Cathelicidin PR-39 peptide inhibits hypoxia/reperfusion-induced kidney cell apoptosis by suppression of the endoplasmic reticulum-stress pathway

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Abstract

Ischemia/reperfusion injury (IRI) is a major cause of acute kidney damage, which often occurs in deceased donor kidney transplants. Cathelicidin PR-39 peptide possesses anti-inflammatory and wound repair effects through tissue angiogenesis and anti-apoptosis. This study assessed the role of PR-39 in anti-apoptosis in vitro using a lentiviral vector with a kidney specific promoter (KSP) to drive PR-39 expression. Our data revealed that PR-39 peptide was specifically over-expressed in kidney-derived HK-2 cells, but was scarcely detected in non-kidney tissue-derived cells. PR-39 over-expression had a protective role in the hypoxia/re-oxygenation (H/R) treated cells. The anti-apoptotic activity of PR-39 peptide was mediated by the inhibition of caspase-2, caspase-12 and caspase-3 activity in the endoplasmic reticulum (ER) stress-induced apoptotic pathway. It was also revealed that the anti-apoptotic effect of PR-39 peptide was mediated by an apoptosis-related protein, cellular inhibitor apoptosis protein-2 (c-IAP-2). Taken together, the current data demonstrate that PR-39 expression driven by KSP could prevent kidney damage (apoptosis) from IRI via the ER stress-induced apoptotic pathway.

Key words: PR-39, ischemia/reperfusion injury, kidney specific promoter (KSP), anti-apoptosis, kidney-derived HK-2 cells

Introduction

Ischemia/reperfusion injury (IRI) is a major cause of damage to acute kidney injury (AKI) or isolated kidney during organ transplantation, and the severity of IRI directly influences graft and patient survival [1,2]. AKI, also called acute renal failure, refers to the abrupt loss of kidney function that develops within seven days [3,4]; and it is diagnosed by the elevated levels of serum urea nitrogen and creatinine and/or the inability to produce sufficient daily amount of urine [5]. AKI can result in a number of clinical complications such as body metabolic acidosis, uremia, high serum potassium level, and at the worst, death [6]. The treatment of AKI depends on the severity of kidney damage and the identification and treatment of its underlying cause (such as crush injury, low blood pressure or contrast agents) [7]. To date, pharmacological interventions have been developed to prevent injury from specific cellular mechanisms; however, these were unable to reduce mortality and the consequent effects associated with AKI in clinical studies [8–10]. Pathophysiologically, a great number of cellular events are involved in AKI development, including excessive renal tubular epithelial cell apoptosis and necrosis which have been increasingly considered as major events in AKI and in the disruption
of kidney function [11]. Mechanistically, kidney hypoxia and subsequent re-oxygenation could lead to kidney epithelium apoptosis and necrosis induced by apoptosis extrinsic (death receptor-mediated), intrinsic (mitochondria-mediated) and/or endoplasmic reticulum (ER) stress pathways [12,13]. Thus, novel strategies for the prevention and treatment of AKI are urgently needed.

PR-39, a member of the cathelicidin-related antimicrobial peptide family isolated from pig intestine, was activated in lysosomes of macrophages, polymorphonuclear leukocytes and keratinocytes in tissues [14]. Cathelicidin plays a critical role in mammalian innate immune defense against bacterial infection, and can stimulate angiogenesis and inhibit inflammatory responses. Previous studies have illustrated that the mechanism of PR-39 action was to specifically block the degradation of hypoxia-inducible factor-1α (HIF-1α)-dependent gene (VEGF, FGF) and nuclear factor-κB inhibitor (IκBα), and in turn, selectively inhibit proteasome degradation in tissues [15,16]. The anti-apoptotic activity of PR-39 also contributed to its anti-inflammatory role during hypoxia/reperfusion (H/R) injury in tissues [17]. Thus, these multiple functions of PR-39 could lead to the prevention and treatment of human diseases such as stroke, myocardial infarction and organ transplantation failures [15,17,18]. However, to the best of our knowledge, previous studies of PR-39 were mainly confined to heart and brain tissues.

