Acidosis promotes cell apoptosis through the G protein-coupled receptor 4/CCAAT/enhancer-binding protein homologous protein pathway

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Abstract. The aim of the present study was to investigate the effects of acidosis on the apoptosis of renal epithelial and endothelial cells, and the molecular pathways responsible for this. A human proximal tubular cell line, HK-2, and human umbilical vein endothelial cells (HUVECs), were transfected with control or G protein-coupled receptor 4 siRNA for 36 h. Cells were exposed to normal (pH 7.4) or acidic (pH 6.4) media. Western blot analysis was used to assess the protein expression levels of G protein-coupled receptor 4 (GPR4), CCAAT/enhancer-binding protein homologous protein (CHOP) and cleaved caspase-3. Cell apoptosis was examined using the TUNEL assay and the lactate dehydrogenase (LDH) release assay. Using these techniques, it was demonstrated that acidosis increased the protein expression levels of GPR4, CHOP, cleaved caspase-3 and intracellular cyclic adenosine monophosphate levels in hypoxia/reoxygenation (HR)-treated cell lines. Knockdown of GPR4 in HK-2 cells and HUVECs markedly reduced the protein expression levels of acidosis-mediated GPR4, CHOP and cleaved caspase-3, as well as the rate of cell apoptosis. Therefore, the results of the present study suggested that acidosis promotes the apoptosis of HK-2 cells and HUVECs by regulating the GPR4/CHOP pathway.

Introduction

Acidosis is associated with various pathological conditions, including ischemia (restricted blood supply), diabetic ketoacidosis, defective blood flow, defective glycolytic metabolism, and lung and renal diseases (1-3). During acidosis, hydrogen ions accumulate in ischemic tissue, leading to intracellular acidosis (4). Within 5 min of the initiation of renal ischemia, the tissue pH drops from 7.2-6.5 and metabolic acidosis persists even with reperfusion (4). Acidosis causes tissue injury and promotes the progression of existing disease, such as stroke (5). However, the specific effects of acidosis on renal epithelial and endothelial cells, and the molecular pathways responsible for these effects, remain unknown.

A previous study suggested that G protein-coupled receptor 4 (GPR4) may be activated by a decrease in extracellular pH, which generates a second messenger protein (6). GPR4 acts as a pH sensor in the kidney, mediating the accumulation of intracellular cyclic adenosine monophosphate (cAMP), which regulates the activity of acid-base transport proteins in the kidney collecting duct (6,7). A previous study using GPR4-knockout mice has also indicated that GPR4 is involved in renal acid-base homeostasis (3).

CCAAT/enhancer-binding protein homologous protein (CHOP) is a transcriptional regulator and is an essential regulator of the endoplasmic reticulum stress (ERS)-mediated apoptosis pathway (8). CHOP is a pro-apoptotic protein and serves key roles in the pathology of liver fibrosis (9). It has been reported that the deletion of CHOP may protect mice from liver and kidney injury (10). A previous study reported that acidosis activates GPR4 in order to increase the expression of ERS-associated genes, including CHOP, in endothelial cells (11). Our previous study demonstrated that knockdown of GPR4 inhibited the expression of CHOP in hypoxia/reoxygenation (HR)-treated human umbilical vein endothelial cells (HUVECs) (12). However, to the best of our knowledge, the effect of GPR4 on the expression of CHOP in renal cells in response to acidosis remains uncharacterized. Therefore, the aim of the present study was to investigate the effects of acidosis on the apoptosis of renal epithelial cells and endothelial cells and the molecular pathways responsible for these effects.

Materials and methods

Cell culture and treatment. The human umbilical vein endothelial cell line, HUVEC, was obtained from All Cells

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LLC. (Alameda, CA, USA), and the human proximal tubular HK-2 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in 5% CO_2 at 37˚C.

