ASIC1a Involves the Acid-Mediated Activation of Pancreatic Stellate Cells Associated With Autophagy Induction

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Received July 21, 2022
Accepted December 8, 2022
Epub Ahead of Print December 22, 2022

Summary

The acidic tumor microenvironment (TME) of pancreatic cancer affects the physiological function of pancreatic stellate cells (PSCs), which in turn promotes cancer progression. Acid-sensing ion channel 1a (ASIC1a) is responsible for acidosis-related physiopathological processes. In this study, we investigated the effect of acid exposure on the activation and autophagy of PSCs, and the role of ASIC1a in these events. The results showed that acidic medium upregulated the expression of ASIC1a, induced PSCs activation and autophagy, which can be suppressed by inhibiting ASIC1a using PcTx1 or ASIC1a knockdown, suggesting that ASIC1a involves these two processes. In addition, the acid-induced activation of PSCs was impaired after the application of autophagy inhibitor alone or in combination with ASIC1a siRNA, meaning a connection between autophagy and activation. Collectively, our study provides evidence for the involvement of ASIC1a in the acid-caused PSCs activation, which may be associated with autophagy induction.

Key words

ASIC1a • Pancreatic stellate cells • Activation • Autophagy • Acidosis

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Introduction

PSCs are in the quiescent state in normal pancreas, and play a significant role in maintaining pancreatic tissue architecture and extracellular matrix (ECM) production. However, a remarkable transition of quiescent PSCs into activated myofibroblast-like phenotype will appear in pancreatic cancer, and the activated PSCs can interact closely with cancer cells as well as other stromal cells to create a favorable microenvironment for tumor growth and metastasis [1]. Increasing researches focus on the potential role of targeting PSCs in pancreatic cancer therapy, and in fact, in the preclinical study, inhibition of PSCs activation or "re-programming" PSCs to an inactive state has been proven to be effective in cutting off the cancer-stroma interaction or decreasing ECM deposition and desmoplasia, which is conducive to improve the outcomes of pancreatic cancer treatment [2,3]. Therefore, exploring the stimulating factors and mechanisms of activating PSCs helps in the search for new interfering strategy for pancreatic cancer treatment.

In general, solid tumors including pancreatic cancer have an acidic TME, which is formed owing to the accumulation of acidic metabolic products and insufficient blood perfusion [4]. A physiological pH (about 7.4) is required for normal cell growth, and too acid or alkaline condition both may affect cell state. It is reasonable to speculate that the biological behavior of

PSCs changes in the acidic TME. Acid-sensing ion channels **(**ASICs), which can be activated by extracellular acidification, are responsible for the acidosis-related pathophysiological processes. As one most-watched member of ASICs, ASIC1a is mainly expressed in mammalian central and peripheral nervous system, and participates in physiological function (e.g. learning and memory) and some neurological disorders including neurodegenerative diseases, stroke, epilepsy, pain and migraine [5]. However, recent studies suggest that, besides neuronal cells, ASIC1a is also expressed in cancer cells, hepatic stellate cells (HSCs), rat articular chondrocytes and synovial fibroblasts [6-8]. In some cancers including pancreatic cancer, glioblastoma, lung cancer, gastric cancer and liver cancer, it is reported that ASIC1a mediates the acidosis-induced cell proliferation, migration and invasion, meaning that ASIC1a might serve as a novel therapeutic target for these cancers [9-14]. Pancreatic cancer cells and their stroma cells (e.g. PSCs) share the same acidic environment. Whether the acidic TME in pancreatic cancer imposes influence on PSCs *via* ASIC1a pathway remains unclear.

In this study, acidic medium (pH 6.5) was used to mimic the acidic TME of pancreatic cancer *in vitro*, and the expression of ASIC1a was detected when PSCs were maintained in culture medium with different pH values. In view the report that autophagy is required for activation of PSCs [15], the activation and autophagy-related markers both were measured after PSCs treatment with acid exposure, and the role of ASIC1a in these two processes was further investigated. Lastly, the association between activation and autophagy of PSCs induced by acidic medium was assessed.

Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) containing high glucose, fetal bovine serum (FBS) and Opti-MEM were purchased from Gibco Company. ASIC1a specific inhibitor Psalmotoxin-1 (PcTx1) was provided by ApexBio Technology. Lipofectamine 2000 reagent was purchased from Invitrogen. Anti-ASIC1a and anti-Collagen I rabbit polyclonal antibody (pAb) were provided by Beijing Bioss Antibodies. Anti-Beclin 1, anti-p62 rabbit pAb, the secondary antibody (HRP-conjugated goat anti-rabbit IgG (H+L)) and RIPA lysis buffer were provided by Beyotime Biotechnology. Cy3-conjugated donkey anti-rabbit IgG and primary antibodies of anti-α-SMA and anti-LC3B rabbit pAb were provided by Servicebio. Anti-GAPDH was obtained from Cell Signaling Technology (CST) Company. Two commonly used autophagy inhibitors chloroquine (CQ) and 3-Methyladenine (3-MA) were purchased from Shanghai Aladdin and MCE Company respectively. Other commonly used agents all were commercially available.

