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Effect of Activation of the Ca^{2+}-Permeable Acid-Sensing Ion Channel 1a on Acid-Induced Vascular Endothelial Cell Injury of Henoch-Schönlein Purpura Children

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Abstract

Acidosis in local environment plays a critical role in cell injury. One key mediator of acidosis-induced cell injury is the acid-sensing ion channels (ASICs), particularly ASIC1a. Herein, we investigated the role of ASIC1a in acid-induced vascular endothelial cell injury of Henoch-Schönlein purpura (HSP) children. Acid-induced ASIC1a, Calpain and Calcineurin expression in vascular endothelial cells pretreated with IgA1 isolated from HSP were detected by real time quantitative polymerase chain reaction and western blot methods, respectively. Cell cytotoxicity was measured by interleukin-8 and nitric oxide production with ELISA. The results showed acid-induced ASIC1a, Calpain and Calcineurin expression in cells increased, especially at PH6.5. The cytotoxicity of vascular endothelial cells was increased by extracellular acidosis. Moreover non-specific or specific blockers of ASIC1a, Amiloride and PcTX-1 could remarkably decrease these parameters. These findings show that increased $[Ca^{2+}]i$, mediated via ASIC1a, might contribute to acid-induced vascular endothelial cell injury of HSP.

Keywords

Acid-Sensing Ion Channels (ASICs), Vascular Endothelial Cell, Henoch-Schönlein Purpura (HSP)

1. Introduction

In biological systems, cells actively partake in maintaining homeostasis of their envi-

ronmental milieu within a precise range of physiological parameters. Cellular systems also foster the unique ability to respond and adapt to physiological stress, preserving survival and function. Signal transduction across cell membrane, through surface receptors is fundamental to detect and respond to changes in the local milieu [1]. Protons (H^+) represent an important component of the extracellular milieu [2]. The extracellular fluids and blood pH are tightly regulated and maintained judiciously at ~7.4 but under many patho-physiological circumstances such as inflammation, ischemia and tumor formation, acidosis occurs in the localized microenvironment [3].

Endothelial cells (EC) line the vasculature and form a barrier between the blood and the underlying tissue. Being present in every organ system in the human body, ECs control the transport of nutrients and oxygen to tissues and organs, and are the first cells to respond to circulating factors such as hyoxemia, PH and inflammatory cytokines that regulate their function under physiological conditions. Henoch-Schönlein purpura (HSP) is a common leukocytoclastic vasculitis with immunoglobulin A (IgA) deposition in children. It typically involves the small vessels of the skin, gastrointestinal (GI) tract, joints, and kidneys. Its wide range of vasculitis resulted in metabolic acidosis with extracellular acidification of vessel microenvironment.

Acid-sensing ion channels (ASICs), which belong to the degenerin/epithelial Na^+ channel (DEG/ENaC) superfamily, are activated by extracellular protons [4]. To date, at least six subunits of ASICs have been identified: 1a, 1b, 2a, 2b, 3, and 4 [5]. Our previous work demonstrated high ASIC1a and ASIC3 expression in the skin vascular endothelium of HSP children [6]. However, the mechanism by which vascular endothelial cells sense proton concentration and transmit their signal remains largely unknown. Intracellular Ca^{2+} ($[Ca^{2+}]i$) represents ubiquitous signaling mechanism and is involved in processes as diverse as cell division and injury [7]. ASIC1a is the only subunit that is permeable to Ca^{2+}, as it differs from other ASIC subunits. Activation of Ca^{2+}-permeable ASIC1a has been demonstrated to be responsible for acidosis-mediated ischemic brain injury caused by Ca^{2+} influx in neurons [8].

Here this study was to firstly investigate whether ASIC1a is involved in the vascular endothelial cell injury of HSP and ASIC1a contributes to cell injury through an acidosis-evoked increase in $[Ca^{2+}]i$.

2. Materials and Methods

2.1. Patients

HSP patients with acute onset and/or active at this hospital during January 2015 to June 2015 were included. The diagnosis of HSP was based on standard classification criteria [9]. The approval and fully informed counseled consent were obtained from the ethical committee of the first affiliated hospital of Anhui Medical University and the children's parents, respectively.

Sera IgA1 was isolated by using the Immobilized Jaclyn isolation kit (Pierce, Rockford, IL, and USA) as described previously [10].

