max}); arterial elastance ($E_a = LVESP/SV$); diastolic decrement (dP/dt_{min}); the relaxation time constant calculated by Weiss method (Tau) were analyzed using Labchart 8 software (AD Instruments, Colorado Springs, CO, USA).

Measurement of Cardiomyocytes Cross-sectional Area

After the completion of 4 weeks' treatment, cardiomyocytes cross-sectional diameters were assessed by hematoxylin-eosin-stained paraffin-embedded sections of left ventricles (5 μ m) longitudinally orientated to the muscle fibers in the sub-endocardium and subepicardium as previously described[1]. Cross-sectional areas were randomly selected in five fields that visualized capillary profiles and nuclei. Images of the left ventricle sections were captured by an Axisoplu image-capturing system (Zeiss) and analyzed by Axiovision Rel. 4.5 image analyzing software. A minimum of 150 cells per animal was chosen for analysis.

Apoptotic Cell Death Detection Using Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) reaction was performed using an *in situ* cell death detection kit (Roche Diagnostics GmbH, Mannheim, Germany) as previously described[4]. The sections were observed in the light microscope by an investigator who was initially blinded to treatment groups, and five randomly selected fields of each slide were analyzed, and the apoptotic index was calculated as a percentage of apoptotic nuclei to total nuclei.

Immunohistochemical Assay for Collagen I, 4-hydroxynonenal (4-HNE), and

8-Hydroxy-2'-deoxyguanosine (8-OHdG)

Paraffin-embedded left ventricular tissue blocks were sectioned at 5 μm . Then, the sections were dewaxed and rehydrated. Slides were incubated in 3% hydrogen peroxide/methanol. Antigen retrieval was performed by heating in 10 mM sodium citrate buffer for 10 min. Sections were incubated in anti-collagen I antibody (abcam, USA), or anti-4-HNE antibody (abcam, USA), or 8-OHdG (Santa Cruz, USA) at 1:500 dilution for 12 hrs at 4 $^{\circ}\text{C}$. 3,3'-Diaminobenzidine Substrate Chromogen System (Dako, S1699) was employed in the detection procedure. Subsequently, the sections were counterstained with hematoxylin for 30s. Finally, the sections were dehydrated in ethanol, cleared in xylene, mounted, and observed in the light microscope. The sections were observed in light microscope by an investigator who was initially blinded to treatment groups, and five randomly selected fields of each slide were semi-quantified and averaged using the software Image J 1.48 (National Institutes of Health) according to its instructions.

Detection of Left Ventricle Tissue Reactive Oxygen Species (ROS) Production by *In Situ* DHE (Dihydroethidium) Staining

Levels of *in situ* O_2^- production were detected by DHE (Sigma-Aldrich) staining as described[24]. DHE binds to DNA upon oxidization by O_2^- to generate fluorescent ethidium bromide, and the resulting fluorescence intensity reflects the quantity of ROS production. Frozen sections of left ventricular tissue (20 μm) were incubated with 7.5 μM DHE at 37 $^{\circ}\text{C}$ for 30min. Fluorescence images of ethidium bromide were obtained with a fluorescence microscope (BX41System microscope; Olympus) and the images were captured by a DP72 digital camera. The fluorescence of DHE-labelled positive nuclei was calculated in each of five randomly selected fields and are expressed as a percentage of the DHE-stained positive myocyte nuclei compared with control by a quantitative morphometric method[25].

Determination of Cardiac 15-F_{2t}-Isoprostane and 8-OHdG

Free 15-F_{2t}-Isoprostane (15-F_{2t}-IsoP), a specific marker of oxidative stress, was measured using an enzyme-linked immunoassay kit as we described[20]. Homogenized heart tissues were purified using Affinity Sorbent and Affinity Column

(Cayman Chemical, Ann Arbor, MI) and then processed for analysis. The values of free 15-F_{2t}-IsoP were expressed as pg/g protein in cardiac homogenates. The lowest level of 15-F_{2t}-IsoP that can be detected by this assay kit is 2.7 pg/mL as described in the assay kit's instruction with the most reliable detection range between 12.5-80pg/ml, and as such our samples were diluted accordingly before performing the assay, and the intra-assay and inter-assay variances (%CV) of this assay were 11.5% and 9.8%, respectively in our study. 8-OHdG, a specific marker of oxidative damage of DNA by ROS, was determined by a commercially available 8-OHdG EIA kit (Cayman chemical, Ann Arbor, MI) according to the manufacturer's instructions[26]. The lowest level of 8-OHdG that can be detected by this assay is 33 pg/mL, and most reliable detection range is between 117 pg/ml and 590 pg/ml according to that described by the assay kit manufacturer. The heart tissue samples were homogenized and processed for assay after proper dilution, and the intra-assay and inter-assay variances (%CV) were 6.8% and 8.6%, respectively in our study.

