Inhibition of Uncoupling Protein 2 Attenuates Cardiac Hypertrophy Induced by Transverse Aortic Constriction in Mice

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Key Words
Uncoupling protein 2 • Hypertrophy • Pressure overload • Genipin

Abstract
Background: Uncoupling protein 2 (UCP2) is critical in regulating energy metabolism. Due to the significant change in energy metabolism of myocardium upon pressure overload, we hypothesize that UCP2 could contribute to the etiology of cardiac hypertrophy. Methods: Adult male C57BL/6J mice were subjected to pressure overload by using transverse aortic constriction (TAC), and then received genipin (a UCP2 selective inhibitor; 25 mg/kg/d, ip) or vehicle for three weeks prior to histologic assessment of myocardial hypertrophy. ATP concentration, ROS level, and myocardial apoptosis were also examined. A parallel set of experiments was also conducted in UCP2-/- mice. Results: TAC induced left ventricular hypertrophy, as reflected by increased ventricular weight/thickness and increased size of myocardial cell (vs. sham controls). ATP concentration was decreased; ROS level was increased. Apoptosis and fibrosis markers were increased. TAC increased mitochondrial UCP2 expression in the myocardium at both mRNA and protein levels. Genipin treatment attenuated cardiac hypertrophy and the histologic/biochemical changes described above. Hypertrophy and associated changes induced by TAC in UCP2-/- mice were much less pronounced than in WT mice. Conclusions: Blocking UCP2 expression attenuates cardiac hypertrophy induced by pressure overload.

Introduction
Ventricular hypertrophy is a compensatory process in response to pressure overload, but could be detrimental in instances [1]. As hypertrophy progresses, energy demand increases. When blood supply does not meet the increasing demand, heart failure occurs. The development and progression of cardiac hypertrophy is associated with changes in the
expression of multiple structural, signaling and metabolic genes [2]. Metabolism changes upon cardiac hypertrophy include substrate oxidative preference and mitochondrial function [3-5]. In particular, dysfunction of complexes I and II of the electron transport chain (ETC) in the mitochondria occurs in early hypertrophy and worsens upon decompensation [6].

Uncoupling proteins (UCPs) in the inner mitochondrial membrane allow protons to flow across mitochondrial membrane, and by doing so, generate heat instead of producing ATP [7]. UCPs have been implicated in the pathogenesis of a variety of diseases with energy metabolism dysfunction, such as type 2 diabetes mellitus, fatty liver disease and degenerative diseases [8-13]. The UCP family includes five UCP homologs. UCP1 is mainly from brown adipose tissue. UCP2 is widely expressed in many tissues. UCP3 is expressed in brown adipose tissue, heart and skeletal muscle. UCP4 and BMCP1 are present only in brain. In mice, UCP2 is expressed in the heart and skeletal muscle at the mRNA but not the protein level [14].

Since energy metabolism dysfunction in myocardium is a core feature upon pressure overload and subsequent heart failure, we examined UCP2 expression in mice subjected to pressure overload (transverse aortic constriction; TAC). Functional role of UCP2 was examined using a selective UCP2 antagonist genipin. A set of experiments was also conducted in UCP2-/- mice to verify the findings with genipin.

Materials and Methods

Animals
Male C57BL/6j mice were from Nanjing University (Nanjing, China). Homozygous UCP2-knockout (UCP2−/−, generated using C57BL/6j mice) mice were generously provided by Professor Chenyu Zhang of Nanjing University [15]. Mice were housed under a 12-hour dark/light cycle, with unlimited access to food and water. All experiments were approved by the IACUC Committee of Nanjing University.

General experimental design
Transverse aortic constriction (TAC) was carried out in adult male C57BL/6j mice. Starting from the first day after the surgery, mice received genipin (a UCP2 selective inhibitor; 25 mg/kg/d, ip) or vehicle for three weeks prior to histologic assessment of myocardial hypertrophy. ATP concentration, ROS level, and myocardial apoptosis in the left ventricle were also examined. A separate set of experiments was also conducted in UCP2−/− mice.

Surgical procedures and treatment protocols
Cardiac hypertrophy was established using TAC, as previously reported [16]. Briefly, mice were anaesthetized with pentobarbital sodium (0.077g/Kg), intubated, and ventilated with a small animal respirator (Harvard Rodent Ventilator, Model 683, Natick, MA). A left thoracotomy was performed. The transverse aorta was constricted to the size of a 27 gauge needle.

