GSH monoethyl ester rescues mitochondrial defects in cystic fibrosis models

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Cystic fibrosis (CF), a multisystem disease caused by CFTR (cystic fibrosis transmembrane conductance regulator) gene mutations, is associated with an abnormal inflammatory response and compromised redox homeostasis in the airways. Recent evidence suggests that dysfunctional CFTR leads to redox imbalance and to mitochondrial reduced glutathione (mtGSH) depletion in CF models. This study was designed to investigate the consequences of mtGSH depletion on mitochondrial function and inflammatory response. mtGSH depletion was confirmed in colonic epithelium of CFTR-null mice and in CFTR-mutated human epithelial cells. GSH uptake experiments performed on isolated mitochondria suggest that mtGSH depletion is not due to a defective GSH transport capacity by CF mitochondria, despite the decreased expression of two mtGSH carriers, oxoglutarate carrier and dicarboxylate carrier. CM-H2DCFDA [5 (and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester] fluorescence and aconitase activity showed an increase in reactive oxygen species levels in CFTR-defective cells and a pro-oxidative environment within CF mitochondria. The activities of respiratory chain complexes were further examined. Results showed a selective loss of Complex I (CI) function in CF models associated with an altered mitochondrial membrane potential (∆ψm). CI analysis showed normal expression but an overoxidation of its NADH-ubiquinone oxidoreductase Fe-S protein 1 subunit. GSH monoethyl ester (GSH-EE) significantly enhanced mtGSH levels in the IB3-1/C38 model and reversed CI inhibition, suggesting that mtGSH depletion is responsible for the loss of CI activity. Furthermore, GSH-EE attenuated ∆ψm depolarization and restored normal IL-8 secretion by CFTR-defective cells. These studies provide evidence for a critical role of a mtGSH defect in mitochondrial dysfunction and abnormal IL-8 secretion in CF cells and reveal the therapeutic potential of mitochondria-targeted antioxidants in CF.

INTRODUCTION

Cystic fibrosis (CF), one of the most common life-threatening autosomal recessive disorders in the Caucasian population (1,2), is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene (3,4). The latter encodes an integral membrane glycoprotein expressed at the apical membrane of exocrine epithelial cells. In the plasma membrane, CFTR functions as a cyclic AMP-activated anion channel and a channel regulator, playing a key role in hydroelectrolytic epithelial transport. CF affects epithelia of the respiratory tract, exocrine pancreas, intestine, male genital tract, hepatobiliary system and exocrine sweat glands, resulting in a complex multisystem disease typically dominated by pancreatic insufficiency and severe lung pathology.

CF lung is characterized by abnormal epithelial lining fluid (ELF) composition and compromised pathogen clearance, generally leading to repetitive infections and inflammation.
that ultimately result in fibrosis and deterioration of pulmonary function. Consequently, respiratory failure is the primary cause of mortality in CF. The exaggerated and prolonged inflammation in regard to bacterial load is characterized by high cytokine levels, such as IL-8, IL-6 and tumour necrosis factor alpha (TNFα) (5,6). Anti-inflammatory therapy is effective in limiting lung deterioration (7), but adverse effects have limited the use of steroidal and non-steroidal anti-inflammatory drugs. Therefore, alternative therapies are expected to reduce the inflammatory response and slow the progressive pulmonary deterioration in CF.

The oxidant/antioxidant imbalance is a key parameter in CF lung pathology. Elevated levels of lipid and protein oxidation products found in bronchoalveolar lavage fluid, exhaled breath condensate and sputum of CF patients (8–11) provide evidence of pro-oxidative imbalance in CF airways. Oxidative stress in CF is assigned to an increased oxidant burden resulting from the release of oxidants by neutrophils and Pseudomonas aeruginosa, a pathogen that chronically infects CF airways (12). In addition, the ELF levels of reduced glutathione (GSH), one of the most important antioxidants in this compartment, are markedly reduced in CF patients and CFTR KO mice (13,14), which may also contribute to the oxidant/antioxidant imbalance in CF lung. Since CFTR participates in the apical export of GSH (15–17), GSH depletion in ELF of CF airways and the consecutive oxidative stress could be seen as direct consequences of the CFTR defect.

A growing body of evidence now suggests that an intracellular imbalance between oxidant production and antioxidant defences in CF epithelial cells is a key component of compromised redox homeostasis in CF. Cultured CF cell lines and, more strikingly, freshly isolated epithelial cells from CFTR KO mice present high levels of reactive oxygen species (ROS) in basal conditions (18). As recently proposed (19), the elevated intracellular hydrogen peroxide (H₂O₂) levels could mediate, at least in part, the exaggerated inflammatory cytokine production in CF epithelia, which implies a potential interest of using antioxidants for CF therapy. Of note, mechanisms sparking off the elevated cellular ROS levels in CF cells are still poorly investigated and it remains unclear whether CFTR loss directly affects the intracellular redox state in CF lung (20).

A compelling body of evidence indicates that mitochondria are an important source of ROS in normal non-phagocytic cells and under a variety of pathological conditions (21). Superoxide anions (O₂⁻) generated along the mitochondrial respiratory chain are converted to H₂O₂ by manganese superoxide dismutase (MnSOD). Subsequently, mitochondrial H₂O₂ is either eliminated by mitochondrial peroxidases or diffuses into the cytoplasm. GSH, the most abundant non-protein thiol and the major thiol-disulphide redox buffer in mammalian cells (22), plays an essential role in maintaining the intracellular and mitochondrial redox environment. Because the main antioxidant mechanisms that dispose peroxides, i.e. peroxiredoxin 6 and glutathione peroxidases, depend on GSH for their reducing equivalent, GSH is a key element in the antioxidant defence (23).

GSH exists as separate pools in cell compartments including cytosol, nuclei and mitochondria. Since GSH synthesis does not occur in mitochondria (24), transport from the cytosol into the mitochondrial matrix is probably the major mechanism that sustains mitochondrial GSH (mtGSH) levels. Membrane-permeant GSH derivatives, such as GSH esters, have been found to provide effective means of increasing cellular GSH in various tissues (25,26). In particular, GSH monooethyl ester (GSH-E) has been proved to increase more specifically the mitochondrial pool of GSH in several models (27–29).

Interestingly, previous data from Velsor et al. (30) suggested that CF epithelial cells may present a decrease in mtGSH levels associated with elevated mitochondrial ROS. However, to the best of our knowledge, the mechanisms underlying mtGSH depletion in CF models, the consequences of mtGSH depletion on mitochondrial and cellular functions and the potential interest of strategies aimed to boost mtGSH levels in CF models have never been investigated. The present study addresses these issues and provides data indicating that decreased mtGSH compromises the redox environment and electron transport chain (ETC) Complex I (CI) function in CF mitochondria. By showing that mtGSH replenishment by GSH-E reduces CI inhibition and restores normal IL-8 secretion in CFTR defective cells, this study suggests a potential interest of mitochondria-targeted antioxidants for CF therapy.

