Enoyl Coenzyme A Hydratase Domain–Containing 2, a Potential Novel Regulator of Myocardial Ischemia Injury

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Background—We reported previously that Brown Norway (BN) rats are more resistant to myocardial ischemia/reperfusion (I/R) injury than are Dahl S (SS) rats. To identify the unique genes differentially expressed in the hearts of these rats, we used DNA microarray analysis and observed that enoyl coenzyme A hydratase–containing domain 2 (ECHDC2) is highly expressed (≈18-fold) in the SS hearts compared with the BN hearts.

Methods and Results—RT-PCR, Western blot, and immunohistochemistry analyses verified that ECHDC2 was highly expressed in SS hearts compared with the BN hearts. ECHDC2 gene locates at chromosome 5 of rat and is expressed in mitochondria of the heart, mainly in cardiomyocytes but not in cardiofibroblasts. Overexpression of ECHDC2 in cells increased susceptibility to I/R injury while knockdown of ECHDC2 enhanced resistance to I/R injury. Furthermore, we observed that left anterior descending coronary artery ligation–induced myocardial infarction was more severe in the SS hearts than in the BN hearts or SSBN5 hearts, which was built on SS rats but had the substitution of chromosome 5 from BN rats. We also demonstrated that ECHDC2 did not alter mitochondrial O2 consumption, metabolic intermediates and ATP production. By gas chromatography–mass spectrometry, we found that ECHDC2 overexpression increased the levels of the cellular branched chain amino acids leucine and valine.

Conclusion—ECHDC2, a mitochondrial protein, may be involved in regulating cell death and myocardial injury. Its deficiency in BN rats contributes to their increased resistance to myocardial I/R compared with SS rats. ECHDC2 increases branched chain amino acid metabolism and appears to be a novel regulator linking cell metabolism with cardiovascular disease. (J Am Heart Assoc. 2013;2:e000233 doi: 10.1161/JAHA.113.000233)

Key Words: branched amino acid metabolism • cell death • ECHDC2 • ischemia/reperfusion injury • myocardial infarction

Brown Norway rats (BN) and Dahl salt-sensitive rats (SS) are unique animal models for studying cardioprotection. SS are recognized as a model for salt-sensitive hypertension study, which shares many similar phenotypic traits seen in African American hypertensive patients.1 The BN, the counterpart of the SS, are salt-resistant and have been widely used in cardiovascular studies. BN were the first rat strain to have their genome fully sequenced. Thus, studies using BN have the potential to provide insights into the genetic basis of responses to physiological and pathophysiological challenges. Among 5 inbred strains of rats (ie, BN, SS, Wistar, Lewis, and Dark Agouti rats), BN are most resistant and SS are most susceptible to myocardial ischemia/reperfusion (I/R) injury compared with other strains.2 Our previous study3 using BN and SS showed that the infarct size in BN hearts was about 63% less than in that in SS hearts when subjected to global ischemia for 25 minutes, followed by 3 hours of reperfusion. The BN also displayed an increased association of heat shock protein 90 with nitric oxide synthase 3 to produce more nitric oxide (•NO). 4 Recently, we also reported that BN showed much higher resistance to lipopolysaccharide-induced myocardial dysfunction than SS both in vivo4 and in vitro.5 However, the detailed molecular mechanisms of the differential resistance between these 2 strains remain unknown. Although the rat is a useful animal model for a lot of scientific inquiries, due to technical challenges, there are much fewer genetically modified rats than mice at present. Therefore, chromosomal substitution strains of (consomic) rats between...
SS and BN strains derived by introgressing individual chromosomes from BN into the background of SS are now available\(^6\) and are commonly used as genetic models. In this case, BN and SS may be suitable as models to dissect the complex mechanisms mediating resistance or susceptibility to myocardial ischemia, respectively. We hypothesize that identification of genetic differences between BN and SS will help elucidate mechanisms of cardioprotection against I/R injury as well as discover new therapeutic targets for treating ischemic heart disease.

Mitochondrial function and metabolism play important roles in myocardial I/R injury.\(^7\) A recent study has shown that mitochondrial proteins related to mitochondrial energy metabolism such as the \(\beta\)-subunit of ATP synthase and mitochondrial aldehyde dehydrogenase contribute to cardioprotection against ischemic injury in the study model of intermittent hypobaric hypoxia.\(^8\) In the current study, we identified that a mitochondrial protein, enoyl coenzyme A (CoA) hydratase domain–containing 2 (ECHDC2), was deficient in BN hearts but highly expressed in the SS hearts. Interestingly, ECHDC2 is highly conserved across species. Rat ECHDC2 is 90.7%, 94.9%, and 90.7% homologous with human, mouse and rabbit ECHDC2, respectively. ECHDC2 is named for its sequence similarity to enoyl CoA hydratase domain. Enoyl CoA hydratase hydrates the double bond between the second and third carbons of acyl CoA.\(^9\) It is essential for metabolizing fatty acids to produce both acetyl CoA and energy.\(^9\) However, ECHDC2 has \(<30\%\) sequence identity with enoyl CoA hydratase, and its physiological function is still unknown.

