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Regulation of lung surfactant secretion by microRNA-150

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ABSTRACT

P2X7 receptor (P2X7R) is a purinergic ion-channel receptor. We have previously shown that the activation of P2X7R in alveolar type I cells stimulates surfactant secretion in alveolar type II cells. In this study, we determined whether miR-150 regulates P2X7R-mediated surfactant secretion. The miR-150 expression level in alveolar type II cells was much higher than alveolar type I cells, which was inversely correlated with the P2X7R protein level. An adenovirus expressing miR-150 significantly reduced the P2X7R protein expression in E10 cells, an alveolar type I cell line. Furthermore, pre-treatment of E10 cells with the adenovirus reduced the surfactant secretion induced by E10 cell conditioned medium. Our study demonstrates that miR-150 regulates surfactant secretion through P2X7R.

1. Introduction

The distal lung epithelium is lined with alveolar epithelial type I and type II cells (AEC I and AEC II). AEC I cover about 95% and AEC II occupy less than 5% of the alveolar surface area [14]. AEC I are large squamous cells responsible for gas exchange, fluid transport and protection against oxidative stress [4,5,11,17]. AEC II are cuboidal and normally located in the corners of alveoli. AEC II also synthesize and secrete pulmonary surfactant, a lipoprotein complex that spreads on the surface of alveoli to form a thin lining layer and reduces surface tension. Insufficient amount of pulmonary surfactant leads to abnormal lung functions [10,22].

The molecular mechanism that regulates surfactant secretion is of great interest. The secretion of surfactant from AEC II can be regulated by many molecules, such as intracellular Ca^{2+}, P_2Y_2 receptor agonists (ATP and UTP), adenosine, platelet activating factor, and IL-1. [1]. Of all these molecules, extracellular ATP is the most effective endogenous regulator. Through binding to P_2Y_2 receptors on the AEC II plasma membrane, ATP activates phospholipase C, which generates diacylglycerol (DAG) and inositol trisphosphate (IP_3), and subsequently activates protein kinase C (PKC) and increases cytoplasmic Ca^{2+} concentration [1].

P2X7 receptor (P2X7R) belongs to the P2X receptor family which is a ligand-gated ion channel activated by ATP [12]. P2X7R shares many properties with other P2X receptors. The binding of ATP causes P2X receptors to open within millisecond and stimulates cell depolarization by an influx of cationic ions, including Na^+, K^+ and Ca^{2+} [8]. P2X7R was initially identified as a unique ATP-gated channel, P2Z due to its low degree of structural homology compared to other family members. P2X7R has a less conserved and unusually long COOH terminus, which forms a large non-selective pore permeable to small molecules such as YO-PRO, Lucifer yellow and ethidium bromide [20]. In comparison with other P2X receptors, the activation of P2X7R needs at least 10-fold higher ATP concentration due to its low affinity for ATP, but the desensitization of P2X7R is much slower. Another unique property of P2X7R is that it mediates a reversible plasma membrane permeabilization. P2X7R activation also enhances the release of endogenous cytokines involved in the immune-response, such as interleukin-1 beta (IL-1β) [16]. However, sustained stimulation of P2X7R leads to membrane blebbing and programmed cell death [9,19].

P2X7R is broadly expressed in multiple mammalian cells, including epithelial cells, endothelial cells, fibroblasts, and macrophages. P2X7R is specifically expressed in AEC I [6]. Our previous studies have shown that the activation of P2X7R in AEC I stimulates surfactant secretion in AEC II [15].

MicroRNAs (miRNAs) are naturally existing small non-coding RNAs that either cleave specific mRNAs or inhibit mRNA translation through complementary base pairing and subsequently silence target genes [13]. miR-150 is a miRNA highly expressed in the tumor cells surrounding the proliferation centers in the bone marrow and lymphoid tissues [23,24]. miR-150 is also present in mature B and T cells. It blocks B cell development by silencing the expression of c-Myb [23]. miR-150 has been reported to target P2X7R [25].

In the current study, we demonstrated that miR-150 inhibited endogenous P2X7R protein expression in lung cells. Then we characterized miR-150 expression in different organs and lung epithelial

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cells. Finally, we examined the effect of miR-150 in P2X7R-mediated surfactant secretion in primary cultured AEC II.

2. Materials and methods

2.1. miRNA viral vector construction

The pENTR/CMV-EGFP-pri-miRNA vector containing the primary transcript of human miRNA was constructed and the miRNA adenoviruses were produced as described previously [3].

2.2. Western blot

The proteins were separated by 10% SDS–PAGE and transferred onto a nitrocellulose membrane. Dilutions for rabbit anti-P2X7R antibody (Sigma) and mouse anti-β-actin antibody (Bio-Rad) were 1:1000. Dilution for horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was 1:2000. The blot was developed with Super-Signal West Pico Chemiluminescent Substrate (Pierce).

