Partial restoration of defective chloride conductance in ΔF508 CF mice by trimethylamine oxide

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Fischer, Horst, Norimasa Fukuda, Pascal Barbry, Beate Illek, Claudio Sartori, and Michael A. Matthay. Partial restoration of defective chloride conductance in ΔF508 CF mice by trimethylamine oxide. Am J Physiol Lung Cell Mol Physiol 281: L52–L57, 2001.—This study was designed to test the in vivo efficacy of the chemical chaperone trimethylamine oxide (TMAO) in correcting the Cl⁻ transport defect in a mouse model of cystic fibrosis (CF). Rectal potential difference (RPD) measurements were done in matched wild-type and ΔF508 CF mice. Mice were treated by subcutaneous injections of TMAO. Wild-type mice demonstrated a forskolin-stimulated, Cl⁻-dependent hyperpolarization of −6.4 ± 0.8 mV (n = 11), which was significantly increased to −13.1 ± 1.4 mV after treatment with TMAO. ΔF508 CF mice showed no significant responses to forskolin. Treatment with TMAO recovered a forskolin-activated RPD in ΔF508 CF mice (−1.1 ± 0.2 mV; n = 17) but not in CFTR null mice. The effects of TMAO were dose dependent, resulting in a slope of −0.4 ± 0.1 mV·g⁻¹·kg⁻¹ in ΔF508 CF mice. The forskolin-stimulated RPD in TMAO-treated ΔF508 CF mice was partially blocked by glibenclamide and further stimulated by apigenin. The total response to forskolin plus apigenin was −2.5 ± 0.45 mV (n = 6 mice), corresponding to 39% of the response evoked by forskolin only in wild-type mice.

apigenin; ΔF508 cystic fibrosis transmembrane conductance regulator; cystic fibrosis transmembrane conductance regulator knockout; glibenclamide; rectum; epithelia

CYSTIC FIBROSIS (CF) is an autosomal recessively inherited disease caused by a mutation of the CF transmembrane conductance regulator (CFTR) gene, which encodes for the cAMP-regulated Cl⁻ channel in the apical membranes of epithelial cells. Numerous CF-causing mutations of CFTR have been described. The most common mutation is a deletion of phenylalanine at position 508 of CFTR (ΔF508 CFTR) that is present on 66% of CF chromosomes; 88% of CF patients carry at least one ΔF508 allele (32). This mutation leads to a misfolded protein that is retained in the endoplasmic reticulum and is targeted for rapid degradation (5, 14, 37). Epithelial cells homozygous for the ΔF508 mutation have an extremely reduced plasma membrane Cl⁻ conductance (25, 29, 35). When ΔF508 CFTR was overexpressed so that it could translocate to the cell membrane, ΔF508 CFTR formed a functional Cl⁻ channel with a reduced open probability (9, 11, 19) and a reduced half-life in the membrane (28).

Currently, there is no treatment for CF that targets the molecular defect of CFTR. Possible targets for pharmacological interventions are 1) the CFTR channel in the membrane, 2) signaling cascades that regulate CFTR, and 3) the maturation and trafficking pathway of CFTR. A number of small, hydrophilic molecules have been recently introduced as chemical chaperones (3, 34), which promoted protein folding and maturation. When 3T3 cells expressing ΔF508 CFTR were treated with the chemical chaperone trimethylamine oxide (TMAO) at concentrations of 50–100 mM, fully glycosylated mature ΔF508 CFTR was found and the Cl⁻ permeability of the cell membrane was partially restored (3). This result suggested that treatment with TMAO was able to correct the processing defect of ΔF508 CFTR. To test the effects of TMAO in vivo, Bai et al. (1) developed a treatment protocol for mice that resulted in serum concentrations of TMAO (50–100 mM) that were effective in cell culture models. The current study was designed to determine the in vivo efficacy of TMAO treatment for the correction of the cAMP-stimulated, Cl⁻-dependent rectal potential difference (RPD) in CF mice. The measurement of the rectal difference was chosen because, unlike humans, CF mice develop CF-related intestinal but not airway pathology (31). Furthermore, Cl⁻ transport in CF mouse intestine and colon is impaired (8, 16–18).

