RAPID COMMUNICATION

Protocols for \textit{in vivo} measurement of the ion transport defects in cystic fibrosis nasal epithelium

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ABSTRACT: New treatments for cystic fibrosis (CF), including gene therapy, are currently being assessed. These aim to correct the basic defects of increased sodium absorption and decreased chloride secretion in airway epithelia. Assessment of these bioelectric parameters, particularly in the nasal epithelium, is likely to be used as a measure of treatment efficacy. However, the optimal \textit{in vivo} protocol to discriminate cystic fibrosis from non-cystic fibrosis subjects is unclear. We have, therefore, compared three protocols for measurement of the cystic fibrosis ion transport defects \textit{in vivo} in the nasal epithelium.

Sodium absorption was measured using both the baseline potential difference and the response to the sodium channel blocker, amiloride. Chloride secretion was assessed in the presence of amiloride, using perfusion with isoprenaline, or terbutaline, or a low chloride solution followed by isoprenaline.

Baseline potential difference (PD) and the absolute response to amiloride clearly differentiated the increased sodium absorption in the cystic fibrosis subjects. The responses both to terbutaline (ΔPD: non-CF: -0.8 (SEM 0.7) mV; CF: -3.6 (0.5) mV) and isoprenaline (non-CF: 1.5 (0.6) mV; CF: -2.9 (0.6) mV) differentiated the two groups of subjects, but there was considerable overlap of values. Perfusion with a low chloride solution (non-CF: 12.6 (1.2) mV; CF: 0.6 (0.4) mV), as well as subsequent perfusion with isoprenaline (non-CF: 10.0 (1.1) mV; CF: -1.4 (0.4) mV) allowed clear separation of the two groups, with no overlap of values. Some CF subjects showed a transient hyperpolarization to these stimuli, which could clearly be differentiated from the sustained responses seen in non-cystic fibrosis subjects.

We conclude that sodium hyperabsorption in the nasal epithelium of cystic fibrosis subjects can be reliably assessed using the baseline potential difference and the absolute change following amiloride. The defect in chloride secretion may be clearly measured (in the presence of amiloride) by perfusion with a low chloride solution followed by isoprenaline. We suggest that this protocol is appropriate for \textit{in vivo} assessment of new treatments for cystic fibrosis. Eur Respir J., 1994, 7, 2050–2056.

Despite advances in medical therapy, most patients with cystic fibrosis (CF) still die from respiratory failure. The disease is characterized by abnormalities in ion transport [1], in particular defective cyclic adenosine monophosphate (cAMP)-regulated Cl\textsuperscript{-} secretion. This relates to the CF gene product, the cystic fibrosis transmembrane conductance regulator (CFTR), which can function as a CAMP-regulated Cl channel. Sodium absorption is also increased, though how this relates to the chloride defect remains uncertain. In turn, how either bioelectric abnormality relates to the respiratory disease is equally unknown. Newer therapeutic approaches aimed at these basic defects include reducing Na\textsuperscript{+} absorption [2], or increasing alternate pathways of Cl\textsuperscript{-} secretion [3], but the most promising recent development may be gene therapy.

Following the isolation of the CF gene [4], gene transfer has been shown to correct the Cl\textsuperscript{-} defect \textit{in vitro} [5, 6]. More recently, this has also been demonstrated in mouse models of CF [7, 8]. This has led to 10 groups worldwide proposing phase I clinical trials of gene therapy for CF. One of the important end-points in these trials will be the measurement of the CF ion transport abnormalities \textit{in vivo}. Although previous studies have assessed components of the Na\textsuperscript{+} and Cl\textsuperscript{-} related changes in nasal potential difference (PD), the optimal protocol for discriminating CF from non-CF subjects is unclear. Measurement of Na\textsuperscript{+} hyperabsorption can be made using the baseline PD and the response to the Na\textsuperscript{+} channel blocker, amiloride [9]. Assessment of chloride secretion across the apical membrane of the respiratory epithelium is more difficult, since Cl\textsuperscript{-} is approximately at equilibrium [10]. Thus, an electrical or chemical gradient for Cl\textsuperscript{-} secretion is required to induce Cl\textsuperscript{-} movement. Perfusion with amiloride provides the former, and subsequent addition of agents elevating cAMP would seem an attractive option to assess CFTR function. Indeed, this protocol was used in the first reported study of

An alternative approach is to induce a larger electrochemical gradient for Cl- secretion through perfusion of a low Cl- solution in the presence of amiloride. Subsequent perfusion with agents that increase cAMP are then likely to induce a greater degree of Cl- secretion. Consequently, the larger changes in PD may then allow clearer quantification of the CF bioelectric defects. In turn, better differentiation of the effects of gene transfer, including dose-dependent changes, may be achieved. This may be of particular relevance if gene transfer produces only partial correction of the bioelectric defect. We have, therefore, assessed three protocols for measurement both of the Na+ and the Cl- abnormalities characteristic of CF.

