Knockdown of Cytochrome P450 2E1 Inhibits Oxidative Stress and Apoptosis in the cTnTR141W Dilated Cardiomyopathy Transgenic Mice

Dan Lu, Yuanwu Ma, Wei Zhang, Dan Bao, Wei Dong, Hong Lian, Lan Huang, Lianfeng Zhang

Abstract—Cytochrome P450 2E1 (CYP2E1) is a cytochrome P450 enzyme that catalyzes the metabolism of toxic substrates. CYP2E1 is upregulated in heart disease, including the dilated cardiomyopathy (DCM) mouse model. Here, knockdown of CYP2E1 significantly ameliorated the dilated left ventricle, thin wall, and dysfunctional contraction in the cTnTR141W and adriamycin-induced DCM mouse models. Interstitial fibrosis, poorly organized myofibrils, and swollen mitochondria with loss of cristae were improved in the myocardium of α-myosin heavy chain (MHC)-cTnTR141W×CYP2E1-silence double-transgenic mice when compared with the cTnTR141W transgenic mice. Oxidative stress, the activation of caspase 3 and caspase 9, the release of cytochrome c, and the apoptosis in the myocardium were significantly decreased in double-transgenic mice compared with the cTnTR141W transgenic mice. In summary, the expression of CYP2E1 is upregulated in heart disease and might be induced by hypoxemia in cardiomyopathy. The overexpression of CYP2E1 can enhance the metabolism of endogenous ketones to meet the energy demand of the heart in certain disease states, but the overexpression of CYP2E1 can also increase oxidative stress and apoptosis in the DCM heart. Knockdown or downregulation of CYP2E1 might be a therapeutic strategy to control the development of DCM after mutations of cTnTR141W or other factors, because DCM is the third most common cause of heart failure and the most frequent cause of heart transplantation. (Hypertension. 2012;60:81-89.) ● Online Data Supplement

Key Words: CYP2E1 ■ silence ■ dilated cardiomyopathy ■ oxidative stress ■ apoptosis

The cytochrome P450 enzymes (CYPs) belong to a family of heme proteins that catalyze the metabolism of a vast array of endogenous and exogenous substrates.1–3 Furthermore, CYPs are functionally associated with some pathophysiological states, including cardiovascular injury, tumorigenesis, neuronal dysfunction, and diabetes mellitus.4–5 Cytochrome P450 2E1 (CYP2E1) is mainly located in the endoplasmic reticulum and plays a critical role in the metabolism and activation of xenobiotics.6–9

In the presence or even the absence of substrates, CYP2E1, with O2 and NADPH, can produce reactive oxygen species (ROS), which leads to oxidative stress and apoptosis. CYP2E1 is among the most active CYPs in producing ROS.9 Overexpression of CYP2E1 is of direct importance to human health and is associated with a range of diseases, including diabetes mellitus, alcoholic liver disease, and cancer.10–12 The expression level of CYP2E1 increases significantly in heart tissues in humans under ischemia; mice, rats, and dogs with dilated cardiomyopathy (DCM); and spontaneously hypertensive rats.13–18 The overexpression of CYP2E1 is associated with several cellular markers of oxidative stress, as well as with decreased viability as a result of both necrosis and apoptosis.19–22 Oxidative stress and apoptosis in myocytes play important roles in the pathogenesis of cardiovascular diseases, such as ischemic heart disease, atherosclerosis, cardiomyopathy, and heart failure.23–28

We hypothesized that CYP2E1 is an important gene in the pathogenesis of DCM and that knockdown of CYP2E1 would inhibit oxidative stress and apoptosis of myocytes, thereby preventing the development of DCM. Therefore, in the present study, we generated a heart-specific CYP2E1-knockdown transgenic mouse to reduce the expression of CYP2E1 in heart tissue of cTnTR141W transgenic mice, a model of DCM, to determine whether and by what mechanism this would inhibit oxidative stress and apoptosis and prevent the development of DCM.
Methods

