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Ablation of *cereblon* attenuates myocardial ischemia–reperfusion injury

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ABSTRACT

Cereblon (CRBN) was originally identified as a target protein for a mild type of mental retardation in humans. However, recent studies showed that CRBN acts as a negative regulator of AMP-activated protein kinase (AMPK) by binding directly to the AMPK catalytic subunit. Because AMPK is implicated in myocardial ischemia–reperfusion (I–R) injury, we reasoned that CRBN might play a role in the pathology of myocardial I–R through regulation of AMPK activity. To test this hypothesis, wild-type (WT) and *crbn* knockout (KO) mice were subjected to I–R (complete ligation of the coronary artery for 30 min followed by 24 h of reperfusion). We found significantly smaller infarct sizes and less fibrosis in the hearts of KO mice than in those of WT mice. Apoptosis was also significantly reduced in the KO mice compared with that in WT mice, as shown by the reduced numbers of TUNEL-positive cells. In parallel, AMPK activity remained at normal levels in KO mice undergoing I–R, whereas it was significantly reduced in WT mice under the same conditions. In rat neonatal cardiomyocytes, overexpression of CRBN significantly reduced AMPK activity, as demonstrated by reductions in both phosphorylation levels of AMPK and the expression of its downstream target genes. Collectively, these data demonstrate that CRBN plays an important role in myocardial I–R injury through modulation of AMPK activity.

1. Introduction

The AMP-activated protein kinase (AMPK), a cellular fuel gauge, plays an important role in the signaling pathways that regulate cellular energy status [1]. For example, AMPK promotes energy production by activating glucose transport and glycolysis [2,3], as well as mitochondrial fatty acid uptake and oxidation [4]. In addition, AMPK is involved in a variety of other cellular processes, such as promoting cell survival by regulating apoptosis [5], autophagy [6,7], and the generation of reactive oxygen species [8].

Myocardial ischemia–reperfusion (I–R) injury is a major health problem in developed countries and is mediated by an increased generation of reactive oxygen species in ischemic cardiomyocytes upon abrupt resupply of oxygen. A number of previous studies have suggested a cardio-protective role for AMPK against I–R injury. In mice lacking the catalytic subunit of AMPK or expressing a dominant negative mutant of this subunit in the heart, the ischemia-induced stimulation of glucose uptake and glycolysis is inhibited, leading to ATP depletion and ischemic contracture [9]. Similarly, the infarct size following coronary ligation is larger in mice expressing a dominant negative form of AMPK than in controls [10,11]. By contrast, activation of AMPK reduces myocardial I–R injury in mice [9–11]. Moreover, AMPK activators, such as 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) [12], A-769662 (a non-nucleoside thienopyridone) [13], and metformin [14], protect hearts against I–R injury.

Cereblon (CRBN), a candidate protein linked with mild mental retardation, is a primary target of thalidomide teratogenicity. CRBN forms an E3 ubiquitin ligase complex with damaged DNA binding protein 1 and cullin 4A to play important roles for proper limb development. Thalidomide exerts its teratogenic effects by binding to CRBN and inhibiting its associated ligase activity [15]. Recently, CRBN was shown to inhibit AMPK activity by binding directly to

Abbreviations: CRBN, cereblon; I–R, ischemia–reperfusion; WT, wild-type; KO, knockout; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; PARP1, poly (ADP-ribose) polymerase 1; AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; A-769662, a non-nucleoside thienopyridone; TTC, triphenyltetrazolium; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; AAR, areas at risk; IA, infarcted area; LV, left ventricular; PGC1α, peroxisome proliferator-activated receptor γ coactivator1 α; SREBP1, sterol regulatory element-binding protein 1; eNOS, endothelial nitric oxide synthase; CPT1, carnitine palmitoyltransferase 1.

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the catalytic subunit of AMPK [16]. The ablation of the *crbn* gene prevents high fat diet-induced obesity and insulin resistance in mice through elevation of AMPK activity [17]. These data suggest that CRBN is an endogenous inhibitor of AMPK.

In the present study, we found that ablation of *crbn* significantly reduced myocardial I–R injury in mice and that this cardio-protective effect was paralleled by an increase in AMPK activity. CRBN modulated AMPK activity in cardiomyocytes, as has been shown in other tissues. Therefore, down-regulating CRBN or disrupting CRBN–AMPK interactions may provide therapeutic protection for hearts against I–R injury.

2. Materials and methods

2.1. Animal care

Male CRBN knockout (KO) mice (8–10 weeks old, weighing 20–25 g) were generously donated by Dr. Chul-Seung Park (Gwangju Institute Science and Technology). Mice were maintained under controlled conditions at 22 °C with 55–56% humidity and a 12 h light:dark cycle. All procedures were conducted in accordance with protocols approved by the Gwangju Institute Science and Technology Animal Care Committee and national guidelines.

