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Original article

# Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of Nrf2 expression and transcription activation

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ABSTRACT

This study was to investigate whether sulforaphane (SFN) can prevent diabetic cardiomyopathy. Type 1 diabetes was induced in FVB mice by multiple intraperitoneal injections with low-dose streptozotocin. Hyperglycemic and age-matched control mice were treated with or without SFN at 0.5 mg/kg daily in five days of each week for 3 months and then kept until 6 months. At 3 and 6 months of diabetes, blood pressure and cardiac function were assessed. Cardiac fibrosis, inflammation, and oxidative damage were assessed by Western blot, real-time qPCR, and histopathological examination. SFN significantly prevented diabetes-induced high blood pressure and cardiac dysfunction at both 3 and 6 months, and also prevented diabetes-induced cardiac hypertrophy (increased the ratio of heart weight to tibia length and the expression of atrial natriuretic peptide mRNA and protein) and fibrosis (increased the accumulation of collagen and expression of connective tissue growth factor and tissue growth factor-β). SFN also almost completely prevented diabetes-induced cardiac oxidative damage (increased accumulation of 3-nitrotyrosine and 4-hydroxynonenal) and inflammation (increased tumor necrotic factor-α and plasminogen activator inhibitor 1 expression). SFN up-regulated NFE2-related factor 2 (Nrf2) expression and transcription activity that was reflected by increased Nrf2 nuclear accumulation and phosphorylation as well as the mRNA and protein expression of Nrf2 downstream antioxidants. Furthermore, in cultured H9c2 cardiac cells silencing Nrf2 gene with its siRNA abolished the SFN's prevention of high glucose-induced fibrotic response. These results suggest that diabetes-induced cardiomyopathy can be prevented by SFN, which was associated with the up-regulated Nrf2 expression and transcription function.



## 1. Introduction

Diabetic cardiovascular complications include both macrovascular and microvascular diseases. Changes in the heart independent of macrovascular disease that may exist at the same time are recognized as diabetic cardiomyopathy. Diabetic cardiomyopathy is the most common complications of diabetes and also a main cause of the mortality for diabetic patients [1]. Accumulating evidence already indicates that to prevent the development and progression of cardiomyopathy in the patients with diabetes could not be done by controlling glucose level or blood pressure, lowing lipid level, and blockading the renin–angiotensin system [1]. Therefore, an effective approach to prevent or delay the development and progression of these lethal complications for diabetic patients are urgently needed.

As three key metabolic abnormalities, hyperglycemia, hyperlipidemia, and inflammation all stimulate the generation of reactive oxygen or nitrogen species (ROS or RNS). Extra production of these species is a causative of the development of diabetic complications, including cardiomyopathy [1–3]. Accordingly, antioxidant prevention or therapy of diabetic complications has been explored, but, to date, there was no exogenous antioxidant that efficiently prevents diabetic cardiomyopathy in clinics. Therefore, the activation of tissue's endogenous antioxidant components has been proposed as an attractive strategy [4].

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The transcription factor NFE2-related factor 2 (Nrf2) as one member of the cap 'n' collar family is a master regulator of cellular detoxification responses and redox status [5]. Under physiological conditions Nrf2 locates in the cytoplasm and binds to its inhibitor kelch-like ECH-associated protein 1 (KEAP1) [6]. KEAP1 could mediate a rapid ubiquitination and subsequent degradation of Nrf2 by the proteasome [6]. Upon exposure of cells to oxidative stress or electrophilic compounds, Nrf2 is free from KEAP1 and translocates into the nucleus to bind to antioxidant-responsive elements in the promoters of gene encoding antioxidant enzymes such as NADPH quinone oxidoreductase (NQO1), heme oxygenase-1 (HO-1), glutathione S-transferase, superoxide dismutase (SOD), catalase (CAT), and γ-glutamylcysteine synthetase, to increase the expression of these antioxidants against oxidative stress and associated inflammation and damage [6,7].

Sulforaphane (SFN) is an organosulfur compound and obtained from cruciferous vegetables such as broccoli, brussels sprouts or cabbages [8]. Emerging evidence indicates the association of the increased consumption of cruciferous vegetables with a decreased risk of several degenerative and chronic diseases, including cardiovascular disease under diabetic and non-diabetic conditions. SFN has garnered particular interest as an indirect antioxidant due to its extraordinary ability to induce the expression of several enzymes via the KEAP1/Nrf2 pathway [8].

Therefore the present study investigated whether the chronic use of SFN can prevent the development of diabetic cardiomyopathy. To this end, we have used a type 1 diabetic mouse model induced with multiple low-dose streptozotocin (MLD-STZ). Diabetic and control mice were treated with SFN for 3 months and then kept for another 3 month without SFN treatment. SFN could almost completely prevent the development of diabetic cardiomyopathy along with an up-regulation of Nrf2 expression and transcription function in the heart. Furthermore, we found that the exposure of the cultured cardiac H9c2 cells to high glucose (HG) increased fibrotic effect, which could be completely prevented by pre-treatment with SFN that also significantly increased Nrf2 expression and transcription. Silencing Nrf2 expression completely abolished the prevention by SFN of HG-induced fibrotic effect, suggesting the important role of Nrf2 in the cardiac protection by SFN against HG in vitro or diabetes in vivo.