2.2. Culture of Human Dermal Microvascular Endothelial Cells

Human dermal microvascular endothelial cells (HDMECs) were obtained from Jennio Biotechnology limited company of Guangzhou and were cultured with RPMI-1640 medium containing 15% fetal bovine serum. The cells were firstly incubated with IgA1 (final concentration 250 μg/ml) from HSP children, and then were treated with various extracellular solutions (pH7.5, 7.0, 6.5, 6.0) and Amiloride (final concentration 100 μmol/L), PcTX1 (20nmol/L) for 3h. Interleukin-8 (IL-8) and nitric oxide (NO) levels were measured in the supernatants to observe the cell cytotoxicity by ELISA and then cells were harvested to detect ASIC1a, Calpain and Calcineurin mRNA and protein expression.

2.3. ASIC1a, Calpain and Calcineurin mRNA Expression

Real time quantitative polymerase chain reaction (qRT-PCR) was performed to determine ASIC1a, Calpain and Calcineurin mRNA expression. Total RNA was isolated from HDMECs using Trizol reagent (Invitrogen, Shanghai, China). Total RNA (1 μg) was reverse transcribed into cDNA using AMV reverse transcriptase (Fermentas). Primers for ASIC1a, Calpain and Calcineurin, β-actin was shown in Table 1. The qPCR was under the following conditions: started at 50˚C for 2 min and 95˚C for 10 min (initial denaturation), followed by a 40-cycle amplification (denaturation at 95˚C for 15s, annealing/extension at 60˚C for 60 s). ASIC1a, Calpain and Calcineurin mRNA levels were normalized with those of β-actin mRNA.

2.4. Western Blot Analysis for Determination of ASIC1a, Calpain and Calcineurin Expression

The protein concentration of lysates was determined by BCA Protein Assay Kit (Beyotime, Shanghai, China). Proteins (20 ug) were separated using 10% (13% for caspase analysis) SDS-PAGE and transferred to polyvinylidene fluoride membranes. Antibodies to ASIC1a, Calpain and Calcineurin (Abcam, HongKong, England) were used as described by the manufacturer. Following washing three times with TBST, membranes were incubated with secondary HRP-conjugated anti-mouse IgG for 1h. Blots were

Table 1. Primers for ASIC1a, destrin, SM-α and β-actin.

Primer	primer sequences	Products length (bp)
ASIC1a	5'-GTCTCTGAAGCGGGCACTG-3' 5'-GCTGCTTTTCATCTGCCATC-3'	287
Calpain	5'-GATGTCCTTCCGGGACTTC A -3' 5'-CCTCCAGCCGCATCTTGAAC-3'	203
Calcineurin	5'-TGCATCTAGCAGTTGGATG-3' 5'-AACGTGTTCATTCCCCTAA-3'	258
β-actin	5'-TGACGTGGACATCCGCAAAG-3' 5'-CTGGAAGGTGGACAGCGAGG-3'	205

again washed with TBST, and then developed by enhanced chemiluminescence detection regents (ECL, Amersham). The protein bands were quantified by the average ratios of integral optic density following normalization to the housekeeping gene.

2.5. Statistical Analysis

Results were expressed as means ± SEM. The comparison among the different groups was done using the analysis of variance (ANOVA) and unpaired student t-test. P values <0.01 were considered significant.

3. Results

3.1. Effect of pH Concentration on the Expression of ASIC1a in Human Dermal Microvascular Endothelial Cells (HDMECs)

To observe the effect of pH concentration on ASIC1a expression in vascular endothelial cells of HSP children, we examined ASIC1a mRNA and protein expression in HDMECs pretreated with IgA1 from HSP children using qRT-PCR and western blot methods, respectively. The results showed that ASIC1a mRNA and protein expression in HDMECs significantly increased with the decrement of pH, especially at pH6.5 ($P < 0.01$) (**Figure 1(a)** and **Figure 1(b)**).

