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Cardioprotective actions of Notch1 against myocardial infarction via LKB1-dependent AMPK signaling pathway

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Abstract

AMP-activated protein kinase (AMPK) signaling pathway plays a pivotal role in intracellular adaptation to energy stress during myocardial ischemia. Notch1 signaling in the adult myocardium is also activated in response to ischemic stress. However, the relationship between Notch1 and AMPK signaling pathways during ischemia remains unclear. We hypothesize that Notch1 as an adaptive signaling pathway protects the heart from ischemic injury via modulating the cardioprotective AMPK signaling pathway. C57BL/6J mice were subjected to an *in vivo* ligation of left anterior descending coronary artery and the hearts from C57BL/6J mice were subjected to an *ex vivo* globe ischemia and reperfusion in the Langendorff perfusion system. The Notch1 signaling was activated during myocardial ischemia. A Notch1 γ -secretase inhibitor, dibenzazepine (DBZ), was intraperitoneal injected to mice to inhibit Notch1 signaling pathway by ischemia. The inhibition of Notch1 signaling by DBZ significantly augmented cardiac dysfunctions caused by myocardial infarction. Intriguingly, DBZ treatment also significantly blunted the activation of AMPK signaling pathway. The immunoprecipitation experiments demonstrated that an interaction between Notch1 and liver kinase beta1 (LKB1) modulated AMPK activation during myocardial ischemia. Furthermore, a ligand of Notch1 Jagged1 can significantly reduce cardiac damage caused by ischemia via activation of AMPK signaling pathway and modulation of glucose oxidation and fatty acid oxidation during ischemia and reperfusion. But Jagged1 did not have any cardioprotections on AMPK kinase dead transgenic hearts. Taken together, the results indicate that the cardioprotective effect of Notch1 against ischemic damage is mediated by AMPK signaling via an interaction with upstream LKB1.

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Disclosure

None

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Keywords

myocardial infarction; Notch1; AMPK; LKB1; cardioprotection

Introduction

Myocardial infarction (MI) happens when coronary blood flow is blocked and the oxygen and nutrients supply is abruptly insufficient for the demand of the myocardium, resulting in irreversible damage to the heart. If blood supply is not rapidly restored, the cardiac tissue undergoes ischemia, necrosis and fibrosis resulting in ventricular dysfunction and heart failure [1, 2]. Despite great advances in the understanding and the new treatment of coronary heart diseases in recent years, myocardial infarction is one of high mortality and morbidity worldwide [3].

The evolutionarily conserved Notch signaling pathway controls tissue formation and homeostasis during embryonic and adult life through local cell-cell interactions. After recognized by ligands (Delta-like1, 3, 4, Jagged1, 2), Notch receptors (Notch1-4) cleaved by TNF- α -converting enzyme (TACE) and the γ -secretase complex. The released Notch intracellular (NIC) domain translocate into the nucleus, binds to the transcription factor CSL (C-promoter binding factor-1/Suppressor of hairless/LAG-1), and regulates the target genes Hes and Hey transcription [4]. In the heart, Notch is expressed in a variety of cell types such as cardiomyocytes, smooth muscle cells and endothelial cells. Notch1 is down regulated during postnatal development, but could be activated in response to myocardial injury [5], suggesting that Notch signaling could have a protective role. The mechanisms underlying Notch1-mediated cardiac protection are complex and not completely clear [6, 7].

AMP-activated protein kinase (AMPK) is an energy barometer that participates in the cellular response to metabolic stress when the AMP/ATP ratio is elevated. AMPK plays a pivotal role in intracellular adaptation to energy stress during myocardial ischemia. Evidently, AMPK activation helps to reserve cellular energy stores, to accelerate ATP generation and attenuate ATP depletion during ischemia and decrease cardiac hypertrophy, apoptosis, and inflammation, and limit deleterious changes in cardiac structure, function, and glycolytic metabolism in the post-MI failing heart [8, 9]. On the contrary, AMPK α knockout or dysfunctional mice subjected to I/R endured greater cardiac injury (larger infarct size, more apoptosis, and poorer cardiac function)[10]. It has been shown that the physiological or pharmacological activation of AMPK can decrease cardiac necrosis, and AMPK signaling pathways are involved in some new drugs induced cardioprotection [11].

Several lines of evidence indicate that Notch signaling in hearts is activated in an animal model of myocardial injury and maybe have a protective role [12]. Moreover, there are some lights on Notch signaling regulating energy metabolism [13, 14]. Another study revealed AMPK plays a pivotal role in intracellular adaptation to energy stress during myocardial ischemia [15, 16]. Based on these studies, we hypothesize the cardioprotective effects of Notch1 may crosstalk with cardiac AMPK signaling pathways and modulate the cardiac energy metabolism. Thus, we examined the role of Notch1 in myocardial infarction induced

by ligation of left anterior coronary artery (LAD) and the relationship between Notch1 and AMPK signaling during myocardial ischemia.