The ECHDC2 gene is located on chromosome 5 of the rat. The consomic SSBN5 are SS with their chromosome 5 replaced with chromosome 5 of the BN. Therefore, the SSBN5 could be used as ECHDC2 knockdown rats to probe for ECHDC2 functions. To exclude potential effects on gene expression from chromosomal switch, we included another strain, SSBN7 rats (BN chromosome 7 placed in SS) as a control for ECHDC2 expression. Because SS were originally bred from the Sprague Dawley rats (SD),\(^10\) and our experience with SS, BN, and SD is that the resistance to myocardial I/R injury in SD is between that of the SS and BN, we also used SD as a “normal” control to SS and BN to determine if the expression pattern of ECHDC2 in BN is unique.

Materials and Methods

Ethics Statement

The Institutional Animal Care and Use Committee of the Medical College of Wisconsin approved all animal protocols. Rats used in this study received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” by the National Research Council.

Materials

The polyclonal antibody against human ECHDC2 was purchased from Abcam. Antibodies against heat shock protein 90, glyceraldehyde-3-phosphate dehydrogenase, and cytochrome c oxidase subunit I were obtained from Santa Cruz Biotechnology. Antibody against pyruvate dehydrogenase was from Cell Signaling Technology. Antibodies against medium-chain acyl-CoA dehydrogenase, ATP synthase, and mitochondrial trifunctional protein subunit \(x\) were purchased from MitoSciences.

Animals and Procedure for Tissue Harvest

Twelve-week-old BN, SS, and SD male rats were obtained from Charles River. Male consomic SSBN5 and SSBN7 at 12 weeks of age were from the Medical College of Wisconsin. Rats were maintained on a normal chow diet with unlimited access to water. Once anesthetized, the rat thoracic cavity was promptly opened to expose the heart and to provide drainage for blood and fluids. A needle connected with a perfusion bottle containing PBS was inserted into the exposed left ventricle through a small incision made in the atrium. The perfusion pressure was maintained at \(\approx 80\) mm Hg. The rat heart was perfused with PBS until the blood was completely cleared from the organs (15 minutes). The brain, heart, liver, lung, kidney, and skeletal muscle specimens were rapidly excised, frozen in liquid nitrogen, and stored at \(-80^\circ C\) until use.

Microarray

The total RNA from perfused BN and SS hearts (blood washed out as described earlier) was extracted by use of TRizol (Invitrogen) and purified by RNeasy kit (Qiagen). Microarray gene expression analysis was performed at the Microarray Core Facility of the University of Texas Southwestern Medical Center (http://microarray.swmed.edu). RatRef-12 Expression BeadChip arrays (Illumina) were used for the microarray analysis, according to the manufacturer’s instructions. Microarray data were extracted using BeadStudio version 3.1, background-subtracted, and normalized using a cubic spline algorithm. Genes differentially expressed between groups were identified using the Illumina custom error model implemented in BeadStudio. Genes were considered significantly differentially expressed when \(P\) values were \(<0.01\) and the change was \(>2\)-fold.

Real-Time PCR

Total RNA was extracted by using TRizol reagent (Invitrogen) and treated with DNase I.\(^11\) cDNA was generated from 1 \(\mu g\) of
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Plasmids, Cell Culture, and Transfection

Human ECHDC2 (hECHDC2) and ECHDC2 shRNA plasmids were from OriGen Technologies. hECHDC2 was incorporated into the pcMV6-AC-GFP vector where green fluorescence protein (GFP) was tagged with the open reading frame clone of human ECHDC2. We also cloned hECHDC2 into pcDNA5/FRT/TO/Topo/TA with a Flag epitope. Flag-ECHDC2 was transfected into Flp-In 293 cells (HEK293 cells) to establish stable cell lines, where the expression of hECHDC2 could be induced by tetracycline (Tet).

Flp-in 293 cells and HepG2 liver cell line (ATCC) were cultured in DMEM with 10% FBS. All plasmids were transfected by use of Lipofectamine LTX and Plus Reagent (Life Technologies).