Cell culture and treatment

The immortalized human PSCs were purchased from Tongpai (Shanghai) Biotechnology CO., LTD and were cultured in DMEM containing 10 % FBS in a humidified air atmosphere with 5 % CO_2 at 37 °C. Acidic DMEM with pH 6.5 was prepared firstly using HCl adjustion to simulate an acid microenvironment. PSCs were cultured in normal DMEM (pH 7.4) or acidic DMEM for 4 h, and then ASIC1a expression in two groups was detected. To investigate the role of ASIC1a in the acid-induced cell physiological function changes, PSCs were divided into five groups: (I) control (pH 7.4), (II) pH 6.5, (III) pH 6.5 + PcTx1 (PSCs were pretreated with 50 nM PcTx1 for 30 min), (IV) pH 6.5 + ASIC1a siRNA and (V) pH 6.5 + NC-siRNA. After maintaining in the corresponding condition for 4 h, the markers of activation and autophagy of PSCs were measured by RT-PCR and Western blotting. Lastly, the acid-induced activation degree of PSCs was further assessed when autophagy was inhibited by the autophagy inhibitor (CQ and 3-MA) alone or in combination with ASIC1a siRNA, which help to explore the association between ASIC1a-mediated activation and autophagy induction under acid condition.

Cell immunofluorescence staining

After culturing in normal DMEM or acidic DMEM, PSCs were washed with PBS three times and then fixed with 4 % paraformaldehyde for 30 min, blocked with 3 % BSA for 1 h at room temperature (RT). PSCs were washed with PBS twice again and then incubated with anti-ASIC1a (1:500) antibody overnight at 4 °C. Subsequently, PSCs were incubated with Cy3-conjugated donkey anti-rabbit IgG in the dark at RT for 50 min. Cell nuclear was stained with DAPI. Lastly, PSCs were washed with PBS twice and cell images were acquired using an inverted fluorescence microscope.

ASIC1a knockdown using siRNA

Gene silencing using siRNA technique was

employed to knockdown ASIC1a expression. ASIC1a siRNA (the sequence: 5'-CGGAGCGUGUGCAG UACUACUTT-3' (forward); 5'-UAGUACUGCACACG CUCCGUGTT-3' (reverse)) and its negative control (NC) siRNA (the sequence: 5'-UUCUCCGAACGUGUC ACGUTT-3' (forward); 5'-ACGUGACACGUUCGG AGAATT-3' (reverse)) were designed and synthesized by JRDUN Biotechnology. PSCs were cultured in Opti-MEM and then ASIC1a siRNA or NC-siRNA were transfected into PSCs using Lipofectamine 2000 reagent following the manufactures' instructions. Six hours later, PSCs were cultured in DMEM containing 10 % FBS for the following experiments. To verify the efficiency of ASIC1a knockdown in acid condition, PSCs were cultured in acidic medium for 4 h, and then ASIC1a expression was detected by RT-PCR and Western blotting. The results showed that, compared with PSCs, the expression of ASIC1a in PSCs/ASIC1a siRNA, but not in PSCs/NC-siRNA, was inhibited significantly, suggesting that ASIC1a knockdown in PSCs was effective (Fig. S1).

Western blotting

After different treatments, PSCs were washed twice with ice-cold PBS, and then were lysed using RIPA lysis buffer according to the manufacturer's instructions. The total protein was collected and further quantified with a BCA protein assay kit. Equal amounts of protein in each group were separated by 10 % SDS-PAGE and then transferred onto a PVDF membrane. After blocking with 5 % non-fat milk in TBST for 1 h at RT, the membrane was subjected to the primary antibody dilution (ASIC1a, 1:1000; α-SMA, 1:2000; collagen I, 1:500; Beclin 1, 1:100; LC3B, 1:1000; p62, 1:1000; GAPDH, 1:2000) overnight at 4 °C. After this, the PVDF membranes were washed with TBST for three times, and then incubated in HRP-conjugated second antibody for 1 h at RT. Lastly, the protein bands of membranes were visualized using Tanon-5200 ECL detection system.