Hemodynamic examination
Heart rate and blood pressure (BP) were measured in conscious mice using a tail-cuff system (BP-98A; Sftron, Tokyo, Japan). Transthoracic echocardiography was performed using a Vevo 770 UBM system containing a single-element mechanical transducer with a centre frequency of 30 MHz and a frame rate of 30 Hz (VisualSonics, Toronto, ON, Canada). The spatial resolution of B-mode imaging was approximately 115 mm (lateral) by approximately 55 mm (axial). The body temperature of mice was monitored using a rectal thermometer and was maintained between 36°C and 38°C, while the heart rate was maintained between 350 and 450 beats/min. Cardiac output, ejection fraction, fractional shortening and left ventricular internal diastolic diameter were calculated.

Histologic analysis
Hypertrophy of the left ventricle was examined using routine HE staining. The extent of fibrosis was estimated using Masson staining and immunochemical assay of collagen I, as described previously [17]. The
size of the myocardial cells (10 cells, randomly selected) was estimated using immunofluorescence staining with an anti-wheat germ agglutinin antibody. Images were captured and recorded using Photoshop version 5.0 (Adobe, San Jose, CA).

**Measurements of ROS, Apoptosis and ATP**

ROS levels (total reactive oxygen species and reactive nitrogen species) in left ventricle were determined with the OxiSelect In Vitro ROS/RNS assay kit (Cell Biolabs). Apoptosis was detected using terminal deoxynucleotidyltransferase–mediated DUTP nick-end labeling staining (Promega). Adenosine triphosphate (ATP) was measured using a kit from Beyotime.

**mRNA of UCP2**

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized using random primers with the Reverse Transcription System (Promega, Madison, WI, USA). The reaction lasted for 30 min at 42°C. Following first strand cDNA synthesis, polymerase chain reaction (PCR) was performed on 1μl aliquots of cDNA using a standard PCR kit, HotStarTaq Polymerase (Qiagen, Valencia, CA), and primer pairs specific for mouse UCP2 and GAPDH. The primer sequences are as follows:

- UCP2: 5′-CTCAGAAAGGTGCCTCCCGA-3′ (sense)
  3′-ATCGCCTCCCCGTGATGTGCTCA-5′ (anti-sense)
- GAPDH: 5′-GGTGAAGGTCGGTGTGAACG-3′ (sense)
  3′-CTCGCCTCTGGAAGATGGTG-5′ (anti-sense)

PCR amplification consisted of 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, followed by a final extension step at 72°C for 10 min. PCR products were size fractionated on agarose gels and detected by NA-green staining (D0133, Beyotime).

**UCP2 protein in mitochondria**

Mitochondria were isolated from the ventricular tissues, as described previously [18]. Fresh ventricular tissues were minced in ice-cold STE buffer (250 mM sucrose, 5 mMTris, 2 mM EGTA, pH 7.4 at 4°C) and disrupted in a Dounce homogenizer. Cell debris was removed by centrifugation of the homogenate at 500 g for 3 minutes. The supernatant was centrifuged at 10,000 g for 8 minutes, and the mitochondrial pellet was resuspended in ice-cold STE buffer. Mitochondria were subjected to another cycle of centrifugation at 750 and 10,000 g. Samples were subjected to SDS-PAGE (50 µg protein per sample). UCP2 was detected by Western blot analysis of using anti-UCP2 antibody (ab77363; Abcam, Cambridge, MA; 1:500 dilution). Data were normalized against the internal cytochrome c control, as detected using cytochrome c antibody (sc-13561; Santa Cruz Biotechnology, Dallas, TX, US; 1:1000).

**Statistical analysis**

Data are presented as means ± SD, and analyzed using one-way analysis of variance (ANOVA), followed by the Dunnett’s t-test. Statistically significance was set at P < 0.05. All analyses were performed using the Sigma Stat software (Jandel Scientific Software, San Jose, CA). The mortality rate was analyzed using the Fisher Exact method.

**Results**

**Effects of TAC in wild type mice**

TAC increased the ventricle/body weight ratio (Fig. 8B), the size of myocardiocytes (Fig. 8C/D), TAC increased IVS and LVPW (Fig. 4C/D), and resulted in interstitial fibrosis (Fig. 8E). ATP concentration was decreased; ROS level was increased; apoptosis was increased (Fig. 11A-D).

