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Original Article

miR-149 reduces while let-7 elevates ASIC1a expression in vitro

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Abstract: Acid-sensing ion channel 1a (ASIC1a) is the key subunit that determines acid-activated currents in neurons. ASIC1a is important for neural plasticity, learning, and for multiple neurological diseases, including stroke, multiple sclerosis, and traumatic injuries. These findings underline the importance for better defining mechanisms that regulate ASIC1a expression. During the past decade, microRNA has emerged as one important group of regulatory molecules in controlling protein expression. However, little is known about whether microRNA regulates ASIC1a. Here, we assessed several microRNAs that have predicted targeting sequences in the 3' untranslated region (UTR) of mouse ASIC1a. Our results indicated that miR-144 and -149 reduced ASIC1a expression while Let-7 increased ASIC1a protein levels. miR-30c, -98, -125, -182* had no significant effect. Since a reduction in ASIC1a expression may have translational potentials in treating neuronal injury, we further asked whether the effect of miR-144 and miR-149, both reduced ASIC1a expression, was through specific targeting of the predicted sites on ASIC1a. We mutated the targeting sequence of miR-144 and miR-149 in ASIC1a UTR. The effect of miR-149 was abolished in the corresponding mutation. In contrast, miR-144 still reduced ASIC1a level when its predicted target sequence was mutated. This result indicates that miR-149 targets the 3'UTR of ASIC1a and reduces its expression.

Keywords: ASIC, ASIC1a, microRNA

Introduction

Acid-sensing ion channels (ASICs) are a family of proton-gated cation channels expressed primarily in neurons [1, 2]. Among all ASIC subunits, ASIC1a is the main determinant of acid-activated currents in brain neurons. ASIC1a localizes to postsynaptic side of the synapse, and mediates acid-induced calcium increase in dendritic spines [3-7]. Deleting or inhibiting ASIC1a attenuates long-term potentiation in slices, and reduces fear-dependent learning in mice [6-10]. In addition, ASIC1a also plays a critical role in neuronal injury. ASIC1a null animals exhibit reduced neuronal injury in rodent models of brain ischemia, multiple sclerosis, and traumatic brain injury [11-13]. These data indicate that ASIC1a is the primary postsynaptic proton receptor, and plays a pivotal role in brain function and in multiple neurological diseases.

MicroRNAs are short non-coding RNAs that have diverse functions [14]. The most common function of microRNAs include regulation of gene expression, especially in gene silencing. In recent years, research in microRNAs has led to many important discoveries in our understanding of brain function [14, 15]. However, little is known about potential regulation of ASIC1a by microRNAs. To start addressing this topic, we screened the 3' untranslated region (UTR) of ASIC1a for potential microRNA targets, and analyzed their effect on ASIC1a expression in Chinese hamster ovary (CHO) cells.

Materials and methods

Constructs and reagents

Mouse ASIC1a-UTR construct was generated by PCR-based subcloning of the entire 3'UTR of ASIC1a into the previously described WT-ASIC1a expression construct [3]. This ASIC1a-UTR construct has a CMV promoter with a Kozak sequence (GCCACC) added before the start codon, followed by the coding and 3'UTR of ASIC1a. Mutant ASIC1a UTR constructs, UTR

