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Selective Activation of Alpha 7 Nicotinic Receptor Antagonizes Apoptosis in Renal Cells via Nrf2 Signaling

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OBJECTIVE: Both the selective agonists of α7 nicotinic cholinergic receptor (α7nAChR) and the activators of nuclear factor E2-related factor 2 (Nrf2) are promising therapeutic agents for renal ischemia-reperfusion (IR) injury. We aimed to investigate the relationship of α7nAChR activation and Nrf2 in antagonizing renal IR injury.

STUDY DESIGN: Using hypoxia-reoxygenation (HR) in the renal epithelial cell line (HK2 cells) as a model of renal IR injury, we examined the effects of an α7nAChR agonist, PHA568487, on the HR injury–induced apoptosis and the apoptotic signaling, and the role of Nrf2 in the effects of the α7nAChR agonist.

RESULTS: The selective agonist of α7nAChR, PHA568487, dramatically attenuated the HR injury–induced apoptotic cell deaths of HK2 cells. The agonist also significantly suppressed the HR-induced elevation of mRNA and protein levels of caspase-3 and Bax and enhanced the expression of the anti-apoptotic molecule Bcl-2. The application of an Nrf2 inhibitor, ML385, significantly but incompletely blocked the effects of PHA568487 on apoptosis and the expression of the apoptotic signaling molecules.

CONCLUSION: These results indicate that the selective activation of α7nAChR exerts significant anti-apoptotic effects in the renal cells via antagonizing the apoptotic signaling and activating the anti-apoptotic signaling, which is partially mediated by the Nrf2 transcription factor. Thus, the activation of α7nAChR may exert strong anti-apoptotic effect during renal IR

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injury through Nrf2-dependent and independent mechanisms. (Anal Quant Cytopathol Histpathol 2020;42: 69–76)

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Ischemia-reperfusion injury is one of the most frequent causes for acute renal failure, with no effective therapy currently. Two closely related categories of the most promising therapeutic agents are the selective agonists of α7 nicotinic cholinergic receptor (α7nAChR) and those activating the nuclear factor erythroid 2–related factor 2 (Nrf2). It was shown that α7nAChR mediated the effect of vagus nerve stimulation in antagonizing ischemia-reperfusion (IR) injury in the kidney,[1,2] and the selective activation of α7nAChR exerted direct attenuation of renal IR injury.[3,4] Furthermore, the protective effect of ultrasound against renal IR injury was found to be mediated by α7nAChR.[5,6]

Similarly, many studies have demonstrated that Nrf2 mediates the effects of multiple agents in alleviating various kidney injuries and diseases, including IR injury.[7-10] The Nrf2 protein is a transcription factor of the heme oxygenase–1 (HO-1) gene and often functions together with HO-1 to exert the effects of anti-inflammation, anti-oxidation, and protection against renal IR injury and nephrotoxicity.[13-15] A recent study[4] showed that the attenuation of IR injury by the activation of α7nAChR was accompanied with an elevated expression of the HO-1 gene, which is a key target gene of Nrf2 in the effects of anti-inflammation, anti-oxidation, and protection against renal IR injury and nephrotoxicity.[11-15] The study also demonstrated that the agonist of α7nAChR suppressed the expression of the high-mobility group box 1 (HMGB1) gene through HO-1. However, because HMGB1 may either promote or suppress apoptosis, a direct role of HO-1 in IR injury remains to be determined.[16,17]

The association of α7nAChR activation and Nrf2 has been demonstrated in microglial cells and astrocytes, in which Nrf2 mediated the anti-inflammatory and neuroprotective effects of α7nAChR activation.[18,19] Therefore, it is highly possible that Nrf2 mediates the role of α7nAChR activation in preventing renal IR injury. In this study, we specifically examined the effects of α7nAChR activation on apoptosis and the apoptotic signaling of human renal epithelial cells (HK2 cells) induced by a hypoxia-reoxygenation (HR) injury, which is a model of renal IR injury.[20] We then determined the role of Nrf2 in the effects of α7nAChR activation on apoptosis and the apoptotic signaling using a specific inhibitor of Nrf2.

