Increased Reabsorption of Alveolar Edema Fluid in the Obese Zucker Rat

GANG MA,1 XITONG ZHAO,1 MASAKATSU UENO,1 MAKOTO TANAKA,1 YUICHIRO MACHIDA,1 HIROKAZU AIKAWA,1 KATSUO USUDA,1 MOTOYASU SAGAWA,1 YOSHIMICHI UEDA2 and TSUTOMU SAKUMA1

1Thoracic Surgery, Kanazawa Medical University, Uchinada, Ishikawa, Japan
2Pathology, Kanazawa Medical University, Uchinada, Ishikawa, Japan

Diabetic patients have a decreased incidence of acute respiratory distress syndrome, but the mechanism responsible for the decreased incidence is uncertain. Reabsorption of alveolar edema fluid (alveolar fluid clearance) has been considered to play an important role in resolution of acute respiratory distress syndrome. However, little is known regarding alveolar fluid clearance in diabetes mellitus. Since the obese Zucker rat has been used as an experimental model for diabetes mellitus, we determined if alveolar fluid clearance increased in the obese Zucker rat. First, we compared alveolar fluid clearance in obese Zucker rats with that in lean Zucker rats and Sprague-Dawley (SD) rats. Then, we determined the role of sodium channel, Na,K-ATPase, and \( \beta_2 \)-adrenoceptor, which drives alveolar fluid clearance, in obese Zucker rats. Alveolar fluid clearance was estimated by the progressive increase in alveolar albumin concentrations in the isolated lungs. We found that basal alveolar fluid clearance in obese Zucker rats was two-fold greater than that in lean Zucker rats and SD rats. The mRNA expression of \( \alpha_1 \)-, \( \beta_1 \)-Na, K-ATPase and \( \beta_2 \)-adrenoceptor, but not mRNA expression of sodium channel, increased in obese Zucker rats. A selective \( \beta_2 \)-adrenergic antagonist, but not a Na, K-ATPase inhibitor, specifically inhibited the increase in alveolar fluid clearance in obese Zucker rats. These results indicate that overexpression of \( \beta_2 \)-adrenoceptor primarily increases basal alveolar fluid clearance in the obese Zucker rat. We speculate that the stimulation of alveolar fluid clearance ameliorates acute respiratory distress syndrome in patients with diabetes mellitus.

Obesity; beta-adrenoceptor; terbutaline; lung fluid balance; diabetes.

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Resolution of alveolar edema lessens alveolar fluid volume and may improve the mortality and morbidity in patients with pulmonary edema and acute lung injury (Matthay et al. 2002). The rate of alveolar fluid resolution depends on the alveolar fluid clearance capacity via apical sodium (ENaC) channel and basolateral Na,K-ATPase on alveolar epithelial cells (Matalon and O’Brodovich 1999; Matthay et al. 1996). \( \beta_2 \)-adrenergic agonists stimulate alveolar fluid clearance in dogs (Berthiaume et al. 1988; Grimme et al. 1997), sheep (Berthiaume et al. 1987), mice (Garat et al. 1998; Mutlu et al. 2004a), rats (Crandall et al. 1986; Pittet et al. 1994; Sakuma et al. 2001;
Saldias et al. 1998), and human lungs (Fang et al. 2002; Frank et al. 2007; Sakuma et al. 1994; Sakuma et al. 1997; Sakuma et al. 2006). Recently, it was reported that β₂-adrenergic receptor overexpression increased alveolar fluid clearance in rats (Dumasius et al. 2001) and mice (McGraw et al. 2001).

Since diabetic patients have a decreased incidence of acute respiratory distress syndrome (Moss et al. 2000), the mechanism responsible for the lower incidence has been studied in obese Zucker rats (Wright et al. 1999) and in leptin resistant mice (Bellmeyer et al. 2007) in association with an experimental model of diabetic mellitus. Interestingly, alveolar fluid clearance decreased in leptin resistant mice, but it was not studied in obese Zucker rats. Therefore, our interest was to determine if alveolar fluid clearance would be changed in the obese Zucker rat.

