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The effect of NO-donors on chloride efflux, intracellular Ca^{2+} concentration and mRNA expression of CFTR and ENaC in cystic fibrosis airway epithelial cells

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ABSTRACT

Since previous studies showed that the endogenous bronchodilator, S-nitrosglutathione (GSNO), caused a marked increase in CFTR-mediated chloride (Cl^-) efflux and improved the trafficking of CFTR to the plasma membrane, and that also the nitric oxide (NO)-donor GEA3162 had a similar, but smaller, effect on Cl^- efflux, it was investigated whether the NO-donor properties of GSNO were relevant for its effect on Cl^- efflux from airway epithelial cells. Hence, the effect of a number of other NO-donors, sodium nitroprusside (SNP), S-nitroso-N-acetyl-DL-penicillamine (SNAP), diethylenetriamine/nitric oxide adduct (DETA-NO), and diethylenetriamine/nitric oxide adduct (DEA-NONOate) on Cl^- efflux from CFBE (ΔF508/ΔF508-CFTR) airway epithelial cells was tested. Cl^- efflux was determined using the fluorescent N-(ethoxycarbonylmethyl)-6-methoxyquinoliniu bromide (MQAE)-technique. Possible changes in the intracellular Ca^{2+} concentration were tested by the fluorescent fluo-4 method in a confocal microscope system. Like previously with GSNO, after 4 h incubation with the NO-donor, an increased Cl^- efflux was found (in the order SNAP > DETA-NO > SNP). The effect of DEA-NONOate on Cl^- efflux was not significant, and the compound may have (unspecific) deleterious effects on the cells. Again, as with GSNO, after a short (5 min) incubation, SNP had no significant effect on Cl^- efflux. None of the NO-donors that had a significant effect on Cl^- efflux caused significant changes in the intracellular Ca^{2+} concentration. After 4 h preincubation, SNP caused a significant increase in the mRNA expression of CFTR. SNAP and DEA-NONOate decreased the mRNA expression of all ENaC subunits significantly. DETA-NO caused a significant decrease only in α-ENaC expression. After a short preincubation, none of the NO-donors had a significant effect, neither on the expression of CFTR, nor on that of the ENaC subunits in the presence and absence of L-cysteine. It can be concluded that the effect of GSNO on Cl^- efflux is, at least in part, due to its properties as an NO-donor, and the effect is likely to be mediated by CFTR, not by Ca^{2+}-activated Cl^- channels.

Introduction

Clinical studies suggest a strong relationship between the reduced lung function and low levels of exhaled nitric oxide (NO) in cystic fibrosis (CF) patients (Balfour-Lynn et al., 1996; Elphick et al., 2001; Grasemann et al., 1997; Keen et al., 2010). It has also been shown that CF patients have low levels of S-nitrosothiols (SNO) (naturally occurring bronchodilators) in their airways (Grasemann, 1999). S-nitrosoglutathione (GSNO) is a low molecular weight S-nitrosothiol produced at some point during oxidation of NO (Zeitlin, 2006). The GSNO level is reduced in airways affected by CF, and GSNO turnover is more rapid in asthmatic airways (Gaston et al., 2006). The folding efficiency of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein is greatly affected by the most common mutation in the CF population, ΔF508 (Riordan et al., 1989). The trafficking of ΔF508-CFTR from the endoplasmic reticulum to the plasma membrane is affected by improper folding due to deletion of F508 (Ward et al., 1995). Although ΔF508-CFTR exhibits altered transport kinetics compared to the normal CFTR, it can mediate partial chloride (Cl^-) conductance after reaching the cell membrane (Haws et al., 1996). A possible therapeutic strategy to treat CF can be based on preventing the degradation of ΔF508-CFTR by increasing the efficiency of intracellular processing and folding (Dalemans et al., 1991; Kopito, 1999). GSNO caused a 4-fold increase in CFTR-mediated Cl^- efflux, improved the trafficking of CFTR to the plasma membrane (Andersson et al., 2002) and increased the maturation and expression of ΔF508-CFTR (Zaman et al., 2001). GSNO is an NO-donor, and this may be relevant for its effect on Cl^- efflux, since another NO-donor, GEA3162, had a similar but smaller effect (Servetnyk et al., 2006). The effect of NO on Cl^- efflux from airway epithelial cells has, however, been controversial. Kamosinska et al. (1997) found that GSNO increased whole-cell Cl^- currents in A549 cells (lung epithelial) but stated that this effect was not mediated by CFTR. Duszyk (2001) found that NO promoted Cl^- efflux via a cGMP-dependent pathway. However, others found that NO had no beneficial effects on the Cl^- efflux defect in CF (Galietta et al., 2000; Ruckes-Nilges et al.,