Materials and Methods

Cell Culture and Treatment

Human renal tubular epithelial cells HK-2 (American Type Culture Collection [ATCC], Manassas, Virginia, USA) were cultured in DMEM medium (Gibco) supplemented with Hams F-12 and 10% heat inactivated FBS, in an incubator at 37°C, with 5% CO_2 and 95% air. The HR model was induced by exposing the cells to the atmosphere of 5% CO_2, 94% N_2, and 1% O_2 for 24 hours in a 37°C humidified incubator, followed by exposure to the reoxygenation condition (21% O_2, 5% CO_2, and 74% N_2) for 12 hours.[20] For experiments, cells were randomly divided into 4 groups: (1) control group: cells were routinely cultured without any treatment, (2) HR-alone group: model was established, (3) PHA568487 group: cells were pretreated with PHA568487 (Tocris Bioscience) for 30 minutes at 10 μmol/L, a concentration showed the maximum effect in cultured cells,[21] followed by establishment of HR model; and (4) Nrf-2 inhibitor group: cells were pretreated with Nrf-2 specific inhibitor ML385 (Axon Medchem LLC; 10 μmol/L) before the application of PHA568487 (10 μmol/L), followed by HR treatment.

Cell Proliferation Assay

Cell proliferation assay was carried out using the Cell Count Kit-8 (CCK-8, Abcam, Cambridge, Massachusetts, USA) according to the manufacturer's instructions. The HK2 cells were incubated at 37°C, 5% CO_2 for 1 hour in 96-well plates; each well contained 100 μL medium with 10 μL of CCK-8 solution added. The absorbance was then measured at 450 nm. There were three repeats for each experiment.

Annex V FITC Apoptosis Assay

Apoptosis was detected using the Annexin-V-FLUOS staining kit (Roche) by measuring the

level of Annexin V binding. Briefly, HK-2 cells were harvested after HR treatment and washed with pre-cooled PBS solution twice, and were re-suspended in the Annexin-V-containing binding buffer and incubated in the dark for 15 minutes. Each measurement was performed in triplicate. The number of Annexin-V-labeled HK2 cells was quantified using the FACS Caliber Flow cytometer (BD Biosciences), and the data were analyzed using the CellQuest software (BD Biosciences).

Reverse Transcription and Real-Time Quantitative PCR (qPCR)

Extraction of total RNA from HK2 cells was carried out using Trizol (Thermo Fisher Scientific), and total RNA was converted into cDNA using the M-MLV Reverse Transcriptase (Thermo Fisher Scientific) per instructions of the manufacturer's protocol. Real-time PCR was performed on the ABI Step-One Real-Time PCR System (Thermo Fisher Scientific) and using the SYBR Premix ExTaq II qPCR kit (TakaRa Bio Inc.) according to the manufacturer's protocol. The reference mRNA for normalization of relative mRNA levels was the β-actin mRNA. The primers used were as follows (human):

Bcl-2 F 5′-CTTGACAGAGGATCATGCTGTAC-3′ (forward) 5′-GGATGCTTTATTTCATGAG GC-3′ (reverse);

Bax 5′-GGGCCCACCAGCTCTGA-3′ (forward) 5′-CCTGCTCGATCCTGGATGA-3′ (reverse);

Caspase-3 5′-CCTGGTTATTATTCTTGGCGAA A-3′ (forward) 5′-GCACAAAGCGACTGGAT GA-3′ (reverse).