For hypoxia/reoxygenation (HR) treatment, $5x10^5$ cells in 2 ml media were placed into each well of 6-well flat-bottomed plates at 37˚C. Cells were exposed to hypoxia (1% O_2) for 4 h, followed by a 6 h re-oxygenation (95% O_2) period. Subsequently, HUVECs and HK-2 cells were subjected to acidosis by adjusting the media to pH 6.4 by applying 0.1 M HCl for 12 h prior to harvesting.

siRNA transfection. Cell transfection was performed as previously described (13). In brief, cells were seeded onto 24-well pates at $1x10^5$ cells/well. Cells were then transfected with 80 nM siRNA using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The non-target scrambled siRNA duplexes (Shanghai GenePharma Co., Ltd., Shanghai, China; cat. no. A06001) and GPR4 siRNA (siGPR4) duplexes were purchased from Invitrogen; Thermo Fisher Scientific, Inc. The sequences of si-GPR4 were as follows: Forward, 3'-CCC UCUACAUCUUUGUCAUTT-5' and reverse, 3'-AUGACA AAGAUGUAGAGGGTT-5' (12). The cells were harvested after 36 h of transfection.

Western blot analysis. Total cell protein was extracted from HUVECs and HK-2 cells using a Nuclear Extract kit (Active Motif, Carlsbad, CA, USA), according to the manufacturer's protocols. Equal amounts of protein (60 μg) were separated by 10% SDS-PAGE, prior to being transferred onto Whatman nitrocellulose membranes (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Subsequent to blocking in 5% skimmed milk for 1 h at 37˚C, the membranes were incubated with primary antibodies against the following overnight at 4˚C: GPR4 (dilution, 1:1,000; R&D Systems, Inc., Minneapolis, MN, USA; cat. no. RDC0299), CHOP (dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA; cat. no. 2895), cleaved caspase-3 (dilution, 1:1,000; Cell Signaling Technology, Inc.; cat. no. 9661) and GAPDH (dilution, 1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-32233). Following 3 washes in Tris-buffered saline with 0.1% Tween-20 (TBST), the membranes were incubated with a horseradish peroxidase-conjugated mouse anti-rabbit secondary antibody (dilution, 1:10,000; Cell Signaling Technology, Inc.; cat. no. 5127) for 1 h at 37˚C. The blot was visualized using an enhanced chemiluminescence kit (GE Healthcare, Chicago, IL, USA). Densitometry was performed using ImageJ software (version 1.43a; National Institutes of Health, Bethesda, MD, USA) and GAPDH was used as a loading control.

Measurement of cAMP levels. Cells were treated with 50 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich; Merck KGaA) for 30 min, followed by HR treatment, performed as aforementioned. Subsequently, 200 μl 0.1 M HCl was added to lyse the cells for 12 h at 37˚C. The lysate was boiled at 90˚C for 5 min and centrifuged at 10,000 x g at 4˚C for 5 min prior to the supernatant being collected. The cAMP levels were measured using a cAMP ELISA kit (cat no. KGE002; R&D Systems Inc.), according to the manufacturer's protocol.

TUNEL assay. A TUNEL assay was performed using the *In Situ* Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland). In brief, cells were fixed with 4% paraformaldehyde for 1 h at room temperature and were permeabilized with 0.1% Triton X-100 in PBS for 2 min on ice. Subsequent to rinsing with PBS, the cells were incubated with the TUNEL reaction mixture for 60 min at 37˚C. Cell nuclei were counterstained with 10 mg/ml 4',6-diamidino-2-phenylindole for 1 h at 37˚C. Finally, the slides were mounted with synthetic resin and observed under a fluorescence microscope (original magnification, x400; BX-53; Olympus Corporation, Tokyo, Japan). The total number of TUNEL positive cells was calculated in ≥3 fields of view.

Measurement of lactate dehydrogenase (LDH) release. At 36 h after cell transfection, the supernatant was collected from both HK-2 and HUVEC cells after centrifugation at 3,000 x g for 5 min at room temperature and was measured using the CytoTox 96 Non-Radioactive Cytotoxicity assay (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. The results are expressed as the percentage of maximal LDH released into the culture medium.

