CFTR improves myocardial ischemic/reperfusion injury through activating FUNDC1-mediated mitophagy and regulating ATP and mitochondrial functions

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Research

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Abstract

Background

To investigate the potential role of CFTR in myocardial ischemic/reperfusion (I/R) injury and its relationship with mitophagy.

Methods

Wild type (WT) and age matched CFTR^{-/-} male mice were used to establish the myocardial I/R model. CFTR activator forskolin (FSK) was used to activate CFTR in mice. Hypoxia/reoxygenation (H/R) treatment was used for *in vitro* model in WT or CFTR^{-/-} cardiomyocytes. The autophagy inhibitor 3-MA and activator rapamycin was used for inhibition or activation of autophagy, respectively. The mitochondrial membrane potential (MMP) and ATP concentration were detected. Immunofluorescence was performed for measurement of mitochondria. Oxidative factors reactive oxygen species (ROS), superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione peroxidase (GSH-PX) were detected. The expression of CFTR, MMP-9, TNF- α , IL-8, LC3 II/I, beclin1, caspase-3, caspase-8, caspase-9, bax, bcl-2, p-62 and FUNDC1 was determined using western blotting or PCR.

Results

Knockdown of CFTR significantly increased the infarction volume and decreased the expression of autophagy related proteins beclin1 and LC3II/I in mice. In H/R cardiomyocytes, deficiency of CFTR by induced dysfunction of mitochondrial, decrease of ATP concentration and enhanced oxidative stress, as well as inhibited mitophagy and increased cell apoptosis related protein levels. When treated with 3-MA, the effects of overexpression of CFTR was remarkably reversed, while treatment of rapamycin significantly reversed the effects of inhibiting CFTR on both mitophagy, oxidative stress and cell apoptosis related proteins. The inhibition of FUNDC1 also reversed the above effects of overexpressing CFTR.

Conclusion

Inhibition of CFTR could promote myocardial I/R injury by suppressing FUNDC1-mediated mitophagy and activating of oxidative stress.

A Brief Summary

CFTR improves myocardial ischemia/reperfusion injury by activating FUNDC1-mediated mitochondrial phagocytosis and regulating ATP and mitochondrial function.

Background

Since reperfusion is still the main treatment method to rescue infarction for myocardial ischemic infarction patients, the ischemic/reperfusion (I/R) injury is still clinical challenge (1–3). Many molecular mechanisms and signaling pathways are involved during the I/R process, including activation of cell apoptosis pathways (4), induction of mitochondrial damage (5) and ATP dysfunction (6) and also the increase of oxidative stress (7).

In recent decades, role of autophagy in I/R injury has also been reported in many researches (8). The autophagy is considered as a “double-edged sword” in I/R injury with both harmful and beneficial effects (9). In general, it is considered that at the ischemic stage, normal autophagy may protect the cells and is essential for cardiomyocyte survival (10). However, at reperfusion stage, the inhibition of mitochondrial autophagy, mitophagy, will damage the cell function and induce further mitochondria and ATP dysfunctions (11–13). Besides, it is also reported that autophagy contributes to proteostasis and can remove cytotoxic ubiquitinated proteins in I/R injury (14, 15). However, deeper insights for how mitophagy participates in I/R injury and the molecular mechanisms are still inadequate.

The cystic fibrosis transmembrane-conductance regulator (CFTR) is a widely known ion channel related gene which belongs to the ATP-binding cassette (ABC) transporter superfamily. Studies show that CFTR plays important roles in regulation of Cl^- channel and ATP channel and also mediates nucleotide-regulated glutathione flux (16, 17). In recent years, role of CFTR in other diseases are also reported. It was found inhibition of CFTR might promote prostate cancer sensitivity to cisplatin by inhibition of autophagy (18). Another study also showed CFTR could inhibit epidermal growth factor receptor-dependent pro-inflammatory chemokine production (19). Interestingly, one study observed that the knockout of CFTR might lead to worse myocardial infarction animals with larger myocardial infarction volume (20). However, up to now, no study focused relationship between CFTR and mitophagy in myocardial I/R injury, as well as the underlying mechanisms.