Adult Rat Ventricular Cardiomyocytes Isolation and Treatment

Calcium-tolerant cardiomyocytes were prepared from rat ventricles via a modified method as previously described[27]. Cells isolated from a single rat heart were plated on Matrigel-coated culture dishes and allowed to recover for 3 hrs. Cultured ventricular cardiomyocytes were incubated in normal glucose (NG, 5.5 mM) or high glucose (HG, 25 mM) or HG in the presence of Nrf2 inhibitor luteolin (Lut, 20 µM) or HO inhibitor zinc protoporphyrin (ZnPP, 10 µM) at 37°C in Medium 199 (Gibco, Grand Island, NY) for 48 hours, some of the subgroup were treated with recombinant globular adiponectin (gAd, 2 µg/mL) for 24 hours before sample collection. Each experiment was performed at least times independently in triplicate.

Nrf2 siRNA, Brg1 siRNA, and HO-1 siRNA Studies in H9C2 Cells

Embryonic rat cardiac H9C2 cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS in a humidified atmosphere (5% CO₂) at 37°C. Commercial Nrf2 small interfering RNA (siRNA), Brg1 siRNA, and HO-1 siRNA (Santa Cruz Biotechnology) were used for the inhibition of Nrf2, Brg1 and HO-1 expression per the manufacturer's protocol as previously described[4]. After

transfection with control siRNA, Nrf2 siRNA, Brg1 siRNA, or HO-1 siRNA, cells were incubated in either NG or HG in Dulbecco's modified Eagle's medium for 36 hours, some of the subgroup were treated with gAd (2 µg/mL) for 24 hours and snap-frozen in liquid nitrogen. Each experiment was performed at least times independently in triplicate.

Measurement of Cardiomyocyte Surface Area

H9C2 cells were stained with phalloidin-tetramethylrhodamine conjugate (Santa Cruz Biotechnology) as previously described[4]. The cell surface area was determined with image analysis software Image J 1.48 (National Institutes of Health) and calculated as the mean of 100 cells of 5 randomly selected fields.

Measurement of Cellular ROS in Cultured Cardiomyocytes

Superoxide generation in cultured cardiomyocytes was estimated by DHE staining as previously described[24]. Briefly, cardiomyocytes were loaded with DHE at a concentration of 10 µM for 30 min at 37 °C. The DHE fluorescence of DHE-labelled positive nuclei were calculated in each of five randomly selected fields and were expressed as a percentage of the DHE-stained positive myocyte nuclei compared with control by a quantitative morphometric method[24].

Determination of Cellular Injury

Cell apoptosis was detected by double immunofluorescence staining of TUNEL using the In Situ Cell Death Detection Kit (Roche, Indianapolis, USA) as described[4]. Mitochondrial membrane potential (MMP) loss was measured using a JC-1 mitochondrial membrane potential assay kit (Cayman chemical) as described[17]. Cell lactate dehydrogenase (LDH) content was measured with a LDH Cytotoxicity Assay Kit (Roche, Indianapolis, USA) as described[27].

Isolation of Cytosolic and Nuclear Fractions

Hearts from control and STZ treated rats were cleared of blood by washing thoroughly in Tyrode buffer and aortic and atrial sections removed from the ventricles. Ventricular tissue was freeze-clamped in liquid nitrogen, and stored until fractionated to isolate cytosolic and nuclear fractions according to the manufacturer's protocol as described in the Nuclear and Cytoplasmic Extraction Kit (Thermo, Chicago, IL).

Extraction of Total RNA and Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA was extracted from using Trizol (Invitrogen Life Technologies, Carlsbad, CA), and quantitative real-time polymerase chain reaction (PCR) was performed with a SYBR green PCR master mix (Applied Biosystems, Foster City, CA) on a 7300 ABI-Prizm Sequence Detector (Applied Biosystems) as described[4]. Gene-specific primers were as follows: rat HO-1 forward: 5'-TGCTCGCATGAACACTCTG-3', reverse: 5'-TCCTCTGTCAGCAGTGCCT-3'; 18s RNA, served as an endogenous control, forward: 5'-CGGCTACCACATCCAAGGAA-3', reverse: 5'-GCTGGAATTACCGCGGCT-3'.