RESULTS

Abnormal mtGSH levels in CFTR KO colonic epithelia

We first analysed mtGSH levels in the colonic epithelium of CFTR KO mice. The results presented in Figure 1A show that GSH concentrations in colonic mitochondria from the CFTR KO mice were 5-fold lower than those in CFTR WT mice, whereas no significant difference was observed in the concentrations of oxidized glutathione (GSGG). The GSH-to-GSSG ratio was therefore decreased, implying a more oxidized state in this compartment. The ambient potential evaluated using the GSH/GSSG ratio and calculated by the Nernst equation, decreased from −228 mV in CFTR WT mice to −192 mV in CFTR KO mice.

Mitochondria do not synthesize GSH de novo, and the mitochondrial pool of GSH is derived from the cytosolic pool by the activity of mitochondrial transporters (24,31). Previous studies have shown that the dicarboxylate carrier (slc25a10, DIC) and the oxoglutarate carrier (slc25a11, OGC) act as the main mtGSH transporters in hepatic, brain and renal mitochondria accounting together for 50–80% of mtGSH uptake (32–34). We examined whether the decrease in mtGSH levels in CFTR KO colonic epithelia could be the consequence of an altered expression of these two transporters.

As shown in Figure 1B, quantitative real-time PCR analysis and quantification of relative mRNA expression revealed a slight but not significant decrease in DIC and OGC mRNA expression in KO compared with WT mice. DIC/hypoxanthine phosphoribosyltransferase (HPRT) relative mRNA expression was 1 versus 0.85 ± 0.19 (P = 0.18) for WT and KO mice, respectively. OGC/HPRT relative mRNA expression was 1 versus 0.83 ± 0.19 (P = 0.15) for WT and KO mice, respectively. However, protein levels of DIC and OGC in
mitochondrial fractions (Fig. 1C) were significantly lower in CFTR KO \((P < 0.05)\).

To investigate whether the altered levels of these two transporters could account for a decrease in mtGSH transport, we analysed the initial rates of mtGSH uptake in freshly isolated mitochondria from colonic epithelium of CFTR WT and KO mice and also from CFTR-deficient IB3-1 and CFTR-sufficient C38 cells, in the presence of increasing concentrations of external GSH. As shown in Figure 1D, GSH uptake by mitochondria increased along with external GSH and presented Michaelis–Menten kinetics between 1 and 5 mM GSH. When external GSH was increased above 7 mM, GSH uptake values were variable and seemed to occur by diffusion as previously reported by Martensson et al. (31).

Maximum velocity \((V_{\text{max}})\) for CFTR KO and WT mice did not differ significantly \((6.22 \pm 2.60\) and \(8.40 \pm 0.91\) nmol GSH/mg protein/15 s, respectively, \(P = 0.07\)). The uptake kinetics displayed a single Michaelis constant \((K_m)\) of \(4.9 \pm 0.7\) and \(2.7 \pm 0.6\) mM for CFTR WT and KO mice, respectively, \(P = 0.037\). Conversely, mtGSH uptake by mitochondria from CFTR WT mice at 5 mM of external GSH was unaffected by 10 \(\mu\)M of the CFTR activator UC82-029 (35) (data not shown). At external GSH concentrations ranging from 1 to 7.5 mM, mtGSH uptake in IB3-1 versus C38 cell lines was unchanged (values for uptake at 5 mM of external GSH were \(2.33 \pm 0.93\) and \(1.95 \pm 1.74\) nmol GSH/mg protein/15 s, respectively). These results show that the in vitro capacity of mitochondria to take up GSH was not

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Figure 1. Mitochondrial glutathione levels and uptake in colonic epithelia from CFTR KO and WT mice. (A) Reduced (GSH) and oxidized (GSSG) glutathione levels in mitochondrial fractions from CFTR WT and KO mouse colonic epithelia. Measurements of GSH and GSSG levels using HPLC with electrochemical detection showed lower mtGSH concentrations in colonic epithelia from CFTR KO compared with CFTR WT mice. Results are expressed as nanomole per milligram protein and are the mean values \(\pm\) SD of \(n = 4\) samples in each group. \(*P < 0.05\) versus CFTR WT group. (B) Quantitative real-time PCR analysis of DIC and OGC mRNA expression in colonic epithelia from CFTR WT and CFTR KO mice. Quantification revealed no significant difference between CFTR WT and KO mice. (C) Immunoblot analysis of DIC and OGC protein levels in mitochondrial fractions from colonic epithelia from CFTR WT and KO mice. Mitochondrial proteins (15 \(\mu\)g of mitochondrial protein per well) were run on SDS–PAGE gels, transferred onto PVDF membranes and then blotted for DIC and OGC; Complex III Core 2 subunit was used as a loading control (upper panel). Quantification of band intensities by the Infrared Imager Odyssey indicated that DIC and OGC steady-state levels, normalized to Complex III Core 2 subunit, were lower in mitochondria from CFTR KO mice. Results are the mean values \(\pm\) SD of \(n = 5\) samples in each group. \(*P < 0.05\) versus CFTR WT group. (D) Initial rates of GSH uptake in freshly isolated mitochondria from colonic epithelia from CFTR WT and KO mice. Mitochondria were incubated with increasing concentrations of external GSH and 1 \(\mu\)Ci of [35S]GSH. GSH uptake was expressed as nanomole GSH/15 s per milligram protein. Kinetic parameters were obtained by fitting the experimental data to the appropriate Michaelis–Menten equation using the non-least squares method. \(V_{\text{max}}\) and \(K_m\) values were determined according to this equation and are given in Results. Values are the mean \(\pm\) SD of \(n = 4–5\) samples in each group.
defective in CF models despite the decrease in the expression levels of GSH carriers DIC and OGC.

Abnormal mtGSH levels in CF lung cells; effect of GSH-EE and NAC

As previously reported, GSH concentrations were significantly decreased by 30% in mitochondria from IB3-1 cells compared with corrected C38 cells (Fig. 2A). No significant difference was observed for the oxidized form GSSG (0.079 ± 0.028 nmol/mg protein in C38 cells versus 0.035 ± 0.030 nmol/mg protein in IB3-1 cells). GSH concentrations were unchanged in the cytosolic compartment of IB3-1 cells versus C38 cells (Fig. 2B), whereas GSSG concentrations were slightly enhanced in the cytosol of IB3-1 cells (2.42 ± 0.28 and 1.14 ± 0.22 nmol/mg protein in IB3-1 and C38 cells, respectively, \( P = 0.011 \)). Cytosolic glutathione homeostasis is therefore altered in IB3-1 cells.

A useful approach to increase cellular GSH concentrations is to incubate cells with GSH-EE, a permeant GSH compound (25). To test the efficacy of this GSH prodrug in our model, IB3-1 and C38 cells were treated for 90 min with 10 mM GSH-EE. As shown in Figure 2A, GSH-EE treatment was able to rapidly enhance mtGSH levels in both cell types, but had little effect on cytosolic GSH (Fig. 2B), suggesting that GSH-EE would represent an effective tool to specifically elevate the mtGSH pool in this model. Comparatively, the commonly used cysteine precursor N-acetyl cysteine (NAC) was able to enhance the cytosolic levels of GSH in both cell lines (Fig. 2B) but failed (Fig. 2A) to significantly increase mtGSH concentrations in both cell types (\( P = 0.47 \) and \( P = 0.88 \) for IB3-1 and C38 cells, respectively).