Animals
Five small interfering RNAs (siRNAs) against the CYP2E1 gene were designed using Invitrogen’s RNAi Designer (www.invitrogen.com/rnai) and were inserted into the pcDNA™6.2-GW/EmGFP-miR vector (Invitrogen, BLOCK-iT Pol II miR RNAi Expression Vector Kit). The knockdown effects on CYP2E1 were screened in 293T cells. Two siRNAs against CYP2E1, 5′ GGGGATA- TTCTCTTCAACAAA (siRNA1) and 5′ CCATCTGGAAACATC- TATA (siRNA2), knocked down the target gene with a high efficiency and showed synergic effects on the knockdown of CYP2E1 in 293T cells (Figure S1A and S1B, available in the online-only Data Supplement). Loops for siRNA1 and siRNA2 were cloned in series into an expression plasmid under the α-MHC promoter. The heart-specific CYP2E1-knockdown transgenic mouse was generated by microinjection26 and was genotyped by the PCR with 2 primers (5′ GGATGGAGCAGCTGTACAA and 5′ CTCTAGATCAACACTTGTG). For genotyping, a 416-bp fragment of the siRNA cassette was amplified with 35 PCR cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The inhibition of CYP2E1 expression was screened by Western blot analysis using a rabbit antimiRNA polyclonal antibody (Abcam). The α-MHC-CtnT141W (referred to as CtnT141W) DCM transgenic mice were generated previously in our laboratory.29 The α-MHC- cTnT141W×CYP2E1-silence double-transgenic (DTG) mice were generated through mating of the α-MHC-CYP2E1-silence (referred to as CYP2E1-silence) with the cTnT141W transgenic mice. The α-MHC-LmnaE82K (referred to as LmnaE82K) DCM transgenic mice were generated previously in our laboratory.30 All of the mice used in this study were housed in a C57BL/6j genetic background, and all were bred in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility. The use of animals was approved by the animal care and use committees of the Institute of Laboratory Animal Science of Peking Union Medical College (GC08-2001).

Echocardiography
M-mode echocardiography was performed at 5 months of age on each transgenic mouse with the small animal echocardiography analysis system (Vevo770), as described previously.29,30

Adriamycin Treatment
The CYP2E1-silence mice and nontransgenic (NTG) littermates at 2 months of age were used for adriamycin (ADR) treatment. In the groups of NTG+ADR and CYP2E1-silence +ADR, ADR was induced by intraperitoneal injection in a constant volume of saline at 4 mg/kg every other day for a total of 2 weeks.31 The groups of NTG+saline and CYP2E1-silence+saline mice received the same amount of saline. Echocardiography was performed on all of the mice at day 0 (the day before the treatment of ADR). All of the surviving mice were chosen for follow-up echocardiographic analysis at 3 months of age (2 weeks after cessation of ADR treatment) and were used to analyze the expression of CYP2E1 in heart tissues.

Measurement of CYP2E1 Activity
CYP2E1 activity was determined by the rate of oxidation of p-nitrophenol (PNP) to p-nitrophenol in the presence of NADPH and O2, as described previously.16 The activity was determined using the following equation: PNP activity (in nanomoles per minute per milligram of protein)=D492/9.53/0.26/0/7.1 × 10⁻⁴16,16

Histological Analysis
For light microscopy, cardiac tissue from mice at 5 months of age was fixed in 4% formaldehyde and mounted in paraffin blocks, and sections were stained with hematoxylin-eosin or Masson trichrome, as described previously.30 For transmission electron microscopy, cardiac tissue was prepared, as described previously.16,30 Myocytes were analyzed by an observer blinded to the genotypes of the mice.

RNA Extraction, Quantification, and RT-PCR
Total RNA was isolated from the heart tissues using TRIZol Reagent (Invitrogen). First-strand cDNA was synthesized from 2 μg of total RNA using random hexamer primers according to the Superscript III reverse transcriptase manufacturer’s protocol (Invitrogen). Procollagen type III α1 (Col3α1) mRNA was detected by RT-PCR using GADPH for normalization under standard conditions (primers: for Col3α1, forward 5′ CTCAGAGCG-GAGAATCTGG and reverse 5′ CAATGTCATAGGGTGTCG-GATA; for GAPDH, forward 5′ CAGGGTCATCCATGA- CAACTTTG and reverse 5′ GTCACACCGCTTTGCTTGTA). levels in Heart Tissue
Heart tissue from mice at 5 months of age was homogenized rapidly in buffer (0.15 mol/L of KCl and 1.0 mmol/L EDTA) to obtain 1:10 (wt/vol) homogenates, then centrifuged at 13 000g (4°C) for 30 minutes, and the supernatant was collected for assays. The protein concentration was determined by the Bradford method using BSA as a standard.32 Malondialdehyde (MDA) was measured by the thiobarbituric acid-reactive substances method.33 H2O2 was measured using hydrogen peroxide assay kit (R&D Systems). Glutathione (GSH) was measured using the GSH-400 colorimetric assay kit (Promega). Total antioxidant capacity (T-AOC) was measured using a total antioxidant capacity assay kit (Abcam).