2.3. Real-time PCR

Total RNAs were isolated using the mirVana™ miRNA Isolation Kit (Ambion). RNase-free™ DNase was used to remove genomic DNA contamination. The RNAs were reverse-transcribed into cDNA using oligo dT and random hexamers and M-MLV reverse transcriptase (Invitrogen). The primers used are: P2X7R: forward, 5′-ACCGTGCCAGTGTTGTTCTG-3′, and reverse, 5′-AGCACCTGTGGTTGTTATTTTGT-3′; 18S rRNA: forward, 5′-ATTGCTCAATCTCGGGTGGCTG-3′ and reverse, 5′-CGTTCTTAGTTGGTGGAGCGATTTG-3′. Real-time PCR was performed as described [21]. 18S rRNA was used as an internal control.

Real-time PCR for miR-150 was carried out as described [18]. Five microgram of total RNA was polyadenylated using A-Plus™ Poly (A) Polymerase tailing kit (Epicenter). The tailed RNA was purified using 1:1 phenol/Chloroform and precipitated with 75% ethanol. One microgram poly(A) tailed RNA was reverse-transcribed into cDNA using oligo dT primers by M-MLV reverse transcriptase. The target mature miRNA sequence (5′-CTGGTACAGGCCTGGGGGACAG-3′) was used as the forward PCR primer and a universal primer (5′-GCGAGCACAGAATTAATACGACTCAC-3′) was used as the reverse primer. Real-time PCR was performed as described above. U2 small RNA was used to normalize the data.

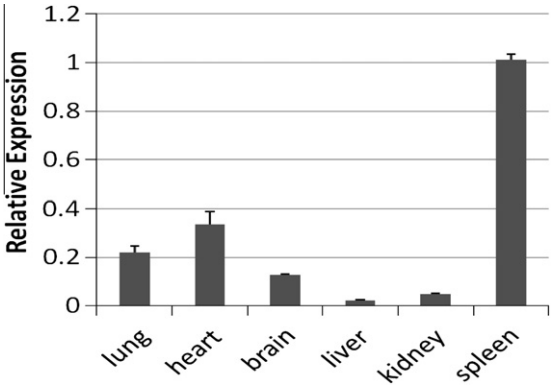

Fig. 2. Expression level of miR-150 in organs. Total RNAs were extracted from various organs, poly (A)-tailed and reversed-transcribed into cDNA. Real-time PCR was used to examine miR-150 levels. The results were normalized to U2. The data were expressed as means ± SE (n = 3 biological replications, assayed in duplicate).

2.4. Surfactant secretion

AEC II were isolated from Male Sprague-Dawley rats (180–200 g) as previously described [2]. E10 cells were cultured in DMEM supplied with 10% FBS and transduced with 100 Multiplicity of Infection (MOI = ratio of infectious virus particles to cells) adenoviruses for 4 days. The cells were then treated with 25 μM BzATP for 2 h. The media were collected as conditional media. AEC II (1×10^6) were cultured overnight with [3H] choline (0.6 μCi/10^6 cells). AEC II were incubated with or without the conditional media for 2 h. Lipids in media and cells were extracted and surfactant secretion was performed [7]. Surfactant secretion (%) was expressed as (dpm in medium/dpm in medium and cells) × 100. A stimulation index was expressed as a ratio of stimulated secretion with a conditioned media to basal secretion without conditioned media.

3. Results and discussion

3.1. miR-150 reduces P2X7R protein level

To determine whether miR-150 down-regulates endogenous P2X7R protein expression, we generated an adenovirus to overex-

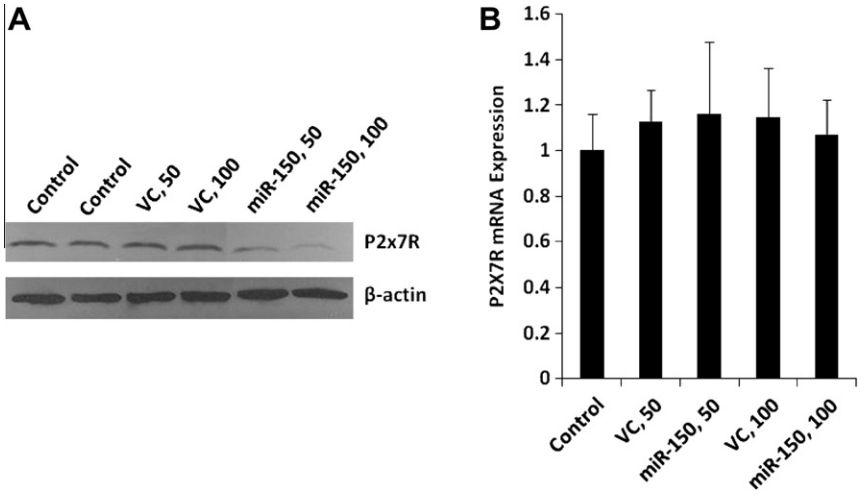

Fig. 1. miR-150 silences the P2X7R protein expression. E10 cells were treated with different doses of miR-150 overexpressing viruses (MOI, 50 and 100). A virus overexpressing GFP was used as a control. Four days after treatment, protein (A) and mRNA (B) levels were examined using Western blot and Real-time PCR. β-Actin were used as an internal control for Western blot. 18S rRNA was used as an internal control for real-time PCR. For panel B, data are means ± SE (n = 3 cell preparations, assayed in duplicate).