**UCP2 in ventricular myocardium is increased upon pressure overload**

In wild-type mice, UCP2 protein was detected by Western blot in the spleen and lungs, but not in the heart (Fig. 1A). TAC increased UCP2 mRNA in the heart (Fig. 1B). UCP2 protein became detectable with Western blot (Fig. 1C/D), as well as immunohistochemical staining (Fig. 1E) in the heart at 3 weeks after TAC. UCP2 protein was not detected in any organs in the UCP2-/- mice.
Genipin attenuates TAC-induced cardiac hypertrophy

Treatment of TAC mice with genipin decreased myocardiocyte hypertrophy and cell size (Fig. 3A-D), attenuated myocardial interstitial fibrosis (Fig. 3E), but did not affect MBP (Fig. 2). Neither TAC nor genipin treatment significantly affected EF and FS (Fig. 4A/B). TAC increased IVS and LVPW (Fig. 4C/D). Genipin also prevented TAC-induced increase in β-MHC mRNA (Fig. 5). Genipin produced a decreasing trend (not statistically significant) in myocardiocyte size, IVS and LVPW in TAC mice.
Genipin increased mitochondrial ATP concentration and inhibited myocardial apoptosis

TAC reduced ATP concentration in both WT and UCP2-/- mice (Fig. 6A/B). Genipin attenuated the effect of TAC on ATP concentration (Fig. 6A), and the effect of TAC on cell apoptosis. Genipin treatment did not alter the effect of TAC on ROS level in wild-type mice.

UCP2 KO attenuates cardiac hypertrophy induced by pressure overload

BP increased significantly after TAC in both WT and UCP2-/- mice, with no difference between the two genotypes (Fig. 7A). Neither WT nor UCP2-/- mice exhibited any abnormalities in cardiac structure or function (Table 1). The survival rate in mice following TAC is higher in the UCP2-/- than in the wild-type control mice for the duration of observation (Fig. 7B).

At 3 weeks after TAC, cardiac hypertrophy was less pronounced in UCP2-/- mice than in the WT mice (Fig. 8A/B). Increased myocardiocyte size upon TAC was also less pronounced in UCP2-/- mice (Fig. 8C/D). Masson's and collagen I staining revealed much less cardiac fibrosis in UCP2-/- mice after TAC (Fig. 8E).

TAC did not affect EF and FS in either UCP2-/- or the WT mice (Fig. 9A/B). Also, genipin treatment did not affect EF and FS. TAC increased both IVS and LVPW, but to a greater extent in the WT mice than in the UCP2-/- mice as reflected by transcardiography. BNP and β-MHC mRNA levels were significantly lower after TAC in UCP2-/- mice than in the WT control (Fig. 10A/C).

Fig. 3. Blocking of UCP2 with genipin attenuates load-induced cardiac hypertrophy in mice. (A-B) Genipin attenuates cardiac hypertrophy caused by TAC. Histological heart sections and heart and body weight ratio are shown. (C) Cross-sections stained with FITC-conjugated wheat germ agglutinin. (D) Quantitation of cross-areas of the cardiomyocytes. (E) Representative hematoxylin and eosin staining or Masson staining or collagen I staining microphages showing heart histology in WT and genipin-treated mice (400× magnification). The data are presented as means ± SD. *P<0.05 vs. the sham control; n=8. #P<0.05 vs. TAC mice receiving vehicle n=8.
Fig. 4. Effects of genipin on cardiac structure and function, as measured by echocardiography. (A-B) Ejection fraction (EF) and fractional shortening (FS) at 3 weeks after vehicle and genipin treatment. *P<0.05 vs. the sham control; n=8. #P<0.05 vs. TAC mice receiving vehicle; n=8. (C-D) Interventricular septum (IVS) and left ventricular posterior wall (LVPW) at 3 weeks after vehicle and genipin treatment. *P<0.05 vs. the sham control; n=8.

Fig. 5. Effects of genipin on the expression levels of cardiac hypertrophic marker genes. (A-C) RT-PCR analysis of ANF, BNP and β-MHC mRNA at 3 weeks after vehicle and genipin treatment. *P<0.05 vs. the sham; n=8. #P<0.05 vs. mice receiving vehicle n=8.

Fig. 6. Effects of genipin on ATP, ROS, TUNEL+ cells. *P<0.05 vs. the sham control; n=8. #P<0.05 vs. mice receiving vehicle n=6.
UCP2 KO increases mitochondrial ATP production and inhibits myocardial apoptosis

TAC reduced ATP, but to a less extent in UCP2-/− mice than in the WT control mice (Fig. 11A/B). The increase of ROS after TAC did not differ between the UCP2-/− and WT control mice. Apoptosis induced by TAC was less prominent in UCP2-/− mice than in the WT control mice.

Discussion

The current study showed: 1) UCP2 is induced after TAC in mice; 2) UCP2 KO attenuates TAC-induced cardiac hypertrophy, without affecting BP. The cardioprotection of UCP2
knockout may be explained by elevated ATP and decreased apoptosis of myocardiocytes. We also showed that the effects of the UCP2 inhibitor genipin are similar to the UCP2 knockout. BP did not differ after TAC between UCP2-/- and WT mice, suggesting that BP is not implicated in the cardioprotective effects of UCP2 knockout. A recent study showed that UCP2 deficiency mimics the effects of hypoxia and triggers pseudo-hypoxic pulmonary vascular remodeling and pulmonary hypertension [19]. In this study, we noticed a mouse

Table 1. Baseline characteristics of WT and UCP2-/- Mice. Data are mean ± SD. MBP, mean blood pressure; EF, ejection fraction; FS, fractional shortening; IVS, interventricular septum; LVID, left ventricle inner dimension; LVPW, left ventricular posterior wall. LV vol, left ventricle volume. Mice were measured in conscious state.