Decrease of mitochondrial oxidative stress by GSH-EE in CF cells

Because GSH is an important regulator of the redox environment of mitochondria, we examined the possible effects of a decrease in mtGSH levels on CF cells redox environment. The presence of an altered redox status was first estimated by the widely used marker CM-H2DCFDA [5 (and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester], which is oxidized to the highly fluorescent DCF by either H2O2 or hydroxyl radical (•OH). As shown in Figure 3A and B, basal levels of cellular ROS were higher in CFTR-deficient IB3-1 cells compared with CFTR-sufficient C38 cells. Pre-incubation of IB3-1 cells with 10 mM GSH-EE for 90 min resulted in a decrease in fluorescence intensity, indicating that mtGSH elevation was sufficient to decrease ROS levels in these cells.

Since the results obtained by the CM-H2DCFDA probe must be taken with caution due to possible self-induced production of O2•−/H2O2 in complex systems possessing peroxidase activity (36), we performed an independent assay consisting of the determination of ROS-sensitive aconitase activity normalized to fumarase activity. Aconitase contains an iron–sulphur cluster in its catalytic site that makes it very susceptible to inactivation by ROS. Fumarase, on the other hand, lacks any distinct features making it vulnerable to ROS. Results presented in Figure 3C show that the aconitase-to-fumarase activity ratio was decreased by 25% in IB3-1 cells versus C38 cells while the steady-state level of aconitase 2 protein was similar in the two cell lines (Fig. 3D). These results are consistent with an increase in ROS levels and a pro-oxidative environment in CFTR-deficient cells. Interestingly, elevation of mtGSH by GSH-EE treatment significantly improved aconitase/fumarase activity in IB3-1 cells (Fig. 3C).

Defective CI activity in CFTR KO mice

Several studies showed that GSH depletion can exert a profound effect on mitochondrial functions, including inhibiting mitochondrial CI activity (EC 1.6.5.3. NADH:ubiquinone oxidoreductase) (34,37). To investigate whether the decreased mtGSH levels in CF models could be associated with mitochondrial dysfunction, enzymatic activities of the five oxidative phosphorylation (OXPHOS) complexes, Complexes I–V, were measured in mitochondria isolated from colonic epithelium of CFTR KO and WT mice. The measurement of the activities of OXPHOS complexes revealed a 50% decline of NADH:ubiquinone oxidoreductase activity of CI in CFTR KO colonic epithelium, compared with CFTR WT.
colonic epithelium, whereas no significant activity losses were seen in Complexes II–V (Fig. 4A). Likewise, CI activity was decreased by 25% in mitochondria isolated from CFTR KO lung tissue (74.4 ± 4.6 versus 100 ± 6.7%, \( P < 0.001 \) versus untreated C38 cells; \( P < 0.001 \) versus untreated IB3-1 cells). Aconitase activity normalized to fumarase activity in CFTR-deficient epithelial cells IB3-1 and their CFTR-sufficient counterpart C38. Cells were treated for 90 min in medium containing 10 mM glutathione monoethyl ester (GSH-EE) or medium alone (control). Aconitase and fumarase activities were determined in parallel from the same samples. Aconitase-to-fumarase activity ratio was significantly decreased in IB3-1 cells versus C38 cells and the ratio was increased in IB3-1 cells, following treatment by GSH-EE. Values are means ± SD of \( n = 3 \) samples in each group. \( P < 0.05 \) versus untreated IB3-1 cells. (D) Immunoblot analysis showing the steady-state level of aconitase 2 protein in CFTR-deficient cells IB3-1 and their CFTR-sufficient counterpart C38. Mitochondrial proteins (15 \( \mu \)g of mitochondrial protein per well) were run on SDS–PAGE gels, transferred onto PVDF membranes and then blotted for aconitase 2 (upper blots); VDAC expression (lower blots) was used as a loading control. Markers on the right indicate sizes on SDS–PAGE in kilodaltons. Quantification of band intensities by the Infrared Imager Odyssey indicated that aconitase 2 steady-state level, normalized to VDAC expression, is similar in the two cell lines. Error bars indicate SD.

Normal CI expression level but abnormal thiol oxidation status of CI subunit NDUFS1

To determine whether an abnormal expression of the multisubunit CI could explain a decrease of its NADH:ubiquinone oxidoreductase activity in CF models, CI expression was further investigated in mitochondria from the CFTR KO and WT mouse colonic epithelium by Blue Native (BN)-PAGE and in-gel CI activity, using nitrotetrazolium blue (NTB) as an electron acceptor. In the experiments presented in Figure 4B, native gels were silver-stained for global analysis of CI pattern, and CI was pinpointed by in-gel activity staining. Silver staining (Fig. 4B) showed similar CI patterns and band intensities in CFTR KO versus CFTR WT mitochondria, whereas, for the same samples, CI in-gel activity was clearly decreased in CFTR KO versus WT gel tracks (Fig. 4B). The defective NADH:NTB oxidoreductase activity of CI suggested a possible defect in its NADH dehydrogenase module, which comprises two important subunits prone to oxidative stress.