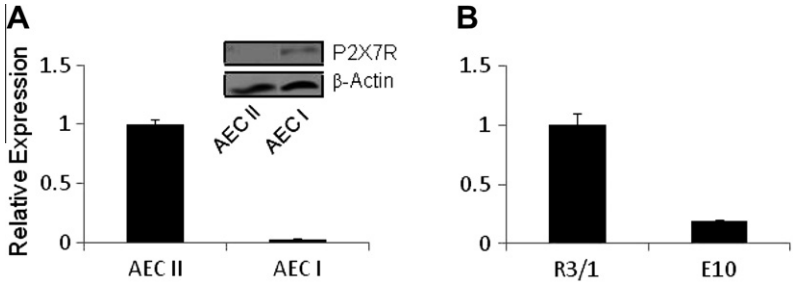

Fig. 3. miR-150 and P2X7R expression in lung cells. Total RNA and/or proteins were extracted from AEC II (freshly isolated type II cells) and AEC I-like cells (trans-differentiated type I cells by culturing AEC II on plastic dishes for 5 days) (A), and E10 and R3/1 cells (B). miR-150 level was determined by real-time PCR. U2 was used as an internal reference. The results were expressed as means ± SE (n = 4–6 cell preparations, all assayed in duplicate). P2X7R protein level was measured by Western blot and β-actin was used a loading control.

press miR-150. The virus was used to transduce E10 cells for 4 days at a MOI of 50 or 100. As shown in Fig. 1A, the P2X7R protein expression was not affected in the control adenovirus-treated cells. However, a significant decrease was observed in miR-150 expressing adenovirus-transduced cells. This decrease was virus dose-dependent. We also examined the P2X7R mRNA expression using real-time PCR. The control and miR-150 viruses had no effects on the P2X7R mRNA expression (Fig. 1B).

3.2. Characterization of miR-150 expression

miR-150 is highly expressed in lymph nodes, spleen, heart, and brain. However, its abundance in the lung is controversial [23,24]. We first examined the expression of miR-150 in various organs by real-time PCR. As previously reported, miR-150 was highly expressed in spleen (Fig. 2). Its expression in the lung was comparable to those in the heart and brain.

miR-150 expression level in type II cells was much higher than type I-like cells, which was inversely correlated with the P2X7R protein level (Fig. 3A). We have previously shown that R3/1 cell, a type I cell line, contains little P2X7R while E10 cell, another type I cell line, has a high level of P2X7R [15]. miR-150 was highly expressed in R3/1 cells and lowly in E10 cells (Fig. 3B).

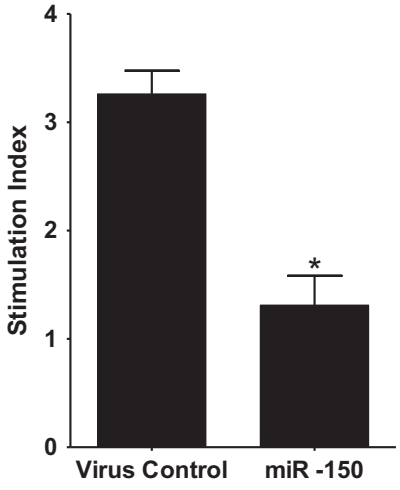

Fig. 4. miR-150 inhibits P2X7R-evoked surfactant secretion. (A) E10 cells were treated with 100 MOI of adenovirus expressing miR-150 for 4 days. Adenovirus only express EGFP was used as a control. E10 cells were stimulated for 2 h with BzATP (25 μM). The E10 cell conditioned media were used to stimulate AEC II for 2 h. PC secretion was measured. Data are means ± S.E. (n = 3, $^*P$ < 0.001 vs. control, Student's test).

3.3. miR-150 reduced P2X7R-mediated surfactant secretion

Previously, we have found that BzATP activates P2X7R in AEC I, resulting in an increase in surfactant secretion in AEC II. We therefore examined whether miR-150 affects surfactant secretion. We pre-treated E10 cells with miR-150 overexpression viruses for 4 days and stimulated E10 cells with BzATP for 2 h. Then the conditioned media were used to stimulate surfactant secretion in AEC II. As shown in Fig. 4, miR-150 significantly decreased the surfactant secretion in AEC II.

In summary, we found that miR-150 had much lower expression in P2X7R-enriched cells, such as type I-like cells and E10 cells. miR-150 expressing adenovirus dramatically decreased the endogenous P2X7R expression in E10 cells. The conditioned media from miR-150 expressing virus-treated E10 cells had a reduced ability to stimulate surfactant secretion in AEC II. Our data suggests a role of miRNA-150 in surfactant secretion.

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