Statistical analysis. All results are expressed as the mean ± standard deviation. The statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between 2 groups were performed using unpaired Student's t-tests, while comparisons between ≥3 groups were performed using one-way analysis of variance, following by Bonferroni's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Acidosis enhances HR-induced GPR4 and CHOP expression, as well as apoptosis. Renal epithelial and endothelial cells are susceptible to ischemic injury, which may lead to renal injury (14). In order to investigate the effect of HR on the expression of GPR4 and CHOP in the two cell types, HK-2 cells were confronted with HR injury and were experimentally assessed. As demonstrated in Fig. 1A and B, the expression levels of GPR4 and CHOP proteins were significantly increased in both HK-2 cells and HUVECs following HR treatment or HCl treatment alone, compared with the control group with a normal pH. The expression levels of GPR4 and CHOP were upregulated in the HR group treated with HCl compared with the cells treated with HR alone. Furthermore, the expression of cleaved caspase-3 was also increased by HCl treatment in the HR group compared with the HR group with a normal pH.

Intracellular cAMP is a downstream effector of GPR4 (15). The expression level of cAMP was increased in the cells exposed to HR and subsequent HCl treatment, compared with the cells treated with HR alone (Fig. 1C). The same changes of

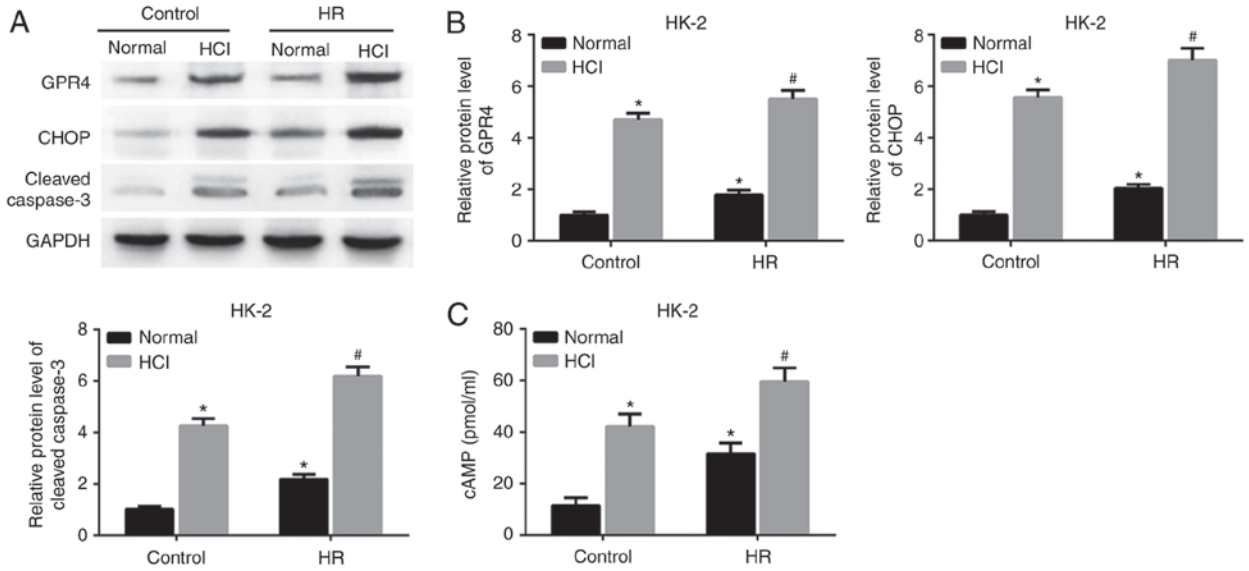

Figure 1. Acidosis activates GPR4 and CHOP expression, and increases cell apoptosis and cAMP levels in HR-treated HK-2 cells. The pH of culture media was adjusted to 6.4 using 0.1 M HCl immediately following HR treatment. (A) The protein expression levels of GPR4, CHOP and cleaved caspase-3 were determined by western blotting. (B) Quantitative analyses of the protein expression levels of GPR4, CHOP and cleaved caspase-3, relative to control cells. (C) The intracellular cAMP levels were measured by ELISA. *P<0.05 vs. control group without pH adjustment. #P<0.05 vs. HR group without pH adjustment. Cells in normal oxygen conditions were used as a control group. GPR4, G protein-coupled receptor 4; CHOP, CCAAT/enhancer-binding protein homologous protein; cAMP, cyclic adenosine monophosphate; HR, hypoxia/reoxygenation.