In the present study, we aimed to illuminate the role of CFTR in myocardial I/R injury and its relationship with mitophagy. We found that inhibition of CFTR could facilitate myocardial ischemic/reperfusion injury by suppressing FUNDC1-mediated mitophagy and activating of oxidative stress. This study might give deeper insights for role of CFTR in I/R injury and might provide a new research target in myocardial I/R treatment.

Methods

Animals and treatment

The wild type and age matched CFTR^{-/-} mice (age 8 ~ 10 weeks, weight 20–30 g, male) were obtained from the Beijing Biocytogen Co., Ltd, Beijing, China. All animals were kept in a light-controlled room under a 12 h/12 h light/dark cycle and controlled temperature (23–25°C), and had free access to food and

water according to the Guide for the Care and Use of Laboratory Animals of Beijing Children's Hospital, Capital Medical University. The whole study was approved by the Institutional Animal Care Committee at Beijing Children's Hospital, Capital Medical University.

The establishment of myocardial I/R model was conducted by temporary ligation of the left anterior descending coronary artery. Briefly, mice were anaesthetized using 4% isoflurane, and the left anterior descending coronary artery was temporarily ligated. After 45 min ligation, the reperfusion was performed for 3 h. The sham group received the same operation with no ligation.

For activation of CFTR, mice received tail vein injection of CFTR activator forskolin (FSK, 2 mg/kg, Sigma-Aldrich, St. Louis, MO, USA). Mice were sacrificed after 72 h of the I/R injury. The 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO, USA)-staining was used for measurement of infarction volume which was calculated by ImageJ software (Rasband, NIH, USA).

Cell culture and transfection

The cardiomyocytes from normal mice or CFTR^{-/-} mice were isolated as reported elsewhere (21). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD, USA) containing 10% Gibco® FBS and 100 µg/mL penicillin-streptomycin (Thermo Fisher Scientific) at 5% CO₂ and 37°C. The hypoxia/reoxygenation (H/R) condition was performed by culturing in the hypoxia cabin under 2% O₂, 93% N₂ and 5% CO₂ for 2 h, followed with culturing under normoxic condition (37°C and 5% CO₂) for 4 h. For control cells, cells didn't receive hypoxia and were cultured under normoxic condition for 6 h.

For cell transfection, the sh-FUNDC1 and the corresponding sh-NC, as well as pcDNA3.1-CFTR and NC (GeneChem Corp) were used for transfecting the cells using the Lipofectamine 3000 (Invitrogen, Waltham, MA, USA) in serum-free Opti-MEM medium according to the manufacturer's instruction. To inhibit autophagy pathway, autophagy inhibitor 3-MA (3 mmol/L) was used to treat the cells and rapamycin (20 nM) was used for activation of autophagy.

Isolation of mitochondria

For isolation of mitochondria, cells were added by mitochondria isolation buffer (0.32 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, 2 mM PMSF, pH 7.4) (22). The samples were then centrifuged at 1200 × g for 3 min at 4 °C, followed with centrifugation at 18000 × g for 10 min at 4 °C to pellet mitochondria. The supernatant labeled as cytosolic fraction was collected.

Mitochondrial membrane potential assay

The mitochondrial membrane potential (MMP) was determined by using a JC-1 flow cytometry probe (BD Biosciences, San Diego, CA, USA). Briefly, after incubation with 10 µg/ml JC-1 in dark for 20 min at 37 °C, the cells were washed with PBS and were observed using a confocal microscope (Leica Microsystems, Heidelberg, Germany). The MMP was quantified by 590/488 fluorescent intensity ratio.