**Figure 3.** Effect of GSH-EE on CM-H₂DCFDA fluorescence and aconitase-to-fumarase activity ratio in IB3-1 and C38 cells. (A) ROS levels were estimated in living cells, using the fluorescent dye CM-H₂DCFDA. Under basal conditions, CFTR-deficient IB3-1 cells (middle panel) had a higher CM-H₂DCFDA signal than CFTR-sufficient C38 cells (upper panel). In contrast, CM-H₂DCFDA signals were lower, and differences between the cell lines were not seen when the cells were pretreated for 90 min with 10 mM GSH-EE prior to imaging (lower panel). (B) Quantification of CM-H₂DCFDA fluorescence intensity, using ImageJ software. Values are means ± SD of a minimum of 40 cells per group. **P < 0.001** versus untreated C38 cells; \( P < 0.001 \) versus untreated IB3-1 cells. (C) Aconitase activity normalized to fumarase activity in CFTR-deficient epithelial cells IB3-1 and their CFTR-sufficient counterpart C38. Cells were treated for 90 min in medium containing 10 mM glutathione monoethyl ester (GSH-EE) or medium alone (control). Aconitase and fumarase activities were determined in parallel from the same samples. Aconitase-to-fumarase activity ratio was significantly decreased in IB3-1 cells versus C38 cells and the ratio was increased in IB3-1 cells, following treatment by GSH-EE. Values are means ± SD of \( n = 3 \) samples in each group. *P < 0.05 versus C38 group; §P < 0.05 versus untreated IB3-1 cells. (D) Aconitase expression normalized to fumarase activity in CFTR-deficient cells IB3-1 and their CFTR-sufficient counterpart C38. Mitochondrial proteins (15 \( \mu \)g of mitochondrial protein per well) were run on SDS–PAGE gels, transferred onto PVDF membranes and then blotted for aconitase 2 (upper blots); VDAC expression (lower blots) was used as a loading control. Markers on the right indicate sizes on SDS–PAGE in kilodaltons. Quantification of band intensities by the Infrared Imager Odyssey indicated that aconitase 2 steady-state level, normalized to VDAC expression, is similar in the two cell lines. Error bars indicate SD.
damage, namely NDUFS1 (NADH-ubiquinone oxidoreductase Fe-S protein 1) and NDUFV1 (NADH-ubiquinone oxidoreductase flavoprotein 1). To investigate whether a misassembly of these subunits into the NADH dehydrogenase module could account for the loss of CI activity, equal amounts of mitochondrial membrane proteins from CFTR KO and WT colons were separated by BN-PAGE and subjected to immunoblot analysis, using antibodies against NDUFS1 and NDUFV1. Similar levels of both subunits in native CI from CFTR KO versus WT mice were found (Fig. 4C), indicating a normal assembly in CI from CFTR KO colon. Oxidative modification of one of CI’s constitutive subunits, i.e. NDUFS1, was shown elsewhere as being responsible for a loss of CI enzymatic activity in response to changes in glutathione redox state via S-glutathionylation (38,39). To determine whether CI inhibition in CF models could be due to oxidative modifications, NDUFS1 thiol redox status was analysed by a redox western blotting method based on AMS
(4'-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid) derivatization. Briefly, alkylation of reduced sulphydryl groups with AMS increases the molecular weight of reduced forms of proteins that can therefore be resolved from the oxidized ones by classical non-reducing SDS–PAGE. Figure 4D shows a representative NDUFS1 redox western blot obtained after AMS derivatization of mitochondrial proteins from CFTR KO and WT colonic epithelia. In CFTR WT and KO mice, NDFSU1 was predominantly in the reduced state. However, there was a significant difference in oxidation levels of NDUFS1 between CFTR KO and WT mice as indicated by oxidized forms of NDUFS1 being 2-fold more abundant in CFTR KO mitochondria (Fig. 4D). These results showing abnormal levels of oxidized NDUFS1 in CFTR KO mitochondria suggested that the loss of CI activity in CF models could be due, at least in part, to an abnormal redox state. This hypothesis implies that CI inhibition in CF cells could be suppressed by modulating the mitochondrial redox environment.

**Restoration of CI activity and mitochondrial membrane potential by GSH-EE in CFTR-deficient cells**

Similar to what was observed in the mouse model, human IB3-1 cells showed a 50% decrease in CI activity compared with C38 cells (Fig. 5A). Defective CI activity was confirmed in a second in vitro model, the CFTR-deficient dCFBE41o-cell line compared with its WT-corrected counterpart corrCFBE41o- (53.5 ± 18.1 versus 100.0 ± 12.2%, respectively, \( P < 0.0001 \)). Figure 5B shows that the mitochondrial membrane was less polarized in IB3-1 cells compared with C38 cells as previously described (40).

As GSH-EE represented an effective tool to increase the mtGSH pool in IB-1 cells (Fig. 2A) and restore a normal redox environment as evaluated by CM-H2DCFDA fluorescence (Fig. 3A and B) and by aconitase-to-fumarase ratio (Fig. 3C), we next investigated whether GSH-EE treatment was able to restore CI activity in the in vitro CF model. As shown in Figure 5A, GSH-EE treatment counteracted the loss of CI activity in IB3-1 cells. Moreover, GSH-EE treatment significantly attenuated the mitochondrial membrane depolarization in IB3-1 cells (Fig. 5B). The reversibility of CI inhibition by GSH-EE supplementation strongly supported our hypothesis that redox-dependent mechanisms were involved in the decrease of CI activity in CF cells.

**Decrease of pro-inflammatory IL-8 cytokine secretion level by GSH-EE in IB-3 cells**

A growing body of evidence suggests that high intracellular ROS levels could be involved in the exaggerated inflammatory cytokine production by CF cells. Given our results suggesting that GSH-EE limits ROS production in CF cells, we therefore tested the effect of GSH-EE on IL-8 production, a major pro-inflammatory cytokine, in IB3-1 and C38 cells. As previously reported (41), IB3-1 cells produce higher levels of IL-8 than C38 cells at the baseline (Fig. 6). This increased production was emphasized upon TNFα stimulation. Pre-treatment of cells with GSH-EE was able to reduce basal and TNFα-induced IL-8 production in CF cells (Fig. 6).

**DISCUSSION**

**GSH depletion in CF mitochondria**

The data presented here confirm and extend a previous report by Velsor et al. (30) which provided evidence for decreased mtGSH levels in the lungs of CFTR KO mice and in CFTR-deficient human lung epithelial cells. In the current paper, we sought to investigate mtGSH levels in the colonic tissue from the CFTR KO mouse since colon displays histological and functional abnormalities in this model. Moreover,
this tissue allows easy recovery of the epithelial cells layer, avoiding extensive contamination of the samples by cell types that do not naturally express CFTR. This may partly explain our observation of a greater mtGSH decrease in CF versus WT colon than that reported in CF versus WT lung (30) (80% in the colon versus 43% in the lung). In the present study, mtGSH concentration was found 10-fold lower in the normal mouse colonic epithelium compared with the lung (30), but within the range of values reported by others in human colonic epithelial cells (42). There are several possible and non-exclusive causes for mtGSH loss, including a decrease in GSH uptake by mitochondria or an increase in the formation of mixed disulphides in oxidative conditions. Considering GSH uptake, mitochondria are devoid of GSH synthesis enzymes and rely on GSH import from the cytosol (24). In renal, brain and hepatic mitochondria (33,34,43), this process is essentially mediated by organic anion transporters located in the inner mitochondrial membrane, namely DIC and OGC. In the course of this study, mtGSH uptake kinetics were investigated in vitro to test the hypothesis of decreased uptake in CFTR-deficient cells. The results presented in Figure 1D show that the kinetics of mtGSH uptake in colonic cells display a single Michaelis–Menten component. Our results, in which experiments were performed at physiological external GSH concentrations, are consistent with those reported by Zhong et al. (44) in rat liver mitochondria in similar conditions.

Notably, kinetic parameters of mtGSH uptake did not support a defective GSH uptake in CF mitochondria (unchanged maximum velocity and smaller $K_m$ value in the CFTR KO mouse colon) in our in vitro conditions despite a decrease in the expression levels of the two GSH transporters DIC and OGC. This apparent contradiction may be explained by a compensatory mechanism involving an alternative, still unknown, GSH carrier. Using a similar experimental approach to measure mtGSH transport in hepatocytes from alcohol-fed rats, it has been shown that defective mtGSH uptake could be the consequence of an altered lipid composition of the mitochondrial inner membrane leading to membrane fluidity loss (29,45). Our results suggest that the latter is not involved in the mtGSH decrease observed in CF models, even though de novo cholesterol synthesis and accumulation in the plasma membrane have been described in CF models (46). The unchanged transport in CFTR WT mitochondria in the presence of CFTR activator UC$_{CF}$-029 does not support the hypothesis of a CFTR channel acting by itself at the mitochondrial membrane as a GSH carrier (30).