Indeed, the introduction of a therapeutic gene into an organ using a tissue-specific gene promoter could induce an organ-specific expression of a gene to: (1) cure some organ diseases and (2) reduce unwanted effects on other tissues and cells. This has become an effective strategy in disease treatment [19,20]. As a tissue-specific member of the cadherin superfamily, KSP has been cloned and isolated from renal cortices of basolateral membrane vesicles and cortical microsomes of male New Zealand White rabbits [21]. KSP can be exclusively expressed in the basolateral membrane of renal tubular epithelial cells. Studies in rabbits, mice and humans revealed that KSP was able to direct a specific gene expression in the kidney in vivo [22,23]. Schievenbusch et al. [24] also employed KSP in combination with a Cytomegalovirus (CMV) enhancer to successfully drive hHGF expression in renal and hepatic tissues, while avoiding its expression in non-target organs such as the lung, heart and spleen. We hypothesize that the over-expression of PR-39 driven by KSP could help prevent kidney epithelium damage during AKI.

In this study, we constructed a lentiviral vector with KSP to drive PR-39 expression in different tissue-derived cell lines and assess the specific PR-39 expression in kidney cells, the activity of PR-39 expression in the protection of hypoxia-induced cell apoptosis and damage, and the underlying molecular events as well.

**Materials and Methods**

**Cell lines, culture and viral infection**

Human umbilical vein endothelial-derived cells (HUVECs), human kidney HK-2 cells and NIH3/T3 cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM/F12 or RPMI-1640 (Hyclone, Logan, USA) supplemented with 10% FBS (Gibco, Grand Island, USA) in a humidified incubator with 5% CO2 at 37°C.

Lentiviral vectors carrying PR-39 (NM-407610) and eGFP cDNA under the control of a KSP or a CMV promoter were constructed by Cyagen Bioscience Inc. (Guangzhou, China), and were named LV-CMV-PR39-eGFP-Puro (CP) and LV-KSP-PR39-eGFP-Puro (KP), respectively. Empty vector LV-KSP-eGFP-Puro (KE) was used as a control. For lentiviral infection experiments, cells were seeded into cell culture plates, incubated for 24h, and randomly divided into three groups: treatment (CP or KP) group, negative control (KE) group and blank control (CON) group. Then, cells were infected at a multiplicity of infection (MOI) of 30 with indicated lentiviral particles in the presence of 2 μg/ml polybrene for 8h. Green fluorescence was observed at 24, 48 and 72h under a fluorescence microscope (DP72; Olympus, Tokyo, Japan) to assess infection efficiency. Cells were then assessed by the following assays.

**Flow cytometric assay**

The proportion of eGFP-expressing cells were determined by fluorescence microscope and quantified by a FACS/Calibur flow cytometer (BD Biosciences, San Jose, USA) five days after lentiviral infection. Briefly, cells were harvested and centrifuged for 10 min at 300g, re-suspended in 200 μl of phosphate buffered saline (PBS), and analyzed by the FACS/Calibur flow cytometer.

**Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)**

qRT-PCR was performed to detect the expression level of PR-39, and GAPDH was used as control. Briefly, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, USA) and converted into cDNA using a TaqMan RNA Reverse Transcription Kit (Thermo, Waltham, USA) according to manufacturer’s protocols. Then, qPCR was performed on an ABI Prism 7500-East Thermocycler Sequence Detection System (Applied Biosystems, Foster City, USA) with SYBR Green Master Mix (DBI Bioscience, Shanghai, China) according to the instructions provided with the kit. Primers used to amplify PR-39 mRNA were 5′-ACCCCCCATATTGCCAAG-3′ (forward) and 5′-ATCAGCCACTCTCATCACC-3′ (reverse). Meanwhile, GAPDH primers were 5′-TTGATTAGTGCTGGCTTTTGT-3′ (forward) and 5′-CGATCCAGGGGCTCCTTACT-3′ (reverse). Relative expression level of the target gene was calculated using 2^−ΔΔCt (ΔΔCt = ΔCt in the CP group − ΔCt in the KP group).