Adult Cardiomyocytes and Cardiac Fibroblasts Isolation

Cardiomyocytes were enzymatically isolated from BN and SS as previously reported. Cardiac fibroblasts were isolated during the isolation of cardiomyocytes. The hearts were excised, mounted on a Langendorff apparatus, and perfused retrogradely with oxygenated isolation buffer, and cardiomyocytes were dissociated with an enzyme solution containing 2.85 mg/mL collagenase type II (Invitrogen), and 0.1 mmol/L CaCl₂ in the isolation buffer at pH 7.35; the temperature was maintained at 37°C. All solutions were continuously bubbled with 95% O₂–5% CO₂ gas mixture. After 25 minutes of enzyme treatment, the ventricles were excised, minced, and incubated in the same enzyme solution for an additional 5 minutes in a shaker bath at 37°C. The cell suspension was filtered through a 200-μm mesh and centrifuged at 125g for 30 seconds. The pellet was resuspended in isolation buffer with 10% BSA, and the precipitate containing the cardiomyocytes was collected; the supernatant was centrifuged again at 1000g for 10 minutes and the pellet contained the cardiac fibroblasts. The fresh isolated cardiomyocytes and cardiac fibroblasts were lysed in RIPA buffer for Western blot analysis.

Western Blot Analysis

Western blot analysis was performed as we previously described. Briefly, 30 μg protein lysates was separated by SDS-PAGE and incubated with primary antibodies overnight at 4°C and secondary antibody for 1 hour at room temperature. The blotted membranes were visualized with HyGLO Quick Spray (Denville Scientific, Inc).

Immunohistochemistry

The hearts from BN and SS were perfused free of blood and then cut into 4 or 5 horizontal slices and fixed with zinc formalin. The samples were then embedded in paraffin and sectioned to 4-μm thickness. The slides were deparaffinized, rehydrated, and subjected to citrate buffer (pH 6) antigen retrieval for 20 minutes at 99°C. Endogenous peroxide was blocked by incubating the slides with peroxidase blocking reagent (Dako). To block nonspecific background staining, the slides were incubated with serum-free Protein Block (Dako). Then, the slides were incubated with anti-ECHDC2 antibody (1:100 diluted with Dako Antibody diluent with blocking reagents for 1 hour at room temperature. MACH 2 Universal HRP polymer (BioCare Medical) was applied as secondary antibody for 15 minutes at room temperature, and DAB was used as chromagen. Slides were counterstained with hematoxylin, dehydrated, and coverslipped with Permount mounting medium. Negative control was included and processed in parallel without primary antibody.

O₂ Consumption

Cells (5 x 10⁵) were seeded into each well of O₂ biosensor fluorescence plate (BD Biosciences) with cytoex3 beads (600 beads/well) (GE Healthcare Life Sciences). The O₂ consumption rate was determined using fluorescent plate reader with excitation and emission wavelength at 485/630 nm.

Mitochondria Isolation

Mitochondria were isolated from dissected organs and tissues according to published protocols. The excised rat organ tissues were minced and rinsed with cold extraction buffer (Tris-MOPS 10 mmol/L, EGTA/Tris 10 mmol/L, and sucrose...
20 mmol/L). After homogenization with Glass/Teflon Potter, the extraction was centrifuged at 600g for 10 minutes and the supernatant was collected and centrifuged at 7000g for 10 minutes. The supernatant was extracted as cytosolic fraction, and the pellet containing mitochondria was washed twice and centrifuged at 7000g for 10 minutes.

**In Vitro Simulated Ischemia or Chemical I/R-Induced Injury in Cells**

After 12 hours of transfection with either vector or hEC-HDC2, cells (HEK293 or HEPG2 cell) were reseeded onto a 96-well plate in DMEM with 10% FBS (control) or DMEM deprived of serum and glucose (in vitro simulated ischemia) for 48 hours; the cells were examined for viability assayed by using MTT (12) (Sigma). In detail, an aliquot of 20 μL MTT solution (5 mg/mL) was added to each well and incubated with the cells for 4 hours. The supernatant was then removed, and the formed formazan product was dissolved in each well containing 150 μL DMSO. The absorbance value was detected at 490 nm using a microplate reader. In a different study, transfected cells were subjected to simulated ischemia (described earlier) or chemical I/R. Chemical I/R was induced in the cells by treating the cells with NaN3 (10 mmol/L) in nutrient-deprived medium (DMEM without glucose and serum) for 1 hour (mimic ischemia) followed by changing the culture medium back to nutrient-rich medium (high-glucose DMEM with 10% FBS) for 2 hours (mimic reperfusion). After simulated ischemia or chemical I/R, the medium was collected and examined for LDH activities. LDH activities in the culture medium were assayed using the LDH-SL kit (Sekisui Diagnostics, Inc) according to the vendor’s manual.