Western Blot

The expression of Nrf-2, HO-1, caspase-3, Bax, and Bcl-2 proteins were detected by western blot in each group after HR treatment. Briefly, the cells were harvested from 6-well plates with trypsin, centrifuged for 5 minutes at 1,500 rpm, and the spin was repeated after suspension in PBS solution. The cells were then lysed with RIPA Lysis Buffer (Beyotime, China) and centrifuged at 12,000 rpm for 30 minutes at 4°C. After being denatured with boiling for 5 minutes in 4× SDS loading buffer, the supernatant was separated on a 12% SDS-PAGE gel. The proteins in the gel were then electrotransferred to PVDF nitrocellulose membranes. Nonspecific binding was blocked overnight at 4°C by 3% nonfat milk in a Tris-buffered saline, containing 0.05% Tween 20. The membranes were then incubated sequentially with primary antibodies of caspase-3, Bax, Bcl-2, and β-actin (Cell Signaling Technology, Beverly, Massachusetts, USA) and corresponding secondary antibodies. Signals were visualized using an ECL detection kit after exposure to X-ray. The signal intensity was processed and quantified using the LabWorks Image Acquisition and Analysis Software (UVP Inc., Upland, California, USA).

Statistical Analysis

Statistical significance was analyzed using the statistic software SPSS 19.0. Differences among groups were detected by one-way analysis of variance (ANOVA), followed by the Bonferroni post-hoc analysis to determine the two group differences. The alpha level was set as 0.05.

Results

PHA568487 Prevented HR-Caused Cell Viability Reduction via Nrf2

The cell viability of the HR group (group 2) (Figure 1) was significantly reduced compared to the control group (group 1 in Figure 1; $p<0.05$). The pretreatment of the α7nAChR agonist PHA568487 before HR (group 3) mostly prevented the HR-induced cell viability reduction ($p<0.05$). However, the Nrf2 inhibitor (group 4) significantly blocked

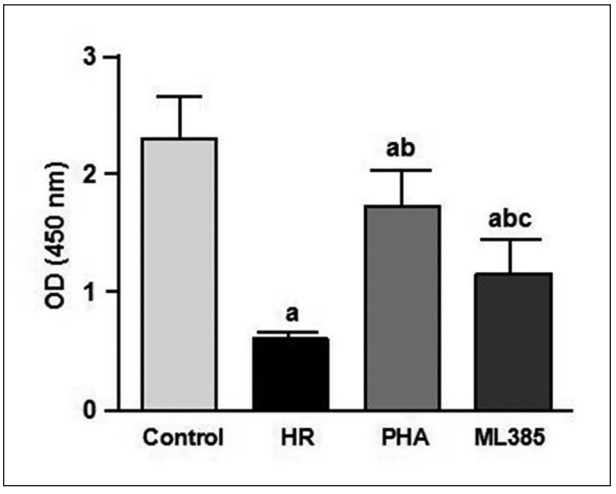

Figure 1 Effects of the α7nAChR agonist, PHA568487, and the Nrf2 inhibitor, ML385, on HR-induced cell viability. Data are expressed as mean±SD. [a]$P<0.05$ compared with the control group. [b]$P<0.05$ compared with the HR group. [c]$P<0.05$ compared with the PHA568487 group.