Zucker rat appeared spontaneously as a cross between 13M strain Sherman and NIH black at the Laboratory of Comparative Pathology of Theodore and Lois Zucker in Stow, MA, and has been continued for many generations of random breeding (Johnson et al. 1971). This colony carries the mutant recessive gene “fatty” (fa/fa), which when present in the homozygous form (fa/fa) produces extreme obesity. Heterozygotes and normal homozygos (fa/Fa and Fa/Fa) are lean and phenotypically indistinguishable. In brown adipose tissue, β-receptor density is apparently better correlated with obesity condition (Raasmaja and York 1988). Since the obese Zucker rat possesses the overexpression of Na,K-ATPase (Ferrer-Martinez et al. 1996), we hypothesized that alveolar fluid clearance increased in the obese Zucker rat and the increase was mediated through the overexpression of Na,K-ATPase.

The objective in this study was to determine whether basal alveolar fluid clearance increased in obese Zucker rats. Since basal alveolar fluid clearance increased in the obese Zucker rat, the mechanism responsible for the increase in alveolar fluid clearance was determined.

**METHODS**

**Materials**

Zucker and Sprague-Dawley (SD) rats were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Amiloride, ouabain, ICI-118,551, and terbutaline were obtained from Sigma (St Louis, MO, USA).

**Experimental Protocol**

All rats received humane care and this study was approved by the Committee for Animal Experiments at Kanazawa Medical University. When we did experiments in Zucker rats, obese and lean Zucker rats with the same birthday were used in the same day.

Alveolar fluid clearance was measured in the isolated rat lungs in the absence of pulmonary perfusion or ventilation (Sakuma et al. 2001; Sakuma et al. 2002; Sakuma et al. 2004). Briefly, 8-10 weeks aged male rats (body weight in Table 1) were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg). An endotracheal tube was inserted through a tracheostomy. The rats were exsanguinated via the abdominal aorta and a trachea, bilateral lungs, and a heart were excised en bloc through a median sternotomy. Isotonic saline solution (2 ml, 37°C) containing 5% bovine albumin was instilled into both lungs, followed by 4 ml oxygen to deliver all the instilled fluid into the alveolar spaces. The lungs were placed in a humid incubator at 37°C and inflated with intraperitoneal pentobarbital sodium (50 mg/kg). An endotracheal tube was inserted through a tracheostomy. The rats were exsanguinated via the abdominal aorta and a trachea, bilateral lungs, and a heart were excised en bloc through a median sternotomy. Isotonic saline solution (2 ml, 37°C) containing 5% bovine albumin was instilled into both lungs, followed by 4 ml oxygen to deliver all the instilled fluid into the alveolar spaces. The lungs were placed in a humid incubator at 37°C and inflated with 100% oxygen at an airway pressure of 7 cm H₂O. Alveolar fluid was aspirated 1 h after instillation. The protein concentrations in the instilled and aspirated solutions were measured by the pyrogallol red protein dye-binding method (SRL Inc., Tokyo, Japan). Alveolar fluid

**Table 1. Laboratory data in rats**

<table>
<thead>
<tr>
<th>Rat</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Blood sugar (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>Adrenaline (pg/ml)</th>
<th>Noradrenaline (pg/ml)</th>
<th>Dopamine (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD</td>
<td>8</td>
<td>285 ± 25</td>
<td>157 ± 16</td>
<td>53 ± 17</td>
<td>67 ± 8</td>
<td>94 ± 61</td>
<td>292 ± 95</td>
<td>66 ± 22</td>
</tr>
<tr>
<td>Obese</td>
<td>8</td>
<td>382 ± 24*</td>
<td>134 ± 35</td>
<td>124 ± 27*</td>
<td>110 ± 9*</td>
<td>54 ± 26</td>
<td>202 ± 44</td>
<td>143 ± 82*</td>
</tr>
<tr>
<td>Lean</td>
<td>8</td>
<td>264 ± 15</td>
<td>133 ± 18</td>
<td>25 ± 6</td>
<td>78 ± 5</td>
<td>83 ± 38</td>
<td>200 ± 49</td>
<td>49 ± 17</td>
</tr>
</tbody>
</table>

* P < 0.05 vs SD rats.
clearance was estimated by the progressive increase in the concentration of albumin (Sakuma et al. 2001; Sakuma et al. 2002; Sakuma et al. 2004). Alveolar fluid clearance (AFC) was calculated as follows:

\[ AFC = \left( \frac{V_i - V_f}{V_i} \right) \times 100 \]  

(1)

where \( V \) is the volume of the instilled albumin solution (i) and the final alveolar fluid (f).

\[ V_f = \left( \frac{V_i \times P_i}{P_f} \right) \]  

(2)

where \( P \) is the concentration of protein in the instilled albumin solution (i) and the final alveolar fluid (f). The term alveolar does not imply that all reabsorption occurs across the alveolar epithelial cells because the distal bronchial epithelia can also transport sodium.