Materials and Methods

Experimental animals

Wild-type (WT) male C57BL/6J mice (12 weeks of age) and AMPK kinase dead mice (AMPK KD, expressing a KD $\alpha 2$ K45R mutation, driven in heart and skeletal muscles by the muscle creatine kinase promoter) [17] were used in the experiments. All animal protocols in this study were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee (IACUC).

Myocardial infarction model

Mice were randomly divided into the following six groups in a blind study: (1) sham-operated group (sham), (2) the DBZ injection group (DBZ, 5 μ mol/kg/day [18, 19], i.p., Axon Medchem LLC, Reston, VA, USA), (3) myocardial infarction group (MI), (4) myocardial infarction with DBZ injection group (MI+DBZ), (5) AMPK KD sham-operated group (AMPK KD-sham), (6) myocardial infarction of AMPK KD group (AMPK KD-MI). Mice were anesthetized with 60 mg/kg of sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, USA) by intraperitoneal injection. Mice were then subjected to operation of MI model as described [20]. Limb-lead electrocardiography (AD Instruments Inc, Colorado Springs, CO, USA) was performed consistently. Mice were anesthetized with 2.0% isoflurane mixed with 40% oxygen, endotracheal intubation was performed with 20-gauge intravenous catheter and ventilated by a volume-regulated respirator (Harvard apparatus, Holliston, MA, USA) during surgery. Myocardial infarction was performed by ligating left anterior descending artery (LAD) at 1.5 to 2.0 mm below the tip of the left auricle with an 8-0 nylon suture. Occlusion of LAD was confirmed by the change of color and the elevation of ST segment on electrocardiogram. After surgery, chest cavity and skin incision were closed. Sham operation was performed via an identical procedure, except that the suture was just passed underneath LAD without occlusion. DBZ or Vehicle (5 % DMSO/H₂O, Sigma-Aldrich, MO, USA) was administered via intraperitoneal injection in DBZ and MI+DBZ groups.

Echocardiography

Echocardiography was performed in all mice at 2 weeks after MI. Mice were anaesthetized using 1.5–2.0% isoflurane (Vedco, St. Joseph, MO, USA) for function measurement with echocardiogram (14.0 MHz, Sequoia 512; Acuson, Germany). Both two-dimensional and M-mode images were recorded. The left ventricle inner diameter during diastole (LVIDd) and left ventricle anterior wall thickness during diastole (LVAWd) were measured. LV fractional shortening (FS) and LV ejection fraction (EF) were calculated as follows [21]: FS (%) = [(LVIDd-LVAWd)/LVIDd] $\times$ 100; EF (%) = [(LVIDd3-LVAWd3)/LVIDd3] $\times$ 100.

Histological analysis and assessment of infarct size

Histological analysis of mouse hearts was performed as described [22]. Sections (5 μ m) were deparaffinized and stained with hematoxylin and eosin (H&E) and Trichrome Stain

(Sigma-Aldrich, St. Louis, MO, USA) according to standard protocols [23]. Myocardial infarct size was evaluated by Evans Blue (Sigma-Aldrich, St. Louis, MO, USA) and 2, 3, 5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, St. Louis, MO, USA) staining as described [24]. The hearts were then excised after 24 hours. The excised heart was injected 1%TTC from the 20 gauge cannula which was retrograded into the aorta. Next the heart was immersed in 1%TTC and incubated in the 37°C incubator for 5 min. After that, the heart was perfused 1% Evans blue gently until the right heart turn blue. The heart stained with TTC and Evans blue dye to delineate the extent of myocardial necrosis as a percent of the area at risk (AAR). The areas were defined as follows: the infarct area consists of the TTC-negative staining region, the AAR consists of the Evan's Blue negatively staining region, including the TTC-positive staining and TTC-negative staining regions, and the remote area consists of the Evan's blue positively staining regions. The area of infarct size and the area at risk were measured digitally using NIH Image J software.

