Klotho overexpression suppresses apoptosis by regulating the Hsp70/Akt/Bad pathway in H9c2(2-1) cells

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Abstract. Early reperfusion is the most effective and important treatment for acute myocardial infarction. However, reperfusion therapy often leads to a certain degree of myocardial damage. The aim of the present study was to identify the role of klotho, and the molecular mechanism underlying its effects, in myocardial damage using a model of myocardial hypoxia injury. Hypoxia/reoxygenation (H/R) was used to mimic ischemia/reperfusion (I/R) injury in vitro. The expression and distribution of klotho in H9c2(2-1) cells was observed by fluorogenic scanning, and the apoptotic rate was determined by Annexin V-FITC/propidium iodide dual staining. Cell viability was determined by MTT assay, and caspase-3, cleaved caspase-3, Bcl-2, Bax, heat shock protein (Hsp) 70 and Akt levels were assessed by western blotting. A lactate dehydrogenase test was performed to determine the degree of H9c2(2-1) cell damage. The results revealed that klotho was primarily located in the cytoplasm of H9c2(2-1) cells. Klotho overexpression markedly suppressed H/R-induced H9c2(2-1) cell apoptosis. Furthermore, cell viability increased, and injury decreased in response to klotho. Klotho also suppressed the activation of caspase-3, upregulated Bcl2 and decreased Bax levels following H/R injury, as well as alleviating H/R injury by upregulating the expression of Hsp70 and increasing the levels of phosphorylated (p)-Akt and Bad. In conclusion, these results indicate that klotho suppressed H/R-induced H9c2(2-1) cell apoptosis by regulating the levels of Hsp70, p-Akt and p-Bad, which suggest that klotho could be a novel agent for the treatment of coronary disease.

Introduction

Globally, acute myocardial infarction (AMI) is associated with high mortality and morbidity rates (1,2). Reperfusion therapy is the most effective way to treat this disease in a clinical setting (3). Though the therapeutic aim is to restore blood flow to the affected area, restoration of the blood supply often activates a cascade of cellular damage mechanisms, which may further aggravate myocardial cell pathology, namely myocardial ischemia/reperfusion injury (MI/R) (4). Cellular ischemic injury involves multiple injury mechanisms, such as abnormal calcium regulation in cells (5,6), free radical production (7), mitochondrial damage (8) and the activation of apoptotic pathways (9). The cytotoxic cascade leads to the excessive production of reactive oxygen species (ROS), and is considered to be the primary factor responsible for myocardial contractile dysfunction, cell death and inflammation (10). These injuries may have serious consequences, such as heart failure or death. Therefore, there is an urgent requirement for further investigation of the pathogenesis of AMI, and to develop new therapeutic compounds and strategies.

The pathological mechanisms underlying ischemia-reperfusion injury (IRI) are complex, and include autophagy, cardiomyocyte apoptosis, cellular infiltration, calcium overload, oxidative stress, energy metabolism disorder and vascular endothelial dysfunction (11-13). There are multiple signaling pathways involved in I/R injury, such as the matrix metalloenzyme-associated, ATP-sensitive potassium channel, angiotensin II and Fas signaling pathways. Klotho is a protein that is involved in human aging and the length of the human lifespan (14,15). A large number of previous studies have revealed that klotho is involved in the occurrence and development of various human diseases, including chronic kidney disease (15,16), arteriosclerosis (17), myocardial hyper trophy (18,19), diabetes and obesity (20-22), as well as various types of cancer (23,24).

Klotho protein has been reported to exert anti- or pro-apoptotic functions in different diseases (25,26). In an acute pancreatitis model, klotho was found to alleviate inflammation and apoptosis (27). Sugiura et al (28) found that klotho is involved in the pathophysiology of renal IRI, and
that it alleviated apoptosis in a renal IRI model via heat shock protein (Hsp) 70. In an oxidative damage model, klotho was reported to attenuate oxidant-induced alveolar epithelial cell apoptosis and mitochondrial DNA damage (29). Moreover, klotho was also found to suppress ROS-induced apoptosis to improve cardiac function (30). In a stress-induced cardiac injury model, klotho inhibited cardiomyocyte apoptosis partly by suppressing the activation of the p38 and JNK pathways (31). Additionally, klotho inhibited the effects of dexamethasone via the NF-κB signaling pathway in MC3T3-E1 osteoblasts (32). Moreover, in human umbilical vein endothelial cells, klotho suppressed apoptosis by reducing the activation of the PI3K/AKT pathway (33). Thus, klotho may be a potential therapeutic target for acute inflammatory disease.

