Erythrocytes of humans with cystic fibrosis fail to stimulate nitric oxide synthesis in isolated rabbit lungs

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It is well established that the endothelium-derived relaxing factor nitric oxide (NO) is produced by endothelial cells and, when released, relaxes vascular smooth muscle (10, 14, 19). Physiologically, an alteration in shear applied to the endothelium of blood vessels has been suggested to be a major stimulus for NO release (3, 22). However, in the pulmonary circulation, a reappraisal of this viewpoint was required when it was reported that, in isolated rabbit (31) and rat (36, 38) lungs perfused with a physiological salt solution (PSS), alterations in shear stress alone did not evoke release of NO. In contrast, when lungs of these species were perfused with either blood (36) or PSS containing washed erythrocytes (30, 38), inhibition of endogenous NO synthesis produced a shift in the slope of the pressure-flow relationship consistent with a decrease in vascular caliber, i.e., NO was a determinant of vascular resistance. The ability of the erythrocyte to stimulate NO synthesis in the pulmonary circulation of the rabbit and rat was reported to be independent of effects on viscosity or pressure (31, 36). The property of the erythrocyte that was associated with the stimulation of endogenous NO synthesis was shown to be the release of ATP (26, 28, 30). In support of the hypothesis that erythrocyte-derived ATP is a stimulus for endogenous NO synthesis in the lung, the addition of ATP to the perfusate of isolated rabbit lungs produces vasodilation that is, in large part, dependent on the stimulation of endogenous NO synthesis (30). Importantly, in addition to rabbit erythrocytes, human erythrocytes were reported both to release ATP in response to physiological stimuli, such as mechanical deformation, and to stimulate endogenous NO synthesis in the isolated perfused rabbit lung (26).

Recently, a signal transduction pathway that relates mechanical deformation to ATP release from human and rabbit erythrocytes has been described (17, 18, 27, 29). This pathway includes the heterotrimeric G proteins Gi (17) and G (18), adenylyl cyclase (29), PKA (29), and the cystic fibrosis transmembrane conductance regulator (CFTR) (27). The activity of CFTR was shown to be required for deformation-induced ATP release from erythrocytes of both rabbits and humans (27). Thus erythrocytes of humans with cystic fibrosis, a condition in which CFTR activity is absent or markedly reduced, and rabbit erythrocytes incubated with inhibitors of CFTR activity failed to release ATP in response to mechanical deformation (27). Here, we demonstrate that CFTR is expressed in the membrane of both rabbit and human erythrocytes and that, in contrast to those of healthy humans, erythrocytes from humans with cystic fibrosis fail to stimulate endogenous NO synthesis in the isolated rabbit pulmonary circulation.

MATERIALS AND METHODS

Isolation of rabbit and human erythrocytes. Rabbit erythrocytes were obtained from New Zealand White rabbits (male, 2–3 kg body wt). The animals were anesthetized with ketamine (12.5 ml/kg im) and xylazine (1 mg/kg im) followed by pentobarbital sodium (10 mg/kg iv). After tracheal intubation, the animals were mechanically ventilated with room air (tidal volume 10 ml/kg, rate 20–25 breaths/min). A catheter was placed into the carotid artery for the administration of heparin (500 units) and for a phlebotomy. Ten minutes after heparin, the animals were exsanguinated. Human erythrocytes were obtained by venipuncture performed in an antecubital vein without the use of a tourniquet. Sixty milliliters of blood were collected into a syringe containing 50 units of heparin. Protocols for removal of blood from rabbits and humans were approved by the Institutional Animal Care and Use Committee and the Institutional Review Board of Saint Louis University, respectively.

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Rabbit and human blood was centrifuged at 500 g for 10 min at 4°C. The plasma, buffy coat, and uppermost erythrocytes were removed by aspiration and discarded. The remaining erythrocytes were washed three times in buffer solution containing 140.5 mM NaCl, 21.0 mM Tris, 4.7 mM KCl, 2.0 mM CaCl2, 1.2 mM MgSO4, 0.1% dextrose, and 0.5% BSA fraction V (ICN Biomedicals; Aurora, OH), with a final pH of 7.4. The hematocrit of the washed erythrocytes was determined using an Autocrit Ultra 3 centrifuge (Becton Dickinson; Bedford, MA).

Preparation of erythrocyte membranes. Washed rabbit or human erythrocytes were diluted 1:100 with ice-cold lysis buffer (5 mM Tris·HCl and 2 mM EDTA, pH 7.4) and stirred at 4°C for 20 min. The resulting mixture was centrifuged at 23,000 g for 15 min at 4°C. The supernatant was removed and discarded. The membranes were resuspended in cold lysis buffer and centrifuged at 23,000 g for 15 min at 4°C a final time. The supernatant was discarded, and the protein concentration was determined (BCA Protein Assay, Pierce; Rockford, IL). Aliquots were frozen at −80°C. Immediately before electrophoresis, membranes were thawed on ice.