METHODS

Study protocol. Transgenic ΔF508 CF mice were bred at the University of California, San Francisco Animal Facility and genotyped with standard protocols (36). Transgenic CFTR knockout mice (31) were bred and genotyped in the laboratory of Dr. B. H. Koller (University of North Carolina, Chapel Hill, NC) and were kindly made available for this study.

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RESULTS

RPD was measured in ΔF508 CF mice and their wild-type littermates before and 24 and 48 h after the start of treatment with TMAO. To amplify the CFTR-dependent RPD, all measurements were done in the presence of amiloride (to block the Na⁺-dependent RPD) and with Cl⁻-free solutions. The change of perfusion solution from a Cl⁻-containing to a Cl⁻-free solution (in the presence of amiloride) did not result in significant changes in RPD in any of the treatment groups (average for all groups, −0.11 ± 0.51 mV; n = 55 mice; P = 0.84 by one-sample t-test). Figure 1A shows representative recordings of the responses to forskolin, and Fig. 1B shows the average responses measured in control and TMAO-treated wild-type mice. Perfusion with forskolin induced a hyperpolarization of RPD, which indicated activation of a Cl⁻ conductance in the rectal epithelium. In control mice, perfusion with forskolin hyperpolarized RPD by −6.4 ± 0.79 mV (n = 11). Treatment with TMAO significantly increased the responses to forskolin in normal mice to −12.6 ± 2.0 mV (n = 7) at 24 h and to −13.5 ± 2.0 mV (n = 10) at 48 h, which were not different from one another (Fig. 1B). Figure 1C and D, shows representative recordings and the average responses from ΔF508 CF mice. In untreated CF mice, RPD was not significantly affected by forskolin (−0.10 ± 0.14 mV; n = 14 mice; not different from zero by one-group t-test). Treatment with TMAO significantly increased the responses to forskolin in ΔF508 mice to −1.3 ± 0.38 mV (n = 9) at 24 h and to −1.0 ± 0.3 mV (n = 7) at 48 h, which were not different from one another (Fig. 1D). These data show that the treatment of mice with TMAO increased the forskolin-activated RPD in both wild-type and ΔF508 CF mice.

The effects of dose and length of treatment with TMAO were tested with a multiple regression analysis. In ΔF508 CF mice, the responses of RPD to forskolin correlated significantly with the administered dose (P = 0.019) but not with length of treatment (P = 0.89). Figure 2 shows the relationship between dosage and forskolin-induced RPD responses, which yielded a slope of −0.40 ± 0.10 mV·g⁻¹·kg⁻¹. To verify the CFTR dependence of the forskolin-induced responses of RPD by TMAO treatment, we
carried out experiments that probe for CFTR by using 1) CFTR knockout mice, 2) the CFTR blocker glibenclamide, and 3) the CFTR activator apigenin. Representative experiments with CFTR knockout mice are shown in Fig. 3. CFTR knockout ("+/−") mice and their heterozygous littermates were treated with 4 g/kg body wt of TMAO for 24 h. In heterozygotes (Fig. 3A) but not in CFTR("+/−") mice (Fig. 3B), forskolin hyperpolarized the RPD, indicating the lack of a Cl− conductance in TMAO-treated CFTR("+/−") mice. In CFTR("+/−") mice, forskolin caused a depolarization of 1.6 ± 1.3 mV (n = 5), which was possibly caused by the activation of K secretion in these mice, similar to that reported for the mouse colon epithelium (8).