The present study aimed to identify whether klotho exerts a protective effect on hypoxia/reoxygenation (H/R) injury in H9c2(2-1) cells, as well as the potential molecular mechanisms underlying this process. The results provide a useful reference for clarifying the molecular mechanisms of klotho during H/R progression, and suggest klotho as a potential therapeutic target for acute MIRI.

Materials and methods

**Regents and cell lines.** The H9c2(2-1) rat heart myoblast cell line (ATCC® CRL-1446™) was purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The pCMV3-C-HA negative control vector (C-terminal HA-tagged) and klotho cDNA ORF clone were purchased from Sino Biological, Inc. The klotho overexpression lentivirus and control plasmid lentivirus were constructed by and purchased from Kilton Biotechnology (Shanghai) Co., Ltd. Klotho shRNA (m) and control shRNA lentiviral particles (containing a scrambled shRNA sequence) were purchased from Santa Cruz Biotechnology, Inc.

**H/R injury model.** H9c2(2-1) cells were precultured in serum-free DMEM overnight prior to H/R injury. Then, the cells were cultured and exposed to hypoxic conditions for 4 h using an Anaeropack pouch (Mitsubishi Gas Chemical). The hypoxic conditions were 37°C, 2% O₂, and 5% CO₂. After 4 h, reoxygenation was achieved by returning the cells to normal culture conditions, which involved incubation at 37°C in a humidified atmosphere containing 95% air and 5% CO₂ for 4 h.

**Transfection.** H9c2(2-1) cells were plated into 6-well plate at the cell density of 4x10⁵ cells/well 10 h prior to transfection. The transfection agent Lipofectamine® 2000 was obtained from Invitrogen; Thermo Fisher Scientific, Inc. For transfection, 1 µg pCMV3-C-HA negative control vector or 1 µg klotho cDNA ORF clone was diluted in 100 µl Opti-MEM (Thermo Fisher Scientific, Inc.); 4 µl of Lipofectamine® 2000 was diluted in 200 µl of Opti-MEM. The mixtures were incubated at room temperature for 5 min. Then, 100 µl plasmid dilution liquid and 100 µl diluted Lipofectamine® 2000 liquid was mixed gently and kept at room temperature for 20 min. The transfection mixture was added into each 6-well plate at room temperature. After 6 h, the transfection medium was discarded and 2 ml completed DMEM medium was added.

The transfected cells were cultured for 24 h and the target proteins such as caspase-3, Bcl2 and Bax were detected by western blotting after H/R treatment.

Additionally, for shRNA lentiviral particles transduction, Klotho shRNA (m) and control shRNA lentiviral particles were used in order to knockdown klotho, H9c2(2-1) cells. Firstly, the cells were grown to ~50% confluency and a mixture of complete DMEM medium with Polybrene® (cat. no. sc-134220; Santa Cruz Biotechnology, Inc.) at a final concentration of 5 µg/ml was prepared. 10 µl klotho shRNA lentivirus or 10 µl control shRNA lentivirus was added and cultured at 37°C for 24 h. Subsequently, the culture medium was removed and the complete DMEM medium (without Polybrene®) was replaced, and the cells were cultured for another 24 h. The stable clones expressing the shRNA were selected and the levels of klotho, p-Akt and total Akt were detected by western blotting. Percentages of apoptotic H9c2(2-1) cells transfected with klotho or control vector were subsequently investigated.