Western Blot Analysis

Equal protein amounts from isolated cardiomyocytes, H9C2 cells, and rat heart homogenate were resolved by 7.5-12.5% SDS-PAGE and subsequently transferred to polyvinylidene nitrocellulose membranes and processed as previously described[27]. The primary antibodies against collagen I, caspase-3, β -actin, and histone H3 were purchased from Cell Signaling Technology (Beverly, MA), Nrf2 and Brg1 antibodies was purchased from Abcam (Cambridge, MA), HO-1 antibody was purchased from Santa cruz (Santa Cruz Biotechnology, Dallas, TX). Immunoreactive bands were visualized by enzymatic chemiluminescence method and quantified with Quantity One image software.

Statistical Analysis

All the values were expressed as mean \pm standard error of the mean (S.E.M). One-way analysis of variance (ANOVA) was used for statistical analyses (GraphPad Prism, USA) of data obtained within the same group of rats and between groups of rats, respectively, followed by Tukey's test for multiple comparisons of group means. $P < 0.05$ was considered statistically significant.

Results

General Characteristics of Control and Diabetic Rats Treated With or Without Adiponectin

As shown in **Table 1**, 4 weeks STZ-induced diabetic rats display hyperglycemia, polydipsia, and polyphagia evidenced as increased plasma glucose, water intake, and food consumption ($P<0.05$ vs. C). Blood glucose was significantly increased associated reduced plasma and heart tissue APN in D group then that in C group ($P<0.05$). Body weight were lower while the heart/body weight ratio was higher in D group than that in the C group ($P<0.05$), APN supplementation significantly reduced heart/body weight ratio but has no impact on body weight despite that it significantly reduced food consumption and water intake. APN treatment significantly reduced plasma glucose level and restored plasma and heart tissue APN content ($P<0.05$ vs. D).

Adiponectin Attenuated Cardiac Dysfunction in Diabetic Rats

As shown in **Table 2**, cardiac function in rats after 4 weeks of diabetes was impaired, evidenced as reductions in basal HR, SV, SW, CO, and prolonged relaxation time constant (Tau, an index of diastolic function) while Ea was increased (all $P<0.05$ vs. control). The Pes, EF, dP/dt_{max} , and dP/dt_{min} were also significantly decreased in diabetic rats, while the Ped was not altered. The diabetes-induced reductions in SV, SW, EF, and Pes were reversed by adiponectin. Also, adiponectin partially recovered CO, dP/dt_{max} , and dP/dt_{min} in diabetic rats although the respective values were still lower than those in C rats ($P<0.05$). Adiponectin significantly attenuated the increases of Ea and Tau in diabetic rats.

Adiponectin Reduced Myocardial Oxidative Stress, Cardiomyocyte Hypertrophy, and Apoptosis in Diabetic Rats

Myocardial levels of 4-HNE (**Fig. 1A and B**)- a marker of lipid peroxidation, cardiac levels of 15-F_{2t}-Isoprotane (**Fig. 1C**)-a specific index of ROS-induced oxidative stress, cardiac levels of 8-OHdG-a marker of oxidative damage to DNA (**Fig. 1D-F**), and

myocardial superoxide anion generation (number of DHE-labelled nuclei, **Fig. 1G** and **H**) were significantly increased in D rats compared to C rats, and this was accompanied with increased myocyte cross-sectional area (**Fig. 1I** and **J**) (an indication of cardiomyocyte hypertrophy) and abundance in myocardial collagen I content (**Fig. 1K**) (an indication of cardiac fibrosis) and cardiac collagen I protein expression (**Fig. 1L**) in D rats compared to C. Cardiomyocyte apoptosis was significantly increased in D rats, evidenced as increased cleaved caspase-3 protein expression (**Fig. 1N**) and increased number of TUNEL-staining positive cells (**Fig. 1M** and **O**). APN supplementation prevented these changes in diabetes (**Fig. 1A-O**).

Adiponectin Increased Cardiac Expression of Brg1, HO-1 and Nuclear Nrf2 in Diabetic Rats

Diabetes significantly increased cytosolic Nrf2 protein retention (**Fig. 2A**) resulting in reduced nuclear Nrf2 protein expression in the myocardium (**Fig. 2B**), wherever APN supplementation significantly reduced cytosolic Nrf2 protein expression and increased nuclear Nrf2 protein expression, indicating an increased Nrf2 nuclear translocation and activation after APN treatment (**Fig. 2A** and **B**). Myocardial HO-1 mRNA and protein expression were significantly decreased in D compared to C (**Fig. 2C** and **D**), this was associated with reduced cardiac Brg1 protein expression in D rats (**Fig. 2E**). APN treatment significantly attenuated the reductions of mRNA and protein expression of HO-1 and attenuated the reduction of Brg1 protein expression in D rats (**Fig. 2C-E**).