Protein Extraction and Immunoblotting
Total protein lysates from mouse heart tissues were prepared as described previously.30 After performing SDS-PAGE and transferring to nitrocellulose (Millipore), the membranes were incubated overnight with antibody to CYP2E1 (Abcam), caspase 3 (Cell Signaling), or caspase 9 (Cell Signaling). After incubation with the appropriate secondary antibody for 1 hour at room temperature, antibody binding was detected with a horseradish peroxidase–conjugated immunoglobulin G (Santa Cruz) using a chemiluminescence detection system (Santa Cruz). GAPDH was used for normalization.

Detection of Mitochondrial Cytochrome C Release
The whole mouse heart was excised and washed in cold PBS, and the cytosolic and mitochondrial fractions were isolated following the mitochondrial/Cytosol Fractionation kit manufacturer’s protocol (DBI Biosciences). The cytochrome c content in cytosol and mitochondria was detected by Western blot analysis using antibody to cytochrome c (Cell Signaling).

TUNEL Assay
TUNEL assay was performed in sections using an In Situ Apoptosis Kit (Millipore), as described previously.30 Six images per heart (3 hearts per genotype group) were acquired, and positive cells were counted individually. The results are expressed as the percentage of apoptotic cells among the total cell population.

Statistical Analysis
Data were analyzed with 1-way ANOVA for multiple groups followed by a Tukey post hoc analysis. Data are expressed as means±SEM from individual experiments. Differences were considered significant at P<0.05.

Results

Generation of α-MHC-CYP2E1-Silence Transgenic Mice and Detection of CYP2E1 Expression
The expression of CYP2E1 is strongly upregulated in the cTnT141W DCM mice model,16 and we found that CYP2E1 was also increased significantly in ADR-induced DCM mice model and LmnaE82K DCM mice (Figure 1A and 1B; n=3; P<0.05; P<0.01). This finding suggests that the increased...
expression of CYP2E1 could be a common event in the development of DCM. The \( \alpha \)-MHC-CYP2E1-silence transgenic mice, with significantly decreased expression of CYP2E1, was detected by RT-PCR (Figure 1C), and the CYP2E1-silence transgenic mice were indistinguishable from their NTG littermates at birth and in youth. The \( \alpha \)-MHC-C\( \text{TnTR}_{141W} \)CYP2E1-silence DTG mice were established to study the effect of CYP2E1 on the DCM model. The increased expression of CYP2E1 in the \( \text{TnTR}_{141W} \) transgenic mice was knocked down significantly by the heart-specific transgenic expression of the siRNAs against CYP2E1 at 1, 3, and 5 months of age (Figure 1D and 1E; \( n=3; P<0.05 \) DTG mice versus c\( \text{TnTR}_{141W} \) mice). In addition, this expression pattern of CYP2E1 in the transgenic mice was further confirmed by immunohistochemistry (Figure S1C). The increased enzyme activity of CYP2E1 was also knocked down significantly in the DTG mice, accompanied by the decrease of the CYP2E1 protein level at 5 months of age (Figure 1F; \( n=3; P<0.05 \) DTG mice versus c\( \text{TnTR}_{141W} \) mice).

Knockdown of CYP2E1 Improves Cardiac Geometry and Dysfunction in Transgenic and Induced DCM Mice Model

The \( \text{TnTR}_{141W} \) transgenic mouse showed typical DCM phenotypes with dilated chambers, thin walls, and cardiac dysfunction.\(^{16,29} \) However, the DCM phenotypes were significantly improved when the increased expression of CYP2E1 in \( \text{TnTR}_{141W} \) transgenic mice was knocked down (Table 1). The amelioration was demonstrated by the 11.2% reduced left ventricular diameter at end systole (\( P<0.01 \), DTG mice versus c\( \text{TnTR}_{141W} \) mice), 40.1% increased left ventricular posterior wall thickness at end systole (\( P<0.001 \), DTG mice versus c\( \text{TnTR}_{141W} \) mice), and 26.3% increased left ventricular percent fractional shortening (\( P<0.05 \), DTG mice versus c\( \text{TnTR}_{141W} \) mice). Three lines of CYP2E1-silence transgenic mice, which were selected from among 18 founders, presented the similar phenotypes as thick-walled ventricles compared with the NTG mice (Table 1, founder 11); left ventricular posterior wall thickness increased 19.8% (\( P<0.001 \)), and left ventricular anterior wall thickness at end systole increased 19.6% (\( P<0.001 \)), producing an opposite phenotype to CYP2E1-overexpressing transgenic mice.\(^{16} \) Please see the online-only Data Supplement regarding the echocardiographic characteristics of the other 2 lines from CYP2E1-silence mice.