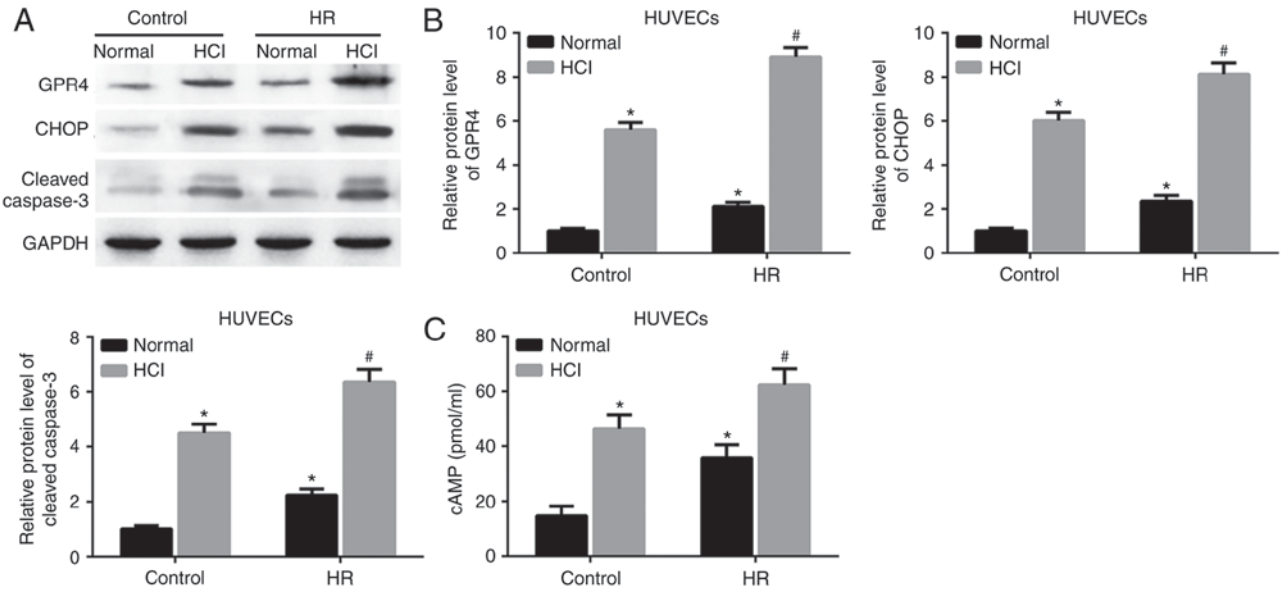

Figure 2. Acidosis activates GPR4 and CHOP expression, and increases cell apoptosis and cAMP levels in HR-treated HUVECs. The pH of culture media was adjusted to 6.4 using 0.1 M HCl immediately following HR treatment. (A) The protein expression levels of GPR4, CHOP and cleaved caspase-3 were determined by western blotting. (B) Quantitative analyses of the protein expression levels of GPR4, CHOP and cleaved caspase-3, relative to control cells. (C) The intracellular cAMP levels were measured by ELISA. *P<0.05 vs. control group without pH adjustment. #P<0.05 vs. HR group without pH adjustment. Cells in normal oxygen conditions were used as a control group. GPR4, G protein-coupled receptor 4; CHOP, CCAAT/enhancer-binding protein homologous protein; cAMP, cyclic adenosine monophosphate; HR, hypoxia/reoxygenation; HUVECs, human umbilical vein endothelial cells.

gene expressions (Fig. 2A and B) and cAMP levels (Fig. 2C) existed in HR-treated HUVECs. These results suggested that the HR-induced increase in GPR4 and CHOP expression and the apoptosis of HK-2 cells and HUVECs may result from acidosis.