Globular Adiponectin Attenuated HG-induced Cardiomyocyte Oxidative Stress and Apoptosis by Enhancing Nrf2, Brg1, and HO-1 Protein Expression

In cultured isolated cardiomyocytes, the number of DHE-labelled positive nuclei was significantly increased in cardiomyocytes exposed to HG than that exposed to NG, and globular adiponectin (gAd) treatment attenuated this change. However the protective effects of gAd were abolished by either Nrf2 inhibition by luteolin (Lut) or HO-1 inhibition by ZnPP (**Fig. 3A** and **B**). HG exposure significantly increased

cellular injury manifested as exacerbated MMP loss, increased TUNEL positive cells, enhanced cleaved caspase3 protein expression, and elevated LDH release and these changes were prevented by gAd. However, the abovementioned cellular protective effects of gAd were cancelled by Lut and ZnPP (**Fig. 3C-H**). gAd significantly increased nuclear Nrf2 protein expression but had no impact on cytosolic Nrf2 protein expression (**Fig. 3I and J**) in cardiomyocytes exposed to HG. These were accompanied with increased HO-1 protein expression (**Fig. 3K**) and Brg1 protein expression (**Fig. 3L**). Lut treatment significantly reduced gAd-induced increased HO-1 protein expression but had no impact on gAd-induced up-regulation of Brg1 protein expression (**Fig. 3K and L**).

Globular Adiponectin Reduced HG-induced Cardiomyocyte Size, Lipid Peroxidation, and Cellular Injury via Brg1/Nrf2 Signaling Pathway

HG significantly increased cultured H9C2 cell size (**Fig. 4A and B**) and elevated cellular 4-HNE expression (**Fig. 4C and D**), which was associated with increased cellular injury manifested as increased TUNEL positive cells and increased LDH release (**Fig. 4E-G**). gAd supplementation prevented these changes. Gene knockdown of Nrf2, HO-1, or Brg1 all respectively cancelled gAd cellular protection (**Fig. 4A-G**). gAd supplementation significantly increased nuclear Nrf2, HO-1 as well as Brg1 protein expression (**Fig. 4H-K**). Similar to HO-1 gene knockdown, Nrf2 or Brg1 gene knockdown attenuated gAd-induced elevation of HO-1 protein expression (**Fig. 4J**). Brg1 gene knockdown had no impact on Nrf2 protein expression both in cytosolic and in nuclear fractions (**Fig. 4H and I**). Similarly, Nrf2 gene knockdown did not affect gAd-induced increased Brg1 protein expression (**Fig. 4K**). HO-1 gene knock down cancelled gAd-mediated attenuation of HG-induced cellular injury (i.e., reduction in apoptotic cell death and LDH release) without affecting gAd-mediated restoration of Brg1 and nuclear Nrf2 protein expression (**Fig. 4I and K**).

Discussion

In the current study, we have demonstrated that adiponectin (APN) supplementation, through concomitantly activating Nrf2 and Brg1, activates myocardial HO-1 and attenuates cardiac hypertrophy and cardiac dysfunction in STZ-induced diabetic rats. We showed that in hearts from STZ-induced diabetic rats, reduced myocardial HO-1, Brg1, and nuclear Nrf2 protein expression were associated with increased myocardial hypertrophy and cardiac dysfunction, which can be partially reversed by APN supplementation. We further demonstrated that in isolated adult rat cardiomyocytes as well as in H9C2 cells, high glucose (HG) exposure decreased HO-1, Brg1, and nuclear Nrf2 protein expression with concomitant increases in lipid peroxidation, cardiomyocyte size, and apoptosis. These changes were prevented by globular adiponectin (gAd) administration. However, cardioprotective effects of gAd were cancelled by Nrf2, Brg1, or HO-1 inhibition/genes knockdown. In addition, Nrf2 or Brg1 gene knockdown attenuated gAd-induced HO-1 induction, while Nrf2 gene knockdown did not affect Brg1 protein expression, nor did Brg1 gene knockdown affect Nrf2, suggesting that HO-1 functions downstream of Brg1 and Nrf2 in cardiomyocytes under hyperglycemic condition. . To our best knowledge, this is the first study examining the relationship between Nrf2 and Brg1, and their interplay with HO-1, in APN mediated cardioprotection against diabetic hypertrophy and cardiac dysfunction.