3.2. Cell Cytotoxicity of HDMECs

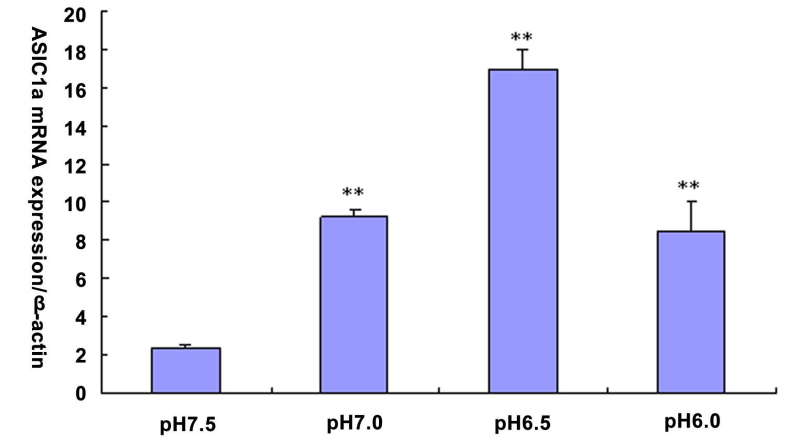
To determine the acid-induced cell cytotoxicity and whether activation of ASIC1a is involved in acid-induced vascular endothelium cells injury of HSP patients, we examined IL-8 and NO release and the effect of ASIC1a blockers on their production when HDMECs were exposed to different pH concentration solution. As shown in **Figure 2**, acid at different pH (especially PH6.5), induced the high IL-8 and NO levels in the supernatants of HDMECs pretreated with aIgA1 from HSP patients and there was significant difference compared with pH 7.5 group ($P < 0.01$). Amiloride and Psalmotoxin 1 (PcTx1), as blockers of ASIC1a, could significantly reduce the acid-induced IL-8 and NO release in the supernatants of HDMECs ($P < 0.01$).

3.3. Effect of PH Concentration and ASIC1a Blockers on the Expression of Calpain and Calcineurin

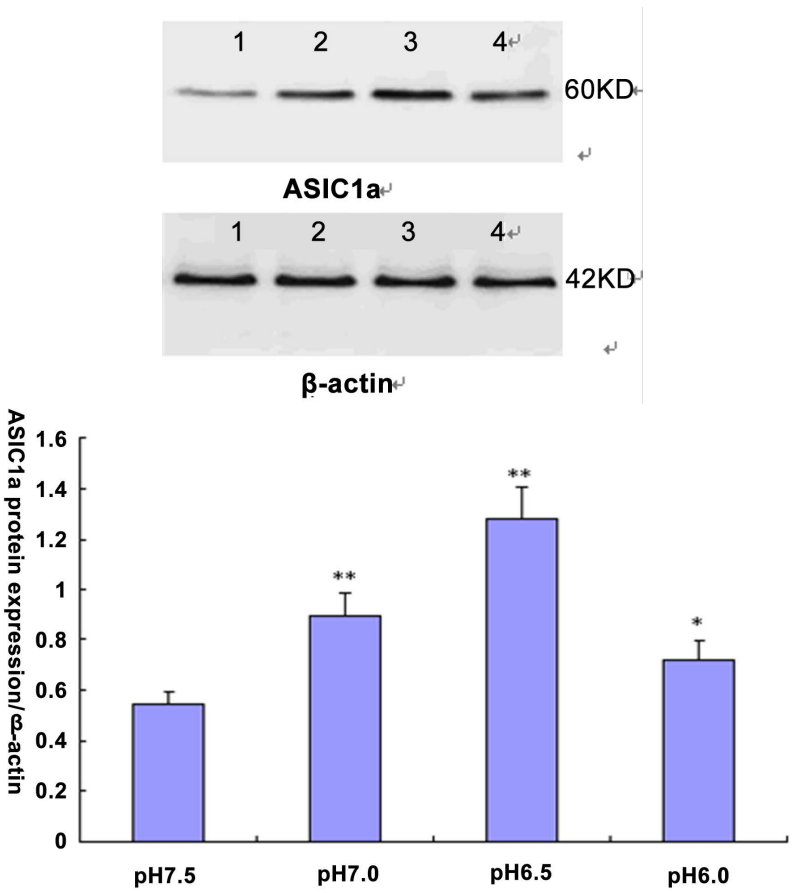
To observe the changes of Ca^{2+}-related protein expression in the vascular endothelial cell injury of HSP children, we observed the Calpain and Calcineurin mRNA and protein expression in HDMECs exposed to different pH concentration. The results showed that extracellular acid could significantly stimulate Calpain and Calcineurin expression with the decrement of pH while Amiloride and PcTx1, obviously inhibited acid-induced Calpain and Calcineurin expression in HDMECs (**Figure 3(a)**, **Figure 3(b)**).