Western blot analysis. Membrane proteins were mixed in sample buffer [1.5% SDS, 6% (vol/vol) glycerol, 0.6% dithiothreitol, 10 mM Tris (pH 6.8), and 0.01% bromophenol blue], heated to 95°C for 2 min, and resolved by electrophoresis in 5% SDS-polyacrylamide precast gels (Bio-Rad; Hercules, CA). After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore; Bedford, MA), vortexed with 5 μl of 10% SDS per 200 μl of sample, and heated at 95°C for 90 s.

Preparation of T84 human colon carcinoma cells. T84 cells (American Type Culture Collection), which express large amounts of CFTR (Amersham) were used as a positive control (4, 33), as T84 cells (American Type Culture Collection), which express large amounts of CFTR (Amersham), were used as a positive control (4, 33).

Identification of CFTR in erythrocyte membranes. If CFTR is a component of the signal transduction pathway for ATP release from rabbit and human erythrocytes, then it must be present in these cell membranes. Membranes prepared from a human colon carcinoma cell line that has been shown to express CFTR (T84 cells) were used as a positive control (4, 33). Membranes of both human and rabbit erythrocytes were found to contain CFTR protein when examined by Western blot analysis (Fig. 1).

Effect of l-NAME on pressure-flow relationships in isolated rabbit lungs perfused with PSS in the absence of erythrocytes. The pressure-flow relationship was determined in isolated rabbit lungs perfused with PSS in the absence and presence of L-NAME at concentrations of either 30, 100, or 300 μM. In the absence of erythrocytes, L-NAME had no effect on the pressure-flow relationship (Fig. 2A).

Perfusion of isolated rabbit lungs with PSS containing washed human erythrocytes. Initial pH, gas tensions, and hematocrit in studies in which lungs were perfused with PSS containing erythrocytes from healthy humans or humans with cystic fibrosis were added.

RESULTS

Identification of CFTR in erythrocyte membranes. If CFTR is a component of the signal transduction pathway for ATP release from rabbit and human erythrocytes, then it must be present in these cell membranes. Membranes prepared from a human colon carcinoma cell line that has been shown to express CFTR (T84 cells) were used as a positive control (4, 33). Membranes of both human and rabbit erythrocytes were found to contain CFTR protein when examined by Western blot analysis (Fig. 1).

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Perfusion of isolated rabbit lungs with PSS containing washed human erythrocytes. Initial pH, gas tensions, and hematocrit in studies in which lungs were perfused with PSS containing erythrocytes from healthy human erythrocytes or from humans with cystic fibrosis are depicted in Table 1. Humans with cystic fibrosis, identified as homozygous for the ΔF508 genotype, were treated with medications including inhaled β-agonists, ipratropium bromide, corticosteroids, antibiotics, DNAse, and inhibitors of mast cell degranulation as well as oral pancreatic enzyme replacement, antibiotics, proton.
pump inhibitors, and vitamins. The addition of washed human erythrocytes to the perfusate of isolated rabbit lungs did not result in increased airway pressure or weight gain over the course of the experiment.

Effect of L-NAME on pressure-flow relationships in isolated rabbit lungs perfused with PSS containing erythrocytes from healthy humans. Under control conditions (perfusion at 80 ml/min), the addition of L-NAME to the perfusate resulted in an increase in perfusion pressure (Fig. 2B). Moreover, the addition of L-NAME resulted in a shift in the pressure-flow relationship such that the pressure difference across the pulmonary circulation \((P_{PA} - P_{LA})\) was increased at each flow rate studied (Fig. 3). Importantly, the slope of this relationship was increased after L-NAME \((P < 0.01)\) consistent with the interpretation that, under these conditions, inhibition of endogenous NO synthesis was associated with an increase in Ohmic resistance (15). Finally, the site of increased resistance was examined by determination of the effect of L-NAME on changes in both upstream \((P_{PA} - P_{MV})\) and downstream \((P_{MV} - P_{LA})\) vascular resistance. The increases in vascular resistance were confined to the upstream vascular segment (Fig. 4, A and B).

**Effect of L-NAME on pressure-flow relationships in isolated rabbit lungs perfused with PSS containing erythrocytes from humans with cystic fibrosis.** In isolated rabbit lungs perfused with PSS containing erythrocytes from humans with cystic fibrosis, L-NAME administration did not result in any change in perfusion pressure under control conditions (Fig. 2B). Moreover, in the presence of these erythrocytes, L-NAME had no effect on pressure-flow relationships (Fig. 5).  