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2000). The stimulatory effect of GSNO on Cl^- efflux has a lag time of several hours (Andersson et al., 2002); this lag time can be avoided if GSNO is added in the presence of L-cysteine (Servetnyk et al., 2011).

The purpose of the present study was to investigate the effect of other NO-donors on Cl^- efflux from CF airway epithelial (CFBE) cells in order to answer the question to what extent the effect of GSNO on Cl^- efflux from CF airway epithelial cells is dependent on NO. In addition, the effect of NO-donors on the expression of mRNA for CFTR and ENaC subunits was investigated.

Methods

Chemicals

The NO-donors, DETA-NO (diethylamine NONOate diethylammonium salt), SNP (sodium nitroprusside dihydrate), SNAP (S-Nitroso-N-acetyl-DL-penicillamine) and DEA-NONOate (diethylenetriamine/nitric oxide adduct) were from Sigma-Aldrich (St. Louis, MO, U.S.A). Ringer's Standard (SR) solution consisted of: 140 mM NaCl, 5 mM KCl, 5 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), 1 mM MgCl2, and 5 mM glucose, pH 7.4. For Ringer's Standard solution without Cl^- (RS0), NO_3^- was used as a substituting anion. The fluorescent probe *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE), nigericin, Medium 199, fetal bovine serum (FBS), penicillin and streptomycin (PEST) were obtained from Invitrogen (Paisley, U.K.), while tributyltin acetate was from Sigma-Aldrich.

Cell culture

The bronchial epithelial CFBE cell line, homozygous for the ΔF508-CFTR mutation (Cozens et al., 1994), a kind gift from Dr. D. Gruenert, San Francisco (CA, U.S.A.), was cultured in Medium 199 with Glutamax supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate at 37 °C in 5% CO_2 in air (Servetnyk et al., 2011). The medium was changed two times weekly. In one experiment, the bronchial epithelial 16HBE cell line (wild-type CFTR) was used as a positive control.

Cl^- efflux measurements

CFBE cells were cultured to confluence in a tissue culture dish with cover glass bottom (23 mm in diameter) (Fluorodish™, World Precision Instruments, Sarasota, FL, U.S.A.) for 3–4 days. After incubation with 100 μM of each NO-donor for 4 h the cells were loaded with 10 mM MQAE for 2 h and rinsed in SR solution. (In some experiments the 4 h preincubation period was omitted). The tissue culture dish was fixed in a perfusion chamber on the stage of an inverted microscope (Nikon, Diaphot, Tokyo, Japan). The temperature was kept at 37 °C by heating the chamber holder and the objective separately. A 75 W xenon lamp and a monochromator, part of a Quanticell 2000 image-processing system (VisiTech International, Sunderland, U.K.), provided excitation light at 353 nm (12 nm bandwidth). The emission was measured at 460 (15 nm half-bandwidth filter) using a CCD camera. Cells were bathed in RS. Images were captured for 16 ms every 3 s (Mendes et al., 2003; Munkonge et al., 2004).