4. Discussion

HSP is a systemic vasculitis of unknown cause, with immune-mediated inflammation of the small vessels and inflammation is often associated with extracellular acidification.



(a)



(b)

Figure 1. Effect of different pH concentration on ASIC1a expression in HDMVEC (X ± S, n = 6), ASIC1a expression was measured in human dermal microvascular endothelial cells (HDMVEC) by using qPCR and Western blot methods, respectively. (a) The ASIC1a mRNA expression was measured by qPCR method; (b) the ASIC1a protein expression was measured by Western blot method. Data are expressed as mean ± SD; **P < 0.01, compared with pH7.5 group, 1: pH7.5 group; 2: pH 7.0 group; 3: pH6.5 group; 4: pH6.0 group.

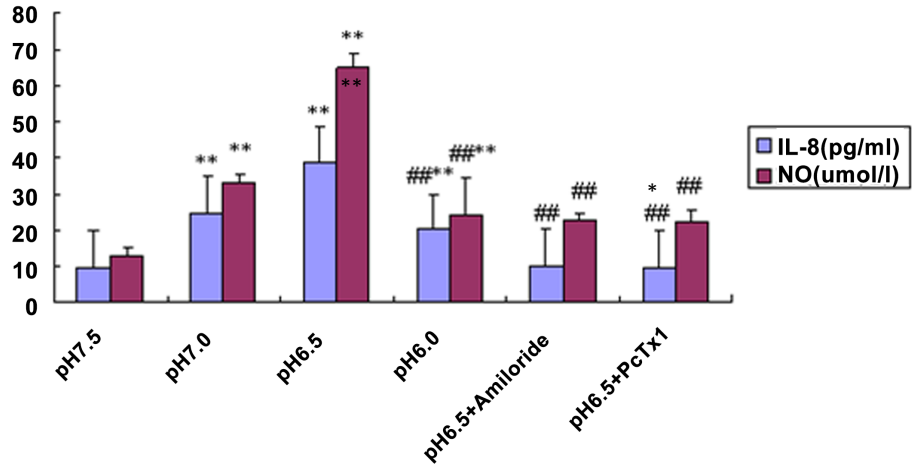
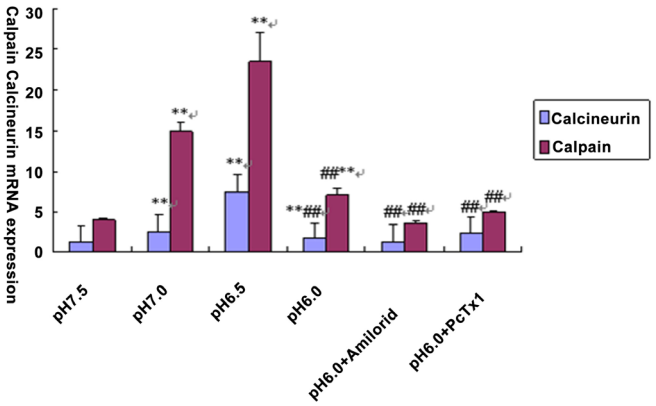


Figure 2. Effect of different pH concentration and ASIC1a blockers on the IL-8 and NO levels in the supernatants of cultured HDMVEC (X ± S, n = 6), IL-8 and NO level was measured in the supernatants of cultured HDMVEC using ELISA methods; Data are expressed as mean ± SD; **P < 0.01, compared with pH7.5 group; ##P < 0.01, compared with pH6.5 group.