The effect of perfusion of the rectal mucosa with 300 μM glibenclamide is shown in Fig. 4. In wild-type mice, glibenclamide partially (39 ± 16%; n = 3) inhibited the forskolin-stimulated RPD (Fig. 4A). In TMAO-treated (4 g/kg body wt, 24 h) ΔF508 CF mice, forskolin hyperpolarized RPD by −1.9 ± 0.1 mV (n = 3) in this set of experiments, of which glibenclamide inhibited 53 ± 7.8% (i.e., 1.0 ± 0.04 mV; n = 3; Fig. 4B). The partial block of CFTR by glibenclamide is comparable to its effects in other CFTR-expressing epithelia such as Calu-3 cells (29 ± 5%) in the presence of a mucosal Cl−-free solution (23). Partial block of CFTR by glibenclamide in presence of mucosal Cl−-free solution is consistent with the voltage-dependent blocker affinity of glibenclamide, which has been shown to be reduced when the membrane potential was depolarized (30).

We also tested the CFTR activator apigenin (21) in TMAO-treated ΔF508 CF mice (4 g/kg body wt, 24 h). The effect of acute perfusion of apigenin is shown in Fig. 5. In this set of experiments, perfusion with forskolin hyperpolarized RPD to, on average, −1.4 ± 0.3 mV (n = 6 mice). The addition of apigenin caused a further hyperpolarization and a total response of −2.5 ± 0.5 mV (P = 0.036 by paired t-test; Fig. 5B). For comparison, in untreated CF mice, rectal perfusion with apigenin did not significantly affect RPD (change in RPD = 0.12 ± 0.19 mV; n = 5 mice; not different from 0, P = 0.56; Fig. 5B).

DISCUSSION

Using ΔF508 CFTR heterologously expressed in cell cultures, Brown et al. (3) reported that the chemical chaperones glycerol (1.2 M) and TMAO (100 mM) corrected the maturation defect of ΔF508 CFTR. These investigators measured the TMAO effect by the appearance of the mature, fully glycosylated band C in Western blots of CFTR and by Cl− efflux in cellular studies. When we initially tested these chemical chaperones in an in vivo mouse model, our goal was to achieve concentrations that were similar to the effective concentrations in the cell culture model used by Brown et al. We found that it was not possible to achieve adequate serum levels using glycerol without severe toxicity. However, with TMAO given in subcutaneous injections every 8 h over 48 h, it was possible to achieve similar serum concentrations of ~50–100 mM with only 50% lethality (1). These high concentrations were necessary to achieve measurable effects in this in vivo study and are similar to the effective TMAO concentration in the cell culture studies (3). Thus the experiments for this study were designed to test the efficacy of this chaperone in correcting the Cl− defect in CF mice.

The data show that TMAO treatment partially corrected the forskolin-activated Cl− conductance in CF mouse rectum. In single-channel recordings, the ΔF508 CFTR mutant expresses a reduced open probability (25–30% of normal value; 9, 11, 19) and the lifetime in
the cell membrane is reduced by ~50% (28). Thus the restoration of normal trafficking of ΔF508 CFTR can be predicted to yield only a partial recovery (~15%) of the Cl− conductance found in wild-type cells. We found that treatment with TMAO recovered a response to forskolin of −1.1 mV in ΔF508 CF mice, which is ~17% of the response in wild-type mice. Although variations in a transepithelial potential difference do not have a direct linear relationship with changes in conductance, the data suggest a significant correction of the trafficking defect of ΔF508 CFTR. Because some studies support the hypothesis that only a small fraction (5–10%) of normal CFTR-mediated Cl− conductance is required to ameliorate clinical CF symptoms (10, 20, 27), it is conceivable that a restoration of ΔF508 trafficking in humans, similar to the effect observed here in ΔF508 mice, could be associated with positive clinical effects in patients. However, the main value of these studies is that they constitute a proof of principle that a chemical chaperone can be effective in vivo. Because TMAO itself was associated with severe toxicity (i.e., the compound was used near its half-maximal lethal dose), it cannot be seriously considered for clinical use. Thus the present study demonstrates the feasibility of a pharmacological approach with a chemical chaperone. It also emphasizes the need to develop better compounds, characterized by higher efficiency and lower toxicity.