The ADR-treated mouse is a typical DCM model. The decreases of LVAWD and left ventricular anterior wall thickness at end systole induced with ADR treatment were reversed by 21.3% (\( P<0.05 \)) and 34.1% (\( P<0.01 \)), and the...
CYP2E1 Knockdown Ameliorates Cardiac Microstructure and Ultrastructure in cTnTR141W Transgenic Mice

Morphological changes of ventricular and myocytes were further observed by histological examination, as demonstrated using hematoxylin-eosin and Masson trichrome staining (Figure 2A through 2C). Knockdown of CYP2E1 in the DTG mice significantly reduced malalignment and collagen accumulation in the interstitial space compared with the typical histological changes in the heart tissues of the cTnTR141W transgenic mice. The fibrosis of the heart tissue was further analyzed by RT-PCR (Figure 2D and 2E), which indicated that the expression of Col3a1 mRNA was increased by 193.3% in the cTnTR141W transgenic mice (n=3; P<0.001 versus NTG mice), an increase that was reduced by 38.2% by CYP2E1 knockdown in the DTG mice (n=3; P<0.01 versus cTnTR141W mice).

Disrupted ultrastructures, including swollen mitochondria with a loss of cristae and vacuolization, poorly organized myofibrils with diffusion, damage, and lysis, were seen in the cTnTR141W transgenic mice compared with the NTG heart. In contrast, both the prominence of swollen mitochondria and poorly organized myofibrils were clearly lessened by the knockdown of CYP2E1 in the DTG mice compared with that of the cTnTR141W transgenic mice. (Figure 3). The Z-line has a fuzzy appearance in the CYP2E1-silence mice compared with the NTG mice, and intercalated disks were further observed; however, no obviously abnormality was found about the intercalated disks in the CYP2E1-silence mice compared with the NTG mice (Figure S2), and we have not found any other obvious changes in the ultrastructure in the CYP2E1-silence transgenic mice through transmission electron microscopy observation.

Table 1. Echocardiographic Characteristics of Mice at 5 Mo of Age

<table>
<thead>
<tr>
<th>Group</th>
<th>NTG</th>
<th>CYP2E1-Silence</th>
<th>cTnTR141W</th>
<th>DTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>31</td>
<td>18</td>
<td>23</td>
<td>15</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.65±0.29</td>
<td>3.61±0.21</td>
<td>4.05±0.29‡</td>
<td>3.90±0.25</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.47±0.30</td>
<td>2.35±0.32</td>
<td>3.15±0.29‡</td>
<td>2.80±0.36‡</td>
</tr>
<tr>
<td>LVPWD, mm</td>
<td>0.63±0.12</td>
<td>0.73±0.13†</td>
<td>0.60±0.14</td>
<td>0.79±0.12‡</td>
</tr>
<tr>
<td>LVPWS, mm</td>
<td>0.86±0.11</td>
<td>1.03±0.20‡</td>
<td>0.73±0.17†</td>
<td>1.03±0.15§</td>
</tr>
<tr>
<td>LVAWD, mm</td>
<td>0.76±0.08</td>
<td>0.87±0.09‡</td>
<td>0.67±0.12</td>
<td>0.93±0.12‡</td>
</tr>
<tr>
<td>LVAWS, mm</td>
<td>0.97±0.12</td>
<td>1.16±0.14‡</td>
<td>0.81±0.14</td>
<td>1.20±0.13‡</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>61.08±7.11</td>
<td>63.98±11.64</td>
<td>44.99±7.58§</td>
<td>54.04±12.63§</td>
</tr>
<tr>
<td>LVFS, %</td>
<td>32.32±4.84</td>
<td>34.85±8.83</td>
<td>22.23±4.55‡</td>
<td>28.08±8.42§</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>440.81±87.81</td>
<td>406.75±80.59</td>
<td>396.85±55.01</td>
<td>422.6±41.95</td>
</tr>
</tbody>
</table>

LVEDD indicates left ventricle (LV) end-diastole diameter; LVESD, LV end-systole diameter; LVPWD, LV posterior wall at end diastole; LVPWS, LV posterior wall at end systole; LVAWD, LV anterior wall at end diastole; LVAWS, LV anterior wall at end systole; LVEF, LV ejection fraction; LVFS, LV fractional shortening; HR, heart rate; NTG, nontransgenic; CYP2E1, cytochrome P450 2E1.