Cardiomyocyte hypertrophy stimulated by enhanced oxidative stress or cell loss resulting from apoptosis/necrosis is the main cause of cardiac dysfunction in diabetic cardiomyopathy both in humans[28] and animal models of diabetes[4, 29]. In the current study, we determined that, in 4-week diabetic rats, APN supplementation prevented the increased myocardial oxidative stress (lipid peroxidation and nucleic acid oxidation) and enhanced HO-1 and Nrf2 protein expression, and concomitantly reduced myocytes apoptosis and the apoptotic activator-cleaved caspase-3 protein expression. These abovementioned APN-mediated beneficial effects in attenuating diabetic cardiac hypertrophy and apoptosis collectively resulted in improved cardiac

performance manifested as increased SV, SW, CO, Pes, as well as EF, and augmented dP/dt_{max} and dP/dt_{min} , with concomitant reductions in Ea and Tau. Our findings were similar to the findings of Tao *et al* who showed that APN attenuated myocardial ischemia reperfusion induced cardiac dysfunction and apoptosis by reducing oxidative/nitrative stress[30].

Oxidative stress induced by hyperglycemia has been considered as the major cause of diabetic cardiomyopathy and that enhancing myocardial antioxidant capacity has been proven to be effective in attenuating diabetic cardiomyopathy[1, 2]. Previously studies show that APN supplementation ameliorates pressure-induced[15] and angiotensin II-stimulated[16] cardiac hypertrophy, which has been assumed to be achieved largely through APN mediated metabolic improvement with AMP-dependent protein kinase (AMPK) as its main downstream target. However, recent studies show that APN reduces post-ischemic myocardial injury mainly through its antioxidative effects rather than through metabolic regulation[30]. In addition, exogenous APN supplementation could attenuate myocardial apoptosis in cardiomyocytes subjected to hypoxic reoxygenation by decreasing NADPH oxidase expression and blocking peroxynitrite formation in an AMPK-independent fashion[31]. Similarly, in our present study, we provided additional evidences showing that APN exerted antihypertrophic and antiapoptotic effects, through its antioxidative properties by inducing HO-1 subsequent to its activation of Nrf2 and Brg1.

Nrf2, by regulating a series of cytoprotective genes (i.e. HO-1 and NQO1, NAD(P)H dehydrogenase, quinone 1) in response to oxidative stress, is central in myocardial cardioprotection against ischemia reperfusion injury[32] and diabetic cardiomyopathy[33], especially in the setting of elevated oxidative stress, such as in diabetes. However, the role of Nrf2 in APN cardioprotection has not been defined. In the current study, Nrf2 inhibition or gene knockdown cancelled APN-mediated antihypertrophic and antiapoptotic effects in cardiomyocytes exposed to HG, this indicates that Nrf2 played a critical role in APN cardioprotective effects against

diabetic cardiomyopathy. We further demonstrated that HO-1 inhibition or gene knockdown cancelled APN-mediated cardioprotection without affecting APN-induced Nrf2 or Brg1 activation (Fig.3 and Fig.4. While Nrf2 inhibition or gene knockdown abolished APN-mediated HO-1 induction and compromised APN cardioprotection. These suggest that APN conferred its antihypertrophic and antiapoptotic properties through Nrf2-mediated up-regulation of HO-1 induction.

The mechanisms whereby Nrf2 mediates HO-1 induction are unclear. In order to activate HO-1, activated Nrf2 translocates into the nucleus, binds to AREs, and then increases the transcriptional activation of HO-1[34]. This process requires the recruitment of Brg1, which facilitates Nrf2 function through chromatin remodeling[11]. Consistent with these observations[11, 34], in the present study, gene knockdown of Brg1, without affecting Nrf2 expression, abolished APN-induced HO-1 induction. Zhang *et al.*[11] reported that Brg1 facilitated Nrf2 to induce HO-1 induction in response to stress, while Brg1 gene knockdown diminished Nrf2-induced HO-1 but had no impact on NQO1, another downstream molecule in Nrf2-mediated antioxidative properties[35]. Interestingly, in the current study, Brg1 gene knockdown not only abolished Nrf2-mediated APN-induced HO-1 induction but also attenuated APN-mediated cardioprotection. These together with the finding in the present study that HO-1 inhibition cancelled APN-mediated cardioprotective effect against diabetic cardiomyopathy, suggest that activation of Nrf2 contributed to the beneficial effects of APN in combating diabetic cardiomyopathy, and that HO-1 served as the main downstream target of Nrf2, and this process needed the recruitment of Brg1.