ATP concentration

The mitochondrial ATP concentration was measured using ATP determination kit according to manufacturer's instruction (Invitrogen, USA) (23). The ATP concentration was normalized to total protein.

Immunofluorescence

The cells were fixed, permeabilized and then incubated with anti-troponin T (ab8295, 1:500, Abcam, USA), anti-Tom20 (mitochondrial marker; ab186735, 1:1000, Abcam, USA) and anti-LAMP1 (lysosome marker; ab24170, 1:500, Abcam) overnight at 4 °C. Samples were then incubated with corresponding secondary antibody for 1 h at room temperature. A Leica TCS-SP laser scanning confocal microscope (Leica Microsystems) was used to observe the staining.

Measurement of oxidative factors

For measurement of oxidative stress, levels of reactive oxygen species (ROS), superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione peroxidase (GSH-PX) were detected. The ROS level was measured using the Fluorometric Intracellular ROS kit (Sigma-Aldrich; Merck KGaA). Levels of SOD, MDA and GSH-PX were determined by commercial kits from Nanjing Jiancheng Bio-Technology Co., Ltd., Nanjing, China.

TUNEL assay

Cell apoptosis was measured by TUNEL assay using an Apoptosis in Situ Detection Kit (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. A Leica TCS-SP laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany) was used to take the photomicrographs.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay

Briefly, total RNA was extracted using the TRIzol reagent (Tiangen Biotech, Beijing, China). A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used for determination of RNA concentration. Then a Prime-Script™ One Step RT-qPCR kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to convert RNA to cDNA. The qPCR reaction was performed in an Applied Biosystems 7500 Real Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Green PCR Master Mix (Solarbio Science & Technology Co., Ltd., Beijing, China). GAPDH was used as an internal reference. The relative expression level was calculated by the $2^{-\Delta\Delta C_q}$ method (24).

Western blotting

Samples were extracted from the cells following standard protocols, loaded on 10% SDS-PAGE and transferred to PVDF membranes. The membranes were then incubated with a primary antibody following with a conjugated secondary antibody. Antibodies used in Western blotting were listed below (all purchased from Abcam): anti-CFTR (ab186735, 1:1000), anti-MMP-9 (ab186735, 1:1000), anti-TNF- α

(ab186735, 1:1000), anti-IL-8 (ab186735, 1:1000), anti-LC3 II/I (ab186735, 1:1000), anti-beclin1 (ab186735, 1:1000), anti-caspase-3 (ab186735, 1:1000), anti-caspase-8 (ab186735, 1:1000), anti-caspase-9 (ab186735, 1:1000), anti-bax (ab186735, 1:1000), anti-bcl-2 (ab186735, 1:1000), anti-p-62 (ab186735, 1:1000), anti-FUNDC1 (ab186735, 1:1000). The films were scanned following with the EasySee Western Blot Kit (Beijing TransGen Biotech, Beijing, China). GAPDH was used as internal control.

Statistical analysis

The measurement data was expressed by mean $\pm$ SD. Comparison between two groups was performed using the Student t-test. Comparison among three or more groups was conducted using one-way analysis of variance (ANOVA). It was considered statistically significant when P-value was less than 0.05. All calculations were made using SPSS 18.0.

Results

Knockdown of CFTR aggravated the myocardial I/R injury and inhibited the autophagy in mice

First, we determined effects of knockdown of CFTR on I/R injury *in vivo*. As shown in (Fig. 1A), in normal mice, when CFTR was activated by FSK, the infarction volume was significantly smaller than the I/R group. However, in CFTR^{-/-} mice, FSK didn't influence the infarction volume which was induced by I/R injury. However, no difference was found between I/R mice with/without FSK in CFTR^{-/-} mice. TUNEL assay showed I/R injury induced cell apoptosis in both wild type and CFTR^{-/-} mice and troponin T was also enhanced in I/R cells (Fig. 1B). However, activation of CFTR by FSK only reduced cell apoptosis and troponin T in wild type mice. Further analysis for inflammatory proteins and autophagy proteins showed I/R injury remarkably increased the levels of inflammatory proteins MMP-9, TNF- α and IL-8, as well as reduced the LC3 II/I level (Fig. 1C-D). The treatment of FSK significantly reduced the levels of inflammatory proteins and enhanced LC3 II/I ratio in wild type mice. However, no significant difference was found in CFTR^{-/-} mice when treated by FSK. All these results indicated that knockdown of CFTR aggravated the myocardial I/R injury and inhibited the autophagy in mice.