Quantitative RT-PCR assay

After the corresponding treatment, total RNA in PSCs was extracted using Trizol reagent, and then was performed reverse transcription using Thermo Scientific Fermentas RevertAidTM First Strand cDNA Synthesis Kit according to the manufacturer' protocol. qRT-PCR assay (SYBR green) was performed on the ABI-7300 Real-Time PCR System. The specific primers were as follows: ASIC1a, 5'-CACATCCCAATATCCGTAAG-3' (F) and 5'-CAAATGAAGTCAGGGAAGAG-3' (R); α-SMA, 5'-CCGGGACATCAAGGAGAAAC-3' (F) and 5'-CGATGAAGGATGGCTGGAAC-3' (R); Collagen I, 5'-TCTTTGACCAACCGAACATGAC-3' (F) and 5'-TTGATTGCTGGGCAGACAATAC-3' (R); Beclin 1, 5'-AGGGATGGAAGGGTCTAAG-3' (F) and 5'-GGG CTGTGGTAAGTAATGG-3' (R); LC3B II, 5'-AACG GCCCTGACTGTAAAC-3' (F) and 5'-TGGTACA CTGCTGCTTTCC-3' (R); p62, 5'-TTACCCACAA CAGACAAG-3' (F) and 5'-ATACAGAAGCCAG AATGC-3' (R); GAPDH, 5'-AATCCCATCACCATC TTC-3' (F) and 5'-AGGCTGTTGTCATACTTC-3' (R). The relative expression amounts of mRNA of the target genes were normalized to GAPDH (the internal reference gene) and calculated using the $2^{-\triangle\triangle CT}$ method.

Statistical analysis

The data were expressed as mean ± SD, and the differences between two groups were compared by ANOVA or non-paired *t*-test. The value of $P<0.05$ was considered statistically significant.

Results and Discussion

Acid exposure promotes ASIC1a expression in PSCs

The expression of ASIC1a in PSCs cultured in medium with different pH values was investigated firstly. As shown in Fig. 1A, when PSCs were exposed to the acidic medium, the red fluorescence of cells became stronger, indicating an increased ASIC1a expression. The phenomenon was further confirmed by the results from RT-PCR and Western blotting, which suggested that the expression of ASIC1a at mRNA level and protein level in pH 6.5 group both was higher than that in control group (pH 7.4) (Fig. 1B-C).

Previous studies focus on the promoting effect of acidic TME on the malignant phenotype of pancreatic cancer cells, but to some extent, ignore the fact that the stromal cells (e.g. PSCs) also can be affected by the acid exposure. It has been reported that higher ASIC1a expression in pancreatic cancer tissues means worse tumor prognosis, and *in vitro*, ASIC1a involves the acidity-induced EMT process of pancreatic cancer cells, which facilitates tumor invasion and metastasis [9]. PSCs are among the most important stromal cells of pancreatic cancer. Our present results showed that, like pancreatic cancer cells, PSCs also had a function expression of ASIC1a, and its expression was enhanced in the acidic TME.