**Hypoxia/re-oxygenation (H/R) treatment of HK-2 cells**

HK-2 cells were seeded, cultured and exposed to CoCl2 stimulation (0, 200, 400, 600 or 800μM) for 24h. Subsequently, cells were grown in complete culture medium (RPMI-1640 containing with 10% FBS) for 12h. For H/R treatment, the culture medium was deprived of FBS for 24h in a tri-gas incubator (Heal Force, Shanghai, China) with ed air containing 5% CO2 and 94% N2 at 37°C to simulate hypoxia and re-oxygenation, according to a previous study [25]. The control (NC) group were cultured in the complete culture medium with humidified air containing 5% CO2 and 94% N2 at 37°C to simulate hypoxia (1% oxygen). Then, cells were further cultured by reintroducing the complete cell culture medium and incubating in a normal culture incubator at 37°C for 12h to simulate re-oxygenation, according to a previous study [25]. The control (NC) group were cultured in the complete culture medium with humidified air containing 5% CO2 at 37°C for 36h without such treatment.

Cell injury (damage) was assessed by LDH release assay to measure the LDH level in the culture medium using an assay kit [Jiancheng Biotechnology, Nanjing, China] according to manufacturer’s instructions. In brief, at the end of H/R incubation, 600 μl of the culture supernatants were collected, equal amounts of supernatants were added into three wells (200 μl/well), and the OD at 450 nm was measured using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, USA) to detect LDH levels (U/ml).
Cell proliferation and viability assays
After gene transfection and H/R treatment, cells of each group were counted under a microscope to assess cell proliferation. In brief, approximately $1 \times 10^4$ or $10^5$ cells were seeded into 24-well plates (Corning Co., Corning, USA) and cultured for up to five days. At the end of each experiment, cells were detached with 0.25% trypsin, collected to count cell numbers daily and proliferation curves of three independent experiments were plotted.

Meanwhile, cell viability MTT assay was also performed. In brief, cells were exposed to CoCl$_2$ stimulation at different concentrations (0, 200, 400, 600 and 800 μM) for 24 h, reintroduced into complete medium, and cultured for 6 h. After that, the cell culture was incubated with 20 μl of MTT solution per well (0.5 mg/ml) at 37°C for an additional 4 h, 150 μl of dimethyl sulfoxide was added into each well to solubilize the formazan crystals for 10 min, and the OD at 490 nm was measured using the SpectraMax M5 microplate reader. Cell viability was also assessed by trypan blue (Merck, Darmstadt, Germany) exclusion assay.

Flow cytometric apoptosis assay
After treatment, flow cytometric apoptosis assay was used to detect HK-2 cell apoptosis using an APC-Annexin-V/7-AAD apoptosis detection kit (KeyGen Biotech, Shanghai, China). In brief, approximately $2 \times 10^5$ cells were washed with PBS after treatment and re-suspended in the Annexin-binding buffer. Subsequently, Annexin V-APC and 7-AAD solution (5 μl each) were added into the cells and incubated for 30 min in the dark at room temperature. The cells were then analyzed using a FACS flow cytometer. Signals were detected in logarithmic mode at FL4. Unstained cells were used as a system control. APC-Annexin-V was set as the horizontal axis. Cells staining positive for Annexin-V and APC were considered apoptotic cells (upper right quadrant and lower right quadrant).

siRNA, Smac cDNA and cell transfection
Short interfering RNA (siRNA) constructed to knockdown c-IAP-2 expression (c-IAP-2-siRNA) was synthesized by Cyagen Biotechnology Inc. with sequences of 5′-AGGAGUCUUGCUCGUGCUGTT-3′ (forward) and 5′-CAGCACGAGCAAGACUCCUTT-3′ (reverse) according to a previous study [17]. The HK-2 cells (KP and KE groups) were transfected with either negative control siRNA (Qiagen Translational Medicine Co., Suzhou, China) or c-IAP-2-siRNA using the Lipofectamine® RNAiMAX reagent (Thermo Fisher, Waltham, USA) in mixture of complete culture medium for 48 h. Knockdown of c-IAP-2 was evaluated by western blot analysis.

Smac cDNA in pcDNA3.0-Smac mCherry (Addgene plasmid # 40880) was a kind gift of Dr. Douglas Green (Department of Immunology, St Jude Children’s Research Hospital, Memphis, USA) [26]. The HK-2 cells were seeded at a density of $4 \times 10^5$ cells per well.
in 96-well plates and then transfected with pcDNA3.0-Smac mCherry using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. And 48 h later, the transfection rate was inspected under the DP72 fluorescence microscopy or the level of m-cherry protein expression, and Smac expression was confirmed by western blotting using a polyclonal antibody against Smac (Proteintech, Wuhan, China).