**In Vivo I/R Injury, Ischemic Area at Risk, and Infarct Size**

Regional myocardial I/R was induced in 3 groups of rats (BN, SS, and SSBN5; n=5 in each group) by left anterior descending coronary artery (LAD) occlusion. Briefly, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg of body weight) and placed on a heating pad to maintain body temperature at 37±0.5°C. After oral intubation, respiration was maintained using a volume-controlled Inspira Advanced Safety Ventilator (Harvard Apparatus) with a mixture of room air and 100% O2 to maintain blood gases within the normal physiological range. The ECG was recorded continuously using Powerlab data acquisition system (AD Instruments). The LAD was occluded for 30 minutes using a 6.0 Prolene suture at about 1 mm below the left atrial appendage, and reperfusion was initiated by loosening the ligature. Successful presence of acute myocardial ischemia or reperfusion was verified by visual inspection (ie, paleness in the area at risk and return of bright red color after release of the ligature) and confirmed by changes in ECG profiles (ie, immediate elevation of ST segment and significant increase in the QRS complex amplitude and width). After reperfusion, the chest wall was closed by layers. Mechanical ventilation was maintained until the rat could breathe on its own. The loose suture was left in place for determination of area at risk and infarct size.

A dual staining protocol with Phthalo blue dye and triphenyl tetrnzolium chloride (TTC) was used for the measurement of ischemic area at risk (IAR) and infarct size. After 48 hours of reperfusion, rats were heparinized intravenously and anesthetized with pentobarbital. A thoracotomy was performed and the ascending aorta was canulated with PE-50 tubing. Pre-warmed TTC (1% in PBS, w/v, pH 7.4) was perfused via the aortic root into the coronary arteries to delineate the infarct area. To delineate IAR, the LAD was reoccluded in situ, and a 5% Phthalo blue dye solution was gently infused retrograde into the aortic root. The heart was then excised, washed with PBS, and frozen at −20°C. Thin transverse sections of the heart (500 µm) were incubated in TTC for 10 minutes at 37°C to further stain viable tissue. This was followed by incubation in 10% PBS-buffered formalin overnight at 2°C to 8°C to terminate the TTC reduction reaction and to preserve tissue morphology. As a result of these procedures, the nonischemic myocardium of the left ventricle was stained dark blue, the viable myocardium within the IAR was stained bright red, and the infarcted tissue was stained light yellow (white).

**Gas Chromatography–Mass Spectrometry (GC/MS)**

Gas chromatography–mass spectrometry (GC/MS) was carried out using the Agilent 5973 MSD/6890 GC (Agilent Corp). An Rtx-5MS (30 m×0.25 mm×0.5-μm film; Restek) column was used for GC separation of metabolites. Ultra high purity helium was the gas carrier at a constant flow rate of 1 mL/min. Each sample had 1 μL injected in splitless mode by use of the Agilent 7683 autosampler. The temperature gradient started at 100°C with a hold time of 4 minutes and then increased at 5°C/min to 300°C, where it was held for 5 minutes. The temperatures were set as follows: inlet 250°C, transfer line 280°C, ion source 230°C, and quadrupole 150°C. Mass spectra were collected from m/z 50 to 600 at 1.4 spectra/s after a 6.5-minute solvent delay. The peaks were analyzed using Agilent data analysis software. Metabolite peak identities were previously defined with standards and verified by mass after each experiment. Data are expressed as ion signal strength for each metabolite normalized to cell numbers or tissue weight.
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Statistical Analysis
Values are expressed as mean±SEM, and the number of animals or number of experiments for each group is indicated. Data were analyzed by nonparametric tests. Mann–Whitney test was used for studies involving 2 groups (studies comparing the ECHDC2 mRNA levels in BN and SS hearts by real-time PCR; studies comparing cell viabilities and LDH activities in HEK cells transfected with empty vector or hECHDC2 and subjected to simulated ischemia/or chemical I/R; studies comparing O2 consumption, cellular ATP concentration, and mitochondrial intermediates in HEK Flip-in cells that expresses vector or hECHDC2; and studies comparing leucine and valine levels in vector or ECHDC2 expressing cells or in SS and BN hearts). For comparison of multiple groups (studies comparing cell viabilities in ECHDC2-overexpressing HEK cells that were transfected with different shRNA clones targeting ECHDC2 and subjected to simulated ischemia or chemical I/R and studies comparing infarction size and area at risk in the hearts of SS, BN, and SSBN5 that were subjected to myocardial I/R induced by LAD ligation), Kruskal–Wallis H test was used. After Kruskal–Wallis H test, Mann–Whitney U test was used as post-hoc test to compare means between 2 groups. All the statistical analysis was conducted in SPSS software 17.0. *P<0.05 was considered to be significant.

Results
ECHDC2 Was Highly Downregulated in the BN Hearts Compared With the SS Hearts
We reported previously that BN were more resistant to myocardial I/R than SS.3,11 To identify the gene expression differences associated with the phenotype, we used microarray to analyze RNAs from BN and SS hearts. There was a significantly different cardiac gene expression profile between these 2 strains (Table S1). Compared with SS, there were 81 genes downregulated and 52 genes upregulated in BN hearts at a very significant level (P<0.001, fold change >2). Among them, ECHDC2 showed the most pronounced downregulation in BN (fold change=−18.6; P<2.4×10^{-23}), and its significance in the disease processing remains unknown (Figure 1A). Therefore, we explored the function of ECHDC2 in the differential sensitivity to I/R injury in the BN and SS rat. To verify our microarray data, we designed 2 sets of primers covering different regions of ECHDC2. As expected, real-time PCR with both set of primers showed that ECHDC2 mRNA was about 6- to 7-fold lower in BN hearts (Figure 1B and 1C). Furthermore, we found that ECHDC2 protein expression levels were very low (almost undetectable) in the BN hearts but was high in the SS hearts as shown by Western blot analysis (Figure 1D).