Immunohistochemistry

Immunohistochemical staining was performed as previously described [22]. The histological sections were localized in the area at risk. Heart sections were stained with primary antibodies against Mac-2 (1:200, Abcam, Cambridge, MA, USA), Caspase-3 (1:200, Cell signaling Technology, Beverly, MA, USA) or IgG control at 4°C overnight, and then with second antibody (Vectastain ABC Kit, VECTOR Laboratories, Inc, Burlingame, CA). Peroxidase activity was visualized with use of diaminobenzidine (Peroxidase Substrate Kit, VECTOR Laboratories, Inc, Burlingame, CA), and the sections were counterstained with hematoxylin. The numbers of Mac-2 and Caspase-3 positive cells were counted blindly and expressed as a percentage of total number of cardiomyocytes in six sequentially cut 5 µm sections of the ischemic lesion for each heart. Digital photographs were taken at 200× magnifications of over 20 random fields from each heart, and the positive areas were calculated by NIH Image J software.

Assay of cell apoptosis

Apoptosis of heart sections and cardiomyocytes after MI 5h was analyzed with TACS[®] 2 TdT In situ Apoptosis Detection Kits, according to manufacturer's instructions (Trevigen Inc. Maryland, USA). The myocardium was labeled with α-actinin antibody and the nucleus was stained with DAPI. Fluorescence staining was viewed by microscopy (ZEISS Axio Imager Fluorescence Microscope, Germany). The number of TUNEL-positive cells was analyzed using NIH Image software as described [25], which were quantified, and at least 200 cells per section were counted.

Quantitative real-time RT-PCR

Quantitative Real Time-PCR (Q-PCR) analysis was as described previously [25]. Total RNA was extracted using the TRIzol method (Invitrogen Life Technologies, Carlsbad, California, USA). RNA samples (1µg) were reverse-transcribed to generate first-strand cDNA. Primers were designed as described previously [25]. Q-PCR was performed in a 20 µl reaction mixture prepared with SYBR GREEN PCR Master Mix (Applied Biosystems, Warrington, UK) containing an appropriately diluted cDNA solution and 0.2 mM of each primer at 95°C for 10 min, followed by 35 cycles at 95°C for 10 s and 60°C for 45 s. The transcript levels of

Hes1, Hey1, IL-6, IL-1 β , TNF- α , MCP-1, Hmox1, GDF-15, TGF- β , MMP-9 and TIMP-1 were detected by Q-PCR system (Bio Rad CFX96 Touch PCR, Hercules, CA, USA). All reactions were conducted in triplicated and the data was analyzed using the delta Ct (DDCt) method. These transcripts were normalized to β -actin.

Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) analysis was as described previously [26]. The heart tissues were homogenate in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.5% NP-40, 0.5 mM DTT, Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany), and Phosphatase Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA). The lysates were incubated on ice for 30 min, and removed by centrifugation at 21,000 $\times g$ for 15 min. The resulting supernatants were incubated with Protein G Sepharose™ 4 Fast Flow (GE Healthcare Life Sciences, Germany) overnight at 4 °C, and the precipitates were washed three times with the lysis buffer. For western blotting, whole tissue lysates and precipitates were separated by SDS-PAGE and subsequent steps were same as western blotting. The membranes were immunoblotted with the following antibodies: anti-Notch1 (Cell Signaling Technology, Beverly, MA, USA), anti-LKB1/STK11 (mouse, Lot: NBP2-14834, Littleton, CO, USA) and anti-AMPK α (Cell Signaling Technology, Beverly, MA, USA).

Immunoblotting

Immunoblots were performed as previously described [27]. Cytosolic proteins and nuclear proteins were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce; Rockford, IL, USA) according to the manufacturer's instructions. The protein concentrations of all samples were measured using Bradford dye-binding method (Dye Reagent Concentrate, Bio-Rad Protein Assay, USA). Protein (100 μ g) from heart were separated by SDS-PAGE, transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA), and probed with primary antibodies against Notch1 (1:1000), Bax (1:1000), Bcl-2 (1:1000), phosphor-JNK (Thr183/Tyr185) (1:1000), JNK, phosphor-AMPK (Thr172) (1:1000), AMPK, phosphor-LKB1 (Ser428) (1:1000), LKB1, and then with horseradish peroxidase-conjugated secondary antibodies (1:2000). To quantify the protein signal, we subtracted background, normalized the value to β -actin. As for the phosphor-specific protein, we normalized the signal to the amount of total target protein and β -actin, and the proteins in nucleus were normalized the value to TBP (TATA binding protein). All the antibodies were purchased from Cell Signaling (Danvers, MA, USA).