*P<0.05 vs NTG mice.
†P<0.01 vs NTG mice.
‡P<0.001 vs NTG mice.
§P<0.05 vs cTnTR141W mice.
¶P<0.01 vs cTnTR141W mice.
##P<0.001 vs NTG mice.

Table 2. Echocardiographic Characteristics of Mice 2 Wk After Cessation of ADR Treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>NTG+Saline</th>
<th>NTG+ADR</th>
<th>CYP2E1-Silence + Saline</th>
<th>CYP2E1-Silence + ADR</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>12</td>
<td>8</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.41±0.24</td>
<td>3.54±0.15</td>
<td>3.23±0.15</td>
<td>3.30±0.59</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.22±0.19</td>
<td>2.49±0.20</td>
<td>2.02±0.21</td>
<td>2.22±0.36</td>
</tr>
<tr>
<td>LVPWD, mm</td>
<td>0.61±0.08</td>
<td>0.48±0.05‡</td>
<td>0.63±0.06</td>
<td>0.58±0.07§</td>
</tr>
<tr>
<td>LVPWS, mm</td>
<td>0.83±0.09</td>
<td>0.69±0.00‡</td>
<td>0.85±0.11</td>
<td>0.90±0.17</td>
</tr>
<tr>
<td>LVAWD, mm</td>
<td>0.73±0.11</td>
<td>0.62±0.07*</td>
<td>0.72±0.06</td>
<td>0.76±0.08§</td>
</tr>
<tr>
<td>LVAWS, mm</td>
<td>0.97±0.10</td>
<td>0.82±0.09†</td>
<td>0.97±0.09</td>
<td>1.10±0.02</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>64.85±5.67</td>
<td>57.54±7.04*</td>
<td>68.39±7.79</td>
<td>62.45±2.01</td>
</tr>
<tr>
<td>LVFS, %</td>
<td>34.76±4.25</td>
<td>29.79±4.54*</td>
<td>37.54±5.97</td>
<td>32.73±1.70</td>
</tr>
</tbody>
</table>

LVEDD indicates left ventricle (LV) end-diastole diameter; LVESD, LV end-systole diameter; LVPWD, LV posterior wall at end diastole; LVPWS, LV posterior wall at end systole; LVAWD, LV anterior wall at end diastole; LVAWS, LV anterior wall at end systole; LVEF, LV ejection fraction; LVFS, LV fractional shortening; ADR, adriamycin; NTG, nontransgenic; CYP2E1, cytochrome P450 2E1.

*P<0.05, NTG+ADR mice vs NTG+saline mice.
†P<0.01, NTG+ADR mice vs NTG+saline mice.
‡P<0.001, NTG+ADR mice vs NTG+saline mice.
§P<0.05, CYP2E1-silence+ADR mice vs NTG+ADR mice.
||P<0.01, CYP2E1-silence+ADR mice vs NTG+ADR mice.
Knockdown of CYP2E1 Inhibits Oxidative Stress in cTnTR141W Transgenic Mice

In the presence or even the absence of substrate, CYP2E1, with $O_2$ and NADPH, can produce ROS, which cause oxidative stress. Therefore, we measured $H_2O_2$, MDA, antioxidant GSH, and T-AOC, which reflect the level of oxidative stress, in the heart tissues from the NTG, CYP2E1-silence, cTnTR141W, and DTG mice (Figure 4). $H_2O_2$ and MDA were increased by 65.1% (n=3; P<0.05) and 129.7% (n=3; P<0.05), respectively, and GSH and T-AOC were decreased by 69.8% (n=3; P<0.05) and 25.9% (n=3; P<0.05), respectively, in the heart tissues from the cTnTR141W transgenic mice compared with the NTG mice. The increased $H_2O_2$ and MDA in the cTnTR141W heart were reversed by 25.3% and 49.3% (n=3; P<0.05), respectively, and the decreased GSH and T-AOC in the cTnTR141W heart were reversed by 172.9% (n=3; P<0.01) and 30.0% (n=3; P<0.01), respectively, in the DTG heart. MDA and T-AOC in the DTG heart were reversed almost to levels in NTG mice. These results suggest that the overexpression of CYP2E1 might be the main reason for the increased ROS in the DCM heart, and reversing the overexpression of CYP2E1 could reverse most of the increased ROS in DCM mice.