The most novel finding of the present study is that enhancement of Brg1 by APN is cardioprotective in diabetic cardiomyopathy. Our findings are in line with the observation by Zhang *et al.*[11] who showed that in response to oxidative stress, Brg1 facilitated the recruitment of RNA polymerase II, a critical component in Nrf2-mediated HO-1 expression, while Brg1 gene knockdown decreased Nrf2-mediated inducible expression of HO-1 in SW480 cells. It is also reported that

Brg1 confers cardioprotection by initiating the activation of STAT3[36], a molecule mediated propofol-induced cardioprotection against diabetic cardiomyopathy as we previously reported[4], and by restoring endothelial nitric oxide synthase (eNOS) protein expression and maintaining eNOS transcription in endothelial cells in response to hypoxic injury[37]. Further, our present study demonstrated that either Brg1 gene knockdown or Nrf2 inhibition/gene knockdown all abolished APN-mediated increase in HO-1 expression, suggesting that both Brg1 and Nrf2 were required in APN-mediated HO-1 induction in combating myocardial hypertrophy and myocardial oxidative stress in diabetes. Of note, in the present study, Nrf2 gene knockdown cancelled APN-mediated antioxidative and anti-apoptotic effects (Fig. 3A-G; Fig. 4C-F) without affecting APN-mediated enhancement of Brg1 expression (Fig. 3L and Fig. 4K), which indicates that in the setting of increased oxidative stress (where Nrf2 is inactivated), like diabetes, the beneficial effects of Brg1 might be diminished or even abolished.

It should be noted that the expression of HO-1 in diabetes seems to be complex since several relevant studies have yielded inconsistent results. We[14] have shown that HO-1 expression is reduced in STZ-induced diabetes 5 weeks after diabetes induction, while others have indicated that HO-1 expression is increased in relative early stage (4 weeks) of diabetes while decreased in the relative late stage (8 week) of the disease[38]. These indicate that HO-1 expression varies at different stages of diabetes (e.g. differences of the severity of the diabetic state and the metabolic profiles after diabetic induction). In our current study, HO-1 expression was reduced *in vivo* in diabetic rats but was increased *in vitro* in isolated cardiomyocytes after HG exposure, one possible explanation could be that the cardiomyocyte HG stimulation in our *in vitro* model mimicked the relatively early stage of hyperglycemic induced pathological adaptation while our diabetic rat model represents the pathological adaptation after prolonged hyperglycemia stimulation. While the consistent findings that APN supplementation reduced oxidative stress and cardiomyocyte hypertrophy in diabetes by increasing HO-1 induction both *in vivo* and *in vitro* indicates HO-1 as a

critical molecule in APN cardioprotection under diabetic condition. Findings from our current study and others suggest that HO-1 may compensatorily increase at the early stage of diabetes, while this compensatory in HO-1 is not sufficient to combat diabetic cardiomyopathy. As such, induction of endogenous HO-1 production both at the early phase and later stage of diabetes represents a major mechanism whereby APN confer cardioprotection in diabetes.

Conclusion

In summary, to our knowledge, our study demonstrated for the first time, that hyperglycemia-induced oxidative stress deactivates Nrf2 and Brg1 which led to the reduced HO-1 induction and, consequently, exacerbated myocardial hypertrophy and apoptosis (Fig.5). APN supplementation attenuated cardiac hypertrophy and dysfunction by concomitantly restoring Nrf2 activation and Brg1 expression and subsequently rescuing HO-1 induction, resulting in reduced myocardial oxidative stress, decreased cardiac hypertrophy, and reduced cardiac apoptosis, which eventually improved cardiac function. Nrf2 and Brg1 activation by APN might therefore represent as novel therapeutic targets in the treatment of diabetic cardiomyopathy.

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Author contribution

H.L., W.Y., T.W., and S.W. performed experiments and analyzed data; H.L., W.Y., T.W., M.G.I., L.Z., and Z.X. interpreted results of experiments; H.L. drafted manuscript; M.G.I. and Z.X. edited and revised manuscript; Z.X. approved final version of manuscript. Z.X. has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure legends