Materials and methods

Nasal PD was measured using methods described previously [12, 13]. Briefly, the exploring electrode consisted of a double lumen silicone rubber tube with the openings of both lumens at the same site, 3 mm from the tip. One lumen was filled with an equal mixture of Ringer's lactate and electrocardiographic (ECG) electrode cream, connected to a high impedance voltmeter via a silver/silver chloride electrode. The second lumen was perfused with the different solutions, as outlined below, using a peristaltic pump which provided a continuous flow of 4 ml·min⁻¹ throughout the perfusion period. The reference electrode consisted of a second silver/silver chloride electrode placed over an area of abraded skin on the forearm, again connected to the voltmeter. Prior to recordings, the offset of the electrodes was measured and appropriate corrections made to recorded values.

The exploring electrode was passed along the floor of the nose, the maximum nasal PD recorded, and the tube then positioned at this site. Perfusion commenced with the diluent alone, KH (Kreb's N-[2-hydroxyethyl]-piperazine-n'-[2-ethane-sulphonic-acid] (HEPES) of composition (mM): Na+ 140, K+ 6, Mg2+ 1, Ca2+ 2, Cl- 152, HEPES 10, titrated to pH 7.4). Following stabilization of the PD, the perfusing solution was then changed according to one of the three protocols outlined below. All solutions were perfused sequentially, such that each new solution included the previous drugs. Due to the 3 ml dead space of the perfusion system, the new perfusate reached the catheter tip approximately 45 s following solution change. A low chloride solution was prepared by substituting NaCl and KCl with equimolar gluconate, giving a final Cl- concentration of 6 mM. Fresh stock solutions (10 mM) of isoprenaline and terbutaline, each in 4% ascorbic acid, were prepared daily and diluted as required.

Perfusion protocols

Protocol 1. Baseline KH - amiloride (100 µM) in KH - amiloride (100 µM) + terbutaline (10 µM) in KH - amiloride (100 µM) + isoprenaline (10 µM) in KH.

Protocol 2. Baseline KH - amiloride (100 µM) in KH - amiloride (100 µM) + isoprenaline (10 µM) in KH - amiloride (100 µM) + terbutaline (10 µM) in KH.

Protocol 3. Baseline KH - amiloride (100 µM) in KH - amiloride (100 µM) in low chloride (6 mM) KH - amiloride (100 µM) + isoprenaline (10 µM) in low chloride solution.

For protocols 1 and 2, the second β-agonist was perfused to help comparison of relative efficacy of the two agents. Each subject had one nostril perfused according to protocol 1 and the other nostril according to protocol 2, in random order, with 10 min between tests. On a separate day, both nostrils were perfused according to protocol 3, with the responses averaged for later analysis. For discussion purposes, increases and decreases refer to the absolute magnitude of the PD (lumen negative).

Subjects

Six nonsmoking subjects (3 males and 3 females, mean age 29 yrs, range 23–41 yrs) with no history of respiratory disease, and six CF subjects, homozygous for the ΔF508 mutation (3 males and 3 females, mean age 26 yrs, range 18–32 yrs) underwent each of the three protocols. In addition, 4 normal volunteers and 2 homozygous ΔF508 CF subjects were studied with protocols 1 and 2, and 6 normal volunteers and 19 homozygous ΔF508 CF subjects with protocol 3. No subject had previous nasal surgery, and all tests were performed at least four weeks following an upper respiratory tract infection. Baseline values for 241 non-CF subjects, including normal volunteers and subjects with bronchiectasis, asthma, sarcoidosis, hypertension, diabetes mellitus, coeliac disease and known CF heterozygotes, are also included, compared with 146 CF subjects of various genotypes. This includes some patients described previously [13], where the reference electrode was a subcutaneous cannula. The study was approved by the Royal Brompton Hospital Ethics Committee, and all subjects gave informed consent.

Statistical analysis

The Wilcoxon signed rank test was used for comparison within the subjects who underwent all three protocols, and the Mann-Whitney U-test for comparison between groups, unless data were normally distributed (Student’s t-test). The null hypothesis was rejected at p<0.05.