Inhibition of CFTR induced dysfunction of mitochondrial function and enhanced oxidative stress in H/R cardiomyocytes

Then we further investigated effect of CFTR on mitochondrial function in H/R cell model. The expression of CFTR was successfully suppressed in cells from CFTR^{-/-} mice (Fig. 2A). It was found treatment with H/R condition significantly decreased MMP level and ATP concentration in mitochondria, and increased the levels of ROS and MDA, as well as decreased levels of SOD and GSH-PX (Fig. 2B-C). When CFTR was knocked down, the alteration by H/R treatment was remarkably heavier compared with NC cells, with

significantly lower MMP level and ATP concentration, higher ROS and MDA levels and lower SOD and GSH-PX levels. All these indicated that the inhibition of CFTR both induced mitochondrial function and enhanced oxidative stress in H/R cardiomyocytes.

Inhibition of CFTR suppressed mitophagy and induced mitochondrial cell apoptosis in H/R cardiomyocytes

Further researches for effects of CFTR on mitophagy and cell apoptosis showed the H/R treatment significantly up-regulated the level of LC3 II/I and beclin1 and decreased p-62 level in cytoplasm, as well as down-regulated the level of LC3 II/I and beclin1 and increased p-62 level in mitochondria, and the inhibition of CFTR aggravated the effects (Fig. 3A). The mitochondrial lysosome double staining also showed that H/R treatment obviously induced mitochondrial dysfunction and when inhibition of CFTR, the dysfunction was heavier (Fig. 3B). The cell apoptosis biomarkers also showed H/R significantly increased the expression of caspase-3, caspase-8, caspase-9 and bax, as well as significantly decreased the expression of bcl-2 (Fig. 3C-D). And knockdown of CFTR remarkably aggravated the effects by H/R, suggesting that inhibition of CFTR induced suppression of mitophagy and cell apoptosis.

CFTR regulated mitochondrial function and oxidative stress through mitophagy in H/R cardiomyocytes

To further study relationship between CFTR and mitophagy, CFTR was overexpressed in cells and rapamycin and 3-MA were used to activate or inhibit autophagy, respectively. As shown in (Fig. 4A), when transfected with pcDNA3.1-CFTR, the expression of CFTR was significantly enhanced, suggesting the successful overexpression. The MMP level and ATP concentration in mitochondria were remarkably decreased in CFTR deficiency cells, and treatment of rapamycin significantly increased the MMP level and ATP concentration (Fig. 4B-C). Meanwhile, when overexpressing CFTR by transfection of pcDNA3.1-CFTR, the decreased levels of MMP and ATP concentration were remarkably enhanced, and inhibition of autophagy by 3-MA significantly reverse the effects. Similar results were also found for oxidative stress. Rapamycin improved the promotion effects of inhibiting CFTR on oxidative stress, and 3-MA reversed the inhibition effects of overexpressing CFTR on oxidative stress (Fig. 4C). These results further demonstrated that CFTR regulated mitochondrial function and oxidative stress through mitophagy.

CFTR regulated cell apoptosis through mitophagy in H/R cardiomyocytes

The effects of CFTR on mitophagy and cell apoptosis related proteins were then investigated. It was observed that the inhibition of CFTR significantly reduced the LC3 II/I ratio and beclin1 level, as well as enhanced p-62 level in H/R cells, while overexpression of CFTR led to opposite results (Fig. 5A).