GPR4-knockdown attenuates the GPR4 and CHOP expression, and the cell apoptosis induced by acidosis. To determine whether GPR4 function is associated with acidosis-induced GPR4/CHOP expression or cell apoptosis, GPR4 expression was knocked down by si-GPR4 in HK-2 cells and HUVECs. Acidosis resulted in an increase in the protein expression levels of GPR4, CHOP and cleaved caspase-3 in the HK-2 cells and HUVECs transfected with scrambled siRNA. The expression of these proteins and the acidosis-induced increase in intracellular cAMP expression

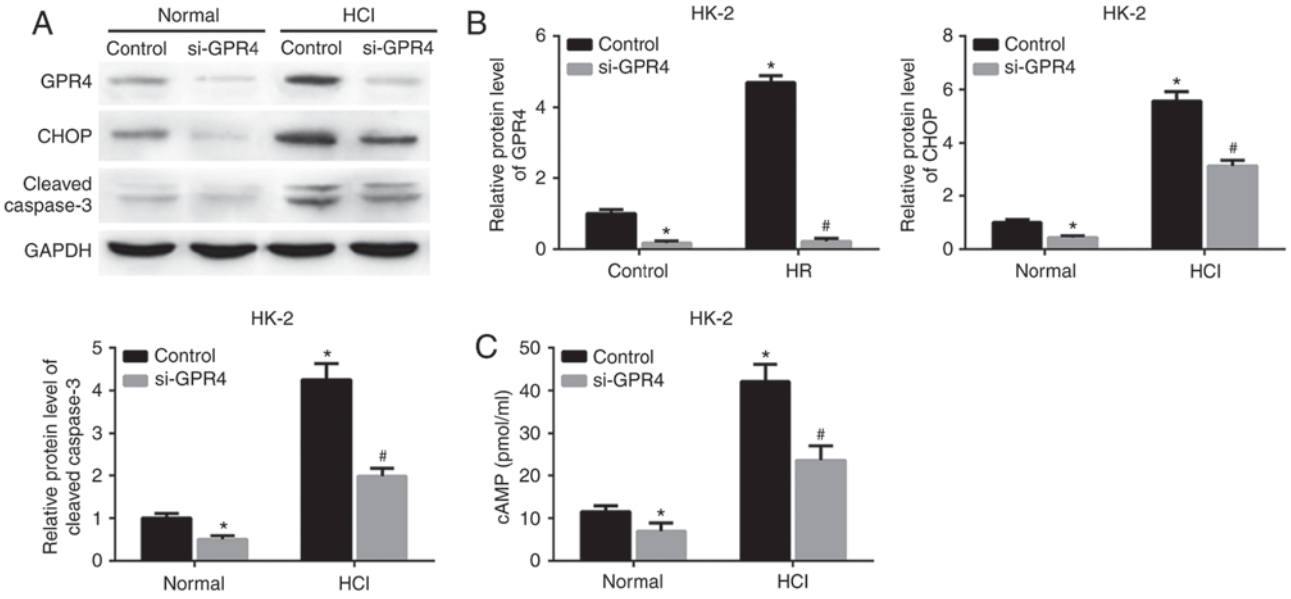

Figure 3. GPR4-knockdown reduces acidosis-induced GPR4 and CHOP protein expression levels, and suppresses apoptosis and cAMP levels of HK-2 cells. HK-2 cells were transfected with control siRNA or siGPR4. After 36 h, cells were cultured in normal culture medium (pH 7.4) or acidic medium (pH 6.4) for 12 h. (A) Protein expression of GPR4, CHOP and cleaved caspase-3 was determined by western blotting. (B) Quantitative analyses of the relative expression levels of proteins. (C) Intracellular cAMP levels. *P<0.05 vs. control group under normal conditions. #P<0.05 vs. control group under acidic conditions. GPR4, G protein-coupled receptor 4; CHOP, CCAAT/enhancer-binding protein homologous protein; cAMP, cyclic adenosine monophosphate; siRNA/si, small interfering RNA; HCl, hydrochloric acid; HR, hypoxia/reoxygenation.