Knockdown of CYP2E1 Inhibits Mitochondrial Pathways of Apoptosis in cTnTR141W Transgenic Mice

The overexpression of CYP2E1 in the heart tissues can cause mitochondrial lesions, release of cytochrome c, and activation of caspase 9 and 3 and can ultimately trigger cell death pathways in the CYP2E1-overexpressing transgenic mice. The release of cytochrome c was increased 49.8% (n=3; P<0.01), and activated caspase 9 and 3 were increased by 33.5% (n=3; P<0.05) and 62.9% (n=3; P<0.01), respectively, in the heart tissues of cTnTR141W transgenic mice compared with NTG mice (Figure 4).
5). Knockdown of CYP2E1 resulted in the inhibition of the release of cytochrome \( c \) and the activation of caspase 9 and 3 in the CYP2E1-silence mice. Knockdown of overexpressed CYP2E1 inhibited the release of cytochrome \( c \) by 15.3% (\( n = 3; P < 0.05 \)) and reduced the expression of activated of caspase 9 and 3 by 19.2% (\( n = 3; P < 0.05 \)) and 16.8% (\( n = 3; P < 0.05 \)), respectively, in the DTG mice compared with cTnTR141W transgenic mice (Figure 5). The levels of activated caspase 3 in heart tissues of NTG, CYP2E1-silence, cTnTR141W, and DTG mice were further confirmed by immunohistochemistry (Figure S1E).

The release of cytochrome \( c \) triggered the apoptosis of myocytes, as detected by TUNEL assay in heart tissues of the NTG, CYP2E1-silence, cTnTR141W, and DTG mice. Apoptotic myocytes were rare in NTG and CYP2E1-silence mice, with the apoptotic indices of 0.07% and 0.04%, respectively. Apoptotic myocytes were increased 38.7-fold, with an apoptotic index of 2.71% in the cTnTR141W transgenic mice compared with NTG mice (\( n = 3; P < 0.05 \)). The apoptosis was inhibited by 67.2% by CYP2E1 knockdown in the DTG mice compared with cTnTR141W transgenic mice (\( n = 3; P < 0.05 \)).

**Discussion**

CYP2E1 is involved in the metabolism of endogenous ketones. Conversely, acetone induces the activation of CYP2E1 and certain other P450s by stabilization of the

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**Figure 4.** Measurements of oxidative stress. Levels of \( \text{H}_2\text{O}_2 \) (A), malondialdehyde (MDA; B), glutathione (GSH; C), and total antioxidation capacity (T-AOC; D) in heart tissues were determined by colorimetric assays in the mice at 5 months of age (\( n = 3; *P < 0.05 \) cTnTR141W vs nontransgenic [NTG] mice; †\( P < 0.05 \) double-transgenic [DTG] vs cTnTR141W mice).

**Figure 5.** Measurement of cytochrome \( c \) release and activation of caspase 9 and caspase 3. A, Mitochondrial cytochrome \( c \) release and the activation of caspase 9 and caspase 3 were measured by Western blot in heart tissues. B, The quantitative analysis of cytochrome \( c \) in cytoplasm and mitochondria; dark grey box, in mito; light grey box, in cyto. C, The quantitative analysis of the activation of caspase 9 using GAPDH for normalization. □, procaspase 9; □, active caspase 9. D, The quantitative analysis of the activation of caspase 3 using GAPDH for normalization. □, procaspase 3; □, active caspase 3. n=3; *\( P < 0.05 \), †\( P < 0.01 \) cTnTR141W vs nontransgenic (NTG) mice; ‡\( P < 0.05 \), §\( P < 0.01 \) double-transgenic (DTG) vs cTnTR141W mice.
CYP2E1 protein but not by direct transcriptional regulation. CYP2E1 is also involved in the pathway leading to gluconeogenesis from ketone bodies. Ketone bodies are produced primarily within the liver and are oxidized predominantly in the heart, skeletal muscle, and brain. The serum concentration of ketone bodies is elevated in poorly controlled diabetes mellitus, heart failure, and hypoxic condition of heart diseases; therefore, in these disease states, ketone bodies can become a more significant fuel source, particularly in the context of cardiomyopathy, in which the use of both fatty acids and glucose is impaired. CYP2E1 activity has been correlated with hypoxemia in the liver, chronic pulmonary disease, and heart failure in vivo and in vitro. Thus, it is possible that hypoxemia in a state of cardiomyopathy triggers the formation of acetone, which then induces CYP2E1 to enhance the metabolism of endogenous ketones to meet the energy demand of the heart.

The expression of CYP2E1 increases significantly in heart disease, and here we found that the expression of CYP2E1 also increased in ADR-induced DCM heart and LmnaE82K DCM mice (Figure 1A and 1B). The previous reports and our results suggest that the upregulation of CYP2E1 might be an important molecular event to meet the energy demand in diseased hearts.