**Flow cytometric analysis.** H9c2(2-1) cells were infected with klotho lentivirus or control plasmid lentivirus for 24 h prior to H/R treatment. Apoptosis was assessed by Annexin V-FITC/prodium iodide (PI) staining according to the kit protocols (Santa Cruz Biotechnology, Inc.). For flow cytometric analysis, the cells were digested with 0.25% trypsin for 1 min and washed twice with precooled phosphate-buffered saline (PBS). The cells were then resuspended in 500 µl binding buffer with Annexin V-FITC (0.1 µg/µl) and PI (0.05 µg/µl) in the dark for 15 min on ice. From each sample, 1x10⁴ cells were collected for detection. The samples were then run through a flow cytometer (BD LSRFortessa X-20; BD Biosciences) and the data was analyzed using FlowJo v10 software (FlowJo LLC).

**MTT assay.** Cell viability was determined with an MTT assay. Briefly, H9c2(2-1) cells were transfected with klotho or negative control lentivirus for 24 h. Then, 3x10⁴ cells/well were plated into 96-well plates for 6 h and treated by H/R injury as aforementioned. Then, 10 µl MTT reagent was added to each well and the target protein was detected by MTT assay. Cell viability = (OD₅₇₀ nm of treated group-OD₅₇₀ nm of blank wells)/(OD₅₇₀ nm of untreated group-OD₅₇₀ nm of blank wells) x100%.

**Lactate dehydrogenase (LDH) assay.** Cellular injury was evaluated using the LDH test. LDH is a stable and abundant cytoplasmic enzyme that is unable to pass through the cell membrane. When a cell is damaged or dead, LDH is quickly released into the cell culture medium. Thus, LDH activity in the supernatant is proportional to the number of dead cells. Briefly, H9c2(2-1) cells were infected with klotho lentivirus or control plasmid lentivirus for 24 h prior to H/R treatment. Cell supernatants were obtained from each group and LDH activity was determined using an LDH release assay according to the manufacturer's protocol (Nanjing KeyGen Biotech Co., Ltd.).

**Scanning confocal microscopy.** H9c2(2-1) cells were infected with klotho lentivirus or control plasmid lentivirus for 24 h prior to H/R treatment. Then, the cells were washed twice with PBS and fixed with 100% methanol for 5 min at room temperature.
Next, the cells were permeabilized with 0.1% Triton X-100 for 5 min and treated with 2% BSA in 0.1% PBS-Tween for 1 h to block non-specific protein-protein interactions at room temperature. Primary Hsp70 antibody was added, and the cells were incubated overnight at 4˚C. The Anti-Hsp70 antibody [EPR16892 (Alexa Fluor® 647; cat. no. ab204691)] was purchased from Abcam. The cell nuclei were stained with DAPI at a concentration of 1.43 µM, and were observed by fluorescence confocal microscopy at x200 magnification. In an alternative assay, H9c2(2-1) cells were infected, permeabilized and blocked as aforementioned, and then stained with Hoechst 33258 (Qcbio Science & Technologies Co. Ltd.) to assess the effect of klotho overexpression on the morphology of H/R-induced apoptotic cells.

Western blot analysis. The levels of caspase-3, Bax, Bcl-2, klotho, Hsp70, phosphorylated (p-)Akt, Akt, p-Bad and Bad were detected by western blotting. Cell lysis buffer was purchased from Beyotime Institute of Biotechnology (cat. no. P0013). Briefly, cell lysates were prepared and total protein concentration was determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). A 20-µg sample of total protein was loaded into each lane and separated by 10% SDS-PAGE. The proteins were then transferred onto PVDF membranes at 300 mA for 2 h. The membranes were blocked with 5% non-fat milk at room temperature for 40 min, and then incubated with primary antibodies at a ratio of 1:2,000 overnight at 4˚C. The following primary antibodies were used: Anti-caspase-3 (cat. no. 3138-100; BioVision, Inc.), anti-Bcl-2 (cat. no. AP1303a-ev; Abgent, Inc.), anti-Bax (cat. no. AP1302a-ev; Abgent, Inc.), recombinant anti-Hsp70 (EPR16892; cat. no. ab181606; Abcam); anti-Klotho (EPR6856; cat. no. ab181373; Santa Cruz Biotechnology, Inc.); anti-p-Akt (B-5; cat. no. sc-271966; Santa Cruz Biotechnology, Inc.); anti-Akt (EPR16798; cat. no. ab179463; Abcam), anti-Bad [phospho S112 (EPR1891(2)); cat. no. ab129192; Abcam]; anti-Bad (cat. no. ab90435; Abcam) and β-actin (cat. no. AM1021B; Abgent, Inc.). The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The protein bands were detected in a dark room using an ECL detection kit (Cytiva).