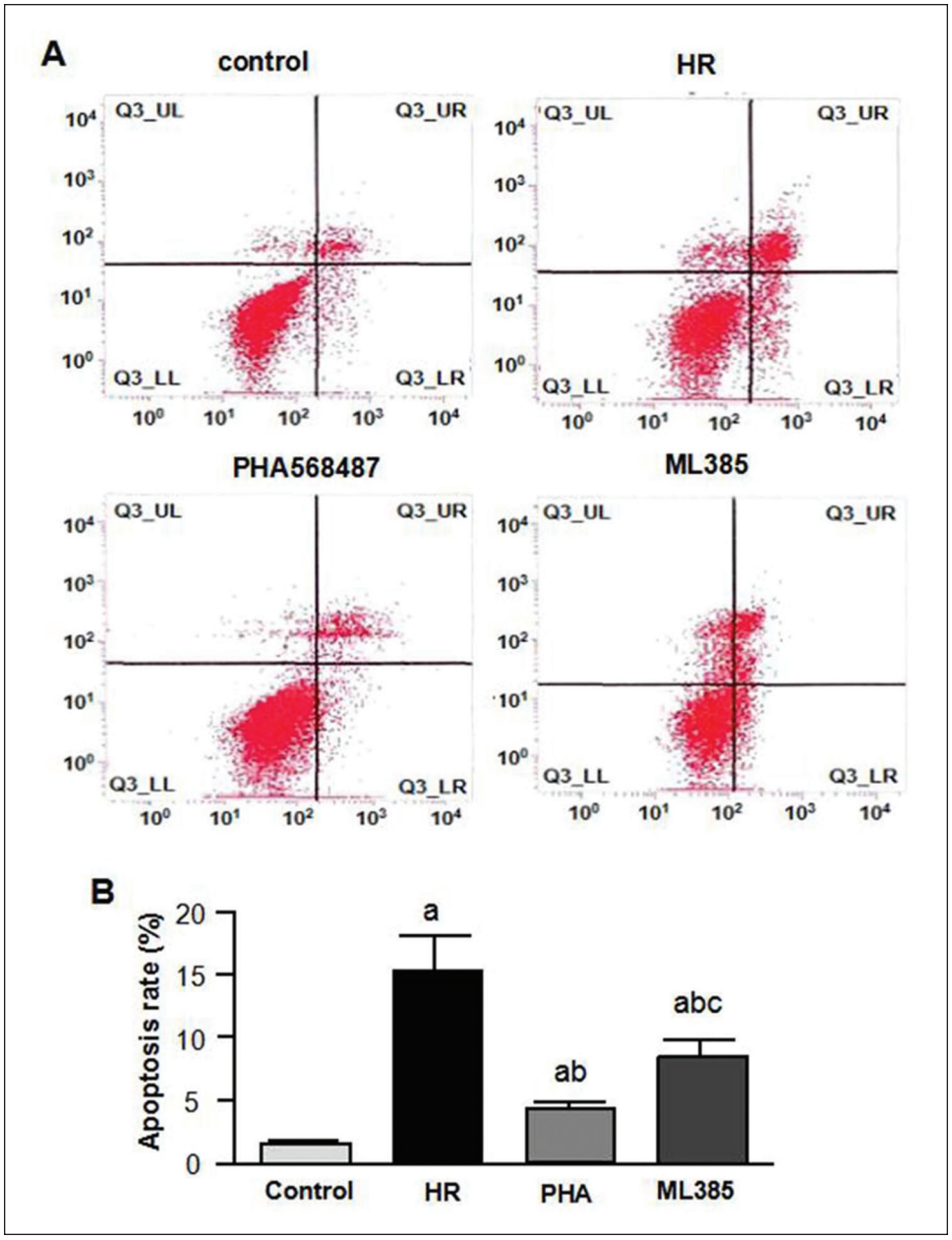

Figure 2
Effects of PHA568487 and ML385 on HR-induced apoptosis. Apoptosis assay was performed using the Annexin V/FITC method. (A) The dots in the upper right quadrant of the scatter plots represent the apoptotic cells. (B) Average rate (%) of apoptosis of three experimental repeats corresponding to the groups in A. Data are expressed as mean ± SD. [a]P<0.05 compared with the control group. [b]P<0.05 compared with the HR group. [c]P<0.05 compared with the PHA568487 group.

the effect of PHA568457 in preventing HR-induced cell viability reduction ($p<0.05$) (Figure 1).

Nrf2 Mediates the Effect of PHA568487 in Preventing HR-Induced Apoptosis

The results of the Annexin V/FITC apoptosis assay are shown in Figure 2. Treatment of HR (group 2) greatly enhanced the rate of apoptotic cell deaths as compared to the control group (group 1; $p<0.05$), which was largely prevented by PHA568487 (group 3, $p<0.05$ as compared with group 2). The prevention of HR-induced apoptosis by PHA568487 was partially blocked by the Nrf2 inhibitor (group 4; $p<0.05$ as compared to group 3).