A second aspect of our work is that treatment with a chemical chaperone combined with a CFTR activator might be beneficial. Several CFTR activators have been previously described (2, 15, 21, 22). We selected apigenin for this study because of its high affinity and low toxicity. We found that in TMAO-treated ΔF508 CF mice, the effects on Cl−-selective potentials were almost doubled after the addition of apigenin (Fig. 5). Previously, Illek and Fischer (21) reported that apigenin is a potent CFTR activator by increasing the open probability of wild-type CFTR, and apigenin can potentiate in vitro the effect of 4-phenylbutyrate on ΔF508 CFTR (24). The present study shows that the combination of a chemical chaperone with a channel activator also exhibits a synergistic effect in vivo.

Clearly, the extraordinarily high concentrations that we employed in this study are likely to have caused various other physiological and cellular effects, such as upregulation of hormones responsible for osmotic homeostasis and induction of cellular stress proteins, possibly including the heat shock proteins (HSPs). Therefore, it may be possible that the effect of TMAO treatment may be indirectly mediated by a response to the osmotic stress. Using the chemical chaperone glycerol (1 M), Brown et al. (3) have shown that treatment of NIH/3T3 mouse fibroblasts with glycerol did not induce expression of the heat-inducible HSP72; however, glycerol treatment supported maturation of recombinant ΔF508 CFTR. When these cells were further heat stressed, expression of HSP72 was induced in control cells but not in glycerol-treated cells (3). Similarly, in HeLa cells, heat shock-induced expression of HSP73 was blocked by glycerol (4), and no effects of glycerol on HSP70 mRNA expression were detected in Madin-Darby canine kidney cells (6). These data show that treatment with the chemical chaperone glycerol did not affect the expression of several HSPs, and the heat-induced expression of HSP did not support ΔF508 maturation. Thus it appears that the effect of chemical
chaperones on ΔF508 CFTR maturation is not related to the expression of HSPs. Although this has not been tested for TMAO, the proposed common mechanism of action (see below) of both TMAO and glycerol supports the notion that HSPs are not involved in the treatment-induced recovery of ΔF508 CFTR. To test for side effects of TMAO in vivo in mice, a panel of serum parameters, including electrolytes and liver enzymes, was measured (1). Only minor, insignificant effects on the concentrations of glucose, NaCl, bicarbonate, phosphate, and albumin were detected, and it was concluded that treatment with TMAO did not impair renal, liver, pancreas, or muscle function (1). Thus these previous measurements did not show significant changes in blood chemistry and on cellular stress protein expression. Although an effect of TMAO on other factors (which may mediate ΔF508 CFTR expression) cannot be ruled out from our study, the simplest explanation of our data is a direct recovery of ΔF508 trafficking by TMAO.

The in vivo measurement of RPD is determined by the ion selectivity of both the transcellular and paracellular pathways, both of which may have been affected by TMAO treatment, and a contribution of the paracellular pathway cannot be excluded. However, the following observations suggest that the TMAO-recovered RPD is mediated in large part by CFTR activity: 1) stimulation by forskolin in ΔF508 but not in knockout mice, 2) sensitivity to glibenclamide, and 3) sensitivity to apigenin.

The mechanism of action of chemical chaperones is not totally understood. These molecules may support the formation of a stable conformation of CFTR in the endoplasmic reticulum. They are thought to act through a mechanism of stabilizing protein structures by affecting the degree of hydration and stabilize intramolecular interactions (7, 12, 13). Interestingly, in our study, TMAO increased the responses to forskolin in both wild-type and ΔF508 CF mice. This observation is consistent with the hypothesis that only a fraction (25%) of wild-type CFTR trafficks correctly to the cell membrane, whereas the majority of wild-type CFTR and probably all of ΔF508 CFTR are targeted for degradation by intracellular proteasomes (26, 33, 37). Thus the stabilization of the CFTR protein in the endoplasmic reticulum by TMAO may increase forskolin-activated RPDs in both normal and CF mice.

In summary, these studies provide evidence that in vivo treatment with TMAO can partially correct the Cl− conductance defect in ΔF508 CF mice. Although TMAO is unlikely to be suitable for clinical use, these studies in CF mice provide support for the potential efficacy of a less toxic chemical chaperone for treatment of human CF.

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