**DISCUSSION**

It was reported previously that erythrocytes from either rabbits (31), rats (36, 38), or healthy humans (26) are required for the demonstration of flow-induced NO synthesis in isolated perfused lungs. These observations, coupled with the findings that physiological stimuli, such as mechanical deformation (18, 26, 27, 29), stimulate ATP release from erythrocytes and that ATP is a stimulus for endogenous NO synthesis in the isolated

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**Table 1. Perfusate pH and gas tensions in isolated perfused rabbit lungs perfused with PSS containing RBCs of healthy humans or humans with cystic fibrosis**

<table>
<thead>
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<th>pH</th>
<th>(P_{O2}), mmHg</th>
<th>(P_{CO2}), mmHg</th>
<th>Hematocrit, %</th>
<th>(n)</th>
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<tr>
<td>Lungs perfused with PSS</td>
<td>7.38±0.01</td>
<td>137±10</td>
<td>46±2</td>
<td>29±2</td>
<td>5</td>
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<tr>
<td>and RBCs of healthy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>humans</td>
<td></td>
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<tr>
<td>Lungs perfused with PSS</td>
<td>7.37±0.01</td>
<td>125±9</td>
<td>35±2*</td>
<td>28±2</td>
<td>4</td>
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<tr>
<td>and RBCs of humans</td>
<td></td>
<td></td>
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<td>with cystic fibrosis</td>
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Values are means ± SE; \(n\), no. of rabbit lungs. PSS, physiological salt solution; RBCs, red blood cells (erythrocytes). *\(P < 0.05\) compared with healthy humans.
rabbit lung (30), suggest a novel mechanism for the control of pulmonary vascular caliber. In this construct, as the erythrocyte is deformed by increments in the velocity of blood flow through a vessel and/or by reductions in vascular caliber, it releases ATP, which stimulates endothelial synthesis of NO resulting in relaxation of vascular smooth muscle and, thereby, an increase in vascular caliber. This vasodilation would then result in a decrease in pulmonary vascular resistance as well as a decrease in the stimulus for additional erythrocyte deformation. Thus erythrocyte-derived ATP would contribute to the low vascular pressure in the healthy lung via stimulation of endogenous NO synthesis.

Recently, a signal transduction pathway that relates mechanical deformation to ATP release from erythrocytes of rabbits and humans has been described. Components of this pathway include the heterotrimeric G proteins Gs (17) and Gi (18), adenyl cyclase (29), PKA (29) and CFTR (27). A role for CFTR in this pathway was suggested by reports that this member of the ATP binding cassette could facilitate the movement of ATP out of cells either by acting as an ATP conduit (21, 22) or by regulating another channel for ATP release (20, 23). The finding that CFTR activity is lost or markedly diminished in humans with cystic fibrosis (5, 6) provided a unique opportunity for investigation of the role of CFTR in the release of ATP from erythrocytes in response to the physiological stimulus of mechanical deformation. It was reported previously that erythrocytes of patients with cystic fibrosis as well as rabbit erythrocytes incubated with two chemically dissimilar inhibitors of CFTR, namely, glibenclamide (24, 25) and niflumic acid (7), failed to release ATP in response to mechanical deformation (27).

A necessary step in establishing that CFTR is a component of a signal transduction pathway for ATP release from erythrocytes is the demonstration of its presence in erythrocyte membranes. Recently, it was shown that CFTR protein is present in erythrocyte membranes of both healthy humans and humans with cystic fibrosis (1, 32). In addition, it was reported that human erythroblasts contain CFTR mRNA (37). However, it must be noted that neither CFTR protein in human erythrocytes nor mRNA in human erythroid progenitor cells were detected in another study (13). In the present work, we confirm the finding that CFTR protein is a component of human erythrocyte membranes (Fig. 1). In addition, we show that the membranes of rabbit erythrocytes also possess CFTR (Fig. 1). Thus CFTR, a necessary component of a proposed signal transduction pathway for ATP release from erythrocytes, is present in the membranes of both rabbit and human erythrocytes, cells that release ATP in response to mechanical deformation (26, 27, 29).

Although CFTR activity is required for deformation-induced ATP release from erythrocytes of humans (27), the loss of CFTR activity may have a different effect on basal ATP release from these cells. It was reported that the concentration of ATP...
was increased in the blood of CFTR knockout mice as well as humans with cystic fibrosis compared with either wild-type controls or healthy humans, respectively (1). It is important to note that, in these studies, ATP release was studied under quiescent conditions, i.e., erythrocytes were not subjected to mechanical deformation. Thus it is possible that, although CFTR is required for deformation-induced ATP release from erythrocytes, another member of the ATP binding cassette, such as the multidrug resistance-associated protein 1, is involved in basal ATP release from these cells (1).