Other than enhancing the metabolism of endogenous ketones, CYP2E1 can also cause toxicity or cell damage through the production of toxic metabolites, oxygen radicals, and lipid peroxide. The transgenic overexpression of CYP2E1 in heart tissue increases oxidative stress and results in myocyte apoptosis. In the present article, we found that knockdown of CYP2E1 significantlyameliorated oxidative stress in the DTG mice by decreasing H2O2 and MDA and increasing GSH and T-AOC when compared with the cTnTR141W transgenic mice (Figure 4). These results indicate that CYP2E1 regulates oxidative stress in the heart, especially in a condition of cardiomyopathy. Based on clinical and animal studies, apoptosis in myocytes plays an important role in the pathogenesis of cardiovascular diseases, such as ischemic heart disease, atherosclerosis, cardiomyopathy, heart failure, and ADR-induced cardiotoxicity. We found that knockdown of the overexpressed CYP2E1 in the cTnTR141W transgenic mice and the ADR-induced mice improved the DCM phenotypes of dilated chamber, thin wall, dysfunctional myocytes, fibrosis, and poorly organized myofibrils (Figures 2 and 3 and Tables 1 and 2). Damage to mitochondria by CYP2E1 is an important mechanism underlying CYP2E1-dependent cytoxicity. The damaged mitochondria and mitochondrial-dependent apoptotic pathway are triggered in CYP2E1-overexpressing mice and cTnTR141W transgenic mice. Our results indicate that the mitochondrial damage was significantly ameliorated by CYP2E1 knockdown in the DTG mice (Figure 3). Furthermore, cytochrome c release, caspase 9 dependent caspase 3 activation, and the apoptosis of myocytes were also inhibited by CYP2E1 knockdown in DTG mice (Figures 5 and 6). The results suggest that CYP2E1 knockdown could reduce oxidative stress and apoptosis and improve the pathophysiological phenotypes of the DCM heart.

Figure 6. TUNEL assay. A. Photomicrographs of heart tissue used for TUNEL assay. Arrows indicate TUNEL-positive cells (magnification, ×400). B. The quantitative analysis of apoptotic cells in the heart tissue of mice at 5 months of age (n=3). *P<0.05 cTnTR141W vs nontransgenic (NTG) mice; †P<0.05 double-transgenic (DTG) vs cTnTR141W mice.

Perspectives
The expression of CYP2E1 is upregulated in heart disease and might be induced by hypoxemia in cardiomyopathy. The overexpressed CYP2E1 enhances the metabolism of endogenous ketones to meet the energy demand of the heart in certain disease states, but the overexpression of CYP2E1 also increases oxidative stress and apoptosis in the DCM heart. Knockdown or downregulation of CYP2E1 might be a valuable therapeutic strategy to control the development of DCM after mutations of cTnTR141W or other factors, because DCM is the third most common cause of heart failure and the most frequent cause of heart transplantation.

Sources of Funding
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Disclosures
None.
References


Novelty and Significance

What Is New?
- The expression of CYP2E1 is upregulated in DCM mice models, and it might be induced by hypoxemia in cardiomyopathy. Knockdown of CYP2E1 significantly ameliorated the DCM phenotypes and inhibited the oxidative stress and apoptosis in the myocardium of DTG mice.

What Is Relevant?
- Because DCM is the third most common cause of heart failure and the most frequent cause of heart transplantation, this study adds a new dimension to understanding the pathogenesis of DCM.

Summary
Knockdown or downregulation of CYP2E1 might be a valuable therapeutic strategy to control the development of DCM after mutations of cTnT[sup][R141W][r] or other factors.
Knockdown of Cytochrome P450 2E1 Inhibits Oxidative Stress and Apoptosis in the cTnT R141W Dilated Cardiomyopathy Transgenic Mice
Dan Lu, Yuanwu Ma, Wei Zhang, Dan Bao, Wei Dong, Hong Lian, Lan Huang and Lianfeng Zhang

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Knockdown of CYP2E1 Inhibits Oxidative Stress and Apoptosis in the cTnT^{R141W} Dilated Cardiomyopathy Transgenic Mice
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4. These authors contributed equally to this work.
Short title: CYP2E1 knockdown inhibits OS and apoptosis
Conflict of interest: The authors have declared that no conflict of interest exists.
Methods

Survival Analysis
Cumulative mortality in each group of mice was calculated every 0.5 weeks from 2 to 3 months of age. Upon the death of each mouse, the body was autopsied by a pathologist and morphological and pathological changes in the heart were recorded. Heart dilation and mural thrombi were observed upon post-mortem examination. Kaplan-Meier curves for survival analysis were compared by log-rank test (Spss 10.0 software; SPSS Inc., Chicago, IL, USA).