Fatty acid/glucose oxidation analysis

A working heart model (Radnoti Glass Technology, Inc, Monrovia, CA) was used to test the cardiac substrate (fatty acid and glucose) metabolism as described [28]. Glucose and oleate oxidation rates were determined simultaneously by the addition of U-¹⁴C-glucose and [9, 10]-³H-oleate to the recirculation perfused buffer (both isotopes from Perkin Elmer, Waltham, MA, USA). Mice were anesthetized with pentobarbital (60 mg/kg i.p.), heparinized (100 U i.p.) (Sigma-Aldrich, St Louis, Mo, USA.), and the hearts excised. When cannulated through the aorta to initiate retrograde Langendorff perfusion, mouse hearts were perfused with 95% O₂/5% CO₂ equilibrated KHB containing 7 mM glucose and 0.2 U/ml insulin (Sigma-Aldrich, St Louis, Mo, USA.). Jagged1 (SRP8012, Sigma-Aldrich, St Louis,

Mo, USA) was added into KHB buffer from balance perfusion in Jagged1 groups, which was extracellular domain of human Jagged1 fused at the C-terminus to the Fc portion of human IgG1. And then followed by cannulation of the pulmonary vein to initiate anterograde perfusion in the working heart mode, hearts were perfused with radiolabeled KHB buffer containing 7 mM glucose, 0.2 U/ml insulin, 1% bovine serum albumin, U-¹⁴C-glucose (20 µci/L) and [9, 10]-³H-oleate (50 µci/L). The flow rate was maintained at 15 ml/min. After addition of radioactive substrates, perfusion was continued under conditions of moderate cardiac work for 20 min, at which time steady oxidation rates had been attained. Followed by 10 min of global ischemia, oxidation rate measurements were continued for an additional 20 min reperfusion. Oxidation rates were determined at 5 min intervals and are expressed as micromoles of fuel oxidized per minute per gram dry heart weight. At the end of the perfusion, hearts were quickly weighed and frozen with tongs cooled in liquid nitrogen. Glucose oxidation was measured by quantifying the rate of ¹⁴CO₂ appearance in the effluent gas and buffer. Aliquots from the 1 M NaOH gas traps were placed directly into scintillation vials and counted. The ¹⁴CO₂ present as bicarbonate was determined by syringe removal of an aliquot of the perfused buffer without exposure to air. The perfused samples were acidified by syringe addition of 0.3 ml of H₂SO₄ through the stopper for overnight to evolve all of the ¹⁴CO₂. The center well was then transferred to scintillation vials for quantification of radioactivity. Oleic acid oxidation was measured simultaneously as the rate of appearance of ³H₂O in the perfused buffer. Tritiated water was separated from [9, 10]-³H-oleate by anion-exchange chromatography on Dowex-1-borate columns. AG® 1-×2 Resin, (chloride form, 106–250 µm mesh, Bio-Rad, Hercules, CA, USA) was converted by activated with 1 M NaOH, and washing with deionized water until pH < 8. Samples (0.4 ml) were loaded onto a 3 ml volume bed of resin and eluted with 2 ml H₂O into scintillation vials. Analysis of standard solutions containing known amounts of ³H₂O and [U-¹⁴C] glucose revealed that >99% of the labeled glucose and <5% of labeled water were retained by the column. Other chemicals and reagents were of analytical grade.

Statistical Analysis

Values are means ± S.E.M. Data analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA). Significance was determined by either a two-tailed, unpaired Student's t test or ANOVA using Tukey's post-test. A value of p < 0.05 was considered statistically significant.

Results

Notch1 signaling pathway is activated during myocardial infarction

In order to study the expression of Notch1 signaling pathway, anti-Notch1 antibody was used to recognize the Notch1 receptor and its intracellular fragments in Immunoblot analysis at post MI 1 week and 2 weeks. There are 3 bands recognized easily on the membrane, which molecular weights were between 50 and 150 KD. The third band about 50–60 KD was focus on in whole study, which significantly increased after myocardial infarction (MI) 1 week and 2 weeks by permanent coronary occlusion compared with Sham group (Fig. 1A). Because Notch intracellular fragments (NIC) could translocate into the nucleus and regulate the target genes Hes1 and Hey1 transcription, the proteins from nuclei and

cytoplasm were analyzed respectively. The degradation fragments of Notch1 about 50–60 KD were significantly increased in nuclei and in cytoplasm (Fig. 1B). Q-PCR revealed that the mRNA levels of Hes1 and Hey1 significantly up-regulated after MI 24h (Fig. 1C). These findings suggested Notch1 signaling pathway was activated after MI compared with Sham group. DBZ an inhibitor of γ -secretase was used to block the Notch1 activation. Treated with DBZ, the expression of Notch1 intracellular fragment was significantly decreased compared with MI group, and the mRNA levels of Hes1 and Hey1 changed at the same way (Fig. 1C). These results suggested DBZ inhibited Notch1 signaling pathway effectively in MI model.