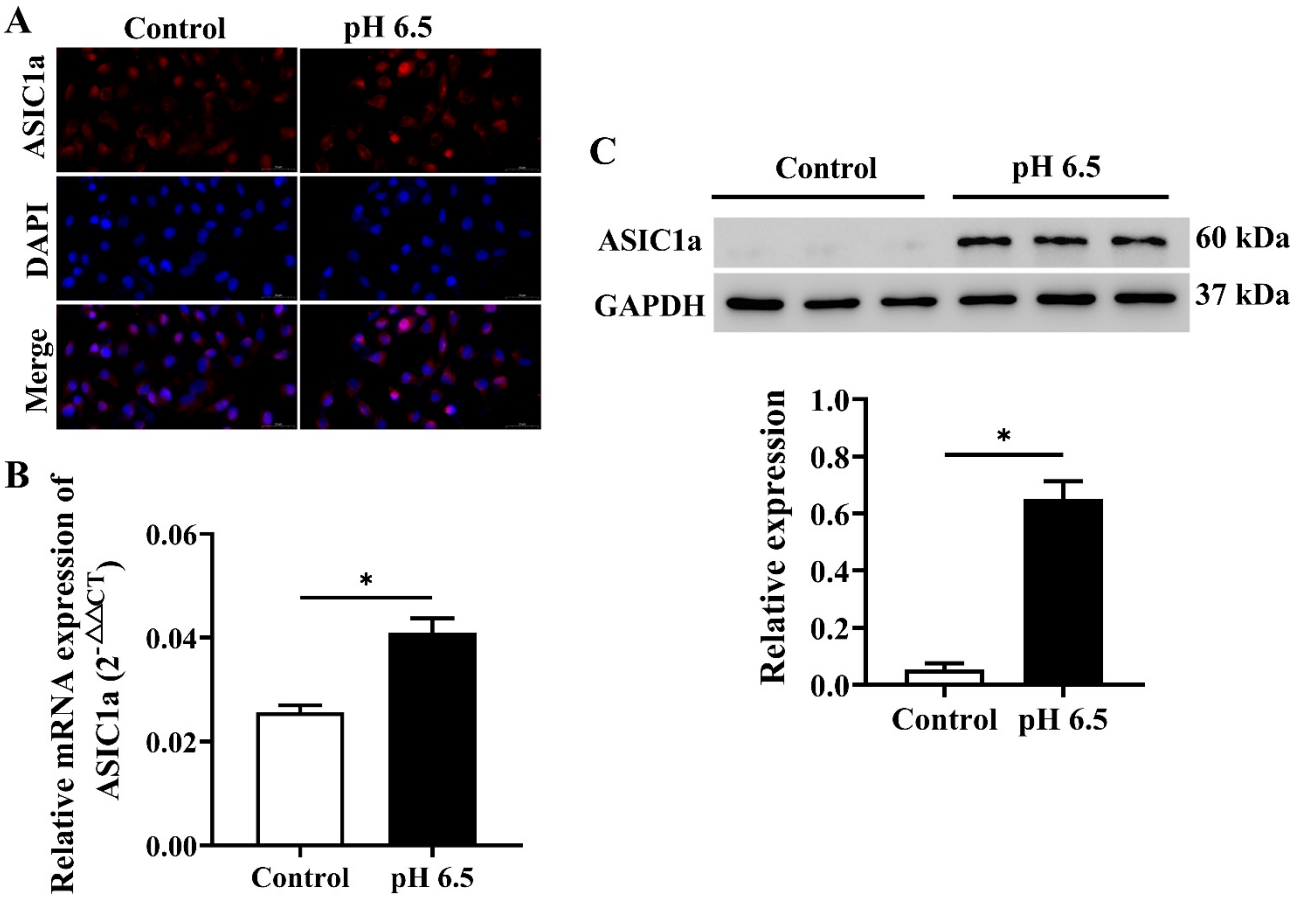

Fig. 1. Exposure to an acidic medium made the expression of ASIC1a in PSCs increase obviously. Immunofluorescence staining of ASIC1a (red) and cell nuclei (blue) (**A**). RT-PCR (**B**) and Western blotting result (**C**) of ASIC1a expression in control group (pH 7.4) and acidic medium group (pH 6.5). * P<0.05 (n=3).

ASIC1a involves PSCs activation induced by acidosis

The activated PSCs are transformed to α-SMA-positive myofibroblast-like cells, and can secrete a variety of cytokines, chemokines and ECM components such as collagen I, fibronectin and MMPs [16]. Therefore, α-SMA and collagen I are often chosen as the marker of PSCs activation. Our results showed that the expression of α-SMA mRNA and collagen I mRNA in pH 6.5 group was higher than that in control group, but the application of PcTx1 or ASIC1a knockdown using siRNA made the acid-induced elevated expression of activation marker decrease significantly (Fig. 2A-B). Furthermore, NC-siRNA could not produce a similar effect, suggesting that it was ASIC1a inhibition that suppressed the acid-induced activation of PSCs. The results of Western blotting showed that the expression tendency of α-SMA and collagen I at protein level in different groups was in line with that at mRNA level, supporting the opinion that acid exposure can induce PSCs activation, in which ASIC1a was involved (Fig. 2C).

PSCs activation is of great value in maintaining the malignant biological behavior of pancreatic cancer cells, and the stimulating factors of PSCs activation include cytokines, transcription factors, non-coding RNA, oxidative stress and calcium signalling [17]. Different from these reported factors, our present study suggested that the acidic TME itself also worked in the activation of PSCs *via* ASIC1a pathway. Much like PSCs, acid exposure also causes the activation of HSCs, which is associated with liver fibrosis, and ASIC1a inhibition can relieve acid-induced activation of HSCs [6]. These results suggested that the activated PSCs and HSCs shared some common characteristics of fibrosis development. It can be inferred that inhibition of ASIC1a is expected to suppress the acidic TME-induced activation of PSCs and further reduce fibrotic responses, producing a positive impact on the therapy of pancreatic cancer.

ASIC1a contributes to the acid-induced autophagy of PSCs

Autophagy is a tightly regulated cellular process in which various biological molecules and dysfunctional