Figure 1. ECHDC2 was highly downregulated in the hearts of BN rats. A, The microarray signal intensity for ECHDC2 from SS (n=3) and BN (n=3) heart samples. B and C, The fold increase of ECHDC2 mRNA in SS hearts over BN hearts by real-time PCR using primer set 1 (B) and primer set 2 (C) (n=6 per group); each experiment was performed in duplicate. D, ECHDC2 protein expression in the SS and BN hearts. Protein lysates from SS and BN rat hearts were immunoblotted with antibodies against ECHDC2 and HSP90 (used as the internal reference protein). Immunoblots were representatives from 4 independent experiments. *P<0.05 vs SS. BN indicates Brown Norway rats; ECHDC2, enoyl coenzyme A hydratase–containing domain 2; HSP90, heat shock protein 90; SS, Dahl salt-sensitive rats.

These data clearly demonstrate that the expression levels of ECHDC2 in BN hearts are dramatically lower than those in SS hearts.

Distribution of ECHDC2 in Different Tissue or Organs in BN and SS
In SS, analysis of both mRNA and protein consistently showed that liver had the highest expression of ECHDC2, followed by heart and kidney. ECHDC2 expression levels in the SS rat lung were much weaker and they were almost undetectable in brain and muscle (Figure 2A and 2B). In BN, the ECHDC2 expression was significantly lower in all tissues examined compared with that in SS: BN rat liver, kidney, and heart had similar levels of ECHDC2 mRNA (Figure 2A); and their ECHDC2 protein expressions were all extremely low (Figure 2B). To determine whether BN were unique in ECHDC2 expression, we tested another strain, SD (“a normal strain”) and revealed that ECHDC2 protein expression levels were relatively lower in the heart and liver but were higher in the kidney in SD than in SS (Figure 2C). It should be noted that similar to SS, ECHDC2 protein expression levels were much higher in SD than in BN in the heart, liver, and kidney (Figure 2C).

To confirm the expression of ECHDC2 in the hearts of SS and BN, we demonstrated that there was much stronger staining of ECHDC2 in SS hearts than BN hearts by immunohistochemistry study of formalin-fixed, paraffin-embedded heart tissue sections (Figure 3A). Similarly, we
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Figure 2. The tissue distribution of ECHDC2 in rats. A, mRNA expression of ECHDC2 in different tissues was determined by real-time PCR and normalized by GAPDH (n=6). B, ECHDC2 protein expression in different tissues from both SS and BN rats were determined by immunoblotting with antibodies against ECHDC2. C, Immunoblots of ECHDC2 protein from heart, liver, and kidney of BN, SS, and SD. B and C, were representative immunoblots from 6 independent experiments. BN indicates Brown Norway rats; ECHDC2, enoyl coenzyme A hydratase; SS, and SD. B and C, were representative immunoblots from 6 independent experiments. BN indicates Brown Norway rats; ECHDC2, enoyl coenzyme A hydratase-containing domain 2; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; SD, Sprague Dawley rats; SS, Dahl salt-sensitive rats.

We also found striking differences in staining of ECHDC2 between SS and BN in liver and kidney by immunohistochemistry (data not shown). To examine the cell sources for ECHDC2 in the heart, we isolated adult cardiomyocytes and cardiac fibroblasts from SS hearts and revealed that ECHDC2 was predominantly expressed in cardiomyocytes but not in cardiac fibroblasts by Western blot analysis (Figure 3B). Since ECHDC2 has the enoyl CoA hydratase-like domain, it was likely to locate in mitochondria. To test this notion, we isolated mitochondria and cytosol fractions from heart and liver of SS and found that ECHDC2 was specifically located in the mitochondria but not in the cytosol (Figure 3C and 3D). As ECHDC2 has the crotonase-like domain and is named after enoyl CoA hydratase, an enzyme that is involved in fatty acid metabolism, we tested the expression of other fatty acid-related mitochondrial proteins, pyruvate dehydrogenase, medium chain acyl CoA dehydrogenase, ATPase, and mitochondrial trifunctional protein in the hearts of these 2 strains. However, our data showed that in the hearts, there were no differences in the expressions of these proteins between BN and SS (Figure S1).