Caspase activity assay
The activity of caspase proteases was assessed using a caspase activation kit (KeyGen, Nanjing, China) following manufacturer’s instructions. In brief, HK-2 cells were harvested and lysed with the RIPA lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS (Heart Company, Shanghai, China), and then centrifuged at 13,000 g for 30 min at 4°C. Protein concentration was measured using a BCA Protein Assay kit (Beytime Biotechnology, Shanghai, China). The harvested protein sample was added to the reaction buffer containing the substrate for caspase-2, -3, -8 and -9, respectively, and incubated at 37°C for 4 h. Absorbance of the chromophore pNA was determined using a microplate reader (Molecular Devices) at 405 nm. The activity of caspases in each of the treated cell groups was normalized to NC control.

Western blot analysis
The methods of cell lysis and determination of protein concentration were as described in the previously caspase activity assay. For western blot analysis, equal amounts of the protein samples (50 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes using a Bio-Rad semi-dry transfer system (Bio-Rad, Hercules, USA). The membranes were blocked in 5% non-fat milk for 1 h at room temperature, and then incubated with the following primary antibodies:

![Western blot analysis](https://academic.oup.com/abbs/article-abstract/48/8/714/2236631/fig2)

**Figure 2. The cyto-protective effects of PR-39 on hypoxic HK-2 cells** (A) Western blot analysis. Upper panel: ER-resident chaperone GRP78 protein expression was up-regulated after H/R or CoCl₂ treatment, compared with the normal control (NC) group. Lower panel: PR-39 expression was absent in the blank (CON) and negative control (KE) groups, whereas in KP groups, PR-39 protein expression was up-regulated in H/R-treated HK-2 cells. (B) Cell counting assay. Cell proliferation curves of the three groups showed that proliferation was not affected by lentivirus infection, but by hypoxic treatment. (C,D) MTT assays. CoCl₂-treated cell viability was assessed by MTT assay. The effect of CoCl₂ was dose-dependent. Group ‘KP’ has high cell viability, compared with the negative control group ‘KE’. (E) Trypan blue exclusion assay of cell survival rate. (F) LDH release assay. These results represent three independent experiments with triplicate samples in each experiment. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. the negative control KE group. ‘KE’ stands for cells infected with empty vector LV-KSP-eGFP-Puro.
polyclonal anti-PR39 antibody (Wan Jiang Biotechnology Inc, Shanghai, China), polyclonal anti-human calreticulin (CRT) antibody (R&D System, Shanghai, China), monoclonal anti-CHOP antibody (ProSci, San Diego, USA), polyclonal anti-caspase-12 antibody (Santa Cruz, Dallas, USA), polyclonal anti-DIABLO/Smac antibody (Proteintech), polyclonal anti-GRP78 antibody (Proteintech) and anti-β-actin antibody (Beyotime) at appropriated dilutions overnight at 4°C. On the next day, the membranes were washed thrice with Tris-based saline-Tween 20 (10 mM Tris, 150 mM NaCl, and 0.05% Tween 20), and further incubated with a secondary antibody for 1 h at 37°C. Protein bands were visualized by incubation with an enhanced chemiluminescence (ECL) solution and quantified with the ChemiDoc System (Bio-Rad).

Statistical analysis
Data were expressed as mean ± SEM from three independent experiments. Statistical analyses were performed with SPSS 17.0 software (SPSS, Chicago, USA) for One-way ANOVA followed by LSD t-test. A P-value of < 0.05 was considered to be statistically significant.

Results
Differential overexpression of PR-39 in HK-2 cells after being infected with KSP-driven lentivirus
HUVECs, NIH3/T3 cells and HK-2 cells were infected with lentiviral particles carrying PR-39 cDNA with a KSP or a CMV promoter. After 48 h of infection with lentivirus carrying PR-39 cDNA with CMV, eGFP was shown to be strongly expressed in all three cell lines. In contrast, infection with lentivirus with the KSP exhibited a high eGFP expression in HK-2 cells, whereas HUVECs or NIH3/T3 cells had a limited eGFP expression (Fig. 1A). Flow cytometry analysis revealed that eGFP expression under the KSP was high in HK-2 cells (77.87% ± 4.22%), but was very low in HUVECs and NIH3/T3 cells, compared with eGFP under CMV promoter (P < 0.001, Fig. 1B). There was no difference in cell lines infected by lentivirus with CMV (Fig. 1B).