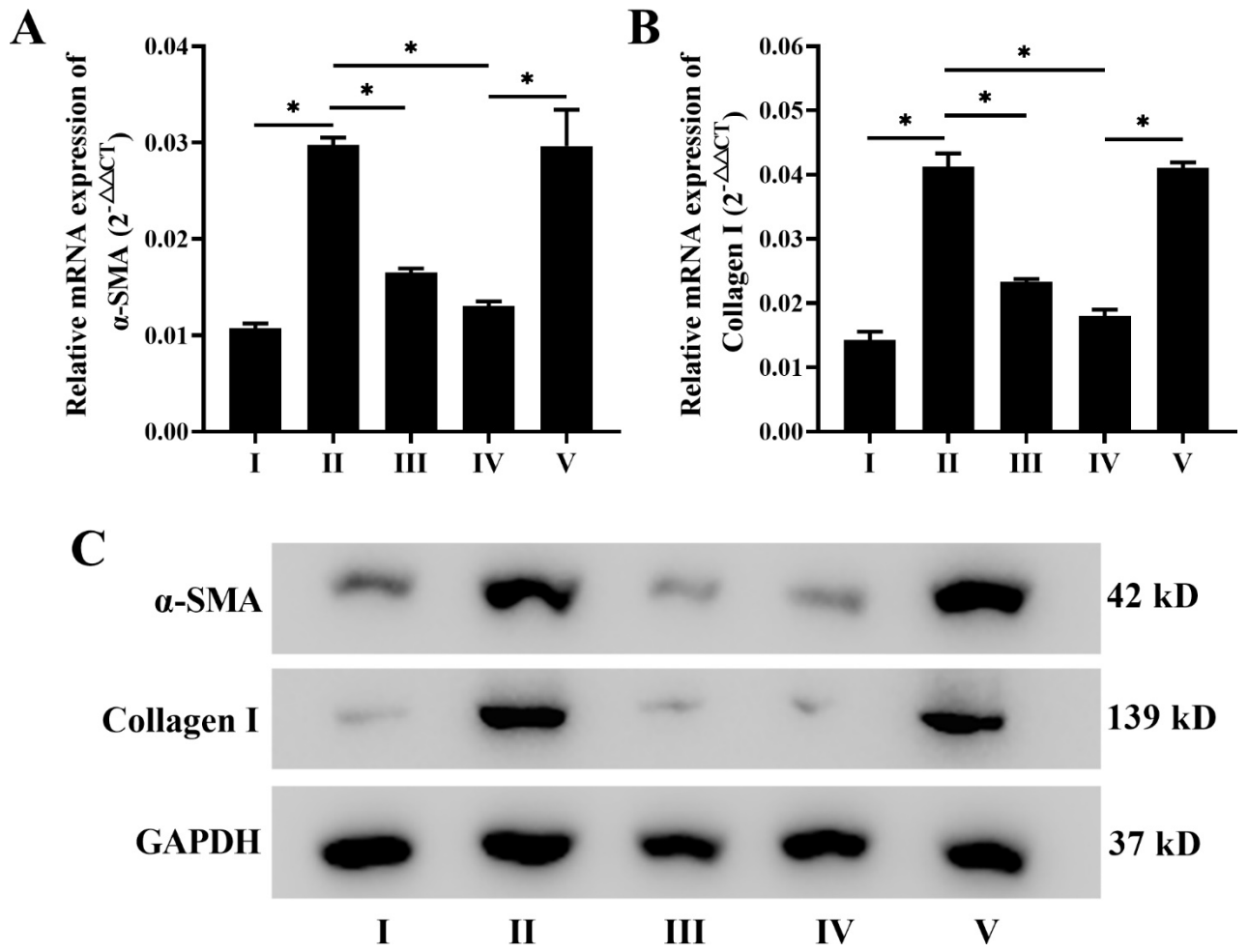

Fig. 2. ASIC1a involved the acid-induced activation of PSCs. The expression of α-SMA and collagen I in PSCs at different conditions was measured at mRNA level by RT-PCR (**A-B**) or at protein level by Western blotting (**C**). * P<0.05 (n=3). Repeated Western blotting experiments showed similar results. I (Control), II (pH 6.5), III (pH 6.5 + PcTx1), IV (pH 6.5 + ASIC1a siRNA), V (pH 6.5 + NC-siRNA).

organelles are delivered into lysosome for degradation and recycling [18]. Autophagy-related molecules work in the different stages. Beclin 1 can initiate autophagy and LC3B I is converted to LC3B II during autophagy activation, while p62, also called sequestosome 1 (SQSTM1), is degraded in autolysosome. Therefore, Beclin 1, LC3B II and p62 are considered as the most representative and reliable markers of autophagy. These three molecules also were used to assess the autophagy of PSCs treated under different treatment conditions. As shown in Figure 3, compared with control group, an upregulated expression of Beclin 1 and LC3B II, and a decreased expression of p62 at mRNA and protein level were observed in pH 6.5 group, suggesting that autophagy occurred due to acid exposure. However, when ASIC1a was inhibited in PcTx1 or ASIC1a knockdown group, the acid-induced up-regulation of Beclin 1 and LC3B II was impaired, and p62 expression was restored partly owing to the blockment of autophagic degradation. These results demonstrated that ASIC1a played an important role in the acid-induced autophagy of PSCs. Similar with our results, the mediating role of ASIC1a in acid-induced autophagy of liver cancer cells and rat articular chondrocytes has been confirmed [19,20]. In pancreatic cancer, the autophagy of PSCs has a special meaning for the nutritional metabolism of cancer cells. A highly hypoxic and malnutrition environment can't meet the metabolic needs of pancreatic cancer cells, but the alanine secreted by PSCs in an autophagy-dependent manner provides an alternative carbon source for cancer cells, which reduces their dependence on glucose and serum-derived nutrients [21]. In this sense, inhibition of PSCs autophagy contributes to exacerbate the starvation of cancer cells. Whether acidic TME-induced autophagy of PSCs can provide nutrients for cancer cells deserves exploration. This study confirmed that ASIC1a took part in the process of autophagy of PSCs, but the underlying molecular mechanism awaited further study.