ECHDC2 Overexpression Increased Susceptibility to I/R Injury in Cells

We reported previously that SS hearts were more susceptible to myocardial I/R injury than BN. On the basis of these reports, we hypothesized that ECHDC2 might contribute to susceptibility to myocardial I/R injury. To this end, we overexpressed human ECHDC2 (hECHDC2) in HEK293 cells. The vector (pCMV6-AC-GFP)–expressing cells had no detectable hECHDC2 expression, but the cells transfected with ECHDC2-containing vector (pCMV6-AC-GFP-hECHDC2) had dramatically increased ECHDC2 expression (Figure 4A, top). The transfection efficacy was similar between the empty vector and hECHDC2-containing vector in the HEK cells as the green fluorescence signals were similar when the cells were checked under fluorescence microscope (data not shown). Under normal conditions, hECHDC2 overexpression did not affect cell viability within 48 hours of culture (data not shown). However, when the cells were subjected to simulated ischemia (culturing in DMEM deprived of serum and glucose for 48 hours), hECHDC2-overexpressing cells had decreased number of viable cells compared with vector-transfected cells (Figure 4A, bottom). When cells were subjected to simulated ischemia, LDH activity did not differ significantly in culture medium from the hECHDC2- or vector-transfected cells (Figure 4B, first 2 columns). However, when the cells were subjected to chemical I/R injury, the LDH activity was higher in culture medium of hECHDC2-overexpressing cells compared with culture medium of vector-expressing cells (Figure 4B, last 2 columns). These data indicate that ECHDC2 overexpression increases the cell’s susceptibility to I/R injury.

In loss-of-function experiments, of 4 different shRNAs (shRNA37, 38, 39, and 40) against ECHDC2, shRNA 37 had the highest potency for knocking down ECHDC2 expression in hECHDC2-overexpressing HEK cells (>90%, Figure 4C, top). When the hECHDC2-overexpressing HEK cells were transfected with shRNA 37 or nonsilencing shRNA control (NS) and subjected to simulated ischemia, cell viability in shRNA 37–transfected cells was significantly lower than that in the cells transfected with NS (Figure 4C, bottom). HEPG2 cells (a perpetual cell line derived from liver tissue with hepatocellular carcinoma) were then used for the following shRNA and simulated ischemia as well as chemical I/R experiments, since they have good expression of endogenous ECHDC2. After transfection and selection, stable HEPG2 cell lines expressing silencing shRNA 37 or NS were established. HEPG2 cells expressing shRNA 37 had obvious deficient

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ECHDC2 expression compared with NS-expressing cells (data not shown). Knockdown of ECHDC2 in HEPG2 cells significantly decreased LDH levels (attenuated LDH leakage from the cells) in the culture medium compared with that from NS-treated HEPG2 cells when the cells were challenged with simulated ischemia or chemical I/R injury (Figure 4D). These results indicate that ECHDC2 might promote cell injury under stress conditions.

**Increased Resistance Against Myocardial I/R Injury in SSBN5 Compared With SS**

The consomic SSBN5 had significantly lower ECHDC2 expression in the heart than did SS or SD (Figure 5A). ECHDC2 expression in SSBN7 was as high as that in SS. Thus, chromosomal switching other than chromosome 5 does not affect ECHDC2 expression. Therefore, the SSBN5 were used as ECHDC2 knockdown rats to probe for ECHDC2 functions. Figure 5C shows that in vivo myocardial I/R using LAD method induced 49.0%, 24.6%, and 24.5% infarct size in SS, BN, and SSBN5, respectively. Both BN and SSBN5 had only half of the infarct size compared with SS (Figure 5C, P<0.05, after correction for multiple comparison), and the infarct pattern in BN and SSBN5 was similar (Figure 5D). Thus, LAD-induced myocardial infarction more severe in the SS hearts than in BN or SSBN5 hearts. The IARs for all 3 groups were not significantly different (Figure 5B).

**ECHDC2 Did Not Increase Mitochondrial O2 Consumption, ATP Production, or Tricarboxylic Acid Cycle Intermediates in Cells**