Fig. 1. Adiponectin reduced myocardial oxidative stress, cardiomyocyte hypertrophy, and apoptosis in 4 weeks streptozotocin-induced diabetic rats. **A** and **B**, The 4-hydroxynonenal (4-HNE) visualization (400 × magnification, stained in brown as indicated by *arrow*) and quantitation in control (C), diabetic (D) rats, and diabetic rats treated with adiponectin adenovirus (D + APN, 1×10^9 pfu) or luciferase as control (D + LacZ, 1×10^9 pfu). **C**, Free 15-F_{2t}-isoprostane (15-F_{2t}-IsoP) in heart tissue was measured by using an enzyme-linked immunoassay kit. **D** and **E**, Cardiomyocyte nucleic acid oxidation assessed by 8-OHdG staining. 8-OHdG positive cells were stained brown as indicated by *arrow*. **F**, Myocardial 8-OHdG level determined by using an enzyme-linked immunoassay kit. **G** and **H**, Myocardial O₂⁻ production assessed by dihydroethidium (DHE) staining (stained in red) and quantification in C, D, D+LacZ, and D+APN. **I** and **J**, The cell size visualization (400 × magnification) and quantitation in C, D, D + LacZ, and D + APN. **K** and **L**, The collagen I visualization (400 × magnification, stained in brown as indicated by *arrow*) and protein expression in heart tissue. **N**, Protein expression of cardiac total caspases-3 and cleaved caspases-3. **M** and **O**, Myocardial cell apoptosis assessed by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). TUNEL-positive cells were stained brown as indicated by *arrow*. Data are shown as means ± SEM, with n=8 animals *per* group. * $P < 0.05$, ** $P < 0.01$. Neg: Negative control.

Fig. 2. Adiponectin increased cardiac expression of Brg1, HO-1 and nuclear Nrf2 in 4 weeks diabetic rats. **A** and **B**, cytosolic and nuclear Nrf2 protein expression in the myocardium. **C**, Cardiac HO-1 protein expression. **D**, Cardiac HO-1 mRNA expression. **E**, Cardiac Brg1 protein expression. Data are shown as means ± SEM, with n=8 animals per group. * $P < 0.05$, ** $P < 0.01$. H3: histone H3.

Fig. 3. Globular adiponectin attenuated oxidative stress and apoptosis in isolated adult cardiomyocytes exposed to high glucose (HG) for 48hr via Nrf2/HO-1

signaling pathway. **A** and **B**, cellular reactive oxygen species production assessed by dihydroethidium (DHE) staining in isolated adult cardiomyocytes exposed to normal glucose (NG, 5.5 mM glucose), HG (25 mM glucose), HG + globular adiponectin (gAd, 2 µg/mL), HG + gAd + Lut (luteolin, Nrf2 inhibitor), or HG + gAd + ZnPP (Zinc protoporphyrin, HO-1 inhibitor). **C** and **D**, mitochondrial membrane permeability (MMP) loss by JC-1 staining. **E** and **F**, cardiomyocyte apoptosis assessed by TUNEL staining. **G**, Protein expression of cardiac total caspase-3 and cleaved caspases-3. **H**, cardiomyocyte death assessed by lactate dehydrogenase (LDH) release. **I** and **J**, protein expression of cytosolic and nuclear Nrf2. **K**, protein expression of HO-1. **L**, protein expression of Brg1. Data are mean ± SEM of two independent experiments each performed in triplicate, * $P < 0.05$ vs. NG, # $P < 0.05$ vs. D + gAd. H3: histone H3.

Fig. 4. Globular adiponectin reduced cardiomyocyte size, lipid peroxidation, and cellular injury in H9C2 cardiomyocytes exposed to high glucose (HG) for 48hr via Nrf2/Brg1/HO-1 signaling pathway. H9C2 cardiomyocytes were pretreated with Nrf2 siRNA, HO-1 siRNA, Brg1 siRNA, or control siRNA and then treated with control medium with normal glucose (NG, 5.5 Mm glucose) or HG (25mM glucose) for 48hr, subgroup were treated with globular adiponectin (gAd) for 24hr before sample collection. **A** and **B**, H9C2 cell size visualization (400 × magnification) and quantitation. **C** and **D**, cellular 4-hydroxynonenal (4-HNE) visualization (400 × magnification) and quantitation. **E** and **F**, H9C2 cardiomyocyte apoptosis assessed by TUNEL staining. **G**, cardiomyocyte death assessed by lactate dehydrogenase (LDH) release. **H** and **I**, protein expression of cytosolic and nuclear Nrf2. **J**, protein expression of HO-1. **K**, protein expression of Brg1. Data are mean ± SEM of two independent experiments each performed in triplicate, * $P < 0.05$ vs. NG, # $P < 0.05$ vs. D + gAd. H3: histone H3.