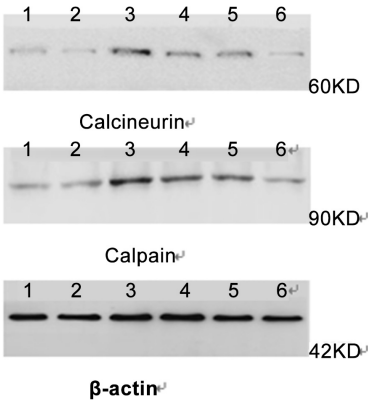
During inflammatory process, the pH value in the local area is always lower than that in normal tissues, ranging from pH 5.5 to 7, while pH_{50} values of 6.2 - 6.7 have been measured for ASIC1a. This present study demonstrated that ASIC1a expression in HDMECs pretreated with IgA1 from HSP patients was increased with the decrement of extracellular pH, and ASIC1a expression reached the maximum level when PH dropped to 6.5, which was consistent with the research previously reported [11].

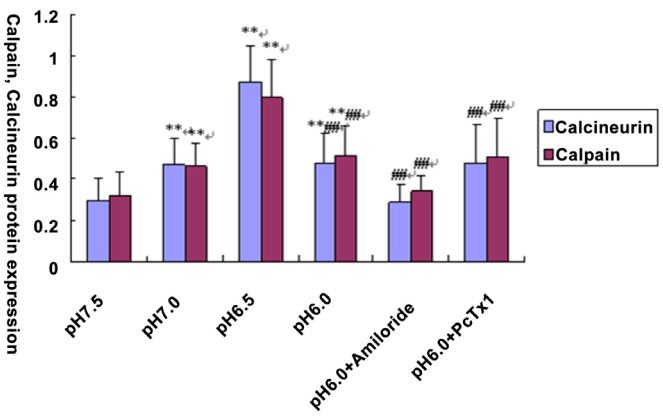
Many researches have demonstrated that children with HSP had raised serum levels of IL-8, NO, TNF-*α* and ET during the acute stage. In this study we found acid-induced IL-8 and NO release in HDMECs could be inhibit by blocking ASIC1a, which suggested that ASIC1a activation by acidosis induced inflammation contributed to further injury of vascular endothelial cells in HSP children.

It has been reported that the prominent regulatory function of annexin-mediated Ca^{2+} influx into vascular endothelial cells can lead to Ca^{2+} overload and cell injury [12]. Acidic stimulation is known to trigger Ca^{2+}-permeable ASIC1a and is essential for neuronal injury. However, no attention has been paid to this process in non-neuronal cells. Calpains, a family of non-lysosomal cysteine proteases play a crucial role in integrating cellular functions of mammals [13]. The activity of calpains is tightly regulated by Ca^{2+}; increased intracellular Ca^{2+} ($[Ca^{2+}]i$) level can increase calpain activity [14]. Calcineurin is a protein phosphatase known as serine/threonine phosphatase and can be activated physiologically by Ca^{2+}/calmodulin during the differentiation of skeletal muscles and the development of osteoclasts in bone [15]. Our present study showed that extracellular acid could significantly stimulate the expression of Calpains and Calcineurin in HDMECs pretreated with IgA1 from HSP children. However their expression was inhibited by blocking ASIC1a. This result suggested that ASIC1a might participant $[Ca^{2+}]i$ influx of the vascular endothelial cells and trigger the expression of Calpains and Calcineurin in HDMECs.



(a)





(b)

Figure 3. Effect of different pH concentration and ASIC1a blockers on the Calpain and Calcineurin in the cultured HDMVEC (X ± S, n = 6), Calpain and Calcineurin expression was measured in HDMVEC using qPCR and Western blot methods, respectively. (a) The Calpain and Calcineurin mRNA expression was measured by qPCR method; (b) the Calpain and Calcineurin protein expression was measured by Western blot method. Data are expressed as mean ± SD; $^{**}P < 0.01$, compared with pH7.5 group; $^{\#\#}P < 0.01$, compared with pH6.5 group, 1: pH7.5 group; 2: pH7.0 group; 3: pH6.5group; 4: pH6.0 group; 5: pH6.5 + Amiloride100umol/L; 6: pH6.5 + PcTX1 20 nmol/L.

5. Conclusion

In conclusion, ASIC1a was involved in acid-induced vascular endothelial cell injury of HSP, which might be related with $[Ca^{2+}]i$ influx mediated via ASIC1a. This result firstly provided us a new strategy for prevention and treatment of HSP children.

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Ph.D. Li-Ping Yuan recruited the subjects. MD. Qi-Di Peng performed the experiment and collected all of the primary data. MD. Yan Bo performed data analysis. Professor Gui Ming helped edit the manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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