Inhibition of Notch1 signaling pathway augments myocardial infarction

In order to detect the main cardiac changes after MI, the hearts were harvested at different time points, for instance, the apoptosis was detected at MI 5 hrs, the myocardial infarction and inflammation were detected at MI 24 hrs, the function and fibrosis were measured at MI 2 weeks. As shown in Fig. 2A, the infarction area significantly increased in MI+DBZ group compared with MI group. However, the proportion of infarct border zone in left ventricle was similar between groups. Similar results were revealed by echocardiography after MI 2 weeks. As compared with Sham group, the left ventricle interior diameter during diastole (LVID d) significantly increased and left ventricle anterior wall thickness during diastole (LVAW d), Ejection fraction (EF) and left ventricular shortening fraction (FS) demonstrated a strong trend toward decreased wall thickness in MI group. Furthermore, as compared with MI group, extra LVID d increase and extra decreased of LVAW d, EF and FS in MI+DBZ group versus MI group (Fig. 2B). It was observed that the fibrosis area and the mRNA levels of fibrosis related genes (TGF- β , MMP-9 and TIMP-1) in MI group was increased versus Sham group, and further increase in MI+DBZ as compared with MI group (Fig. 2C and 2D). Heart apoptosis were increased in MI group as compared with Sham group and extra increased in MI+DBZ group as compared with MI group (Fig. 2D). MI treatment significantly increased caspase-3-positive cells, Bax/Bcl ratio, Mac-2-positive cells, proinflammatory cytokines (including IL-6, IL-1 β , MCP-1, TNF- α , GDF-15, and Hmox-1) as compared with Sham group and additionally increased in MI+DBZ group more than MI group (Fig. 2D and 2E). Compared with Sham group, DBZ alone treatment did slightly changed in these main cardiac injuries, but there were no statistical difference. All these findings demonstrate Notch1 signaling pathway is a critical component to maintain cardiac function during MI.

Notch1 modulates cardiac AMPK signaling via interacting with LKB1

In order to determine whether AMPK signaling pathway is involved in the effects of Notch1, the levels of AMPK, one of AMPK phosphorylating kinase LKB1 and AMPK downstream acetyl CoA carboxylase (ACC) were analyzed. The results revealed that the phosphorylation levels of LKB1, AMPK and ACC were elevated respectively after MI as compared with Sham groups (Fig. 3A). The inhibition of Notch1 signaling by DBZ treatment results in the decreased levels of p-LKB1, p-AMPK and p-ACC (Fig. 3B). These results suggested that Notch1 maybe modulated AMPK signaling pathway during MI. In order to further characterize the relationship between Notch1 and AMPK signaling in response to ischemic insults, co-immunoprecipitates were implemented. The resulting immunoprecipitates were subjected to western blotting with anti-AMPK and anti-LKB1 antibody. As shown in Figure

3C, the Notch1 intracellular subunit (50–60 KD) co-precipitated with LKB1, but not co-precipitated with AMPK. Additionally, the interaction between Notch1 and LKB1 was verified by another co-immunoprecipitation assay with anti-LKB1 antibody. We also found that Notch1 intracellular fragment (50–60 KD) did co-precipitated with LKB1 (Fig. 3C). Intriguingly, the Notch1 inhibitor DBZ attenuates the interaction between Notch1 and LKB1 (Fig. 3C). Thus, the results suggest that Notch1 intracellular fragment (50–60 KD) forms a complex with LKB1, which modulates cardiac AMPK signaling pathway to prevent the heart from ischemic injury.

AMPK mediates the cardioprotection of Notch1 against myocardial infarction

To determine the importance of Notch1-AMPK signaling cascade in cardioprotection against ischemic injury during MI, we used the AMPK kinase dead (AMPK KD) transgenic mice to examine cardiac functions and the Notch1 level alterations. Reduction in EF and FS was evident in AMPK KD mice as compared with WT-MI group (Fig. 4A). The results of TTC staining shown myocardial infarction area significantly increased in AMPK KD mice as compared with WT-MI group (Fig. 4B). Moreover, the numbers of apoptosis positive cells and Mac-2 positive cells significantly increased in AMPK KD mice as compared with WT-MI group (Fig. 4B). Intriguingly, as shown in Figure 4C, the expression of Notch1 intracellular fragment in AMPK KD hearts after MI was significantly decreased as compared with WT-MI hearts. Moreover, p-LKB1 and p-ACC levels were not accordingly increased in AMPK KD mice (Fig. 4C). These results indicate that AMPK play a cardioprotective role after MI. When AMPK signaling pathway was blocked the Notch1 signaling pathway was blunted, indicating there be a feedback control mechanism existing between Notch1 and AMPK signaling in the heart.