GSH uptake is believed to be modulated by mitochondrial energetics (47). mtGSH is transported by the same carriers as intermediates from the citric acid cycle, therefore competing with them, which suggests that the nutritional status can influence mtGSH transport. Consistent with this assumption, mtGSH transport activity has been found to be modulated by the respiration state (47). Our experimental in vitro conditions do not bring into play the metabolic component of GSH uptake control. Thus, the altered GSH uptake hypothesis in CF mitochondria remains plausible in vivo, where the energetic status may differ in CF versus normal mitochondria. Of interest, a recent study by Wetmore et al. (48) demonstrated significant metabolic perturbations in primary human airway epithelial cells from CF patients, suggesting lower malate levels in CF cells.

The decrease in mtGSH was not associated with a significant increase in mtGSSG as it has also been shown in lung cells (30). Given that mitochondria are thought to be devoid of GSSG efflux systems, mtGSH decrease concomitant with no significant changes in mtGSSG content may partly result from thiol-disulphide exchange reactions between GSSG and reactive protein thiolates, leading to the formation of protein-mixed disulphides and G$^-$ release. Transient S-glutathionylation of protein thiols is thought to limit GSSG increase, participate in maintaining the GSH pool and therefore buffer the GSH/GSSG ratio in mitochondria, the reaction may involve a large amount of protein thiols (49), including those from NDUF51 CI subunit and ATP synthase (47,50).

**Consequences of GSH depletion on mitochondrial redox homeostasis**

According to the Nernst equation, the decrease in mtGSH, even with no changes in mtGSSG (or in the GSH/GSSG ratio), oxidizes the ambient redox potential by 35 mV in colonic epithelia of CFTR KO mice. However, this variation was not as large in IB3-1 cells. Consistently, a study by Schwarzer et al. (51) showed no changes in cell and mitochondrial redox potential in CF15 cells. Compared with colonic epithelia, the IB3-1 lung epithelial cell line may present a different mtGSH transport and/or metabolism, which could account for the smaller decrease in the steady-state level of mtGSH. Although ambient redox potential was only slightly changed in IB3-1 mitochondria, the decrease in mtGSH was associated with a
pro-oxidative state as shown by an increase in cellular ROS level and a decrease in redox-sensitive aconitase activity, in agreement with previous observations in IB3-1 cells and in the lung of the CFTR-KO mice (30).

CI dysfunction in CF models
Depletion in GSH and more specifically in mtGSH has been correlated with alterations of the respiratory chain in several pathologies, including liver (52) and neurodegenerative diseases, such as Parkinson’s (37,53) and Alzheimer’s disease (55). In several models, the decrease in mtGSH affects more specifically CI, which is believed to be more prone to inactivation by ROS and/or GSH/GSSG variations than other OXPHOS complexes (54). In agreement with these outcomes, we found a significant and selective loss of CI activity in both the in vitro and in vivo CF models. It has to be noted that a decrease in CI activity has been reported in fibroblasts from CF patients (55) in the 80s but it has never been investigated since the cloning of the CFTR gene. Given that the loss of CI activity in CF mitochondria was not associated with a detectable decrease in complex expression, we suspected a possible defect in the CI assembly. It has been shown that redox-dependent systems, such as MIA40 in Arabidopsis thaliana (56), are involved in the assembly of CI subunits. Our data presented in Figure 4 suggest that no major CI assembly defect occurs in CF mitochondria, even though modifications of CI composition due to the loss of low molecular weight subunits like those reported in Parkinson’s disease cortex samples (57) cannot be excluded.

Post-translational oxidative modifications are known to be involved in CI inhibition. For example, it has been shown that oxidative modifications of thiol groups such as S-glutathionylation of NDUFS1 (58) are involved in the regulation of CI activity (59). Therefore, our results showing a higher proportion of NDUFS1 in an oxidized state in CF mitochondria support the hypothesis that oxidative modifications are responsible for CI inhibition. Oxidative modifications of other CI subunits such as NDUFV1 (50), or of lipid constituents of the mitochondrial inner membrane such as cardiolipin (60), cannot be overruled.

Whether CI inhibition is the primitive defect or secondary to GSH depletion, and which mechanism comes first, is difficult to be answered, since it is generally assumed that both CI inhibition and GSH depletion result in increased ROS levels, which in turn consume GSH and alter the GSH/GSSG ratio, leading to CI inhibition by oxidative modifications. A decrease in mtGSH and CI activity is inextricably linked and will lead to a vicious cycle resulting in ROS elevation.

We propose that mtGSH depletion and CI deficiency, together with altered expression and/or activity of peroxidases, including peroxiredoxin-6 (18) and peroxiredoxin-1 (19), and increased SOD activity are the underlying mechanisms of the increase in H2O2 steady state in CF cells.

Consistent with respiratory chain inhibition, mitochondrial membrane potential was depolarized in IB3-1 cells as recently reported in the same cells (40) and in CFTR-deficient tracheal serous gland cells (61). Mitochondrial membrane depolarization probably plays a role in deregulated Ca2+ homeostasis (61–63) by limiting Ca2+ buffering (61) and may contribute to mitochondrial permeability transition pore opening (61).

Another important aspect of respiratory chain inhibition is its impact on ATP production. One could expect a 50% decrease in CI activity to be insufficient by itself to significantly decrease ATP synthesis in basal conditions. However, this basal defective capacity to synthesize ATP may be amplified by different factors such as ceramides, which have been shown to accumulate in the CF lung (64) and target the ETC (65), or by Pseudomonas aeruginosa pyocyanins, which were shown to decrease both cellular GSH and ATP levels in lung cells (12,66). Mitochondrial dysfunctions could therefore increase the vulnerability of CF cells in conditions where ATP-dependent mechanisms are strongly required to maintain cellular integrity. It is interesting to note that the enteric epithelium, under metabolic stress compromising ATP production, perceives normally innocuous bacteria as a threat, resulting in loss of barrier function and increased penetration of bacteria into the mucosa (67).

Effects of GSH-EE
Our findings show that GSH-EE raises GSH levels and is more efficient at increasing mtGSH than cytosolic GSH in IB3-1 cells, as previously reported in other cell types. In contrast, NAC, a GSH precursor, was unable to increase mtGSH as reported by Colell et al. (68) in a model of liver disease. This was explained by a deficient mtGSH uptake system that would be required for NAC to increase mtGSH. NAC failure to increase mtGSH in IB3-1 cells thus favours the hypothesis of defective mtGSH uptake in intact CF cells. Moreover, our results show that GSH-EE is able to restore the redox balance, as indicated by the decrease in CM-H2DCFDA fluorescence and the increase in aconitase activity. Consistent with restoration of CI activity, the mitochondrial membrane potential was corrected by GSH-EE, further supporting the hypothesis that GSH depletion is an essential causing factor of mitochondrial dysfunction in CF cells. Importantly, GSH-EE was also effective in reducing basal and TNFα-stimulated IL-8 secretion by CF cells. This result is in agreement with studies showing that ROS produced upon TNFα stimulation are primarily generated in the mitochondria (69).