Rapamycin remarkably increased the LC3 II/I ratio and beclin1 level, as well as decreased p-62 level in mitochondria and thus reversed the effects of CFTR inhibition. And treatment of 3-MA led to opposite results and thus reversed the effects of CFTR overexpression. Similar results were also found for apoptosis related proteins. Rapamycin significantly decreased the levels of caspase-3, caspase-8, caspase-9 and bax, as well as remarkably increased the expression of bcl-2, which were changed by inhibition of CFTR, while 3-MA remarkably reversed the effects of overexpression of CFTR on apoptosis proteins (Fig. 5B). All these results suggested that CFTR might regulate cell apoptosis through regulation of mitophagy.

Activation of CFTR improved mitochondrial function and inhibited cell apoptosis through FUNDC1-mediated mitophagy in H/R cardiomyocytes

At last, we investigated the relationship between CFTR and FUNDC1 signaling in the above process. As shown in (Fig. 6A-B), FUNDC1 was inhibited by sh-FUNDC1. When transfected with pcDNA3.1-CFTR, the expression of FUNDC1 was remarkably increased. Meanwhile, the activation of mitophagy by CFTR was dramatically suppressed by inhibition of FUNDC1. Besides, the inhibition effect for cell apoptosis proteins caspase-3, caspase-8, caspase-9 and bax was also reversed by transfection of sh-FUNDC1, while bcl-2 showed opposite results (Fig. 6C). These results indicated CFTR could activate mitophagy through activation of FUNDC1.

Discussion

Despite numerous studies on myocardial ischemic/reperfusion injury, the molecular mechanisms for myocardial I/R injury are still unclear. As an old ion channel related protein, the role of CFTR in many other bioprocesses have also been noticed in recent years, such as cancer development and lung function (25, 26). However, to our best of knowledge, few studies reported the relationship between CFTR and mitophagy in myocardial I/R injury. In the present study, we demonstrated for the first time that inhibition of CFTR might aggravate the myocardial I/R injury by inhibition of FUNDC1-mediated mitophagy and promotion of oxidative stress.

The autophagy plays both detrimental and beneficial roles in I/R injury. And generally, mitophagy is considered to be beneficial to recover ATP function and clean up damaged mitochondria during I/R process (27). Zhang et al reported that FUNDC1-mediated mitochondria protected heart from I/R injury through maintaining the mitochondrial homeostasis (28). In a recent study, Yu et al found through inhibition of FUNDC1-mediated mitophagy, MST1 could facilitate the cardiac I/R injury (29). In the present study, we also observed obvious inhibition of mitophagy during the I/R or H/R process. Moreover, we confirmed that the inhibition of CFTR would aggravate the mitophagy inhibition.

Role of CFTR in cardiovascular and cerebrovascular diseases has been reported in several studies. An early research reported that deficiency of CFTR in mice would remarkably enhance the myocardial infarction volume (20). Another study also showed the activation of CFTR could reduce the myocardial I/R injury (30). Thus, it can be suspected that CFTR plays important roles in protection against I/R injury. Meanwhile, it is also observed that in cerebral I/R injury, CFTR could reduce the I/R injury by recovering the mitochondrial function and reducing oxidative stress (22). However, whether and how CFTR influences myocardial I/R injury is still unknown. In our research, we demonstrated that the activation of CFTR could activate FUNDC1-mediated mitophagy and reduce oxidative stress in myocardial I/R injury.

Relationship between CFTR and autophagy has also been noticed in previous researches. Tazi et al demonstrated that CFTR function was correlated with autophagy in cystic fibrosis macrophages (31). Xia et al showed by promoting CFTR ubiquitination, MARCH2 could activate autophagy both *in vivo* and *in vitro* (32). The activation effect of CFTR on autophagy was also found in prostate cancer. (18) However, relationship between CFTR and mitophagy is rarely reported. Here, we observed CFTR could also activate mitophagy in cardiomyocytes, and deficiency of CFTR might result in worse I/R injury.