Because ECHDC2 is specifically located in mitochondria (Figure 3C and 3D) and it has crotonase-like domain, we postulated that ECHDC2 might be involved in fatty acid metabolism and affect mitochondrial bioenergetics. We established a tetracycline-inducible hECHDC2 cell line (see Materials and Methods) to test the effects of ECHDC2 overexpression on O2 consumption. This cell line offers the advantage of seeding the cells from the same dish, which excludes the factors from different culture conditions such as cell confluence, numbers, and generation. Tetracycline significantly induced hECHDC2 expression at 24 hours (Figure 6A). As the cells proliferate, O2 consumption by these cells increased over time (Figure 6B). However, overexpression of hECHDC2 in these cells did not significantly affect the O2 consumption by these cells increased over time (Figure 6B). However, overexpression of hECHDC2 in these cells did not significantly affect the O2 consumption of the cells (Figure 6B). In addition, the induced hECHDC2 did not affect ATP production (Figure 6C, first 2 columns). To confirm these data, in a different experiment we transiently transfected the Flp-In 293 cells with hECHDC2 vector and obtained similar results (Figure 6C, last 2 columns). Furthermore, overexpression of hECHDC2 did not significantly affect the tricarboxylic acid cycle intermediates, including citrate, fumarate, malate, and succinate (Figure 6D); although there was a slight increase in fumarate and...
was highly conservative across species (Figure S2) and human protein database is one of the most complete and annotated databases. We found that the highest identical protein to ECHDC2 was AU RNA binding protein/enoyl-CoA hydratase (AUHM) with a 44.2% sequence match (Figure 7A). AUHM is also known as methylglutactony-CoA hydratase and is critical in leucine degradation. The mutation of AUHM may cause 3-methylglutaconic aciduria type I disease. The ECHDC2 protein contains an enoyl CoA hydratase domain, but its sequence only matches 29.8% of that of enoyl CoA hydratase (Figure S3). Thus, we questioned whether ECHDC2 had functions similar to AUHM. In this study, we determined the levels of branched chain amino acids (BCAAs) leucine and valine in the ECHDC2 overexpressing cells. Interestingly, ECHDC2 overexpression significantly increased both leucine and valine in HEK cells (Figure 7B). Furthermore, when we checked the tissue level of these amino acids, we found that there was a tendency of increased leucine and valine levels in SS hearts versus in BN hearts (Figure 7C). All these data suggest that ECHDC2 may be involved in the BCAA metabolism.

**Discussion**

To our knowledge, this is the first report that shows ECHDC2 is deficient in BN rat and negatively regulates cardioprotection. ECHDC2 is located in the mitochondria of cardiomyocytes and increases susceptibility to I/R injury, in those cells where it is highly expressed. It is deficient at both mRNA and protein levels in BN hearts. SSBN5, a strain generated based on SS genetic background with the BN chromosome 5 substitution, which contains ECHDC2 of SS rat, has increased cardioprotection compared with SS. Further, we have also demonstrated that ECHDC2 does not increase mitochondrial energy production; however, it might be involved in BCAA metabolism.

The ECHDC2 expression might be regulated at multiple levels. The rat ECHDC2 sequence in Genbank (NM_001106675.1) is predicted based on BN rat genome, which is still not validated. We cloned and sequenced the ECHDC2 cDNA in the SS heart and found it exactly matched the sequence of BN rat in the Genbank (not shown). The significantly lower levels of ECHDC2 mRNA expression in the BN hearts might be related with the differences in the gene’s transcriptional regulatory regions. The analysis of the promoters and their transcriptional factors from both strains would be helpful to reveal the cause of this differential expression. Indeed, we found >50 SNPs in the BN ECHDC2 promoter region compared with SS ECHDC2. Additionally, ECHDC2 protein is more drastically differentially expressed between SS hearts and BN hearts (SS/BN ≈18-fold) than its mRNA (SS/BN ≈6- to 7-fold), indicating that this gene may also be regulated at translational and posttranslational levels. For example, there are at least 7 potential ubiquitination sites.

**The Effect of ECHDC2 on Branched Chain Amino Acid Metabolism**

To search for the potential substrate of ECHDC2, we blasted the human ECHDC2 proteins against the human protein database. We used the human database because ECHDC2 succinate production. These results suggest that ECHDC2 does not significantly alter mitochondrial energy production under normal conditions.
in rat ECHDC2 genes according to computational prediction (http://bdmpub.biocuckoo.org/).

ECHDC2 has unique distribution and regulates cell death and myocardial I/R injury. Our data clearly showed that ECHDC2 is highly expressed in liver, kidney, and heart in rats (SS and SD). It is consistent with the microarray data from different mouse tissues.16,17 These organs are mitochondria-rich organs, and ECHDC2 specifically localizes in mitochondria (Figure 3). Besides working as a powerhouse of the cell, mitochondria are also involved in fatty acid metabolism, steroids synthesis, calcium signaling, and apoptosis.18 Mitochondrial dysfunction plays critical roles in myocardial I/R.18 Our findings corroborate the hypothesis that ECHDC2 positively regulates the events of cell death and myocardial injury. Overexpression of ECHDC2 increased cell death and exacerbated I/R injury, while knockdown of ECHDC2 protected the cells from injury. More interestingly, when we studied the SSBN5 rat, which is genetically similar to the SS rat, we found that SSBN5 with deficient ECHDC2 expression had higher resistance against myocardial I/R injury compared with the SS rat. Because there is no experimental report available on ECHDC2, we mined the published gene array databases and the protein interacting databases and found that ECHDC2 may be associated with some cell death proteins and pathways. In melanoma cells, ECHDC2 was shown to interact with the melanoma antigen gene (MAGE), which is activated in many tumors and associated with poor outcome.19 We speculate that ECHDC2 may regulate cell death through modulation of cell metabolism, particularly the BCAA metabolism. ECHDC2 increased leucine in both ECHDC2-overexpressed cells and heart. Interestingly, increasing evidence showed that BCAA metabolism is a culprit rather than a protector in heart diseases.20,21 The patients with cardiometabolic risk are associated with increased BCAA levels.22 The physiological role of ECHDC2 in the heart or other organs at this time is not clear. However, our data suggest that ECHDC2’s role in the heart might be related to the BCAA metabolism, which guarantees our future studies in this area.