Lung water volume was measured by drying lungs to a constant weight at 70°C for 48 h (Sakuma et al. 2002; Sakuma et al. 2004). Lung water-to-dry lung weight ratio (LW/DL) was calculated as LW/DL = (wet lung weight – dry lung weight) / (dry lung weight).

Blood samples (4 ml) were collected before exsanguination (obese Zucker rats; \( n = 8 \), lean Zucker rats; \( n = 8 \), SD rats; \( n = 8 \)). The samples of plasma, serum, instilled albumin solution, and final alveolar fluid were stored at –80°C until analysis. Plasma catecholamine (adrenalin, noradrenalin, and dopamine) levels were measured as reported in a prior study (Sakuma et al. 2002). Serum blood sugar, triglyceride, and cholesterol concentrations were measured at a clinical laboratory (SRL Inc., Tokyo, Japan).

Real-time quantitative PCR was used for the measurement of lung mRNA expression (Wang et al. 2007). The distal lung tissue samples were freshly frozen in liquid nitrogen and stored at –80°C. Total RNA was extracted from the lung tissue with RNA isolative reagent (Isogen, Wako, Osaka, Japan) according to the manufacturer’s manual. cDNA was synthesized from 5 µg of total RNA in the DNA engine (PTC-200, MJ Research, Watertown, MS, USA). Then 3.5 µl cDNA was performed with a one-step RT-PCR reagent (TaqMan, Applied Biosystems, Foster, CA, USA) in a final volume of 20 µl containing 1 µl TaqMan probes, DEPC water 5.5 µl and TaqMan universal PCR master mixture 10 µl, at 50°C 2 mins, 95°C for 10 mins, 95°C for 15 secs and 60°C for 1 min, totally 40 cycles in sequence detection system (ABI PRISM 7700, Applied Biosystems, Forster, CA, USA). Oligonucleotide primers and TaqMan probes of \( \alpha -, \beta -, \gamma -\text{ENaC}, \alpha_1-, \beta_1-, \text{Na,K-ATPase}, \beta_2-\text{adrenoreceptor} \) and GAPDH genes were purchased from Assays-On Demand Gene Expression Products (Applied Biosystems). We picked the sample with lowest Ct value, a threshold cycle value representing the PCR cycle number at which the fluorescence was detected above an arbitrary threshold, in all of samples as the standard sample. The standard curve was made of the degressive concentration of standard samples at 5-fold. Gene expression levels, quantified using the standard curve method according to the manufacture’s instructions and standardized with the expression levels of GAPDH gene, were used to analyze the relative amount of target mRNA expressions. We previously confirmed the validity of RT-PCR by the measurement of protein expression using immunohistochemistry (Zhou et al. 2001) and Western blotting (Hatano et al. 2006).

**Experimental groups**

**Basal alveolar fluid clearance in Zucker and SD rats:** Basal alveolar fluid clearance was measured in obese Zucker rats (\( n = 12 \)), lean Zucker rats (\( n = 12 \)), and SD rats (\( n = 18 \)). Alveolar fluid was aspirated 1 h after instillation.

**Lung water volume in rats:** We determined if increased basal alveolar fluid clearance changed lung water volume in obese Zucker rats. Lung water volume was measured in obese Zucker rats (\( n = 5 \)), lean Zucker rats (\( n = 5 \)), and SD rats (\( n = 8 \)).