Furthermore, the relative level of PR-39 mRNA was also high in HK-2 cells infected with lentivirus with KSP and was low in HUVECs and NIH3/T3 cells, compared with those infected with lentiviral particles with CMV (P < 0.001, Fig. 1C). The same is true for the protein level of PR-39 in these cells (Fig. 1D).

PR-39 protects H/R-induced HK-2 cell injury by inhibiting apoptosis
Next, H/R treatment experiments were performed in lentivirus-infected HK-2 cells. Results revealed that PR-39 expression was high in KP groups compared with blank control CON and negative KE groups; and H/R treatment increased the KP-induced PR-39 expression in HK-2 cells (Fig. 2A, lower panel). However, as revealed by cell counting, there was no significant difference in cell proliferation when HK-2 cells were infected with KP (with or without H/R treatment), KE and CON vectors (Fig. 2B). HIF-1α mRNA level was up-regulated in HK-2 cells after H/R treatment (data not shown), suggesting that hypoxia treatment was effective in cells. At the same time, GRP78 protein (an ER-resident chaperone) was up-regulated after 48 h of hypoxia-reperfusion treatment (Fig. 2A, upper panel), suggesting that ER stress was indeed induced.

Furthermore, cell viability MTT assay results revealed that CoCl2 decreased the cell viability of HK-2 cells dose-dependently, while PR-39 significantly inhibited the CoCl2-induced reduction of HK-2 cell viability (P < 0.05, Fig. 2C,D). It is evident that more HK-2 cells survived in the KP group than in the KE group (P < 0.01, Fig. 2E) with H/R or CoCl2 (400 μM) treatment as revealed by trypan blue staining. In addition, LDH levels in the KP group were lower than that in the KE group (P < 0.01, Fig. 2F) with H/R or CoCl2 (400 μM) treatment. Flow cytometric apoptosis assay revealed that the percentage of apoptotic HK-2 cells was significantly decreased in the KP groups (P < 0.001, Fig. 3) with H/R or CoCl2 (400 μM) treatment when compared with KE groups. However, the difference in apoptosis rates among these three groups (KP, KE and CON) was not statistically significant under normoxic conditions. This finding confirmed that PR-39 was able to significantly inhibit HK-2 cell apoptosis under hypoxic conditions (H/R or CoCl2 treatment).

PR-39 inhibits caspase activation in HK-2 cells after H/R-induced injury
Caspase colorimetric assay data revealed that PR-39 expression markedly attenuated H/R-induced caspase activation in HK-2 cells. Specifically, caspase-2 activity in the KP group was significantly inhibited when compared with the control group (P < 0.05, Fig. 4A)

![Figure 3](https://academic.oup.com/abbs/article-abstract/48/8/714/2236631) PR-39 protects kidney cells from hypoxia-induced apoptosis
(A) Flow cytometric Annexin V-APC and 7-AAD double-staining assay. The data were representative of three independent experiments. (B) Summarized data of (A). PR-39 significantly decreased H/R-induced HK-2 cell apoptosis. Data are expressed as mean ± SEM of three independent experiments. ***P < 0.001 vs. negative control KE group.
under H/R or CoCl₂ treatment. Caspase-3 activity had a similar trend to caspase-2 activity (P < 0.001, Fig. 4A). However, the activities of caspase-9 and caspase-8 were not significantly altered among these three groups (P > 0.05, Fig. 4A). These results suggest that the anti-apoptosis activity of PR-39 in H/R-induced cell injury could be due to the suppression of caspase-2 and -3 activities.