Notch1-AMPK cascade optimizes cardiac energy metabolism during I/R

Since keeping the balance of energy production during stress conditions is the major function of the cardiac AMPK signaling pathway, the working heart model was used to determine the modulation of substrate metabolism by Notch1-AMPK signaling cascade under stress conditions. When the Notch1 ligand Jagged1 (10^{-7} M) was added in the heart perfusion, the expression of Notch1 was significantly increased in both WT mice and AMPK KD hearts. The expression of Notch1 intracellular fragment was slight increase in WT mice compared with AMPK KD mice, but there was no statistical difference between them (Fig. 5A). These results suggested cardiac Notch1 signaling pathway was activated by Jagged1. The isolated hearts of WT mice and AMPK KD mice were subjected to 10 min ischemia and 20 min reperfusion after 20 min balance perfusion. After IR, the level of Notch1 intracellular fragment was decreased mildly, and this decrease in WT mice was less than that in AMPK KD mice, with no statistical difference between these two kinds of mice. However, the use of Jagged1 as a ligand of Notch1 clearly triggered the phosphorylation of AMPK downstream acetyl-CoA carboxylase (ACC) in WT mice, but not in AMPK KD mice (Fig. 5B). During IR, glucose oxidation rates in WT vehicle hearts were significant lower than rates observed under aerobic conditions, and the oleate oxidation significantly increased (Fig. 5C and 5D). The effect of Jagged1 on glucose and fatty acid utilization rates in the presence of a physiological level of long chain fatty acid substrate oleate was determined. Intriguingly, Jagged1 treatment significantly inhibited oleate oxidation rates and augmented

the glucose oxidation rates (Fig. 5C and 5D). However, this metabolic-shift function of Jagged1 was abolished in the AMPK-KD heart perfusion experiments (Fig. 5C and 5D). Thus, the results suggested that Jagged1 activated Notch1 signaling pathway that modulates cardiac AMPK signaling cascade, which is a critical regulator of cardiac metabolic-shift under stress conditions, and Notch1 signaling may be feedback regulated by AMPK signaling pathway.

Discussion

This study for the first time demonstrates that the cardioprotection of Notch1 against myocardial infarction carry through LKB1-dependent AMPK signaling pathway. The results demonstrated that myocardial infarction can induce up-regulation of Notch1 expression in both cytosol and nucleus. It is interesting that the cardiac energy sensor AMPK signaling is associated with Notch1 signaling pathway. When Notch1 receptor was blocked by the inhibitor DBZ, the cardiac damage caused by myocardial infarction was significantly aggravated. Meanwhile, the phosphorylation of AMPK upstream LKB1, AMPK and AMPK downstream ACC are attenuated by inhibition of Notch1 signaling. This could be explained by the directly interaction between Notch1 intracellular fragment (50 KD–60 KD) and LKB1 which was found in co-Immunoprecipitation approaches. The cardiac damage by MI in AMPK KD mice are more severe than that in WT mice, but the compensatory increase of Notch1 couldn't reverse this dilemma. Furthermore, Notch1 ligand Jagged1 can trigger cardiac AMPK activation and modulate the energy metabolism during ischemia and reperfusion *ex vivo*, but Jagged1 did not have any effects on the metabolic regulation in AMPK KD hearts. The results suggest that Notch1 plays a cardioprotective role during myocardial infarction via interacting with LKB1 that modulating the activation of AMPK signaling pathways in the hearts.

LKB1 phosphorylates and activates AMPK when energy levels are low. In cells, LKB1 is found in a 1:1:1 heterotrimeric complex with the pseudokinase STRAD (STe20-Related ADaptor) and the scaffolding MO25 (MOuse protein 25). Zehiraj E's study reveals the structure of the LKB1-STRAD-MO25 complex and how LKB1 is activated. Activation of LKB1 is thought to be mediated through a conformational change triggered by binding to STRAD and MO25. The horseshoe-shaped MO25 α acts as a scaffold for assembly of the heterotrimer, by binding both LKB1 and STRAD α . And STRAD α adopt an active conformation, stabilized through ATP and MO25 to activate LKB1 [29]. However, there are other ways to activate LKB1. For example, Gaude's results establish that the Hsp90-Cdc37 complex controls both the stability and activity of the LKB1 kinase [30]. In our study, the Co-IP results demonstrated Notch1 complex with LKB1 in response to stress, but not with AMPK and p-LKB1. The supplementary data showed Notch1 do not complex with MO25 and STRAD α . We guess the complex Notch1 and LKB1 maybe could directly promote the phosphorylation and activation of LKB1.