Conclusion

In conclusion, the present study demonstrates that inhibition of CFTR can aggravate myocardial ischemic/reperfusion injury by inhibition of FUNDC1-mediated mitophagy and activation of oxidative stress. This study provides a new molecular mechanism for development of myocardial ischemic/reperfusion injury. And CFTR also has the potential to be a treatment target for I/R injury in future researches. What's more, the results provide potential significance to human health and disease, CFTR agonist may present new strategies for clinical application and maybe in the foreseeable future, myocardial ischemic/reperfusion injury would be treated as well as possible.

Abbreviations

The ischemic/reperfusion (I/R);The cystic fibrosis transmembrane-conductance regulator (CFTR);Wild type (WT);forskolin (FSK);The mitochondrial membrane potential (MMP);reactive oxygen species (ROS), superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione peroxidase (GSH-PX).

Declarations

Ethics approval and consent to participate

The ethic approval was obtained from the Ethic Committee of Beijing Children's Hospital, Capital Medical University, National Center for Children's Health.

Consent to publish

All of the authors have Consented to publish this research.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

All authors declare no conflict of interest.

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Authors' contributions

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Knockdown of CFTR aggravated the myocardial I/R injury and inhibited the autophagy in mice. (A) TTC staining of mice heart tissues for different groups. (B) TUNEL staining and Immunofluorescence for troponin T. (C) Expression of CFTR in different groups of mice. (D) protein levels of MMP-9, TNF- α , IL-8, LC3 II/I by western blotting. ***P<0.001.

Figure 2

Inhibition of CFTR induced dysfunction of mitochondrial function and enhanced oxidative stress in H/R cardiomyocytes. (A) Expression of CFTR by RT-qPCR and western blotting. (B) Immunofluorescence of Tom2, mitochondrial marker, LAMP1, lysosome marker. (C) MMP and ATP levels, as well as ROS, MDA, SOD and GSH-PX levels. **P<0.01, ***P<0.001.

Figure 3

Inhibition of CFTR suppressed mitophagy and induced mitochondrial cell apoptosis in H/R cardiomyocytes. (A) Expression of LC3 II/I, beclin1, and p62 in cytoplasm and mitochondria. (B) Immunofluorescence of Tom2, mitochondrial marker, LAMP1, lysosome marker. (C) and (D) Protein levels of caspase-3, caspase-8, caspase-9, bax and bcl-2 by western blotting. **P<0.01, ***P<0.001.

Figure 4

CFTR regulated mitochondrial function and oxidative stress through mitophagy in H/R cardiomyocytes. (A) Expression of CFTR by RT-qPCR and western blotting. (B) Immunofluorescence of Tom2, mitochondrial marker, LAMP1, lysosome marker. (C) MMP and ATP levels, as well as ROS, MDA, SOD and GSH-PX levels. **P<0.01, ***P<0.001.

Figure 5

CFTR regulated cell apoptosis through mitophagy in H/R cardiomyocytes. (A) Expression of LC3 II/I, beclin1, and p62 in cytoplasm and mitochondria. (B) Protein levels of caspase-3, caspase-8, caspase-9, bax and bcl-2 by western blotting. **P<0.01, ***P<0.001.

Figure 6

Activation of CFRT improved mitochondrial function and inhibited cell apoptosis through FUNDC1-mediated mitophagy in H/R cardiomyocytes. (A) expression of CFTR and FNDC1, as well as LC3 II/I, beclin1, and p62 in mitochondria. (B) MMP and ATP levels, as well as ROS, MDA, SOD and GSH-PX levels. (C) Protein levels of caspase-3, caspase-8, caspase-9, bax and bcl-2 by western blotting. **P<0.01, ***P<0.001.