PR-39 inhibits caspase-12 activation and up-regulates c-IAP-2 to suppress H/R-induced HK-2 cell apoptosis

ER stress-induced apoptosis is one of the most common mechanisms that contribute to the pathogenesis of H/R injury in the kidney. Thus, the expression of ER stress-induced apoptosis-related proteins was assessed. As shown in Fig. 4B, chaperone calreticulin (CRT) expression in HK-2 cells was significantly decreased in the KP group, compared with the KE group subject to H/R or CoCl₂ treatment. Cleaved caspase-12 was also decreased in the KP group compared with the KE group subject to H/R or CoCl₂ treatment. Furthermore, c-IAP-2 expression was up-regulated in the KP group subject to H/R or CoCl₂ treatment. However, the protein level of CHOP showed no significant change between KP and KE groups subject to H/R or CoCl₂ treatment. From these data, it was speculated that the inhibitory effect of PR-39 on H/R-induced apoptosis in HK-2 cells was due to its ability to protect the organelle membrane by the inhibition of proteolytic caspase-12 activation and up-regulation of c-IAP-2 expression.

c-IAP-2 mediates the inhibitory effect of PR-39 on caspase-12 proteolytic activity and caspase-2 and -3 activities

In order to clarify whether c-IAP-2 is the key mediator of ER stress inhibition induced by PR-39, the expression of c-IAP-2 was knocked down and blocked. Our data revealed that c-IAP-2 was successfully knocked down by c-IAP-2-siRNA or inhibited by Smac
overexpression (Fig. 5A). However, PR-39 protein expression was not affected. After HK-2 cells received H/R treatment, the cleaved caspase-12 was not significantly decreased in the c-IAP-2-knockdown or the Smac-overexpressed KP groups, compared with the KP group. Moreover, unlike those in the KP group, caspase-2 and -3 activities were not inhibited in c-IAP-2-knockdown or Smac-overexpressed groups, even though PR-39 was overexpressed (Fig. 5B). These data indicated that c-IAP-2 played an important role in the anti-apoptotic mechanism of PR-39.

Discussion

Apoptosis and necrosis have been previously demonstrated to be the major cause of damage to the renal tubular epithelia during AKI [27,28]. In order to control AKI, a number of pharmacological interventions have been developed to minimize cell apoptosis or necrosis. However, to date, there are no effective therapies available. In the current study, we explored the utilization of a lentivirus to overexpress PR-39 peptide using KSP to protect kidney tubular epithelial cells from H/R-induced injury in vitro. We found that KSP was able to specifically induce PR-39 expression in HK-2 cells, but hardly in HUVECs and NIH3T3 cells. In addition, this specific exogenous PR-39 expression was able to inhibit H/R-induced apoptosis in HK-2 cells. At the gene level, PR-39 alleviates H/R-induced apoptosis through the caspase-12-dependent ER stress-induced apoptotic pathway.

Indeed, tissue-specific promoters could induce the expression of therapeutic proteins into the specific organ [29,30], which leads to the enhancement of gene products in targeted cells or tissues, but with the reduction of unwanted side effects in other cells. Whyte et al. [31] showed that KSP could specifically induce the expression of a luciferase reporter gene in renal epithelial cell lines, but not in mesenchymal cell lines, which confirmed that the KSP-cadherin promoter was specific to kidney tubular epithelial cell in vitro. Other studies confirmed that KSP was able to induce the kidney-specific expression of different genes in many kinds of species in vivo [22,23]. Our present data are consistent with these previous studies.

PR-39 is a cell-permeable proline- and arginine-rich antibacterial peptide that has shown different biological activities in tissues [32–34]. Our current study further confirmed the effects of PR-39 in kidney-derived HK-2 cell apoptosis after H/R treatment. Here, we found that the overexpression of PR-39 protected HK-2 cells from H/R-induced apoptosis by the inhibition of caspase-2 and -3 activities, which is consistent with a previous study showing that the anti-apoptotic effect of PR-39 was associated with the decrease in caspase-3 activity [35].