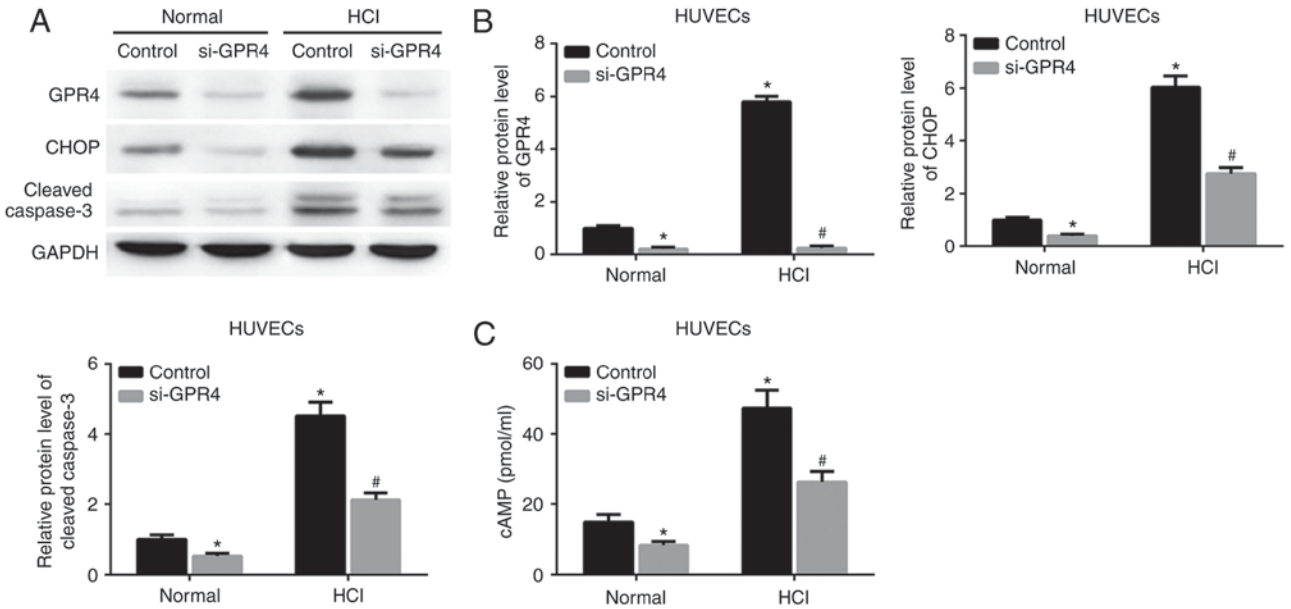

Figure 4. Knockdown of GPR4 reduces acidosis-induced GPR4 and CHOP expression, and suppresses cell apoptosis and cAMP levels in HUVECs. Cells were transfected with control siRNA or siGPR4 for 36 h, prior to being cultured in normal culture medium (pH 7.4) or acidic medium (pH 6.4) for 12 h. (A) Protein expression levels of GPR4, CHOP and cleaved caspase-3 were determined by western blot assay. (B) Quantitative analyses of the relative expression levels of GPR4, CHOP and cleaved caspase-3. (C) Intracellular cAMP levels. *P<0.05 vs. control group under normal conditions. #P<0.05 vs. control group under acidic conditions. GPR4, G protein-coupled receptor 4; CHOP, CCAAT/enhancer-binding protein homologous protein; cAMP, cyclic adenosine monophosphate; HUVECs, human umbilical vein endothelial cells; HCl, hydrochloric acid; siRNA/si, small interfering RNA.

levels in HK-2 cells and HUVECs were inhibited by knockdown of GPR4 (Figs. 3A-C; 4A-C).

GPR4-knockdown leads to a decrease in acidosis-induced apoptosis. To confirm the role of GPR4 in acidosis-induced cell apoptosis, a TUNEL assay was performed. TUNEL staining indicated an increased number of TUNEL positive HK-2/vector cells in response to acidic pH compared with the control group under normal pH conditions (Fig. 5A), whilst the increase in TUNEL positive cells induced by acidosis was attenuated by si-GPR4 treatment. These results were confirmed by measuring the release of LDH, which is a marker of cellular injury. Under acidic conditions, LDH release was significantly reduced in cells transfected with si-GPR4 compared with