**Effect of a sodium channel inhibitor and a Na,K-ATPase inhibitor on alveolar fluid clearance in Zucker rats:** Since basal alveolar fluid clearance was greater in obese Zucker rats than in lean Zucker and SD rats, we determined if the greater rate of basal alveolar fluid clearance was mediated via sodium channels and Na,K-ATPase in obese Zucker rats. Isotonic 5% albumin solution containing amiloride (a sodium channel inhibitor, \( 10^{-3} \) M, \( n = 4 \)) or ouabain (a Na,K-ATPase inhibitor, \( 10^{-3} \) M, \( n = 4 \)) was instilled into the lungs of obese Zucker rats. As control, isotonic 5% albumin solution containing amiloride (\( 10^{-3} \) M, \( n = 4 \)) or ouabain (\( 10^{-3} \) M, \( n = 4 \)) was instilled into the lungs of lean Zucker rats. Alveolar fluid was aspirated 1 h after instillation.

**Effect of \( \beta_2-\text{adrenoceptor agonist and antagonist on alveolar fluid clearance in Zucker rats:** Since basal alveolar fluid clearance increased in obese Zucker rats, we determined if the increased rate of basal alveolar fluid clearance was mediated by a \( \beta_2-\text{adrenoceptor} \) in obese Zucker rats.**
Isotonic 5% albumin solution containing ICI-118,551 (a selective β<sub>2</sub>-adrenergic antagonist, 10<sup>-4</sup> M, n = 4) or terbutaline (a selective β<sub>2</sub>-adrenergic agonist, 10<sup>-5</sup> M, n = 8) was instilled into the lungs of obese Zucker rats. As control, isotonic 5% albumin solution containing ICI-118,551 (10<sup>-4</sup> M, n = 4) or terbutaline (10<sup>-5</sup> M, n = 8) was instilled into the lungs of lean Zucker rats. Alveolar fluid was aspirated 1 h after instillation.

Statistics

Data are summarized as the mean and standard error (mean ± s.d.). The data were analyzed by a one-way analysis of variance (ANOVA) with the Student-Newman-Kouls post hoc test (GraphPad Prism 4, GraphPad Software Inc, San Diego, CA, USA). A value with P < 0.05 was regarded as a significant difference.

RESULTS

Body weight increased greater in obese Zucker rats than in the same aged lean Zucker rats (Table 1). Serum levels of triglyceride and cholesterol, but not blood sugar, were higher in obese Zucker rats than in lean Zucker rats and SD rats. Plasma levels of dopamine, but neither adrenaline nor noradrenaline, were higher in obese Zucker rats than in lean Zucker rats.

Basal alveolar fluid clearance was two-fold greater in obese Zucker rats than in SD rats and lean Zucker rats (Fig. 1). Lung water volume to dry lung weight ratio in obese Zucker rats was significantly smaller than in SD and lean Zucker rats.

Fig. 1. Basal alveolar fluid clearance in SD rats, obese Zucker rats, and lean Zucker rats. Alveolar fluid clearance was significantly greater in obese Zucker rats than in SD and lean Zucker rats. *P < 0.05 vs values in SD and lean Zucker rats. SD, SD rat; obese, obese Zucker rat; lean, lean Zucker rat.

Fig. 2. Lung water volume-to-dry lung weight ratios in SD rats, obese Zucker rats, and lean Zucker rats. Lung water volume-to-dry lung weight ratio was significantly smaller in obese Zucker rats than in SD and lean Zucker rats. *P < 0.05 vs values in SD and lean Zucker rats.

Fig. 3. mRNA expression of ENaC, Na,K-ATPase, and β<sub>2</sub>-adrenoceptor in SD, obese Zucker, and lean Zucker rats. The expression of α<sub>1</sub>-Na,K-ATPase, β<sub>1</sub>-Na,K-ATPase, and β<sub>2</sub>-adrenoceptor increased in obese Zucker rats. *P < 0.05 vs values in SD rats. †P < 0.05 vs values in SD rats and lean Zucker rats.
6.7% lower than that in SD rats ($P < 0.01$) and 4.8% lower than that in lean Zucker rats ($P < 0.05$, Fig. 2).

Alpha-ENaC mRNA expression in obese Zucker rats was not different from that in SD rats or lean Zucker rats (Fig. 3). However, $\beta$-ENaC mRNA expression in obese Zucker rats was lower than that in SD rats. Gamma-ENaC mRNA expression in obese Zucker rats was lower than that in SD rats and lean Zucker rats. In contrast, mRNA expression levels of $\alpha_1$-, $\beta_1$-Na,K-ATPase, and $\beta_2$-adrenoceptor in obese Zucker rats were greater than those in SD rats and lean Zucker rats.