CSL is the key transcriptional regulatory factor in the Notch signaling pathway, and the Notch/CSL-dependent pathway is called canonical Notch signaling pathway. Several observations indicate that canonical signaling is just the tip of the iceberg in the regulation of Notch. More and more ligand/CSL-independent Notch functions have been reported in

various systems across species, suggesting the existence of a CSL-independent Notch pathway [31, 32]. Many of these non-canonical Notch ligands contain EGF-like domains that characterize the canonical ligands, but some share very little similarity to the canonical ligands, which do not require the CSL interacting domain of Notch1 and was not mediated by CSL or known Notch target genes. Wnt/ β -catenin signaling is one such regulator which plays an important role in stem/progenitor cell self-renewal and differentiation processes, and in oncogenesis [33]. This form of β -catenin is dephosphorylated at Ser37 and Thr41 of membrane Notch and normally constitutes a small fraction of total β -catenin [48]. Active β -catenin has emerged as a conserved mediator of a ligand/CSL-independent Notch pathway across species.

In this experiment, there were three bands of Notch1 degradation products in the western blot results, which molecular weight were about 100 KD–150 KD, 60 KD–75 KD and 50 KD–60 KD. The third band about 60 KD -50 KD was the obvious change trend, though less quantity than the others. Some studies suggested that Notch levels are inversely correlated with active β -catenin, and increased levels of membrane Notch decrease active β -catenin levels and decreased levels of Notch increase active β -catenin levels [34]. It is surprising because γ -secretase inhibitors are widely used as a potent inhibitor of canonical Notch signaling. It could be explained that the expression of active NIC1 decreased after DBZ treatment in this study, and inhibited the quantities of NIC1/LKB1 complex, and reduced the cardioprotective effects. On the contrary, Jagged1 could up-regulate active NIC1 expression in both WT and AMPK KD hearts. The results suggest this active NIC1 maybe was a kind of active β -catenin, furthermore it could form complex with LKB1 to regulate AMPK signaling pathway, which possibly is a new non-canonical Notch signaling pathway (Fig. 6).

In conclusion, it is critical to identify the role of Notch1 in the LKB1-AMPK signaling pathway. In the next study, we will devote to find out the structure and functions of this active NIC1, and how it promotes activation of LKB1. As such, future investigation of the biological function and mechanism of the active NIC1/LKB1/AMPK pathway would greatly expand our fundamental knowledge of the cardioprotective effects of Notch1 signaling cascades.

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Figure 1. Notch1 signaling pathway was activated during myocardial infarction and could be suppressed by a γ -secretase inhibitor

(A) The mouse hearts were subjected to MI 1 week and 2 weeks, Western Blot analysis of production of the Notch1 intracellular fragments in AAR of the heart. β -actin was used as a loading control. (B) Western Blot analysis of the expression of Notch1 degradation fragment in nucleus and cytoplasm of AAR. β -actin and TATA-binding protein (TBP) were used as a loading controls. (C) Pretreated with DBZ an inhibitor of γ -secretase, the expression of Notch1 intracellular fragment and the transcription of target genes Hes1 and Hey1 after MI were analyzed by Western Blot and Q-PCR. β -actin was used as an internal control. All values expressed as mean $\pm$ SEM (n=6). *p<0.05 vs. Sham group; #p<0.05 vs. MI group.

Figure 2. Inhibition of Notch1 signaling pathway augmented the cardiac injuries during myocardial infarction

(A) The infarct size (pale white) and area at risk (red) in the heart were measured by postmortem dual dyes with triphenyl tetrazolium chloride (TTC) and Evans blue. INF/AAR ratio and AAR/LV (red/deep blue) ratio were analyzed. Bar=1 cm. (B) H&E staining of heart sections ($\times 10$) and representative M-mode echocardiograms in each group at 2 weeks after MI were showed. The left ventricle inner diameter during diastole (LVID d), left ventricle anterior wall thickness during diastole (LVAW d), ejection fraction (EF), and fractional shortening (FS) were measured. (C) The areas of fibrosis were measured in Masson staining sections after MI ($\times 200$). The mRNA levels of TGF- β , MMP-9 and TIMP-1 in the heart were measured by Q-PCR. (D) Apoptosis in the area at risk of the heart tissue was determined by TUNEL assay, myocardial tissue were identified by α -actinin antibody staining (red), and nuclei by DAPI staining (blue) ($\times 200$). The expression of caspase-3 protein in the heart was determined by immunohistochemistry. The numbers of TUNEL-positive cells and caspase-3-positive cells were quantified, and at least 200 cells per section were counted. Western blots analysis of expression of Bax and Bcl-2 and quantitative