Potential benefits of mitochondrial antioxidant therapy in CF
A number of data accumulated within the last few years place oxidant/antioxidant imbalance in CF epithelial cells as a key factor involved in the excessive production of inflammatory mediators and progressive lung failure. ROS accumulation contributes to the upregulation of inflammatory cytokines in CF cells by different pathways ranging from NF-κB activation and Nrf2 dysfunction (19) to post-translational modifications of histones in cytokine promoters (70) and SUMOylation of transglutaminase 2, which impairs autophagy (40). All these studies suggest the use of antioxidants as a complementary approach to anti-inflammatory therapies to normalize the inflammatory response and slow down the decline in lung function in CF patients.
Several clinical trials have been performed in CF adults and children, consisting of inhalation or oral administration of GSH or NAC (71). Although these treatments were shown to decrease sputum elastase activity and IL-8 levels, lung function was only slightly improved by short-term administration, suggesting that these antioxidants were not properly targeted to the right compartment. Among antioxidants, GSH-EE has been proved to efficiently restore mtGSH levels and to correct cellular damage (72), but the finding of its toxicity at high doses due to ethanol formation during GSH release implies the need for a cautious use in vivo (25). Our results highlight that more potent mitochondria-targeted antioxidants, some of which are currently in clinical trial phase in chronic inflammatory diseases (73), could be useful in CF. Following this approach, a more promising outcome than that observed in NAC and GSH trials could be expected.

MATERIALS AND METHODS

Materials

Metaphosphoric acid and Lichrosolv-grade methanol were obtained from VWR (Fontenay sous Bois, France). Glutathione monoethyl ester (GSH-EE) and TNFα were from Bachem (Weil am Rhein, Germany). [35S]GSH (840–950 Ci/mmol) and UltimaGold™ liquid scintillation fluid were purchased from Perkin Elmer (Courtaboeuf, France). AMS and TMRM (tetramethyl rhodamine methyl ester perchlorate) were purchased from Molecular Probes (Invitrogen, Cergy Pontoise, France). Unless otherwise stated, all reagents for cell culture were purchased from Gibco (Invitrogen, Cergy Pontoise, France). All other chemicals were from Sigma (Lyon, France).

Mice

Wild-type C57BL/6 (CFTR WT) and CFTR-deficient C57BL/6 cfrtm1UNC (CFTR KO) 3-week-old male mice were obtained from CDTA (Orléans, France). They were fed standard rodent chow (Teklad 2018S, Harland, France) and housed with circadian light–dark cycles. To reduce intestinal obstruction and increase survival in the CFTR KO mice, Movicol® (Norgine Pharma, Paris, France), an osmotic laxative, was added in the drinking water at a concentration of 55 g/l. All mice were allowed food and water ad libitum until the time of sacrifice by cervical dislocation. All procedures were approved by the University’s animal care and use committee.

Cell culture

The IB3-1 cell line was derived from bronchial epithelial cells from a patient with CF (F508del/W1282X) and was immortalized by viral transformation. The corrected C38 cell line was generated by stable transfection of the IB3-1 line, with a cDNA encoding a wild-type human CFTR (74). Both cell lines were purchased from ATCC (Manassas, VA, USA) and were cultured in LHC-8 medium (Eurobio, Les Ulis, France) supplemented with 5% fetal calf serum and 100 U/ml of penicillin–streptomycin. The CFBE41o- bronchial epithelial cell line was derived from a patient with CF (F508del/F508del).

Its isogenic wild-type CFTR-complemented counterpart (corrCFBE41o-) and the (F508del) control cell line (dfCFBE41o-) were generous gifts from Dr D. Gruenert (San Francisco, CA, USA). They were cultured in MEM medium supplemented with 10% fetal calf serum and 100 U/ml of penicillin–streptomycin. Hygromycin was added for complemented cells at a concentration of 4 μg/ml for corrCFBE41o- cells and 10 μg/ml for dfCFBE41o- cells. All five cell lines were grown in cell monolayers in a humidified CO2 incubator (37°C, 5% CO2) in pre-coated flasks (0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen type I and 0.01 mg/ml bovine serum albumin in LHC basal culture medium).

Mitochondria isolation

Mitochondria were isolated by differential centrifugation as previously described by Velsor et al. (30) with minor modifications. Cells were harvested with trypsin, washed in ice-cold phosphate buffer saline (PBS) and pelleted at 700g for 5 min at 4°C. The cell pellet was suspended in 750 μl of isolation buffer (210 mm mannitol, 70 mm sucrose, 5 mm Tris, 1 mm EDTA, pH 7.5) and homogenized on ice for 60 s at 1600 r.p.m. with a Potter–Elvehjem Teflon homogenizer (Fisher Scientific, Illkirch, France). The glass potter was rinsed once with 250 μl of isolation buffer, which was added to the previous fraction. The homogenate was centrifuged at 1300g for 10 min at 4°C to eliminate debris and nuclei. Mitochondria were pelleted at 17 000g for 10 min at 4°C. Centrifugation steps were performed only once to avoid possible oxidation of mitochondrial components during mitochondrial isolation. The mitochondrial pellet was frozen at −80°C until use. For mitochondria from the colonic epithelium, a similar procedure was used. Colons were rinsed in cold PBS, cut open longitudinally and placed flat on an ice-cold glass surface. Colonic epithelium was isolated by gently scraping the mucosal surface with a glass slide. Efficiency of mitochondrial preparation was estimated by immunoblotting of the exclusive mitochondrial protein, prohibitin, in the different fractions (crude extract, cytosol and mitochondria). No band was observed in the cytosolic fraction, whereas the intensity of the band was increased by 3.6-fold in the mitochondrial extract compared with the crude extract. By this procedure, mitochondrial pellets reached ~100 μg of protein/T75 flask of cells and 100–150 μg of protein for colonic epithelium preparations from one mouse. Mitochondria purity was assessed by determination of lactate dehydrogenase (LDH) activity, which is an enzyme exclusively found in the cytosol. LDH activity in the different fractions (crude extract, cytosol and mitochondria) was determined using an assay developed for the multiparameter analyser ARCHITECT cSystems™ (Abbott, Rungis, France), based on the transformation of lactate into pyruvate. Briefly, diluted samples were mixed with l-lactate and β-NAD+, and enzyme activity was monitored by the increase in the absorbance of NADH at 340 nm for 10 min. The proportion of the total LDH activity in the mitochondrial fractions did not exceed 10%.
Immunoblot analysis

Samples were diluted with Laemmli buffer and heated at 95°C for 3 min. Proteins were resolved by SDS–polyacrylamide gel electrophoresis and electrotransferred onto a PVDF membrane. After transfer, analysis was performed following the manufacturer’s recommendations for the Odyssey infrared imaging system (LI-COR Biosciences, NE, USA). Dilutions of primary antibodies were the following: rabbit polyclonal antibody to DIC (Abcam, Paris, France) was set at 1:1000; mouse monoclonal antibody to OGC (Abcam) was set at 1:1000; rabbit polyclonal antibody to aconitase 2 (Euromedex, Souffelweyersheim, France) was set at 1:500; goat polyclonal antibody to NDUFV1 (ProteinTech, Manchester, UK) was set at 1:500; and rabbit polyclonal antibody to prohibitin (Abcam) was set at 1:750. Fluorescent secondary antibodies IRDye® TM680 (for DIC, aconitase, NDUFV1 and prohibitin) or TM800 (OGC and NDUFV1) (ScienceTec, Paris, France) were used at 1:5000, and images were acquired with the Odyssey infrared imaging system. Quantification of protein bands was performed with Odyssey software. Membranes were also probed with mouse monoclonal antibody to Complex III subunit Core 2 (Mitosciences, Oregon, USA) at 1:1000 or mouse monoclonal antibody to VDAC (Abcam) at 1:1000 and IRDye® TM800 secondary antibodies (1:5000) to correct for protein loading.