The relationship between the MQAE fluorescence and the cytoplasmic concentration of chloride ($[Cl^-]_i$) can be expressed by the linear function:

$$F_0/F_{Cl} = 1 + [Cl^-]_i^* K_{SV} \quad (1)$$

where F_0 and F_{Cl} are the fluorescences in the absence and presence, respectively, of intracellular Cl^- after subtraction of background fluorescence and K_{SV} is the Stern–Volmer constant for collisional quenching (Verkman, 1990). For determination of K_{SV} the MQAE signal was calibrated against $[Cl^-_i]$ by exposing the cells to a K^+-rich (120 mM) HEPES buffer (pH 7.0), containing various Cl^- concentrations with NO_3^- as the substituting anion (the sum of Cl^- and NO_3^- was always 120 mM). Two ionophores were used to equilibrate the intracellular and extracellular Cl^- concentrations; the Cl^-/OH^- anti-porter tributyltin (10 μM) and the K^+/H^+ anti-porter nigericin (10 μM). Background fluorescence was obtained by quenching the MQAE signal with 150 mM KSCN. The Stern–Volmer constant for the CFBE cells was calculated to 22.6 M^{-1}. The rate of efflux, dCl/dt, was calculated from (Chao et al., 1990):

$$(dCl/dt) = F_0/\left[K_{SV} * (F_{Cl})^2\right] * dF_{Cl}/dt. \quad (2)$$

Since attempts to acquire F_0, the fluorescence in the absence of Cl^-, can meet with difficulties (Eberhardson et al., 2000), F_0 was calculated from F_{20} (the fluorescence at 20 mM Cl^-), the intracellular Cl^--concentration and Eq. (1). F_{Cl} is the fluorescence measured at the time when the Cl^--free buffer was applied. The fluorescence intensity decreased in a linear fashion over time due to dye leakage and photobleaching of less than 10% during an experiment. All values were corrected for background fluorescence and loss of MQAE fluorescence. The efflux rate, dF_{Cl}/dt, was determined from the initial changes in F_{Cl} after changing to Cl^--free buffer (Duszyk, 2001).

Experimental design

After incubation with 100 μM of each NO-donor for 4 h, the Cl^- efflux was induced by changing from a 150 mM Cl^- buffer to a Cl^--free buffer with NO_3^- as the substituting anion with or without cAMP elevating agents. At the end of the MQAE experiments F_{20} and autofluorescence were determined in the presence of ionophores with the help of a quenching solution containing 150 mM KSCN. Each experiment refers to the average of 10–20 cells. GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, U.S.A) was used to determine the value of the Cl^- efflux rate. Results are expressed as means ± standard error of the mean (SEM).

Intracellular Ca^{2+} measurements

Intracellular Ca^{2+} measurement was performed using the Fluo-4 AM probe (Molecular Probes, Eugene, OR, U.S.A.) as described previously (Gee et al., 2000; Ito et al., 2004). The cells were cultured to confluence on tissue culture dishes with a glass bottom and loaded with 2.5 μM Fluo-4 AM in Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco, Invitrogen) for 20 min. The dishes were placed in a perfusion chamber on the stage of a Leica Confocal Systems microscope (Leica Microsystems, Heidelberg, Germany). Recording of the Fluo-4 signal was done using an excitation wavelength of 494 nm and emission was measured at 516 nm. Calibration of the Fluo-4 signal was performed by exposing cells to DPBS containing 1 mM Ca^{2+} in the presence of 10 μM ionophore 4-bromo A-23187 and to DPBS containing no Ca^{2+} + 5 mM EDTA. Images were captured every 5 s and data expressed as arbitrary fluorescence units were analyzed. The average response from all cells in the optic field (15–30 cells) was counted as one experimental data point and raw fluorescence data were converted into Ca^{2+} concentrations.

$$\left[Ca^{2+}\right]_i = K_d \cdot (F - F_{min})/(F_{max} - F) \quad (3)$$

where F_{min} is the fluorescence intensity in the absence of calcium (Ca^{2+}), F_{max} is the fluorescence under Ca^{2+}-saturated conditions, and K_d is the dissociation constant (347 nM).

The $[Ca^{2+}]_i$ was determined separately for untreated cells and for cells exposed to 100 μM of SNAP, DETA-NO and SNP for 4 h. For each NO-donor 6–8 experiments were performed and the mean value determined as one data point.