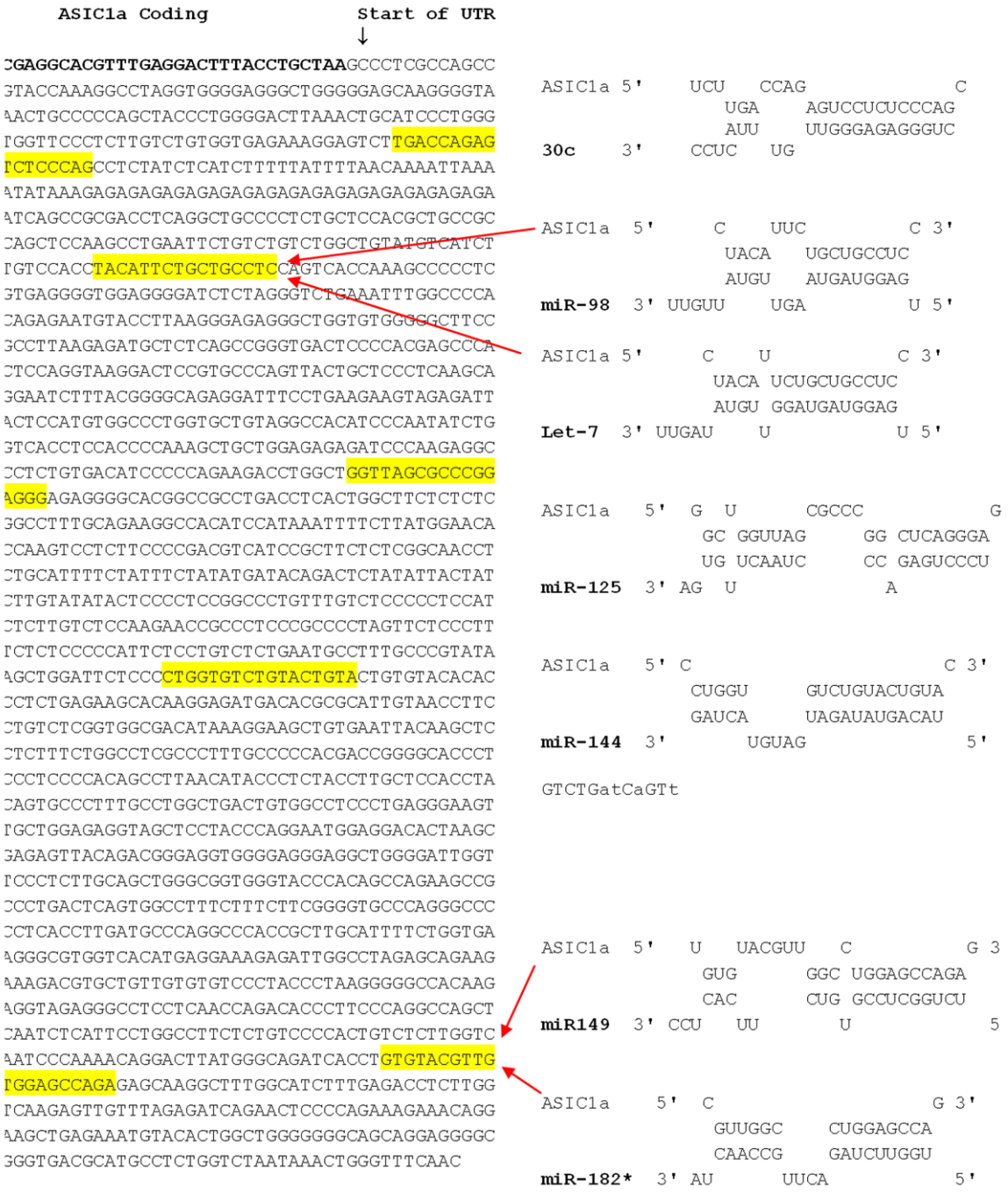

Figure 1. The alignment of candidate microRNA targets within the 3'UTR of mouse ASIC1a. Left panel shows the sequence of the 3'UTR region of mouse ASIC1a. The end of coding region was in bold and the arrow indicates the start of 3'UTR. Right panel illustrates the alignment of predicted microRNAs and ASIC1a. The corresponding target regions on UTR were highlighted on the sequence in the left panel. As illustrated by the arrows, miR-98 and let-7 target the same region, and miR-149 and miR-182* also target the same region.

mt 1 (with the miR-144 binding site mutated) and UTR mt 2 (with the miR-149 binding site mutated), were generated with a Quickchange mutagenesis kit (Stratagene, La Jolla, CA), following manufacturer's instructions. MicroRNA expression constructs were generated by subcloning the microRNA sequences into an IRES-eGFP vector. All constructs were verified by

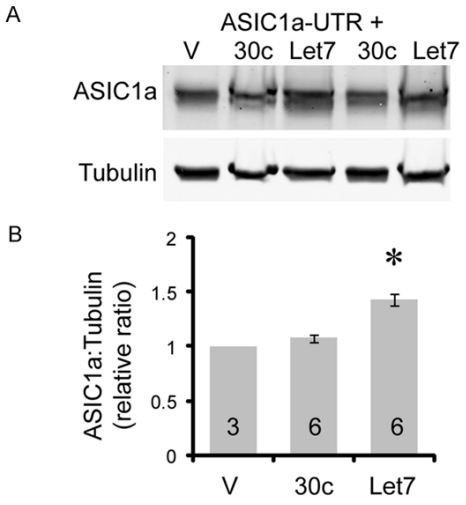

Figure 2. Effect of 30c and Let-7 on ASIC1a expression. CHO cells were transfected with ASIC1a-UTR together with the vector control, or expression constructs for miR-30c or let-7. Two days after transfection, cells were lysed and analyzed by Western blot using an anti-ASIC1 and an anti-b-tubulin antibodies. Representative blots and quantification of ASIC1a: tubulin ratio was shown. Asterisk indicates significant ($p<0.05$, ANOVA with Tukey's post hoc correction) difference from empty vector (V).