It is known that hypoxia can induce ER stress to subsequently activate caspase-12 [36,37]. Caspase-12 is a cytochrome c-independent [38] and ER-specific caspase that participates in apoptosis under ER stress [39]. Indeed, a previous study has revealed that severe H/R caused ER stress and CRT induction [40]. When H/R induces ER stress in cells, a disturbance in Ca2+ homeostasis initiates an unfolded protein response that subsequently activates the caspase cascade [41]. The possible activation mechanism of caspase-12 involved in the ER stress-induced apoptotic pathway is that caspase-12 is released to the cytosol from the ER lumen, and activates the effector caspase-3 [38]. Our current data showed that there

Figure 5. PR-39 suppresses ER stress-induced apoptotic-related proteins mediated by c-IAP-2. (A) Western blot analysis. When c-IAP-2 expression was inhibited, the inhibition of caspase-12 proteolytic by PR-39 vanished after HK-2 cells underwent hypoxic incubation. (B) Caspase colorimetric assay. Caspase-2 and -3 activities were assessed in HK-2 cells before and after c-IAP-2 expression was knocked down and blocked. **P < 0.01, ***P < 0.001 vs. negative control KE group.
was no difference in CHOP protein level or in caspase-8 and -9 activities between PR-39 (KP) and control (KE) cells, indicating that PR-39 inhibited ER-stress-induced apoptosis, but this inhibitory effect was independent of the death-receptor and mitochondria apoptotic pathway [42]. Moreover, PR-39 reduced CRT release from ER and decreased the activity of caspase-12 for the subsequent inhibition of caspase-3 activity [36].

PR-39 overexpression also up-regulates c-IAP-2 expression, which can direct the ubiquitylation of caspase-3 [43]. This is consistent with a previous study showing that exogenous PR-39 inhibited H/R-induced apoptosis in endothelial cells, and that the anti-apoptotic effect of PR-39 was mediated by the increase in c-IAP-2 expression [17]. Caspase-12 and caspase-2 both contain a protein interaction motif termed caspase recruitment domain (CARD) [44], which is activated in response to ER stress; and in this ER stress-induced apoptotic pathway, c-IAP-2 inhibits caspase-2 by its baculovirus IAP repeat (BIR) and CARD domain binding to suppress apoptosis [45]. From these results, we can deduce that the proteolytic caspase-12 inhibition induced by PR-39 might be associated with the up-regulated expression of c-IAP-2. In order to further test this assumption, c-IAP-2 was knocked down with siRNA or inhibited by Smac, which is a main inhibitor of c-IAP-2 [46]. Then, ER stress-induced apoptotic-related caspas (caspase-12, -2 and -3) activities were also investigated. When c-IAP-2 protein expression was inhibited, the inhibitions of caspase-12 proteolytic activity and caspase-2 and -3 activities by PR-39 were abolished, which demonstrates that c-IAP-2 is also the key mediator in PR-39 inhibition of ER stress-induced apoptosis. Therefore, our current study demonstrates that during the course of hypoxic reperfusion injury, the anti-apoptotic role of PR-39 was mediated by up-regulating the expression of c-IAP-2, which inhibits the proteolytic activation of the ER stress-induced apoptosis related biomarkers caspase-12, -2 and -3 (Fig. 6).

However, when PR-39, a porcine-derived cathelicidin, is expected to be applied in humans to cure some related diseases, its immunogenicity has to be considered. Thus, as an alternative member of the cathelin family, LL-37 has been highly expressed in human psoriatic keratinocytes [47]. As the human homolog to PR-39, LL-37 peptides not only possess a broad antibacterial spectrum, but also have angiogenesis and anti-apoptotic effects [48,49]. Vázquez-Sánchez et al. [50] reported that the anti-apoptotic effect of LL-37 is also mediated through the up-regulation of c-IAP-2 via the caspase-3 pathway. Therefore, further studies on therapeutic strategies to protect kidney tissues from ischemia injury in vivo are needed. In future in vivo studies, LL-37 should be used as a target gene in ischemia-reperfusion injury kidney tissues in combination with KSP to find a better protective strategy during the course of IRI.

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**References**


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**Figure 6. Schematic diagram of apoptosis signaling pathways after H/R treatment and the role of PR-39**

Under H/R conditions, the ER stress-apoptosis pathway was activated to induce HK-2 cells to undergo apoptosis. PR-39 overexpression in these HK-2 cells after infection with a lentivirus carrying PR-39 cDNA driven by KSP could inhibit H/R-induced HK-2 cell apoptosis by suppressing caspase-2, -3 and -12 activation. This was mediated by the up-regulation of c-IAP-2 expression. In addition, CHOP protein, caspase-8 and caspase-9 activities were not inhibited by the overexpressed PR-39, which revealed that PR-39 selectively inhibited ER stress-induced apoptotic-related proteins.