Statistical analysis. All results were analyzed with SPSS 20.0 software (IBM Corp) using one-way analysis of variance followed by Tukey's post hoc test. The independent samples were analyzed using an unpaired t-test, including the comparison between klotho protein expression in H/R-injured cells and control H9c2(2-1) cells. P<0.05 was considered to indicate a statistically significant difference.

Results

Klotho is primarily located in the cytoplasm of H9c2(2-1) cells. In order to determine the role of klotho in the H/R cardiomyocyte model, H9c2(2-1) cells were transfected the klotho cDNA ORF clone and its negative control pCMV3-C-GFPSpark vector (with C-terminal GFPSpark-tag). After 24 h, the distribution of klotho in H9c2(2-1) cells was determined by confocal microscopy. As shown in Fig. 1, klotho was primarily distributed in the cytoplasm of H9c2(2-1) cells.

Overexpression of klotho markedly inhibits the H/R-induced apoptosis of H9c2(2-1) cells. Klotho levels were first detected in H/R-treated H9c2(2-1) cells by western blotting. As shown in Fig. 2A, klotho protein expression was reduced by H/R injury. Additionally, to further confirm the role of klotho in apoptosis, Hoechst 33258 was used to stain the nuclei and to
detect the effects of klotho overexpression on the morphology of H/R-induced H9c2(2-1) cells. The results revealed that the nuclear membrane of some cells shrank, the nuclear chromatin appeared dense with enhanced fluorescence staining, and nuclear fragmentation and apoptotic body formation were apparent in a number of cells. Compared with those of the control cells, the nuclei of the klotho lentivirus-infected H9c2(2-1) cells were smooth, intact and uniform in density, and the chromatin in the nucleus was evenly stained. These results revealed that klotho overexpression alleviated apoptosis induced by H/R injury (Fig. 2B).

To determine the role of klotho in H/R-induced apoptosis, H9c2(2-1) cells were infected with klotho lentivirus or the control vector lentivirus for 24 h followed by H/R treatment. The apoptotic rate in each group was determined by Annexin V-FITC/PI dual staining. As shown in Fig. 2C, under
H/R conditions, the apoptotic rate was significantly higher in control vector lentivirus-infected cells. However, infection with klotho plasmid lentivirus significantly inhibited the apoptosis with H/R injury. These results revealed that H/R treatment markedly increased the number of apoptotic cells, while klotho overexpression inhibited the H/R-induced apoptosis of H9c2(2-1) cells.

Klotho suppresses the activation of caspase-3, upregulates Bcl2 and downregulates Bax expression following H/R treatment. In order to investigate the inhibitory effects of klotho on H9c2(2-1) cell apoptosis after H/R treatment, the levels of pro- and cleaved caspase-3 were detected by western blotting. As shown in Fig. 3A, H/R treatment markedly increased the levels of pro- and cleaved caspase-3 in H9c2(2-1) cells, while infection with the klotho lentivirus inhibited the increase in these proteins, compared with those in the control plasmid-infected group. Moreover, the ratio of Bcl-2 to Bax is a key factor in reflecting apoptosis induced by various stimuli. Thus, the levels of Bcl-2 and Bax were also detected in H/R-treated H9c2(2-1) cells after infection with klotho lentivirus or control vector lentivirus. As shown in Fig. 3B, klotho induced a higher level of Bcl-2 and decreased the level of Bax in H9c2(2-1) cells compared with the control vector lentivirus after H/R injury. These results demonstrate that H/R treatment promoted the activation of pro-caspase-3, and that klotho overexpression inhibited H/R injury-induced apoptosis by inhibiting caspase-3 activation and increasing the ratio of Bcl-2 to Bax. Taken together, these data reveal that klotho has an anti-apoptotic effect on H/R-induced apoptosis in H9c2(2-1) cells.