Results

Baseline nasal PD values for non-CF and CF subjects are shown in figure 1. Both populations describe normal distributions, with little overlap between the two groups (p<0.0001). Responses to perfusion with the Na+
channel blocker, amiloride (100 µM), both as the absolute change in PD and the percentage change in the baseline PD, are shown in figure 2. Although the absolute response was significantly (p<0.0001) larger in the CF subjects, the proportional response, expressed as a percentage of baseline, was not significantly different between the two groups. Thus, both the baseline PD and the absolute response to amiloride clearly discriminate CF from non-CF subjects on the basis of Na⁺ hyperabsorption.

Neither isoprenaline nor terbutaline produced a significant effect in non-CF subjects, when added either after amiloride or following perfusion with the other agent. However, comparison of the two β-agonists showed that isoprenaline produced a small degree of hyperpolarization, irrespective of order of addition (table 1). In CF subjects, both agents, irrespective of order of addition produced a depolarization (fig. 3a and b). On this basis, both isoprenaline (p<0.01) and terbutaline (p<0.05) could significantly distinguish CF from non-CF subjects. However, in the CF subjects who underwent all three protocols, 4 of the 6 responses to terbutaline, and 1 of the 6 responses to isoprenaline, were within the non-CF range (fig. 4).

To attempt to increase the discrimination between CF and non-CF subjects, we then assessed the effect of

![Graph](image1)

Fig. 2. – The change in baseline potential difference (ΔPD) produced by amiloride, 100 µM, in CF (n=25) and non-CF (n=12) subjects. a) absolute effect (p<0.0001); b) effect expressed as a percentage of the baseline value (p=NS). CF: cystic fibrosis; NS: non-significant.

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perfusion with a low chloride solution, followed by isoproterenol. In the 6 non-CF subjects, low chloride (6 mM) perfusion produced a significant (p<0.05) hyperpolarization, maximal at approximately 5 min; subsequent addition of isoproterenol (10 µM) induced a further significant (p<0.05) hyperpolarization. In contrast, the 6 CF subjects showed no significant changes at 5 min to perfusion with either low chloride or isoproterenol solutions (fig. 3c and table 1). The difference between the non-CF and CF responses both to low Cl⁻ and isoproterenol were both significant (p<0.01). No responses in the 6 CF subjects to either solution overlapped with those of the 6 non-CF subjects (fig. 4).

Typical responses to the three protocols are shown in figure 5. Although the overall response (measured at 5 min) in the CF subjects showed no hyperpolarization, many subjects exhibited an initial transient response to perfusion with isoproterenol or terbutaline alone. In 6 out of 16 responses to terbutaline and 9 out of 16 responses to isoproterenol in 8 CF subjects, these initial hyperpolarizations were greater than 1 mV in magnitude. None of the 20 responses to either isoproterenol or terbutaline in 10 normal subjects, exhibited these initial transients.

Similar, though larger, transient hyperpolarizations were seen in the CF subjects following perfusion with the low chloride or subsequent isoproterenol solutions (fig. 5). These transient responses were greater than 1 mV in 64 out of 86 individual recordings from 25 CF subjects. In some subjects, it was consistently more marked in one nostril than the other when measured three times at weekly intervals (fig. 6). In 28 recordings from 12 non-CF subjects, no such initial hyperpolarization was seen, though in view of the rapid onset of the normal response to both low chloride and isoproterenol (fig. 5f), small transients obscured by the large normal response cannot be excluded.
Fig. 5. – Typical responses in one cystic fibrosis (CF) (a–c) and one non-CF (d–f) subject. In each case the change in potential difference (ΔPD) is shown subsequent to perfusion with amiloride, 100 µM. a) and d) sequential perfusion with terbutaline (T), 10 µM, and isoprenaline (I) 10 µM; b) and e) sequential perfusion with isoprenaline (I), 10 µM, and terbutaline (T) 10 µM; c) and f) sequential perfusion with a low chloride (Cl) solution 6 mM, and isoprenaline (I) 10 µM.

Fig. 6. – Typical responses in one cystic fibrosis (CF) individual to sequential perfusion with a low chloride solution (Cl) 6 mM and isoprenaline (I), 10 µM, in the presence of amiloride, 100 µM, at weekly intervals (a–c). Responses were measured both in the left (●) and right nostril (○). ΔPD: change in baseline potential difference.
Discussion

In preparation for studies of CFTR complementary deoxyribonucleic acid (cDNA) transfer to the nasal epithelium, we sought to develop a protocol for in vivo measurement of nasal PD which would best discriminate the Na+ and Cl- abnormalities characteristic of CF. The clearest distinction between the CF and non-CF subjects was found with the sequential perfusion of amiloride, low chloride and isoprenaline solutions.