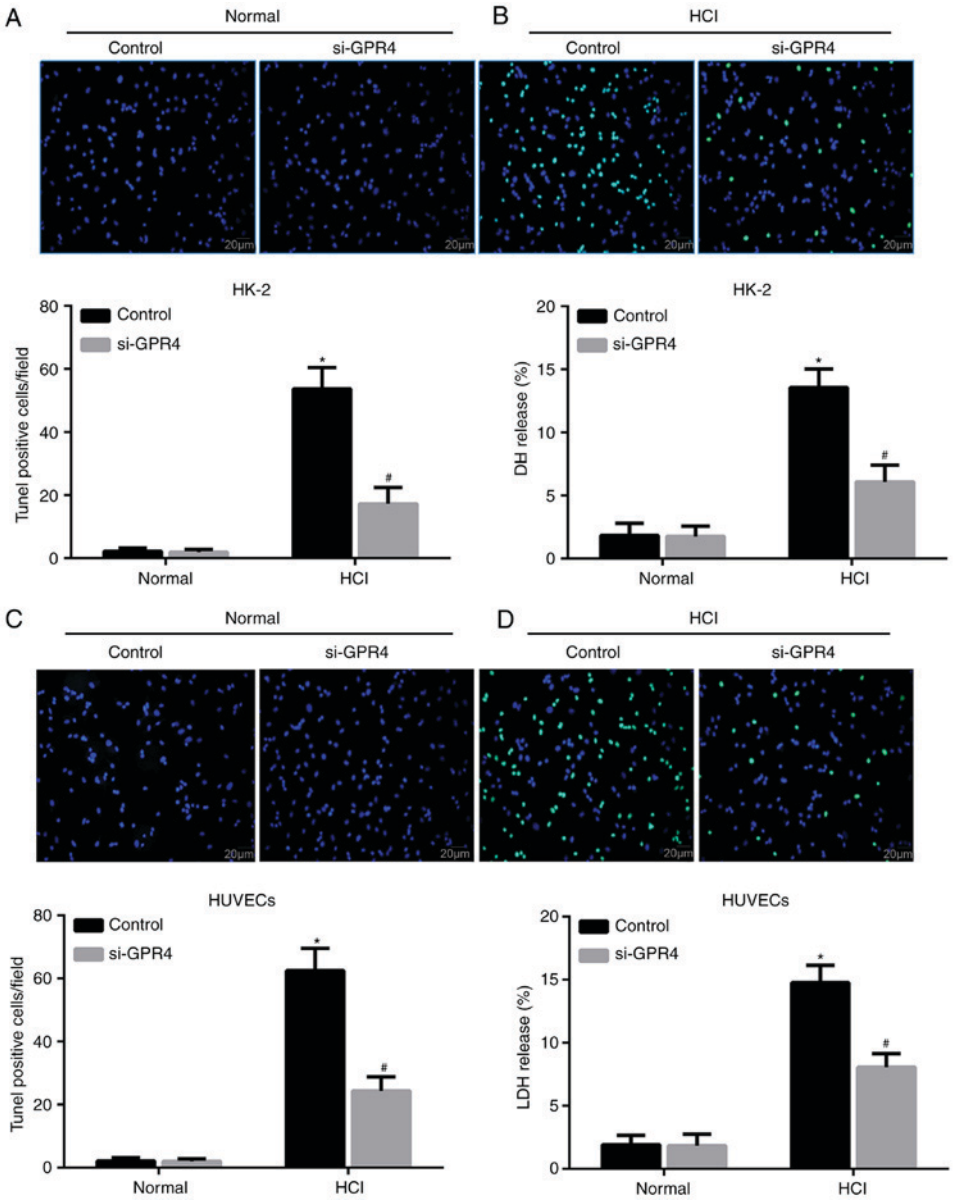

Figure 5. Knockdown of GPR4 reduced the acidosis-induced cell apoptosis of HK-2 cells and HUVECs. (A) Representative images of TUNEL staining and quantification of TUNEL-positive HK-2 cells. Scale bar=20 μm. (B) Percentage of LDH release in the supernatant of HK-2 cells (expressed as a percentage of the maximal value). (C) Representative images of TUNEL staining and quantification of TUNEL-positive HUVECs. Scale bar=20 μm. (D) LDH release into the supernatant of HUVECs (expressed as a percentage of the maximal value). *P<0.05 vs. control group under normal conditions. #P<0.05 vs. control group under acidic conditions. GPR4, G protein-coupled receptor 4; LDH, lactate dehydrogenase; HCl, hydrochloric acid; HUVECs, human umbilical vein endothelial cells; si, small interfering RNA.