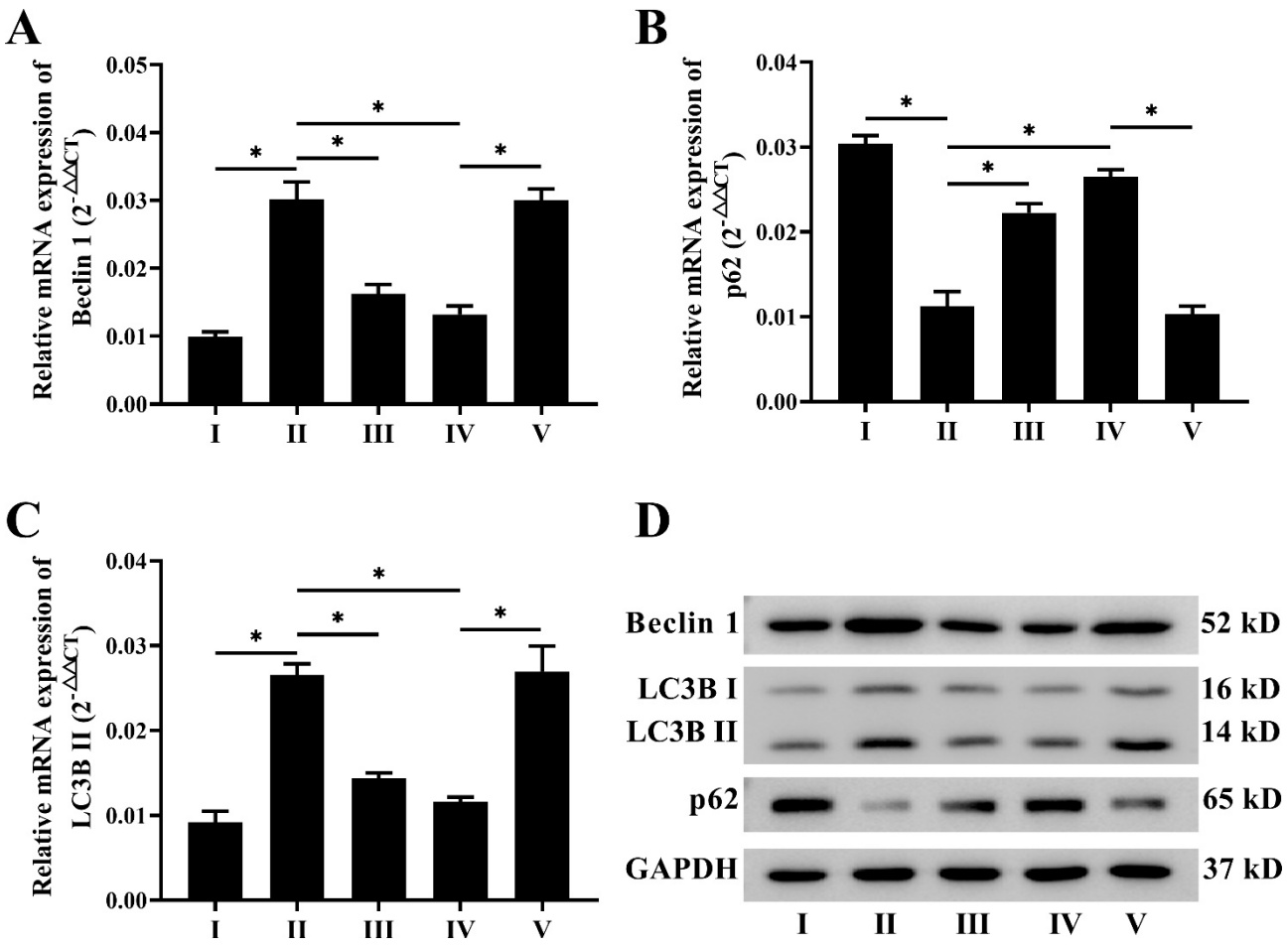

Fig. 3. ASIC1a mediated the acid-induced autophagy of PSCs. After the corresponding treatment, autophagy-associated important molecules, Beclin 1, LC3B and p62 expression in PSCs were detected by RT-PCR (**A-C**) and Western blotting (**D**). * $P<0.05$ (n=3). Repeated Western blotting experiments showed similar results. I (Control), II (pH 6.5), III (pH 6.5 + PcTx1), IV (pH 6.5 + ASIC1a siRNA), V (pH 6.5 + NC-siRNA).