Activation of α7nAChR Inhibits HR-Induced mRNA Expression of Apoptotic Signaling via Nrf2

The treatment of HR dramatically enhanced the

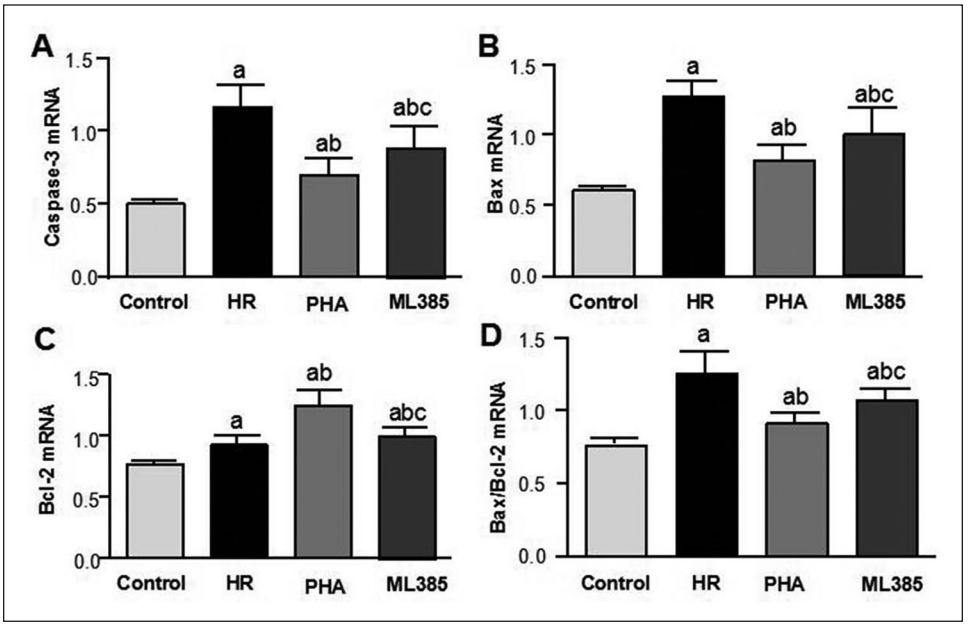

Figure 3
Effects of PHA568487 and ML385 on the relative mRNA expression of caspase-3, Bax, and Bcl-2 gene (in relation to β-actin mRNA levels). Data are expressed as mean±SD. [a]P<0.05 compared with the control group. [b]P<0.05 compared with the HR group. [c]P<0.05 compared with the PHA568487 group.

mRNA expression of caspase-3 and Bax (p<0.05) (Figure 3A–B). The mRNA level of Bcl-2 was slightly increased after HR treatment (p<0.05) (Figure 3C). As a result, the ratio of Bax/Bcl-2