Glutathione determination

Mitochondrial preparations were allowed to thaw on ice, and mitochondria were lysed by addition of 85 μl of cold water. A 10 μl fraction was kept for protein determination with a DC Protein Assay kit (Bio-Rad, Marnes la Coquette, France), and 10 μl of 40% metaphosphoric acid was added to the remaining lysate. The suspension was incubated on ice for 15 min, with brief vortexing every 5 min. Precipitated proteins were eliminated by a 5 min centrifugation at 15 000 g at 4°C. The supernatant was filtered and transferred to a chilled glass vial for GSH and GSSG determination. GSH and GSSG were simultaneously measured by reverse-phase high-performance liquid chromatography (HPLC) coupled with electrochemical detection as previously described with some modifications (75). The mobile phase (10 mM sodium phosphate, 1.25% Lichrosolv-grade methanol, pH 2.7) was delivered at an isocratic flow rate of 1 ml/min through an Uptisphere column and a guard column (UP5ODB 25QK, 120 Å, 250 × 4.6 mm, 5 μm column, Interchim, Montluçon, France) placed in an oven at 30°C. A five-point standard curve with GSH and GSSG was prepared, and standards and samples were placed at 4°C in a cooled sample tray and injected using a 717plus autosampler (Waters, Guyancourt, France). GSH and GSSG detection was obtained by a Coulochem III detector (ESA Eurosep Instruments, Cergy Pontoise, France). The analytical cell (ESA, Model 5010) was set at 400 mV for the first potential and at 900 mV for the second, both with a range set at 50 μA. The guard cell was set at 1000 mV (ESA, Model 5020). Under these conditions, typical retention times for GSH and GSSG were ~6 and 17 min, respectively. Limits of linearity were 0.03 and 0.08 μM for GSH and GSSG, respectively.

Experimentally determined amounts of GSH and GSSG were converted into molar concentrations, and the Nernst equation was used to assess the glutathione redox potential with the following equation: $E_b = E_0 - (59.1/2) \log \left(\frac{[GSH]^2}{[GSSG]}\right)$, assuming $E_0$ at pH 7.8 was $-288$ mV at 25°C.

ROS-level assay

Cellular ROS levels were estimated using the cell-permeant probe CM-H$_2$DCFDA as previously described (20). Briefly, cells were plated on Lab-Tek chambers mounted on glass slides (Nunc, Thermo Scientific, Cergy-Pointoise, France) and grown until 80% confluence. Cells were pre-treated with medium containing 10 μM GSH-EE for 90 min or medium alone. Cells were incubated for 30 min at 37°C with 10 μM CM-H$_2$DCFDA diluted in serum-free medium. Then, cells were washed twice in a salt-balanced solution, and live-cell images (at 37°C) were obtained by laser confocal scanning microscopy (Zeiss, LSM 510) with a 488 nm excitation light from an argon laser and a 505–530 nm band-pass barrier filter. Z-series images were captured, and fluorescence intensity was quantified on maximal projection images, using ImageJ software. A minimum of 40 cells were analysed per experiment.

Respiratory chain complexes enzymatic activities

Enzymatic activities of respiratory chain complexes and citrate synthase were measured by standard spectrophotometric methods as previously described (76). A similar method was then automated on a Roche Diagnostics/Hitachi 912 analyser (Meylan, France). The assays were based on methods described by Kramer et al. (77) and Krabbenhøft et al. (78), slightly modified to match the analyser’s requirements. Briefly, NADH-ubiquinone reductase activity (CI) was measured following NADH disappearance, using rotenone as a specific inhibitor. Complex II activity (succinate–ubiquinone reductase) was assayed through the reduction of DCPIP as the final electron acceptor, after the addition of succinate. The activity of Complex III, ubiquinone-cytochrome c reductase, was followed with the change of reduction of cytochrome c. Cytochrome c oxidase (Complex IV) activity assessment was based on the same statement, with the use of potassium cyanide to inhibit the activity of this enzyme. The activities of all the complexes in each sample were normalized with the corresponding amount of protein to allow sample comparison.

BN-PAGE and CI expression

BN-PAGE was performed as described by Wittig et al. (79); mitochondria pellets were allowed to thaw on ice and were resuspended in 45 μl of extraction buffer (10% dodecyl malto-side, 50 mM imidazole, 5 mM NaCl, 0.1% glycerol, 0.004% Ponceau red, pH 7) at 4°C. After incubation on ice for 20 min with frequent vortexing, the suspension was clarified by ultracentrifugation at 40 000g for 15 min at 4°C.
(Beckman Coulter, Optima™ MAX ultracentrifuge). A fraction was kept for protein determination, and 4 μl of Native PAGE™ 5% G-250 Sample Additive (Invitrogen) was added to the remaining supernatant. Samples were resolved on a 1 mm thick NativePAGE™ 3–12% Bis-Tris gel (Novex®, Invitrogen). The gel was run at 4°C at 150 V. The anode buffer was NativePAGE™ Running Buffer 1× (50 mM Bis-Tris, 50 mM Tricine, pH 6.8), and three successive cathode buffers were used, containing 0.02% Coomassie® G-250, 0.002% Coomassie® G-250 and anode buffer alone. Cathode buffers were changed a third and two-thirds of the way through the migration. Each sample was loaded in duplicate. After electrophoresis, gel slabs were cut and used either for CI localization with in-gel activity or for CI expression estimated by silver staining. In-gel NADH:NTB reductase activity for CI was determined by incubating the native gels in 1 mg/ml NTB, 0.14 mM NADH, 100 mM Tris/HCl, pH 7.4, for 40 min at 37°C as described by Wittig et al. (80). Silver staining was performed according to the method described by Schevchenko et al. (81). CI expression was also analysed by immunoblotting; native gels were incubated in NuPAGE transfer buffer (Invitrogen) and electrotransferred onto a PVDF membrane in NuPAGE transfer buffer at 8 mA/cm² for 1 h. The blots were blocked with 5% milk and incubated with primary antibody against CI (Biocides, Millipore, Molsheim, France) overnight. Blots were then incubated with secondary antibody conjugated to horseradish peroxidase and developed using ECL chemiluminescence reagents (Pierce, Rockford, IL, USA). Blots were then washed and exposed to X-ray film. Blots were also analysed by immunoblotting; native gels were incubated with primary antibody against CI (Biocides, Millipore, Molsheim, France) overnight. Blots were then incubated with secondary antibody conjugated to horseradish peroxidase and developed using ECL chemiluminescence reagents (Pierce, Rockford, IL, USA). Blots were then washed and exposed to X-ray film. Blots were also analysed by immunoblotting; native gels were incubated with primary antibody against CI (Biocides, Millipore, Molsheim, France) overnight. Blots were then incubated with secondary antibody conjugated to horseradish peroxidase and developed using ECL chemiluminescence reagents (Pierce, Rockford, IL, USA). Blots were then washed and exposed to X-ray film.