analysis of Bax/Bcl-2 ratio. β -actin was used as a loading control. (E) H&E staining of heart sections after MI ($\times 200$). The expression of Mac-2 protein in the heart was determined by immunohistochemistry. The numbers of Mac-2-positive cells were quantified and at least 200 cells per section were counted. The mRNA levels of IL-6, IL-1 β , MCP-1, TNF- α , and Hmox-1 in the heart were measured by Q-PCR. Western blot analysis of protein levels of total and phosphorylated JNK1/2. β -actin was used as an internal control. Data are mean $\pm$ SEM (n=6). *p < 0.05 vs. Sham group, #p < 0.05 vs. MI group.

Figure 3. The interaction between Notch1 and LKB1 modulates cardiac AMPK signaling pathway

(A) The levels of phosphor- and total- LKB1 and AMPK in AAR of the heart after MI were analyzed in each group. The ratios of p-LKB1/LKB1 ratio and p-AMPK/AMPK were analyzed. (B) Pretreated with DBZ, the protein levels of phosphor- and total- LKB1, AMPK and ACC were analyzed by Western blot. The ratios of phosphor- and total- were analyzed. (C) Co-Immunoprecipitation was performed using anti-Notch1 antibody and anti-LKB1 antibody, and the precipitates were analyzed by western blot. β-actin was used as a loading control. Data are mean ± SEM (n=6). *p < 0.05 vs. Sham group, #p < 0.05 vs. MI group.

Figure 4. The cardiac damages were augmented in AMPK kinase-dead (KD) mice during myocardial infarction

(A) H&E staining of heart sections ($\times 10$) and representative M-mode echocardiograms in both WT and AMPK KD mice were showed after MI 2 weeks. EF and FS were measured by echocardiography. (B) The infarct size (pale white) and AAR (red) in the heart were measured by postmortem dual dyes with TTC and Evans blue ($\times 10$). INF/AAR ratio and AAR/LV ratio were analyzed. Bar=1 cm. The expression of Mac-2 protein in the heart was determined by immunohistochemistry ($\times 200$). Apoptosis in the AAR was determined by TUNEL assay, myocardial tissue were identified by α -actinin antibody staining (red), and nuclei by DAPI staining (blue) ($\times 200$). The numbers of TUNEL-positive cells and Mac-2-positive cells were quantified, and at least 200 cells per section were counted. (C) Western blot analysis of protein levels of Notch1 intracellular fragment, p-LKB1, LKB1, p-ACC and ACC in WT and AMPK KD mice, and β -actin was used as an internal control. Data are mean $\pm$ SEM (n=6). *p < 0.05 vs. Sham group, #p < 0.05 vs. WT MI group.

Figure 5. Jagged1 activated Notch1-AMPK signaling cascade and modulated energy metabolism in the isolated hearts

(A) Treated with ligand of Notch pathway Jagged1, the level of Notch1 intracellular fragment was analyzed in the *ex vivo* heart perfusion system by Western blot, and β-actin was used as a loading control. (B) The levels of phosphor- and total- ACC of WT and AMPK KD heart after 10 min ischemia and 20 min Jagged1 reperfusion were analyzed by western blot. (C) The hearts isolated from WT mice and AMPK KD mice suffered 30 min of equilibration, 10 min of ischemia and 20 min reperfusion with ¹⁴C-Glucose. The oxidation of glucose was measured. (D) Myocardial oxidation of fatty acid after 30 min of equilibration with the radio-labeled fatty acid (³H-Oleate) in the hearts isolated. The ³H-Oleate utilization was measured after 10 min of ischemia and 20 min reperfusion. Values are mean ± SEM (n=6). *p < 0.05 vs. Basal/vehicle group, #p < 0.05 vs IR vehicle.

Figure 6. Graphic representation of the crosstalk between Notch1 and AMPK in noncanonical signaling pathway

Canonical Notch signaling was activated through cleavage by TACE and γ -secretase and release the Notch intracellular fragments (NIC). NIC translocated to the nucleus and then binded with CSL to direct target genes expression. LKB1, one kind of upstream AMPK phosphorylating kinase initiated AMPK activation and AMPK phosphorylated ACC, whose process keeps the balanced between the Fatty acid oxidation and glycolysis during myocardial ischemia. Crosstalk between Notch and AMPK can occur through binding of LKB1 to NIC and activation of ACC. Collectively, these findings shed light on the new strategies for prevention of ischemic heart disease.