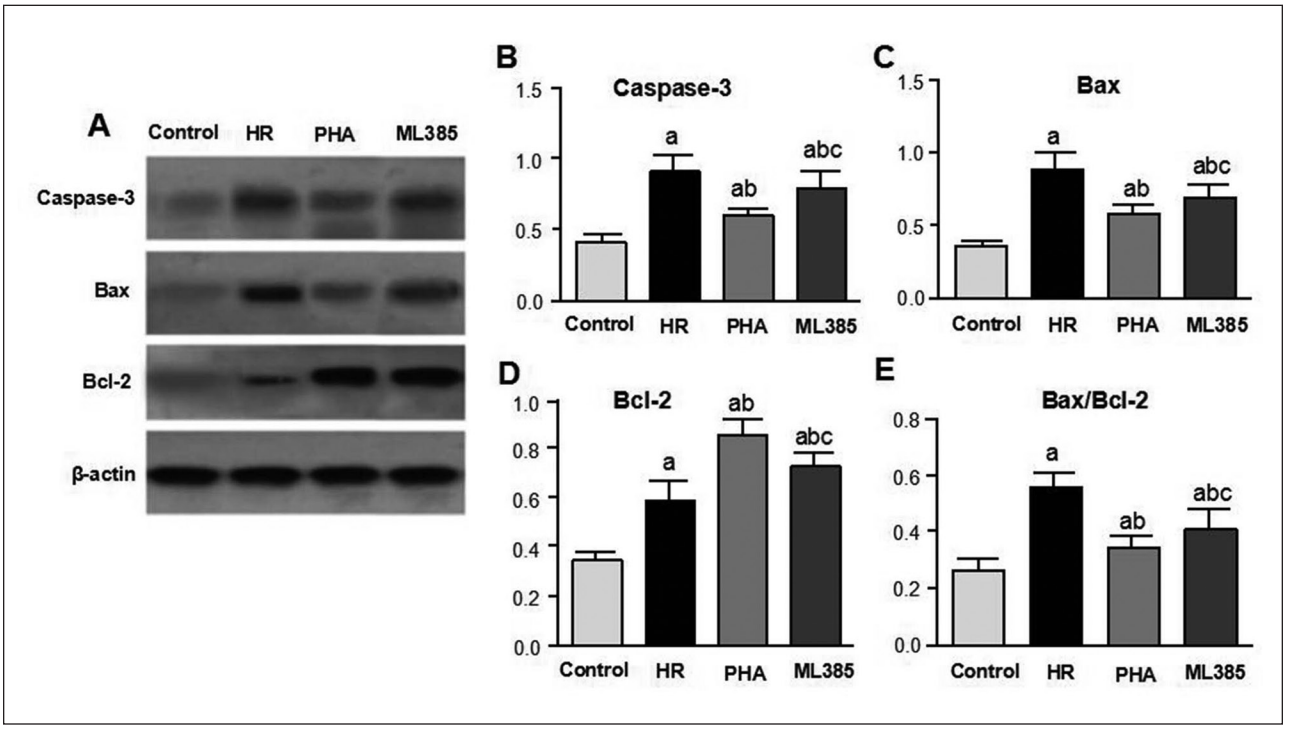

Figure 4 Effects of PHA568487 and ML385 on the relative protein expression of caspase-3, Bax, and Bcl-2 (in relation to β-actin protein levels). Data are expressed as mean±SD. [a]P<0.05 compared with the control group. [b]P<0.05 compared with the HR group. [c]P<0.05 compared with the PHA568487 group.

expression levels was also significantly elevated ($p<0.05$). The application of PHA568487 before HR blocked most of the overexpression of caspase-1 and Bax mRNAs ($p<0.05$) (Figure 3D). In addition, PHA568487 further elevated the Bcl-2 mRNA expression compared to the HR treatment. The application of Nrf2 inhibitor partially compromised the effects of PHA568487 on the mRNA expression caspase-3, Bax, and Bcl-2 ($p<0.05$).

Activation of α7nAChR Inhibits HR-Induced Protein Expression of Apoptotic Signaling via Nrf2

The changes of protein expression after HR treatment observed a trend similar to that of the mRNA expression. The treatment of HR greatly enhanced the protein levels of caspase-3 and Bax ($p<0.05$) (Figure 4B–C). However, the Bcl-2 protein level had a greater increase as compared to the mRNA levels after HR ($p<0.05$) (Figure 4D). The ratio of Bax/Bcl-2 protein levels was also significantly elevated ($p<0.05$). The application of PHA568487 before HR largely prevented most of the overexpression of caspase-1 and Bax proteins ($p<0.05$) (Figure 4E). The Bcl-2 protein expression was further increased by PHA568487 in comparison to that after HR treatment. The application of Nrf2 inhibitor partially blocked the effects of PHA568487 on the protein expression of caspase-3, Bax, and Bcl-2 (group 4; $p<0.05$).