As the nasal epithelium both demonstrates the CF bioelectric defects and is easily accessible, this area provides a convenient site for the assessment of treatment efficacy. We have previously described a simple technique for the measurement of nasal PD along the floor of the nasal cavity [13]. Both baseline PD and the absolute response to amiloride provided good discrimination of the increased Na+ absorption in CF. The amiloride effect, expressed as a percentage of baseline PD was, however, variable and did not differentiate between CF and non-CF subjects. Previous studies have shown that perfusion of amiloride onto the inferior surface of the inferior turbinate produced a significantly greater inhibition of the baseline PD in CF subjects, measured either as the absolute change in PD, or as a percentage of baseline [9, 14]. The greater proportion of ciliated epithelial cells at this site may explain the different findings [15], but differences in technique, including rate and composition of the perfused solution may also be important. The baseline PD may be reduced by infection [16] and mild trauma [15]; therefore, changes following gene transfer to CF subjects may not necessarily indicate correction of the Na+ hyperabsorption seen in CF. Thus, assessment of Cl- secretion will also be required.

Stimulation of Cl- secretion through cAMP-regulated pathways should provide the optimal index for assessing the CF bioelectric defect. However, perfusion of terbutaline or isoprenaline, in the presence of amiloride alone, produced only small responses in either group, with considerable overlap of values. In a recent study measuring PD along the medial aspect of the inferior turbinate, the responses to terbutaline in CF and non-CF subjects were similar to those seen in our study, although clear discrimination was found between the two groups of subjects [11]. Under baseline conditions, there is little driving force for Cl- secretion [10]. Although amiloride will increase the electrical gradient for Cl- secretion, this appears to be insufficient to allow β-agonists to produce large responses in vivo. Given this and the unknown effect on the electrophysiological profile of partial correction of the CF defect, we assessed the effect of perfusion with a low Cl- solution [12]. This will increase efflux through all open Cl- channels, and if, therefore, less specific for the cAMP-regulated Cl- defect [17]. However, similar techniques have been used to demonstrate the CF Cl- impermeability in both respiratory [14] and sweat gland [18] epithelia, and clearly differentiated CF subjects, with no overlap with non-CF values.

Following low Cl- perfusion, the effect of isoprenaline increased approximately seven-fold in the non-CF subjects. This suggests that the relatively small response to isoprenaline alone, in these subjects, was not due to failure of drug delivery. Rather, it reflects the small driving force for Cl- secretion induced by perfusion with amiloride alone. In the CF subjects, the response to isoprenaline was not altered by low chloride solution, confirming that Cl- secretion remains defective despite large gradients for secretion. Thus, the largest Cl- secretory response in non-CF subjects was induced through sequential perfusion with low Cl- and isoprenaline solutions. Our in vivo measurements of nasal PD include variation in the signal up to approximately 1 mV (fig. 5), due to respiration and movement artefacts. Although averaging the response from both nostrils, and repeating the measurements in an individual subject will tend to minimize this noise, increasing the magnitude of the signal with low Cl- may represent the optimal protocol for assessment of the Cl- defect in the CF nasal epithelium in vivo.

The initial transient hyperpolarization in the CF subjects following either isoprenaline, terbutaline or low Cl- solutions was an unexpected finding. This is unlikely to be an artefact, since it did not occur with perfusion of either diluent or amiloride, nor was it seen in the non-CF subjects. Perfusion with a low Cl- solution will induce a junctional potential with both the gel around the exploring electrode and the interstitium of the nasal mucosa. However, these are likely to be approximately equal and opposite, causing little net effect, and to be similar in both CF and non-CF subjects. Furthermore, this would not explain the transients seen with the β-agonists alone. Previous in vitro studies have demonstrated that the CF respiratory epithelium can produce small, transient increases in Cl- secretion, following addition of membrane permeable analogues of cAMP [19], which were abolished following depletion of Ca2+ stores. Both because of this, and since isoprenaline transiently increases intracellular Ca2+ [20], we speculate that the β-agonist induced responses may represent Cl- secretion through a Ca2+-regulated pathway. Both this and the origin of the hyperpolarization following perfusion with a low Cl- solution requires further investigation. Irrespective of the origin of this initial response, CF and non-CF subjects could be clearly distinguished on the basis of the sustained response to a low chloride solution as measured at 5 min.

In conclusion, we suggest that the Na+ and Cl- defects in CF nasal epithelium can be clearly distinguished by sequential perfusion of amiloride, a low Cl- solution and isoprenaline. Optimization of the discrimination between CF and non-CF subjects is likely to be important in the assessment of the effects of CFTR gene transfer.

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References