We have found that there are 133 genes expressed differently (81 genes downregulated and 52 genes upregulated) between the 2 strains; thus, we definitely could not attribute ECHDC2 alone to the differential sensitivity to acute myocardial I/R injury in these 2 strains of rats. We acknowledge this limitation in our study. However, it is very challenging to evaluate the effects of all the 133 genes in our model. Besides, there might be also posttranslational

Figure 5. The SSBN5 had higher resistance to myocardial ischemia/reperfusion (I/R) compared with SS, which have a similar genetic background. A, Heart lysates from SS, BN, SSBN5, and SSBN7 were immunoblotted with antibody against ECHDC2. B and C, In vivo study of myocardial I/R induced by LAD ligation showed that 30-minute ischemia and 48-hour reperfusion induced myocardial infarction that was more severe in SS hearts than in BN or SSBN5 hearts (B). The ischemic areas at risk (IAR) for all the hearts are similar (C). The infarct size was 24.6%, 49.0%, and 24.5% for BN, SS, and SSBN5, respectively. D, Representative images of SS, BN, and SSBN5 heart slices stained with Phthalo blue and TTC as illustrations of infarct size (n=5/group; *P<0.05 vs SS rats). BN indicates Brown Norway rats; ECHDC2, enoyl coenzyme A hydratase-containing domain 2; HSP90, heat shock protein 90; IS, infarct size; LAD, left anterior descending coronary artery; LV, left ventricle; NS, no significant difference; SD, Sprague Dawley rats; SS, Dahl salt-sensitive rats; TTC, triphenyl tetrazolium chloride.

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Figure 6. ECHDC2 did not increase mitochondrial function. A, Stable Flip-in cells expressing human ECHDC2 (hECHDC2) were induced with tetracycline (Tet) (1 μg/mL) for 24 hours, and protein lysate was immunoblotted with ECHDC2 antibody. B, The rECHDC2 stable cell line was induced with Tet for different time points to measure O₂ consumption (n=4 per group). C, Cellular ATP concentration was determined from ECHDC2 stable cell line induced with/without Tet (the first 2 columns) or cell transiently transfected with vector or hECHDC2 for 24 hours (the last 2 columns) (n=4 per group). D, Mitochondrial intermediates were determined by GC-MS from HEK Flip-in cells transiently transfected with vector or hECHDC2 for 24 hours (n=4 per group). ECHDC2 indicates enoyl coenzyme A hydratase-containing domain 2; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GS–MC, gas chromatography–mass spectrometry; NS, no significance compared with the group with vector.

Figure 7. The effect of ECHDC2 on branched chain amino acids metabolism. A, An alignment of human ECHDC2 protein with human AU RNA binding protein/enoyl-CoA hydratase (AUHM) protein. B, The leucine and valine levels from vector- or ECHDC2-expressing cells (fold over control, n=3 per group). C, The leucine and valine levels from SS and BN hearts (n=3 per group). *P<0.05 vs vector group. BN indicates Brown Norway rats; ECHDC2, enoyl coenzyme A hydratase–containing domain 2; SS, Dahl salt-sensitive rats.
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modification difference in regulating expressions of the genes accounting for the different response of the hearts from the 2 strains to I/R. However, in this study, we revealed that ECHDC2 does play critical roles in the myocardial I/R. Another limitation of our study is that we did not use the adenovirus for overexpressing ECHDC2 in adult cultured cardiomyocytes. Instead, for technical convenience, we used HEK cells and HEPG2 cell lines. After screening different cell lines, we found HEK cells did not have detectable ECHDC2 expression on PCR and Western blotting and that HEPG2 cells have higher expression of ECHDC2. Thus, we chose HEK cells for overexpression and HEPG2 cells for knockdown studies.

In conclusion, ECHDC2 is a mitochondrial protein regulating cell death and myocardial injury. Its deficiency in BN contributes to their increased resistance against myocardial I/R. This makes BN an attractive naturally ECHDC2-deconjugated strain for future research into this enzyme’s role in cardiovascular disease. As ECHDC2 increases BCAA metabolism in heart, it might be a novel regulator linking cell metabolism with cardiovascular disease.

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Disclosures

None.

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