Discussion

In this study, we found that the IR-like injury, HR, significantly reduced cell viability and caused a considerable amount of apoptotic cell deaths in the HK2 cells, accompanied with dramatically increased mRNA and protein expression of caspase-3 and Bax. The application of the α7nAChR agonist, PHA568487, counteracted most of the effects of HR on cell viability, apoptosis, and the apoptotic signaling. All of these effects of PHA568487 were mostly but incompletely blocked by the Nrf2 inhibitor ML385, which was shown to be a highly specific and effective Nrf2 inhibitor.[22] These data suggest that the activation of α7nAChR strongly antagonizes the HR-produced cell deaths, which is partially mediated by the Nrf2 signaling. Thus, our data indicate that Nrf2 is an essential downstream signaling of α7nAChR in the protection against renal IR injury.

Previous studies have focused on the anti-inflammatory effects of cholinergic pathway, while few have examined its influences on apoptosis and the signaling. Here for the first time, we demonstrate that the α7nAChR activation significantly attenuated HR-induced apoptosis through suppressing the apoptotic signaling and enhancing the anti-apoptotic factor Bcl-2 in the HK2 cells. Both actions of α7nAChR were significantly blocked by the Nrf2 inhibitor, suggesting that Nrf2 is crucial for the role α7nAChR in antagonizing apoptosis. These findings lend further support for α7nAChR agonists as potential therapeutic agents for IR renal injury and acute renal failure. Nevertheless, here we provide direct evidence that the inhibition of the Nrf2 signaling blocked the effects of the α7nAChR agonist on both suppressing apoptotic signaling and enhancing the anti-apoptotic factor.

It might be argued that anti-apoptotic effect of the cholinergic activation is the result of anti-inflammation. This is possible, as many studies have demonstrated that inflammation can generate radical oxygen species and thereby lead to apoptosis, which could be prevented by the cholinergic activation.[18,23,24] Moreover, both the anti-inflammatory and anti-apoptotic effects of cholinergic activation could be mediated by Nrf2/HO-1.[24] However, activation of Nrf2 may also directly antagonize apoptosis. Studies have shown that Nrf2 can also bind to the Bcl-2 gene promoter, thereby enhancing Bcl-2 gene expression and preventing apoptosis.[25] In another study, increased Nrf2 expression directly resulted in the decreased caspase-3/Bax expression and elevated Bcl-2 expression, with corresponding apoptosis change.[26] In our study, the clear Nrf2 dependent changes of Bcl-2 and caspase-3/Bax gene expression support a direct role of Nrf-2 in regulating the apoptotic pathway.

It should be mentioned that the Nrf2 inhibitor only partially blocked the effects of the α7nAChR agonist, suggesting the cholinergic activation may also antagonize apoptosis via an Nrf2-independent pathway. One candidate signaling is the PI-3 kinase/Akt pro-survival pathway. Previous reports showed that cholinergic activation, including α7nAChR agonists, could activate Akt and exert anti-apoptotic effects.[4,27] Interestingly, one of the downstream effectors of Akt is HO-1, and the interaction of Akt and Nrf2 can regulate the HO-1 gene expression in renal epithelia during HR injury.[28] However, Akt can also exert pro-survival and anti-apoptotic effects by direct inhibition of the caspase cascade or

by inhibiting P53-mediated apoptosis.[29,30] Future studies may involve the use of Akt inhibitor and Nrf2 inhibitor together and evaluate their influence on the anti-apoptotic effects of α7nAChR agonists.

In conclusion, our results indicate that the selective activation of α7nAChR strongly antagonized HR-induced apoptosis through inhibiting the apoptotic signaling and enhancing the anti-apoptotic signaling. In addition to the Nrf2 signaling, the protective effects of α7nAChR against IR injury may also involve other signaling pathways. Our results lend further support for α7nAChR selective agonists as potential therapy for renal IR injury, which might exert stronger protection than the agents targeting on Nrf2 alone.

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