Amiloride decreased basal alveolar fluid clearance by 67% and 57% in obese Zucker rats and in lean Zucker rats, respectively (Fig. 4). There was no difference in alveolar fluid clearance in the presence of amiloride between obese Zucker rats and lean Zucker rats. Ouabain decreased alveolar fluid clearance by 69% and 62% in obese Zucker rats and lean Zucker rats, respectively. There was no difference in alveolar fluid clearance in the presence of ouabain between obese Zucker rats and lean Zucker rats.

ICI-118,551 decreased basal alveolar fluid clearance in obese Zucker rats, but did not decrease basal alveolar fluid clearance in lean Zucker rats (Fig. 5). In the presence of ICI-188,551, the alveolar fluid clearance in obese Zucker rats was decreased to the basal level observed in lean Zucker rats and SD rats. In contrast, terbutaline increased alveolar fluid clearance in obese and lean Zucker rats. There was no difference in alveolar fluid clearance in the presence of terbutaline between obese Zucker rats and lean Zucker rats.

**DISCUSSION**

We determined if basal alveolar fluid clearance increased in obese Zucker rats because Na,K-ATPase plays a primary role in alveolar fluid clearance (Matalon and O’Brodovich 1999; Matthay et al. 1996) and because the upregulation of Na,K-ATPase had been indicated in obese Zucker rats (Ferrer-Martinez et al. 1996). Although the expression of $\alpha_1$- and $\beta_1$-Na,K-ATPase mRNA increased in obese Zucker rat in this study, it is indicated that the upregulation of $\beta_2$-adrenoceptors played a primary role in the increase in basal alveolar fluid clearance in obese Zucker rats.

There are some explanations accounting for the increase in basal alveolar fluid clearance in the obese Zucker rat. The first explanation is that responsiveness to endogenous catecholamine is increased in the obese Zucker rat. It was indicat-

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**Fig. 4.** Effects of amiloride and ouabain on basal alveolar fluid clearance in obese and lean Zucker rats. Both amiloride and ouabain decreased alveolar fluid clearance in obese and lean Zucker rats. $^*P < 0.05$ vs corresponding control value.

**Fig. 5.** Effects of a $\beta_2$-adrenergic agonist and a $\beta_2$-antagonist on basal alveolar fluid clearance in obese and lean Zucker rats. ICI-118,551 decreased alveolar fluid clearance only in obese Zucker rats. Terbutaline increased alveolar fluid clearance in obese and lean Zucker rats. $^*P < 0.05$ in corresponding control values. $^{†}P < 0.05$ vs corresponding control values. ICI: ICI-118,551, Terb: terbutaline.
ed that a β-adrenoceptor did not play a role in basal alveolar fluid clearance in the absence of its stimulation (Matthay et al. 2002; Mutlu et al. 2004b). Recently, it was reported that the rate of alveolar fluid clearance was up to 40% greater in β2-adrenoceptor overexpression mice than in non-transgenic mice (McGraw et al. 2001) and up to 100% greater in β2-adrenoceptor overexpression rats than in sham-infected controls and rats infected with an adenovirus expressing no cDNA (Dumasius et al. 2001). In addition, alveolar fluid clearance in β2-adrenoceptor overexpression lungs from propranolol, a non-selective β-adrenergic antagonist, -treated rats revealed that the clearance rates were similar or less than normal, untreated, sham-infected control (Dumasius et al. 2001). We found that basal alveolar fluid clearance in obese Zucker rats was 100% greater than that in lean Zucker rats and SD rats and that β-adrenergic antagonists decreased basal alveolar fluid clearance in obese Zucker rats. Our results are consistent with the prior reports (Dumasius et al. 2001; McGraw et al. 2001).