The hypothesis that ATP, released from erythrocytes of healthy humans in response to deformation, stimulates endogenous NO synthesis in the isolated rabbit lung is supported by previous studies. Erythrocytes of healthy humans release ATP in response to mechanical deformation as would be encountered as they pass through the pulmonary circulation. The amount of ATP released in response to deformation was shown to be stimulus dependent (26, 27, 29). In addition, the inclusion of human erythrocytes in the perfusate of isolated rabbit lungs resulted in the stimulation of endogenous NO synthesis (26). Finally, the addition of ATP to the perfusate of isolated rabbit lungs resulted in concentration-dependent decreases in total pulmonary vascular resistance that were inhibited by pretreatment with the NO synthase inhibitor L-NAME (30).

In view of the findings that CFTR is expressed in human erythrocyte membranes, that it is a component of a signal transduction pathway that relates erythrocyte deformation to ATP release (27), and that ATP release from erythrocytes is a stimulus for endogenous NO synthesis in the rabbit pulmonary circulation (30), we hypothesized that erythrocytes of humans with cystic fibrosis, a condition in which CFTR activity is impaired, would fail to stimulate NO synthesis in the isolated perfused rabbit lung. In the presence of washed erythrocytes from healthy humans, the administration of the NO synthase inhibitor L-NAME to the perfusate of isolated lungs resulted in a shift in the pressure-flow relationship in a manner consistent with an increase in Ohmic resistance (Fig. 3) (15). This increase in vascular resistance was confined to the upstream vascular segment (\(P_{PA} - P_{MV}\) as a function of flow rate; Fig. 4A). In contrast, in the presence of erythrocytes of patients with cystic fibrosis, L-NAME was without effect on the pressure-flow relationship, i.e., NO was not a determinant of pulmonary vascular resistance (Fig. 5).

Several studies suggest that endogenous NO synthesis in the lung may be decreased in humans with cystic fibrosis. It was reported that amounts of NO detected in exhaled gas are decreased in humans with cystic fibrosis compared with healthy humans (2, 15, 35). It must be recognized that the source of NO that is measured in exhaled gas is unknown and could derive from the endothelium of blood vessels, the airways, or other resident cells. However, it was reported that exhaled NO is reduced in neonates with cystic fibrosis before the development of detectable respiratory symptoms, suggesting that the decrease in exhaled NO is not the result of increased mucus accumulation and/or inflammation (9). Thus the decrease in measurable NO in exhaled gas in cystic fibrosis patients compared with healthy controls could reflect, in part, the failure of erythrocyte-derived ATP to stimulate endogenous NO synthesis.

Importantly, it was reported that isolated blood vessels obtained from lungs of humans with cystic fibrosis, when precontracted with phenylephrine, relaxed in response to the application of ADP, the first degradation product of ATP (8). This response was shown to be endothelium dependent, although the nature of the endothelium-derived relaxing factor was not determined. Thus it is reasonable to hypothesize that if erythrocyte-derived ATP were released into the pulmonary circulation of humans with cystic fibrosis, endogenous NO synthesis could be stimulated.

Failure of deformation-induced ATP release from erythrocytes and, thereby, the loss of a physiological stimulus for endogenous NO synthesis could be expected to lead, ultimately, to the development of pulmonary hypertension. Pulmonary hypertension is seen in humans with cystic fibrosis; however, this condition has most often been attributed to the destructive lung disease and the subsequent loss of pulmonary capillaries and/or hypoxic pulmonary vasoconstriction (11, 34). The results presented here suggest that a defect in ATP release from erythrocytes of cystic fibrosis patients as they traverse the pulmonary circulation could be an additional factor leading to the development of pulmonary hypertension in these individuals.

In summary, we demonstrate that CFTR is a component of the erythrocyte membrane of rabbits and humans. Erythrocytes from humans in whom this protein is functional stimulate endogenous NO synthesis in the isolated rabbit lung. In contrast, erythrocytes of humans with cystic fibrosis, a condition in which CFTR activity is impaired, do not stimulate NO synthesis in this model. These findings provide support for the hypothesis that erythrocyte-derived ATP, released in response to a physiological stimulus, such as mechanical deformation, could serve to aid in the maintenance of normal pulmonary vascular resistance via the stimulation of endogenous NO synthesis.

Fig. 5. Effect of L-NAME (100 \(\mu\)M) on the relationship between flow rate and the pressure gradient across the pulmonary circulation (\(P_{PA} - P_{LA}\)) in lungs perfused with PSS containing washed erythrocytes of humans with cystic fibrosis (n = 4). Values are means ± SE.
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GRANTS

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