Fig. 5. Schematic of proposed signaling involved in APN protects against diabetic-induced cardiac hypertrophy and cardiac dysfunction via activation of

Nrf2 and Brg1. Hyperglycemia-induced oxidative stress by increasing lipid peroxidation inactivates Nrf2 by inhibiting its nuclear translocation which associated with reduced Brg1, and subsequently reduced HO-1 induction, resulting in cardiomyocytes apoptosis and cardiac hypertrophy. APN supplementation activates Nrf2 by increasing its nuclear translocation and concomitantly increases Brg1 expression, which in turn facilitates myocardial HO-1 induction, and reduced myocardial oxidative stress, improve cardiac function, and attenuates cardiac hypertrophy. 4-HNE: 4-hydroxynonenal; 8-OHdG: 8-Hydroxy-2'-deoxyguanosine; Keap1: Kelch-like ECI-associated protein 1; ARE: antioxidant response elements.

Table1. General Characteristics after streptozotocin treatment at termination of study

Parameters	C	D	D + LacZ	D + APN
Water intake (mL/kg/d)	131.60 ± 2.80	753.40 ± 14.10*	780.90 ± 11.60*	449.10 ± 9.40** [#]
Food consumption (g/kg/d)	70.30 ± 2.60	179.20 ± 5.90*	180.00 ± 4.20*	136.30 ± 3.10** [#]
Body weight (g)	447.50 ± 6.70	298.80 ± 2.80*	296.30 ± 1.60*	317.50 ± 3.70*
Heart weight (g)	1.50 ± 0.10	0.90 ± 0.10*	1.00 ± 0.10*	1.20 ± 0.10 [#]
Heart/body weight ratio (g/kg)	3.30 ± 0.10	3.98 ± 0.10*	3.96 ± 0.00*	3.54 ± 0.10** [#]
Blood glucose (mM)	6.70 ± 0.20	29.30 ± 1.50*	28.90 ± 1.30*	22.30 ± 0.40** [#]
Plasma APN levels (mg/mL)	15.10 ± 2.00	11.30 ± 0.80*	9.80 ± 1.10*	15.20 ± 1.60 [#]
Heart tissue APN (ng/mg)	270.50 ± 12.00	140.00 ± 14.50**	140.00 ± 8.80**	217.00 ± 8.80** [#]

All values are expressed as mean ± SEM. n=8 per group. Water intake and food consumption were the average value of 4 weeks. Body weight and plasma glucose were measured at 5 weeks after STZ injection in these five groups. All of the indices were measured among groups. * $P < 0.05$, ** $P < 0.01$ vs. C, [#] $P < 0.05$ vs. D.

Table 2. Hemodynamic variables and indices of cardiac systolic and diastolic function by pressure-volume conductance catheter system

Parameters	C	D	D+LacZ	D+APN
Heart rate (beats/min)	413.40 ± 3.60	314.90 ± 6.40*	314.90 ± 6.90*	340.70 ± 12.90*
Stroke volume (μL)	276.50 ± 5.40	155.10 ± 10.20**	165.10 ± 9.40**	261.50 ± 4.60 [#]
Stroke work (mm Hg/μL)	13,974.00 ± 238.00	9,421.00 ± 706.00*	9,738.00 ± 298.00*	12,778.00 ± 838.00 [#]
Cardiac output (mL/min)	109.70 ± 3.70	47.33 ± 1.80**	49.00 ± 1.90**	82.70 ± 2.70 [#]
End-systolic pressure (mm Hg)	114.30 ± 2.80	84.00 ± 2.30*	81.00 ± 4.40*	96.90 ± 1.90 [#]
End-diastolic pressure (mm Hg)	5.10 ± 0.20	4.80 ± 0.20	4.70 ± 0.30	4.80 ± 0.20
Ejection fraction (%)	61.00 ± 2.30	46.10 ± 0.70*	45.90 ± 1.00*	61.10 ± 2.00 [#]
Arterial elastance (mm Hg/μL)	0.56 ± 0.02	0.80 ± 0.02*	0.76 ± 0.02*	0.58 ± 0.03 [#]
Maximal slope of systolic pressure increment (mm Hg/s)	15,459.00 ± 418.00	6,975.00 ± 207.00**	7,185.00 ± 330.00**	8,518.00 ± 323.00 [#]
Diastolic decrement (mm Hg/s)	6,609.00 ± 324.00	4,918.00 ± 177.00**	4,938.00 ± 206.00**	5,938.00 ± 227.00 [#]
Tau (ms)	9.30 ± 0.30	16.9.0 ± 1.30**	16.80 ± 0.70**	8.20 ± 0.30 [#]

All values are expressed as mean ± SEM. n=8 per group. All of the indices were measured among groups. * $P < 0.05$, ** $P < 0.01$ vs. C, [#] $P < 0.05$ vs. D.