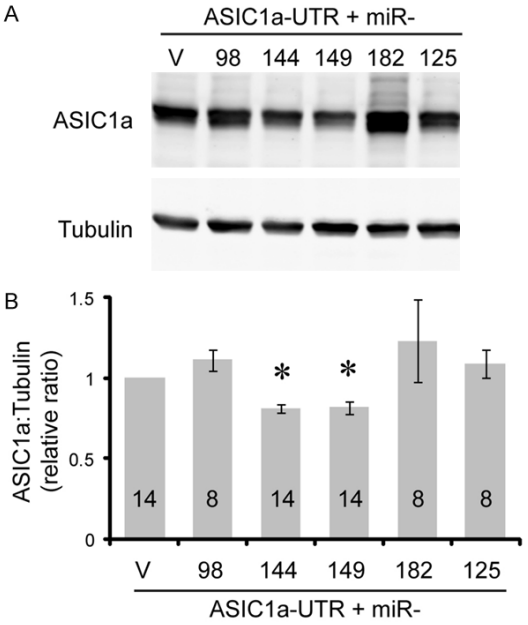

Figure 3. MicroRNA 144 and 149 reduces ASIC1a expression in CHO cells. CHO cell transfection and Western blot analysis were performed as described in **Figure 2** and Methods. Representative blots and quantification of ASIC1a: tubulin ratio were shown. Asterisks indicate significant differences ($p<0.01$ for miR-144; $p<0.05$ for miR-149; ANOVA with Tukey post hoc) from vector (V).

sequencing. ASIC antibodies used: a rabbit anti-ASIC1 antibody [16] and a goat anti-ASIC1 (Santa Cruz SC-13905). Antibody specificity was verified by the lack of signals in corresponding ASIC knockout mice [16, 17]. Secondary antibodies include Alexa 680-, and 800 and dylight 680- and 800-conjugated (ThermoFisher and Rockford). Culture media and serum were purchased from HyClone or Invitrogen. Lipofectamine 2000 were purchased from Invitrogen.

CHO cell culture and transfection

CHO-K1 cells were purchased from ATCC. CHO cell culture and transfection was performed similar to what was described in our previous studies [5, 18]. Briefly, CHO cells were grown in F-12K supplemented with 10% fetal bovine serum in a humidified 5% CO_2 incubator. The cells were transfected using Lipofectamine 2000 following the manufacturer's instructions.

Western blot

Gel electrophoresis and Western blotting were performed similar to what have been described earlier [19]. Briefly, two days after transfection, cells were lysed in lysis buffer, and cleared by centrifugation. The samples were separated by 8% or 10% SDS-PAGE and transferred to nitrocellulose membranes. Blotting was performed according to instructions of the Odyssey Imaging System (Li-cor). Membranes were blocked in blocking buffer (0.1% casein in 0.2×PBS pH 7.4) for 1 hr. Primary antibodies were diluted with blocking buffer containing 0.1% Tween-20 and incubated at 4°C overnight or at room temperature for 2 hrs. Secondary antibodies were diluted in blocking buffer containing 0.1% Tween-20 and 0.01% SDS and incubated at room temperature for 1 hr. Antibody dilutions were: rabbit anti-ASIC1a 1:5K-30K; goat anti-ASIC1 1:1000; monoclonal anti-tubulin, 1:30,000; Secondary antibodies were used at 1:10K-16K dilutions. Blots were imaged using an Odyssey Infrared Imaging System according to manufacturer's instructions. Densitometry of imaged bands was performed as described previously [16, 17].

Statistical analysis

For paired comparisons, we used two tailed Student's t-test. For multiple comparisons, we