Inhibition of autophagy impairs ASIC1a-involved PSCs activation under acidic environment

Numerous studies show that the autophagy of PSCs is closely related with the activation, and high autophagic activity is capable of facilitating PSCs activation *via* different signalling pathways such as JAK2/STAT3 and PI3K/Akt/mTOR [15,22-25]. Based on the previous reports, we tried to investigate the association between acid-induced autophagy and activation of PSCs. As shown in Figure 4, the expression of α-SMA and collagen I at mRNA and protein level in pH 6.5 group both was higher than that in pH 7.4 group, suggesting acid-induced PSCs activation. 3-MA and CQ as two conventional autophagy inhibitors work by blocking the formation of autophagosome and decreasing the fusion of autophagosome-lysosome respectively. The elevated expression of α-SMA and collagen I was suppressed after the application of 3-MA or CQ, suggesting that autophagy inhibition can restrain the activation of PSCs induced by acid medium. Interestingly, the combination of ASIC1a knockdown and autophagy inhibitor (3-MA or CQ) can further suppress the acid-induced PSCs activation, but NC-siRNA can't produce this effect. These results support the inference that, under the condition of this experiment, autophagy and activation of PSCs are not two independent processes, and ASIC1a may participate in the process of acid-induced activation through the induction of autophagy at least partially.

It has been reported that autophagic PSCs can secrete IL-6, which has a regulatory role on the activation of PSCs, and inhibition of autophagy can promote the transformation of PSCs from an activated phenotype to a quiescent state [15]. Our present study also suggested that the autophagy may be indispensable for PSCs activation, and the suppression of the former impaired the latter, but the pathway by which autophagy affects activation is still unknown. The acidic TME of pancreatic