IL-8 production

Cell lines were grown until 80% confluence and then serum-starved for 18 h before stimulation. Cells were pre-treated with 10 μM GSH-EE for 90 min before the inflammatory stimulation with 50 ng/ml TNFα. The amount of IL-8 produced in the supernatants over 24 h was quantified using a commercial kit (Millipore, Molsheim, France). The assay was carried out according to the manufacturer’s instructions, and quantification was determined with a microplate reader at 450 nm.

Aconitase and fumarase activities

Aconitase activity was measured using a commercial kit (Cayman, Ann Arbor, MI, USA) by monitoring the conversion of citrate to α-ketoglutarate, using the coupled reduction of NADP to NADPH by isocitrate dehydrogenase. The assay was performed according to the manufacturer’s instructions with minor modifications. Samples were suspended in pH 7.1 phosphate buffer and assays were carried out in the Cayman Aconitase Assay Buffer containing 1 mM sodium citrate, 1 mM MgCl₂ and 0.2 mM NADP⁺. NADPH formation was monitored at 340 nm for 40 min at 25°C.

Fumarase was assayed in the malate-forming direction as described elsewhere (82). Samples were the same as those used for the aconitase assay. The ratio of the specific activities of aconitate to fumarase was then calculated and expressed relative to C38 cells.

Glutathione transport measurement

Initial rates of [35S]GSH uptake were measured in freshly isolated mitochondria as previously described (31,83). Briefly, freshly isolated mitochondria were resuspended in uptake buffer (220 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, 5 mM pH 7.2 HEPES buffer, 5 mM succinate, 1 mM potassium phosphate and 0.1% fatty acid free bovin serum albumin) at a final concentration of ~10 mg/ml. Uptake was initiated by adding 10 μl of the mitochondrial suspension to 240 μl of uptake buffer to which increasing concentrations of GSH, 3 mM ATP and 1 μCi [35S]GSH were added. After 15 s, the reaction was stopped by adding 1 ml of ice-cold uptake buffer and quickly vacuum-filtering the mitochondria on a 0.45 μm cellulose filter (Millipore, Molsheim, France). Filtered mitochondria were rinsed twice with 2.5 ml of ice-cold uptake buffer. Filters were dried and placed in 3 ml of UltimaGold™ liquid scintillation fluid, and radioactive counts were recorded on a Wallac 1409 liquid scintillation counter. To determine non-specific binding to mitochondria and filters, blanks were performed by adding ice-cold buffer to mitochondria before adding [35S]GSH. The suspension was immediately filtered and rinsed in the same manner as samples. Blanks were subtracted from sample transport measurements. Preliminary experiments showed that maximal uptake rates were observed within 15–30 s at an external GSH level of 5 mM at 23°C. In agreement with previous observations, ATP seemed necessary for optimal uptake, and initial studies in the presence or absence of 3 mM ATP showed an increase in GSH transport compared with samples without ATP.

Quantitative real-time PCR experiments

Total RNAs were isolated from colonic epithelia, using TRizol reagent, according to the manufacturer’s recommendations (Invitrogen). cDNA was obtained from 5 μg of RNA by reverse transcription using the First-Strand cDNA Synthesis Kit (Amersham, GE Healthcare, Orsay, France) according to the manufacturer’s instructions. Gene expression was studied by quantitative real-time PCR using the cycle-threshold-based method and the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Applied Science, Meylan, France). Pre-incubation was performed at 95°C for 10 min. Amplification of the target cDNA was carried out for 45 cycles, with denaturation for 10 s at 95°C, annealing at 62°C for 7 s and elongation at 72°C for 12 s. Samples were then cooled to 35°C. Each reaction was performed in triplicate and the expression of each gene was normalized to HPRT expression. The following primer sequences were used and were designed via Primerbank (pga.mgh.Harvard.edu/primerbank): DIC forward 5’TCAGTGGTTTAACCTGGAGGTCT and reverse 5’ATGAGA GTAGTTCGTCGTGTG; OGC: forward 5’AAGAACC GGATTGCAGTTG and reverse 5’CACCAGTGA ATGCCCTTCA; HPRT: forward 5’TCAGTCAACGGG GACATATAA and reverse 5’GGGCTGTAATCGCTT AACCAG.
Redox western blotting

The redox status of NDUFS1 was assessed using AMS labeling as previously described by Chen et al. (84) with some modifications. Freshly isolated mitochondria were solubilized in lysis buffer (62.5 mM Tris–Cl, pH 6.8, 1% SDS, 5 mM urea) with 1 mM AMS. The lysates were incubated for 15 min on ice, followed by 15 min incubation at 37°C. After derivatization, redox isoforms were separated by non-reducing SDS–PAGE and electrotransferred onto a PVDF membrane. Immunoblot analysis was performed as described above. The redox status of NDUFS1 is presented as the percentage of oxidized protein to total protein.

Measurement of mitochondrial membrane potential (ΔΨm)

ΔΨm was estimated using the cationic fluorescent probe TMRM as previously described (20). Cells were plated on Lab-Tek® chambers and grown until confluent. Cells were then pre-treated with medium containing 10 mM GSH-EE for 8 h and subsequently incubated for 20 min at 37°C in serum-free medium containing 20 nM TMRM. For fluorescence analysis, medium was replaced by serum-free medium containing 20 nM TMRM and 1 mM AMS. The lysates were incubated for 15 min on ice, followed by 15 min incubation at 37°C. After derivatization, redox isoforms were separated by non-reducing SDS–PAGE and electrotransferred onto a PVDF membrane. Immunoblot analysis was performed as described above. The redox status of NDUFS1 was assessed using AMS labeling as previously described (84) with some modifications. Freshly isolated mitochondria were solubilized in lysis buffer (62.5 mM Tris–Cl, pH 6.8, 1% SDS, 5 mM urea) with 1 mM AMS. The lysates were incubated for 15 min on ice, followed by 15 min incubation at 37°C. After derivatization, redox isoforms were separated by non-reducing SDS–PAGE and electrotransferred onto a PVDF membrane. Immunoblot analysis was performed as described above. The redox status of NDUFS1 is presented as the percentage of oxidized protein to total protein.

Statistical analysis

Results were compared using unpaired Student’s t-test. A P-value of <0.05 was considered statistically significant. Unless stated otherwise, all results are expressed as means ± SD.

Conflict of Interest statement. None declared.

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