There seems to be a difference between the effect of exogenous β-adrenergic agonists on alveolar fluid clearance in β2-adrenoceptor overexpressing rats and that in β2-adrenoceptor overexpressing mice. In β2-adrenoceptor overexpressing rats, procaterol, a selective β2-adrenergic agonist, did not increase alveolar fluid clearance further (Dumasius et al. 2001). In β2-adrenoceptor overexpressing mice, formoterol, a selective β2-adrenergic agonist, increased alveolar fluid clearance in a dose-dependent manner (McGraw et al. 2001). Interestingly, since adrenalectomy inhibited the increase in alveolar fluid clearance in β2-adrenoceptor overexpressing mice, basal endogenous catecholamine may stimulate alveolar fluid clearance in β2-adrenoceptor overexpressing mice. In obese Zucker rats in this study, terbutaline increased alveolar fluid clearance by approximately 40% basal alveolar fluid clearance. Therefore, it is likely that response to β-adrenergic agonist in obese Zucker rats is similar to that in β2-adrenoceptor overexpressing mice.

The second explanation is that Na,K-ATPase played a role in increased alveolar fluid clearance in the obese Zucker rat. Since mRNA expression of α1- Na,K-ATPase and β1-Na,K-ATPase increased in obese Zucker rats, we tested if ouabain, a Na,K-ATPase inhibitor, inhibited the increase in basal alveolar fluid clearance. Since ouabain decreased alveolar fluid clearance in obese Zucker rats, Na,K-ATPase played a role in the increase in basal alveolar fluid clearance in obese Zucker rats. However, since there was no difference between the effects of ouabain in obese Zucker rats and lean Zucker rats, Na,K-ATPase overexpression did not primarily play a role in increased alveolar fluid clearance in obese Zucker rat. Therefore, it is likely that Na,K-ATPase played a role in combination with the β2-adrenoceptor in obese Zucker rats.

Amiloride is a potent blocker of apical sodium channels that regulate ion transport and alveolar fluid clearance (Matalon and O’Brodovich 1999). In the present study, β-ENaC mRNA expression in obese Zucker rats was lower than that in SD rats and γ-ENaC mRNA expression in obese Zucker rat was lower than that in SD and lean Zucker rats. However, since the effect of amiloride was present in obese Zucker rats, it is unlikely that the decreased expression of ENaC mRNA plays a role in alveolar fluid clearance in obese Zucker rats.

The third explanation is that increased plasma dopamine levels stimulated basal alveolar fluid clearance in obese Zucker rats. Earlier reports indicated that dopamine increases alveolar fluid clearance in rats (Barnard et al. 1999). However, increased alveolar fluid clearance in the presence of dopamine was not mediated via β-adrenoceptors, but dopaminergic receptors. In addition, dopamine did not affect short-term Na,K-ATPase mRNA levels in rats (Saldias et al. 2002). Since those results are inconsistent with the results in this study, it is unlikely that dopamine played a role in the increase in basal alveolar fluid clearance in obese Zucker rats.

There are some limitations in this study. First, since serum glucose levels were not increased in obese Zucker rats, the effect of diabetes on the alveolar fluid clearance could not be studied in this model. Second, the expression of β2-adre-
nocceptors may change the expression and function of sodium channel and Na,K-ATPase and affect the ability of alveolar fluid clearance (Dumasius et al. 2001; McGraw et al. 2001). The mechanism between \( \beta_2 \)-adrenoceptors and sodium channel and Na,K-ATPase was not determined. Third, since mRNA expression was measured in lung tissue, the expression does not represent the expression in alveolar epithelial cells. Fourth, the protein expression of \( \beta_2 \)-adrenoceptors was not analyzed in obese Zucker rats.

In summary, basal alveolar fluid clearance in obese Zucker rats was greater than that in lean Zucker rats and SD rats. The expression of \( \alpha_1 \)-, \( \beta_1 \)-Na,K-ATPase mRNA and \( \beta_2 \)-adrenoceptor mRNA, but not the expression of amiloride-sensitive ENaC mRNA, increased in obese Zucker rats. Both ICI-118,551 and ouabain inhibited the increase in alveolar fluid clearance in obese Zucker rats. The effect of ICI-118,551 was present only in obese Zucker rats, but not in lean Zucker rats. These results indicate that \( \beta_2 \)-adrenoceptor overexpression in combination with Na,K-ATPase overexpression primarily increases basal alveolar fluid clearance in the obese Zucker rat.

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**References**