cells transfected with scrambled siRNA (Fig. 5B). Similarly, GPR4 interference led to a decrease in the number of TUNEL positive HUVEC/vector cells (Fig. 5C) and LDH release from HUVECs (Fig. 5D). The results indicated that GPR4 expression was required for acidosis-induced apoptosis of the HK-2 cells and HUVECs.

Discussion

Acidosis is a pathological condition whereby acidity over-accumulates in the body fluid (16). The condition aggravates ischemia-associated tissue injury (17), and metabolic acidosis has been demonstrated to exacerbate the renal injury induced by ischemia/reperfusion (IR) (18). In addition, acidosis has been demonstrated to induce cell apoptosis and inhibit cell proliferation (19). It has been demonstrated that during stroke, the pH of brain tissue may drop to 6.0-6.5 and that this acidotic stress increases neural cell apoptosis (20). Our previous study indicated that HR-induced apoptosis was alleviated by neutralization of acidity (12). The results of the present study demonstrated that acidosis promoted HR-induced apoptosis. Taken together, these results suggest that HR-induced cell apoptosis in HK-2 cells and HUVECs may result from acidosis.

It has been reported that GPR4 is involved in the regulation of acid-base balance and kidney acid secretion (6). Acidosis may activate the expression of GPR4 in order to regulate the expression of the genes involved in different biological pathways, including the ERS response and cell metabolism (3,21). A previous study demonstrated that acidosis/GPR4 regulated

endothelial cell adhesion through cAMP production and served a role in the inflammatory response of vascular endothelial cells (3). In the present study, it was demonstrated that acidosis enhanced HR-induced GPR4 protein expression in HK-2 cells and HUVECs. CHOP is the key inducer of apoptosis in the ERS response (22), and is induced in hypoxia-treated renal tubular epithelial cells and promotes apoptosis (23). Our previous study indicated that hypoxia-induced CHOP expression is mediated by GPR4 (12). This result is supported by those of the present study, in which acidosis was demonstrated to induce the expression of CHOP. However, this effect was diminished by GPR4 knockdown in HK-2 cells and HUVECs. Therefore, acidosis-induced CHOP expression was regulated by GPR4 in HK-2 cells and HUVECs.

An association was identified between HR and cell apoptosis *in vitro*. Furthermore, the observation that inhibition of GRP4 expression reduces the cell apoptosis induced by acidosis contributes to the characterization of the role of the GPR4/CHOP pathway in the pathogenesis of IR injury, and suggests a potential molecular candidate for IR injury therapeutics. cAMP is a ubiquitous second messenger protein that regulates numerous cellular processes (24). Previous studies have demonstrated that an acidic pH activates GPR4 in order to induce the production of cAMP (15). Chen *et al* (3) reported that activation of GPR4 by acidosis increased endothelial cell adhesion via the cAMP pathway. Our previous study demonstrated that a cAMP analog, 8-bromo-cAMP, induced CHOP expression in HUVECs (18). The present study demonstrated that GPR4 expression reduced acidosis-induced cAMP and CHOP expression levels in HK-2 cells and HUVECs. Taken together, these results suggested that acidosis-induced CHOP expression may result from GPR4 activation and cAMP accumulation.

In conclusion, the present study demonstrated that HR-induced cell apoptosis in HK-2 cells and HUVECs may result from acidosis. The CHOP pathway serves a key role in acidosis-induced cell apoptosis. These results indicated that GPR4 inhibitors may be promising and effective therapeutic tools for the treatment of ischemic renal injury.

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Availability of data and materials

All data that were generated or analyzed in this study are included in this manuscript.

Authors' contributions

BD designed the present study. XLZ and YF conducted the experiments. SC performed the statistical analysis. XPZ interpreted the statistical analysis, and reviewed and approved the final version to be published. All authors read and approved the manuscript.

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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