Klotho retains cell viability and decreases H9c2(2-1) cell injury. MTT and LDH assays were used to evaluate the effects of klotho on myocardial cell viability and damage after H/R treatment. Cardiomyocytes were treated with H/R for 4 h, and the cells were then transfected with klotho or control plasmid for 24 h. As shown in Fig. 4A, cell viability decreased after H/R treatment for 4 h, and klotho retained H9c2(2-1) cell viability following H/R treatment (W0.01 compared with the control group). The level of LDH can represent the degree of cardiomyocyte damage, and cardiomyocyte injury results in increased LDH levels. As shown in Fig. 4B, LDH levels were significantly higher in H/R-treated cells than in normal cells, suggesting that H/R results in cellular injury. The level of LDH in the cardiomyocyte culture medium

Figure 3. Klotho suppresses the activation of caspase-3, upregulates Bcl2, and decreases the level of Bax after H/R treatment. H9c2(2-1) cells were transfected with klotho or control plasmids for 24 h, and were then treated with H/R. (A) Levels of pro-caspase-3 and cleaved caspase-3, and (B) Bax and Bcl-2 were detected by western blotting. β-actin was used as the internal reference gene. H/R, hypoxia/reoxygenation; Ctrl, control.

Figure 4. Klotho retains viability and decreases injury in H9c2(2-1) cardiomyocyte cells. H9c2(2-1) cells were transfected with a klotho plasmid or control vector for 24 h prior to H/R treatment. (A) MTT was used to assess cellular viability in each group. **P<0.01 and ##P<0.01. (B) LDH assay was performed to determine the degree of cardiomyocyte damage. LDH levels were tested using cultured medium **P<0.01 and ##P<0.01. H/R, hypoxia/reoxygenation; Ctrl, control; LDH, lactate dehydrogenase.
was significantly lower in the klotho-transfected group than in the control group (P<0.01). These results demonstrate that klotho reduces myocardial cell damage.

Overexpression of klotho in H/R-treated H9c2(2-1) cells increases the levels of Hsp70, p-Akt and p-Bad. It has been reported that klotho mitigates apoptosis in cells involved in ischemic acute kidney injury by regulating the level of Hsp70 (28). The present study investigated whether Hsp70 was involved in IRI in klotho-overexpressing H9c2(2-1) cells. Firstly, cells were infected with klotho lentivirus for 24 h prior to H/R treatment, and Hsp70 expression was detected by confocal laser scanning microscopy and western blotting. As shown in Fig. 5A, Hsp70 and klotho were colocalized in the cytoplasm of H/R-treated cells. Moreover, western blotting revealed that klotho overexpression in H9c2(2-1) cells with H/R injury was accompanied by increased levels of Hsp70, p-Akt and p-Bad; the level of total Akt was not markedly altered, and total Bad was markedly decreased in klotho lentivirus-infected cells (Fig. 5B). Conversely, klotho-knockdown with H/R injury resulted in decreased expression of Hsp70 and p-Akt, though total Akt levels were unchanged in klotho shRNA lentivirus-infected cells (Fig. 5C). These results show that klotho overexpression alleviated H/R injury in H9c2(2-1) cells by upregulating the levels of Hsp70, p-Akt and p-Bad.

Discussion

AMI is a disease with high global incidence and mortality rates (34). MIRI, which refers to the interruption of blood...
supply to the myocardium during pathological injury, is the most common cause of AMI (35). After the blood supply is restored, the original ischemic myocardial injury does not improve, but instead shows more serious damage than that prior to blood supply recovery. In the present study, a H/R injury model was constructed using H9c2(2-1) cells to mimic acute MIRI. The protective role of Klotho on myocardial injury was subsequently identified, and klotho was found to suppress apoptosis by upregulating the levels of Hsp70, p-Akt and p-Bad.