2.2. Ischemia–reperfusion protocol

Myocardial I–R was induced as previously described [18]. Briefly, the left anterior descending coronary artery was ligated using 7–0 silk sutured approximately 2 mm below the level of the tip of the normally positioned left auricle. Polyethylene (PE) 10 tubing with a diameter of 1 mm was placed on top of the vessel, and the suture was tied. After 30 min of occlusion, reperfusion was established by cutting the knot and removing the PE10 tubing. The chest wall was closed using 5–0 suture. Mice were sacrificed, and their hearts were removed after 24 h of reperfusion.

2.3. Determination of cardiac infarct size

Evan's blue dye (0.1%) was injected retrogradely via the abdominal aorta. The heart was removed and sectioned transversely into five slices from the level of the coronary ligature to the apex. Each slice was placed in 1.5% triphenyltetrazolium (TTC) blue dye. The slices of the heart were viewed using a dissecting microscope (400ES, Nikon). Images underwent computer enhancement using Adobe Photoshop, and the areas at risk or infarcted areas were calculated using MetaMorph software.

2.4. Masson's trichrome staining

Hearts were fixed with 4% paraformaldehyde, embedded in paraffin, and sliced into 6 μm thick sections (RM2135 microtome, Leica). Masson's trichrome staining was conducted as directed in the manufacturer's instructions (HT15 kit, Sigma–Aldrich). Images were captured and the fibrotic areas were quantified using MetaMorph software.

2.5. TUNEL assay

Apoptotic changes in DNA were identified with a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using fluorescein-labeled dUTP (Roche Diagnostics). TUNEL staining was performed on 8 μm-thick cryosections from hearts fixed with 4% paraformaldehyde perfusions and processed according to the manufacturer's instructions. Slides were examined for apoptotic nuclei using a fluorescence microscope. The number of TUNEL-positive nuclei was counted in three or four fields for each section.

2.6. Neonatal cardiomyocyte culture and transfection

Primary cardiomyocyte cultures were generated from 1-day-old Sprague–Dawley rats. The whole heart was prepared and digested

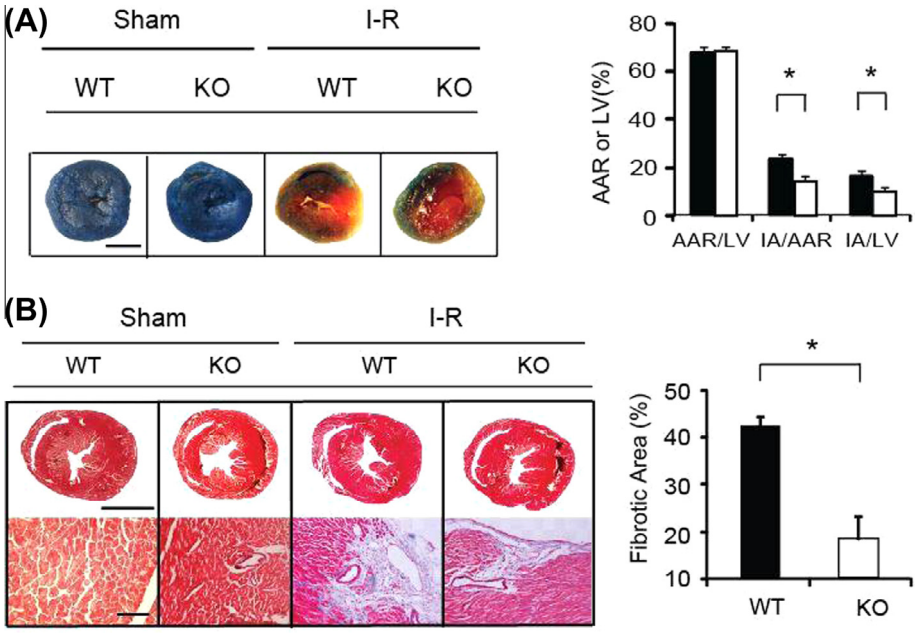

Fig. 1. Ablation of *crbn* attenuates myocardial infarction and fibrosis following I–R. (A) Wild-type (WT) and *crbn* knockout (KO) mice were subjected to 30 min of ischemia followed by 24 h of reperfusion. Heart sections were stained with Evan's blue and triphenyltetrazolium (TTC) blue dye. Healthy areas, areas at risk (AAR), and infarcted areas (IA) are distinguished in blue, red, and whitish colors, respectively. AAR and IA are normalized to whole left ventricular (LV) areas or AAR. Black bar, WT; white bar, KO. Scale bar = 5 mm. $n = 3–7$. $^{*}p < 0.05$. (B) Trichrome staining was performed 4 weeks after I–R. Fibrotic area is blue. Scale bars = 5 mm (upper row) and 5 μm (bottom row). $n = 3$. $^{*}p < 0.05$.