## 2. Materials and methods

### 2.1. Animals

FVB male mice, 8–10 weeks of age, were purchased from the Jackson Laboratory (Bar Harbor, Maine) and housed in the University of Louisville Research Resources Center at 22 °C with a 12-h light/dark cycle with free access to standard rodent chow and tap water. All experimental procedures for these animals were approved by the Institutional Animal Care and Use Committee of the University of Louisville, which is compliant with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

For induction of the type 1 diabetes, mice were injected intraperitoneally with MLD-STZ [Sigma-Aldich, St. Louis, MO, dissolved in 0.1 M sodium citrate (pH 4.5)] at 50 mg/kg body weight daily for 5 consecutive days while age-matched control mice were received multiple injections of the same volume of sodium citrate buffer. Five days after the last injection of STZ, mice with hyperglycemia (blood glucose levels ≥ 250 mg/dl) were defined as diabetic, as before [9]. SFN (Sigma-Aldich) was subcutaneously given at 0.5 mg/kg for five days in each week for 3 months. At the end of 3-month SFN treatment, some mice were sacrificed for experimental measurements, and the rest of these SFN-treated control and diabetic mice were kept for additional 3 months without SFN treatment and then sacrificed for experimental measurements. Dose of SFN used was based on the previous study [10]. Mice were randomly allocated into four groups (n = 6 at least per group): Control, SFN, diabetes (DM) and DM plus SFN (DM/SFN). Since SFN was dissolved in 1% dimethyl sulfoxide (DMSO) and diluted in PBS, mice serving as vehicle controls were given the same volume of PBS containing 1% DMSO.

### 2.2. Non-invasive blood pressure

Blood pressure (BP) was measured by tail-cuff manometry using a CODATM non-invasive BP monitoring system (Kent Scientific Corporation, Torrington, CT). Mice were kept in warm on the heating pad to ensure sufficient blood flow to the tail. Mice were restrained in a plastic tube restrainer. Occlusion and volume-pressure recording cuffs were placed over the tail. Each mouse was allowed to adapt to the restrainer for 5 min prior to BP measurement. The BP was measured for 10 acclimation cycles followed by 20 measurement cycles. After three days of training for the BP measurement, formal measurements for the unanesthetized BP and heart rate (HR) were collected (Table 1), as described previously [11].

### 2.3. Echocardiography

Transthoracic echocardiography (Echo) was performed for Avertin anesthetized mice at rest using a high-resolution imaging system for small animals (Vevo 770, VisualSonics, Canada), equipped with a high-frequent ultrasound probe (RMV-707B). All hair was removed from the chest using a chemical hair remover and the aquasonic clear ultrasound gel (Parker Laboratories, Fairfield, NJ) without bubble was applied to the thorax surface to optimize the visibility of the cardiac chambers. Parasternal long-axis and short-axis views were acquired. Left ventricular (LV) dimensions and wall thicknesses were determined from parasternal short axis M-mode images. The anesthetized HR was collected. Meanwhile, ejection fraction (EF), fractional shortening (FS), and LV mass were calculated by Vevo770 software (Table 2). The final data represent averaged values of 10 cardiac cycles [12].

### 2.4. Heart pathology, immunohistochemical and immunofluorescent staining

After anesthesia, hearts were isolated and fixed in 10% buffered formalin and then dehydrated in graded alcohol series, cleared with xylene, embedded in paraffin, and sectioned at 5 μm thickness. Tissue sections were dewaxed and then incubated with 1 × target retrieval solution (Dako, Carpinteria, CA) in a microwave oven for 15 min at

**Table 1**
Effect of SFN on diabetes-induced unanesthetized blood pressure change and heart rate.

| | Control | SFN | DM | DM/SFN |
|---|---|---|---|---|
| *3 M* | | | | |
| HR (beats/min) | 657 ± 35 | 644 ± 5 | 626 ± 17 | 627 ± 10 |
| Diastolic BP (mm Hg) | 76.7 ± 1.37 | 74.09 ± 2.85 | 85.83 ± 1.11 | 78.45 ± 1.22 |
| Systolic BP (mm Hg) | 105.02 ± 1.19 | 100.29 ± 2.35 | 120.59 ± 1.47 | 106.53 ± 5.63 |
| Mean BP (mm Hg) | 86.14 ± 0.53 | 82.82 ± 3.31 | 97.42 ± 1.63 | 87.81 ± 3.93 |
| *6 M* | | | | |
| HR (beats/min) | 637 ± 26 | 644 ± 9 | 626 ± 12 | 609 ± 13 |
| Diastolic BP (mmHg) | 78.68 ± 2.64 | 76.57 ± 2.57 | 92.42 ± 3.04* | 80.91 ± 1.28# |
| Systolic BP (mm Hg) | 107.45 ± 1.96 | 101.07 ± 0.81 | 125.75 ± 3.85* | 109.97 ± 3.55# |
| Mean BP (mm Hg) | 88.27 ± 2.26 | 84.73 ± 1.75 | 103.53 ± 3.13* | 90.60 ± 1.94# |

Notes: Data were presented as means ± SEM. HR = heart rate; BP = blood pressure.
* $p<0.05$ vs. control.
\# $p<0.05$ vs. DM group.