Immunohistochemical analysis
The sections of hearts were prepared in a standard pathological procedure. The sections were dewaxed, rehydrated, unmask the epitope, blocked, then incubated with anti-CYP2E1 (Abcam), or anti-activated caspase-3 (Cell Signaling) antibody overnight at 4°C. Sections were washed with PBS and incubated with appropriate secondary antibody for 1 h at room temperature. The slides were washed and DAB peroxidase substrate was added, covered with glass cover slips, and observed in a bright-field microscope.
<table>
<thead>
<tr>
<th>Group</th>
<th>NTG</th>
<th>CYP2E1-silence (F 9)</th>
<th>CYP2E1-silence(F 13)</th>
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<tr>
<td>Number</td>
<td>n = 31</td>
<td>n = 19</td>
<td>n = 16</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.65 ± 0.29</td>
<td>3.48 ± 0.27*</td>
<td>3.61 ± 0.36</td>
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<tr>
<td>LVESD, mm</td>
<td>2.47 ± 0.30</td>
<td>2.26 ± 0.44*</td>
<td>2.37 ± 0.29</td>
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<tr>
<td>LVPWD, mm</td>
<td>0.63 ± 0.10</td>
<td>0.69 ± 0.07†</td>
<td>0.71 ± 0.05†</td>
</tr>
<tr>
<td>LVPWS, mm</td>
<td>0.86 ± 0.11</td>
<td>0.92 ± 0.11</td>
<td>1.00 ± 0.09†</td>
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<tr>
<td>LVAWD, mm</td>
<td>0.76 ± 0.08</td>
<td>0.82 ± 0.07</td>
<td>0.85 ± 0.11*</td>
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<tr>
<td>LVAWS, mm</td>
<td>0.97 ± 0.12</td>
<td>1.06 ± 0.12</td>
<td>1.09 ± 0.14*</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>61.08 ± 7.11</td>
<td>64.57 ± 12.01</td>
<td>63.64 ± 7.83</td>
</tr>
<tr>
<td>LVFS,%</td>
<td>32.32 ± 4.84</td>
<td>35.42 ± 9.27</td>
<td>34.29 ± 6.06</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>440.81 ± 87.81</td>
<td>436.57 ± 106.69</td>
<td>433.30 ± 61.16</td>
</tr>
</tbody>
</table>

LVEDD: left ventricle (LV) end-diastole diameter; LVESD: LV end-systole diameter; LVPWD: LV posterior wall at end-diastole; LVPWS: LV posterior wall at end-systole; LVAWD: LV anterior wall at end-diastole; LVAWS: LV anterior wall at end-systole; LVEF: LV ejection fraction; LVFS: LV fractional shortening; HR: heart rate. *P < 0.05, †P < 0.01 versus NTG mice.
Figure S1. (A) The expression of CYP2E1 in 293T cells, 1: pcDNA3.1-CYP2E1; 2: pcDNA3.1-CYP2E1 + siRNA1-siRNA2 in series in pcDNA6.2-GW/EmGFP-miR vector; 3: pcDNA3.1-CYP2E1 + siRNA1 in pcDNA6.2-GW/EmGFP-miR vector; 4: pcDNA3.1-CYP2E1 + siRNA2 in pcDNA6.2-GW/EmGFP-miR vector; 5: pcDNA6.2-GW/EmGFP-miR vector. GAPDH was used for normalisation. (B) The quantitative analysis of the expression of CYP2E1 using GAPDH for normalisation (n = 3, *P<0.001 versus pcDNA3.1-CYP2E1). (C) Immunodetection of CYP2E1 in the NTG, CYP2E1-silence, cTnTR141W and DTG transgenic mice at 5 months of age. (D) Kaplan-Meier survival curves. Observation for NTG+ADR (n=8) and CYP2E1-silence+ADR (n=6) mice was recorded cumulatively every 0.5 week from 2 months to 3 months of age. (E) Immunodetection of activated caspase-3 in the NTG, CYP2E1-silence, cTnTR141W and DTG transgenic mice at 5 months of age.
Figure S2. Transmission electron micrography analysis of intercalated discs (white arrow) of left ventricular free walls from mice at 5 months of age. Scale bars = 1 μm.