H/R injury significantly increased the apoptotic rate of H9c2(2-1) cells and also resulted in decreased klotho expression. Apoptosis was flow cytometrically evaluated by Annexin V-FITC/PI dual staining, and the results showed that the apoptotic rate of H9c2(2-1) cells was markedly increased in the H/R injury group compared with that of the control group. Furthermore, klotho overexpression prior to H/R treatment significantly inhibited H/R-induced apoptosis. The effect of Klotho on H9c2(2-1) cell viability was subsequently assessed using MTT. The results indicated that H/R treatment reduced the survival rate of cardiomyocytes and that klotho overexpression significantly inhibited cell injury and death. Additionally, when myocardial cells are damaged, LDH level increases, thus LDH level represents the degree of myocardial cell damage. An LDH assay was performed to assess H9c2(2-1) cell injury following H/R; the results showed that H/R treatment increased LDH release, suggesting that H/R results in cardiomyocyte injury. However, the LDH level in the cardiomyocyte culture medium of the klotho overexpression group was significantly lower than that in the control group (P<0.01), suggesting that klotho overexpression inhibited H9c2(2-1) cell damage.

Next, the molecular mechanism by which klotho inhibits apoptosis was investigated by western blotting. The results showed that klotho overexpression inhibited the activation of caspase-3, increased the Bcl-2/Bax ratio, as well as the level of p-Bad in H9c2(2-1) cells, compared with those in control vector lentivirus-infected cells. Furthermore, colocalization of Hsp70 and klotho was noted in the cytoplasm of H/R-treated cells via confocal laser scanning microscopy and western blotting. These results suggest that klotho and Hsp70 were co-localized in the cytoplasm and inhibited the apoptosis of H/R-treated cells via hsp70. This was consistent with the findings of Sugiuira et al (28), which showed that in an ischemic acute kidney injury model, klotho inhibited apoptosis via Hsp70. Previously, Hsp90 was found to exert a profound ischemic postconditioning cardioprotective effect and to alleviate I/R-induced myocardial injury and apoptosis in vivo (36). Notably, the western blotting results of the present study showed that klotho overexpression in H9c2(2-1) cells prior to H/R treatment upregulated the levels of p-Akt and p-Bad. The total Akt level was not notably altered, and the total Bad level was markedly decreased in klotho-overexpressing cells. Together, these data suggest that klotho inhibited apoptosis by interacting with Hsp70 and decreasing the levels of Bad in H9c2(2-1) cells.

As mature cardiac myocytes are unable to proliferate, their survival is important in maintaining cardiac health and function. The proliferative abilities and apoptosis of cardiomyocytes after MIRI have been widely studied. Although the antiaging protein klotho reportedly possesses a protective role in cardiac diseases, the precise mechanisms underlying this effect remain unknown. A previous study indicated that klotho inhibited angiotensin II-induced cardiomyocyte hypertrophy by suppressing the angiotensin II type I receptor/β-catenin pathway (1). Another group found that klotho suppressed cardiomyocyte apoptosis in mice with stress-induced cardiac injury by downregulating endoplasmic reticulum stress (2). In the present study, klotho and Hsp70 were found to co-localize in the cell cytoplasm, and klotho overexpression was accompanied by Hsp70 upregulation. Thus, how klotho exerts its protective role in the H/R cardiomyocytes by regulating the expression of Hsp70 was further investigated. However, as the present study was comprised solely of in vitro experiments, in vivo functions should be investigated in the future.

In the present study, the role of klotho in H/R injury-induced proliferation and apoptosis after MIRI was investigated, and the associated molecular mechanism was further clarified. Collectively, the results indicate that klotho reduces apoptosis by upregulating Hsp70 and p-Akt after IRI, suggesting that klotho may serve as a potential therapeutic target for I/R injury and repair.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

JH checked the references and performed several western blotting assays. BS performed the flow cytometry and several western blotting experiments. XL and JZ performed the MTT assay, acquired the data and prepared the manuscript. YL designed the experiments and wrote the manuscript. All authors read and approved the final manuscript. JH and YL were responsible for the authenticity of the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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