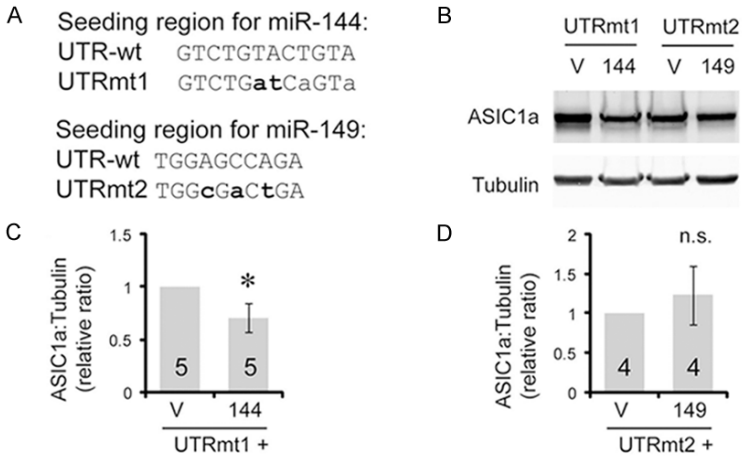

Figure 4. Mutating the target region of miR-149 on ASIC1a UTR abolishes the inhibition by miR-149. CHO cells were transfected with ASIC1a-UTR mutant constructs as indicated, together with either the vector control or the corresponding microRNA. A. Illustrates the WT and mutated UTR sequence. B-D. Typical Western blots and analysis showing the effect of miR-144 and miR-149 on the expression of the corresponding UTR mutant.

used ANOVA followed by Tukey HSD post hoc analysis. Data were reported as mean ± s.e.m.

Results and discussion

To start answering whether microRNAs regulate ASIC1a expression, we first did an in silico analysis, primarily using miRWalk and miRanda. In this initial analysis, we focused on the 3'UTR of mouse ASIC1a, because most microRNA targeting sites are within the 3'UTR region of a given gene [20]. From the list that returned from the predicting programs, we visually inspected the potential targeting sites, and selected seven candidate microRNAs which show good pairing between the microRNA and the target sequence. **Figure 1** illustrates the 7 microRNAs and their predicted targeting sites in ASIC1a.

Next, we tested whether these microRNAs alter ASIC1a expression. In the literature, some have used a reporter gene with the 3'UTR of the interest to perform this kind of analysis. However, the reporter gene may behave differently from the protein of interest. Since we have specific antibodies to ASIC1a, we performed this analysis on ASIC1a itself. To do so, we subcloned the entire 3'UTR region of ASIC1a into an ASIC1a expression construct. In our first experiment, we co-expressed this ASIC1a construct, which has intact 3'UTR, together with either a vector control or miR-30c and let-7 in CHO cells. Two days after transfection, we perform Western blot analysis using an anti-ASIC1 and anti-beta-tubulin antibody, and quantified the ratio of ASIC1a:tubulin in different conditions. miR-30c had no significant effect on ASIC1a protein level while let-7 led to an increase of ASIC1a expression (**Figure 2**).

Using the same approach, we then analyzed the effect of several other microRNAs. miR-98, miR-125, miR-182 had no significant effect. In contrast, miR-144 and miR-149 reduced ASIC1a expression (**Figure 3**). Since ASIC1a is an important mediator of acid-related neuronal injury, we found that the reduction in ASIC1a expression is of particular interest because it provides a potential approach to reduce ASIC-mediated injuries. We then asked whether the effect of miR-144 and -149 is through targeting the predicted sites in 3'UTR of ASIC1a. We generated corresponding ASIC1a-UTR mutants, ASIC1a-UTR-mt1 and ASIC1a-UTR-mt2, which have the miR-144 or miR-149 seeding sequence mutated, respectively (**Figure 4A**). We co-transfected CHO cells with ASIC1a-UTR-mt1, together with either vector control or miR-144 expression construct, and performed Western blot analysis. miR-144 reduced the expression of ASIC1a-UTR-mt1 (**Figure 4B**, **4C**). This result suggests that miR-144 either has additional seeding site(s) on ASIC1a-UTR, or reduces ASIC1a expression through a secondary mechanism that is independent of the seeding on ASIC1a mRNA. In contrast, when we performed similar experiment using ASIC1a-UTR-mt2, miR-149 no longer affected its expression (**Figure 4B**, **4D**). This result indicates that miR-149 reduces ASIC1a through targeting the predicted miR-149 seeding region within the 3'UTR of ASIC1a.

Our data here demonstrate that miR-149 targets to the 3'UTR of ASIC1a and reduces ASIC1a expression. Since we started here with

a limited number of candidates, it is conceivable that additional microRNAs can target ASIC1a. Nevertheless, our finding suggests that up-regulation of miR-149 can provide an alternative approach to attenuate acid-induced injuries in disease. Future experiments are necessary to determine whether miR-149 has similar effect on endogenous ASIC1a expression in neurons, and whether miR-149 overexpression has a protective effect in acidotoxicity. One important note is that our study here provides one example for microRNA regulation of expression of mouse ASIC1a. However, the UTR region is less conserved between species. Therefore, to facilitate targeting of ASIC1a in human patients, it will be important to determine the microRNAs that target human ASIC1a gene in a future study. Given the lack of specific small molecule inhibitor that can be administered clinically [21, 22], studies along this line may provide one potential alternative to manipulate ASICs and acidosis-related responses in human subjects.

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