<table>
<thead>
<tr>
<th></th>
<th>WT (n=8)</th>
<th>UCP2-/- (n=6)</th>
</tr>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>24.6±3.5</td>
<td>23.7±4.7</td>
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<td>LV weight/body weight, mg/g</td>
<td>2.97±0.50</td>
<td>3.05±0.61</td>
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<td>Heart rate, bpm</td>
<td>525±75</td>
<td>486±90</td>
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<tr>
<td>Systolic BP, mmHg</td>
<td>104.1±6.42</td>
<td>116±10.26</td>
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<tr>
<td>Diastolic BP, mmHg</td>
<td>74.25±4.83</td>
<td>78.5±7.39</td>
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<tr>
<td>Mean BP, mmHg</td>
<td>84.2±7.62</td>
<td>91.7±6.0</td>
</tr>
<tr>
<td>EF, %</td>
<td>66.49±4.41</td>
<td>73.97±8.72</td>
</tr>
<tr>
<td>FS, %</td>
<td>36.36±3.05</td>
<td>42.6±8.64</td>
</tr>
<tr>
<td>IVS;d mm</td>
<td>0.67±0.04</td>
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<td>IVS;s mm</td>
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<td>0.7±0.05</td>
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<td>1.19±0.15</td>
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<tr>
<td>LV vol;s ul</td>
<td>21.53±5.76</td>
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Fig. 9. Effects of TAC on cardiac function and structure by echocardiography. (A-B) Ejection fraction (EF) and fractional shortening (FS) at 3 weeks after s TAC. *P<0.05 vs. the sham control; n=6. #P<0.05 vs. TAC alone; n=6. (C-D) Interventricular septum (IVS) and left ventricular posterior wall (LVPW) at 3 weeks after TAC. *P<0.05 vs. the sham control; n=6. #P<0.05 vs. TAC alone; n=6.
with mild right ventricular hypertrophy in UCP2-/- mice by transthoracic echocardiography. UCP2 is part of the antioxidant defense against endothelial dysfunction in hypertension [20]. However, in this study, we did not observe any abnormalities in cardiac function in the UCP2-/- mice (Table 1).

Studies of UCP2 mRNA expression in the development of cardiac hypertrophy yielded contradicting results. A previous study in rats reported reduced UCP2 and UCP3 mRNAs at 1 week after ascending aortic constriction [21]. In another rat study, UCP2 mRNA was increased significantly by aortic regurgitation [22]. A human study failed to shown significant change in UCP2 mRNA in heart failure caused by either ischemic or non-ischemic cardiomyopathy [23]. In the current study, we showed increased UCP2 mRNA and protein following TAC in WT mice. The discrepancies could apparently be attributed to differences in subject species and the causes of cardiac hypertrophy.

In a previous study from this laboratory [24], we found that UCP2 knockout could alleviate in a Unilateral ureteral obstruction (UUO) fibrosis model of the kidneys. The heart consumes more oxygen than the kidney, and primarily depends on ATP as the immediate energy carrier. In vitro studies indicated that UCP2 overexpression could reduce mitochondrial ROS, and
prevent mitochondrial Ca\(^{2+}\) overload in myocardiocytes [25]. This represents an undesirable aspect of the UCP2 knockout. A potential advantage of blocking of UCP2 is the enhanced coupling between citric acid cycle flux and mitochondrial ATP synthesis, which in turn could provide critically important benefits to myocardiocytes upon energetic stress. Attenuation of ATP decrease after TAC by UCP2 knockout, as shown in the current study, apparently could result in cardioprotection. In contrast to the connection between cell apoptosis and ROS [26, 27], we showed increased ROS, but not increased apoptosis after TAC, possibly due to enhanced oxygen utilization after UCP2 knockout [28]. Exact mechanisms underlying such discrepancy, however, needs further studies.

In summary, we demonstrated that UCP2 is a component of the morphological, functional, and cellular-level responses of the heart to pressure overload using both genetic and pharmacological tools. The findings also encourage further research to examine whether UCP2 could be developed as a potential target in the treatment of cardiac hypertrophy and heart failure. Whether UCP2 plays a role in cardiac hypertrophy caused by pathological factors other than pressure overload, such as neural–hormonal stimuli, remains unknown.

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**Disclosure Statement**

The authors have declared that no competing interests exist.

**References**