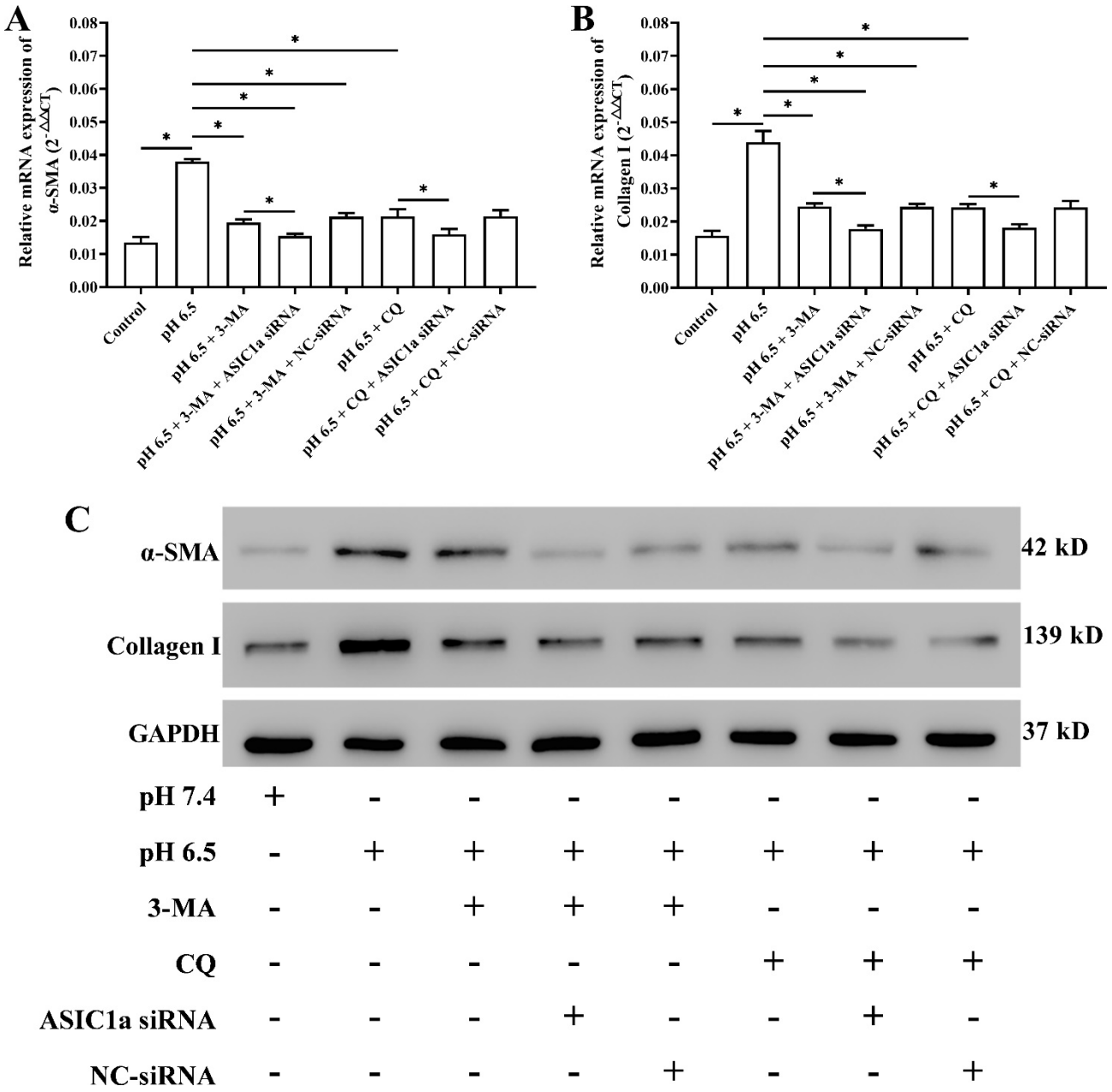

Fig 4. Involvement of autophagy in ASIC1a mediated acid-induced PSCs activation. After treatment with autophagy inhibitor alone or together with ASIC1a knockdown, the expression of α-SMA and collagen I in PSCs was detected by RT-PCR (**A-B**) and Western blotting (**C**). * P<0.05 (n=3). Repeated Western blotting experiments showed similar results.

cancer can induce PSCs activation and autophagy, which in turn, accelerate pancreatic cancer progression. It is imagined that, inhibition of ASIC1a in PSCs will lead to kill two birds with one stone: it alleviates the acid-mediated autophagy and activation of PSCs simultaneously. Our present results not only help to better understand the role of ASIC1a in the acidosis-related biological processes of PSCs, but also provide support for the opinion that ASIC1a is a potential target for pancreatic cancer therapy.

Conclusions

In conclusion, we used acidic medium to simulate the acidic TME of pancreatic cancer, and our results suggested that ASIC1a involved the acid-caused activation of PSCs, which may be associated with autophagy induction. In view of the fact that the activation and autophagy of PSCs has an important pathophysiologic significance in pancreatic cancer, blockade of ASIC1a in PSCs may become a novel anti-tumor therapeutic strategy.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This study was supported by grants from the Staring Foundation for Young Researcher of Changhai Hospital (No. CH201714).

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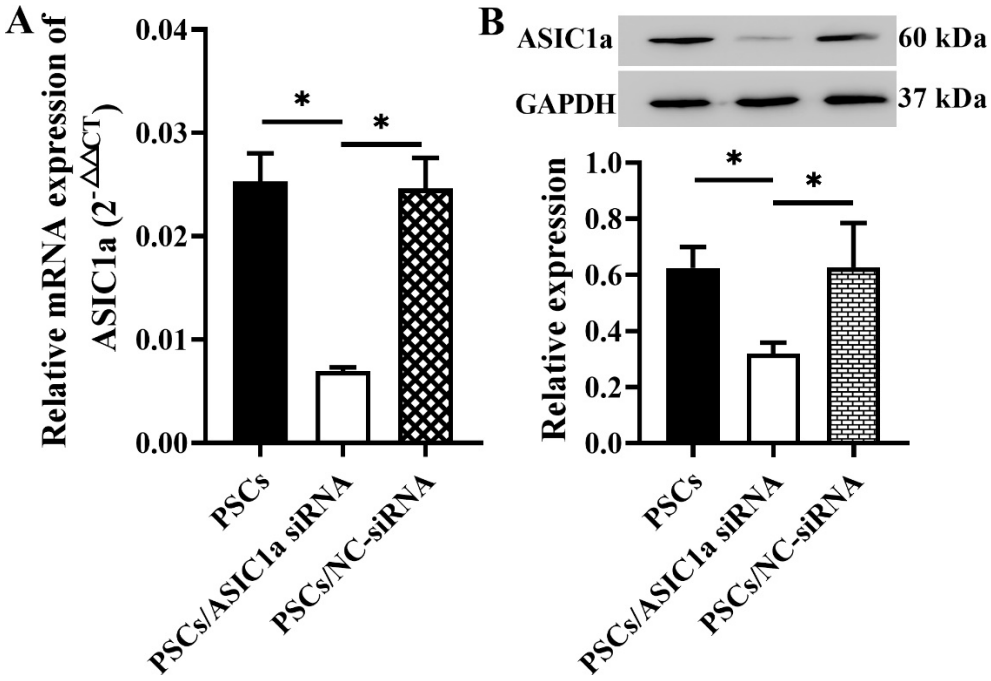

Supplementary Fig. 1. The expression of ASIC1a in PSCs with different treatment in acidic medium (pH 6.5) was assessed using qRT-PCR (**A**) and Western blotting (**B**).