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Modulation of the unfolded protein response pathway as an antiviral approach in airway epithelial cells

Aline Schögler[a], Oliver Caliaro[a], Melanie Brügger[b,c,d], Blandina I. Oliveira Esteves[c,d], Izabela Nita[e,f], Amiq Gazdhar[e,f], Thomas Geiser[e,f], Marco P. Alves[a,c,d,*]

[a] Division of Respiratory Medicine, Department of Paediatrics, University Hospital Bern, Bern, Switzerland
[b] Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland
[c] Institute of Virology and Immunology, Bern, Switzerland
[d] Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Bern, Switzerland
[e] Department of Pulmonary Medicine, University Hospital Bern, Bern, Switzerland
[f] Department of Biomedical Research, University of Bern, Bern, Switzerland

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ABSTRACT

Introduction: Rhinovirus (RV) infection is a major cause of cystic fibrosis (CF) lung morbidity with limited therapeutic options. Various diseases involving chronic inflammatory response and infection are associated with endoplasmic reticulum (ER) stress and subsequent activation of the unfolded protein response (UPR), an adaptive response to maintain cellular homeostasis. Recent evidence suggests impaired ER stress response in CF airway epithelial cells, this might be a reason for recurrent viral infection in CF. Therefore, assuming that ER stress inducing drugs have antiviral properties, we evaluated the activation of the UPR by selected ER stress inducers as an approach to control virus replication in the CF bronchial epithelium.

Methods: We assessed the levels of UPR markers, namely the glucose-regulated protein 78 (Grp78) and the C/EBP homologous protein (CHOP), in primary CF and control bronchial epithelial cells and in a CF and control bronchial epithelial cell line before and after infection with RV. The cells were also pretreated with ER stress-inducing drugs and RV replication and shedding was measured by quantitative RT-PCR and by a $TCID_{50}$ assay, respectively. Cell death was assessed by a lactate dehydrogenate (LDH) activity test in supernatants.

Results: We observed a significantly impaired induction of Grp78 and CHOP in CF compare to control cells following RV infection. The ER stress response could be significantly induced in CF cells by pharmacological ER stress inducers Brefeldin A, Tunicamycin, and Thapsigargin. The chemical induction of the UPR pathway prior to RV infection of CF and control cells reduced viral replication and shedding by up to two orders of magnitude and protected cells from RV-induced cell death.

Conclusion: RV infection causes an impaired activation of the UPR in CF cells. Rescue of the ER stress response by chemical ER stress inducers reduced significantly RV replication in CF cells. Thus, pharmacological modulation of the UPR might represent a strategy to control respiratory virus replication in the CF bronchial epithelium.

1. Introduction

Respiratory viruses are significantly associated with pulmonary morbidity of cystic fibrosis (CF) patients characterized by a deleterious decrease in pulmonary function and aggravation of respiratory symptoms (de Almeida et al., 2010; Flight et al., 2014). At present, there are only limited therapeutic approaches available for the treatment or prevention of the virus-associated deteriorations of CF lung disease. The current management consists of the annual influenza vaccination, which is available and advised to all CF patients, and passive immunoprophylaxis by Palivizumab against respiratory syncytial virus (RSV) infection, which is recommended in some countries (Giebels et al., 2008). Specific antiviral treatments against influenza, such as zanamivir and oseltamivir, or against RSV infection such as ribavirin are available as therapeutic strategy and prescribed to CF patients in specific clinical situations (Renk et al., 2014). However, there are no effective treatments available specifically against rhinovirus (RV) infection, which is the most prevalent virus found during CF

* Corresponding author. Institute of Virology and Immunology, University of Bern, Switzerland.
E-mail address: marco.alves@vetsuisse.unibe.ch (M.P. Alves).

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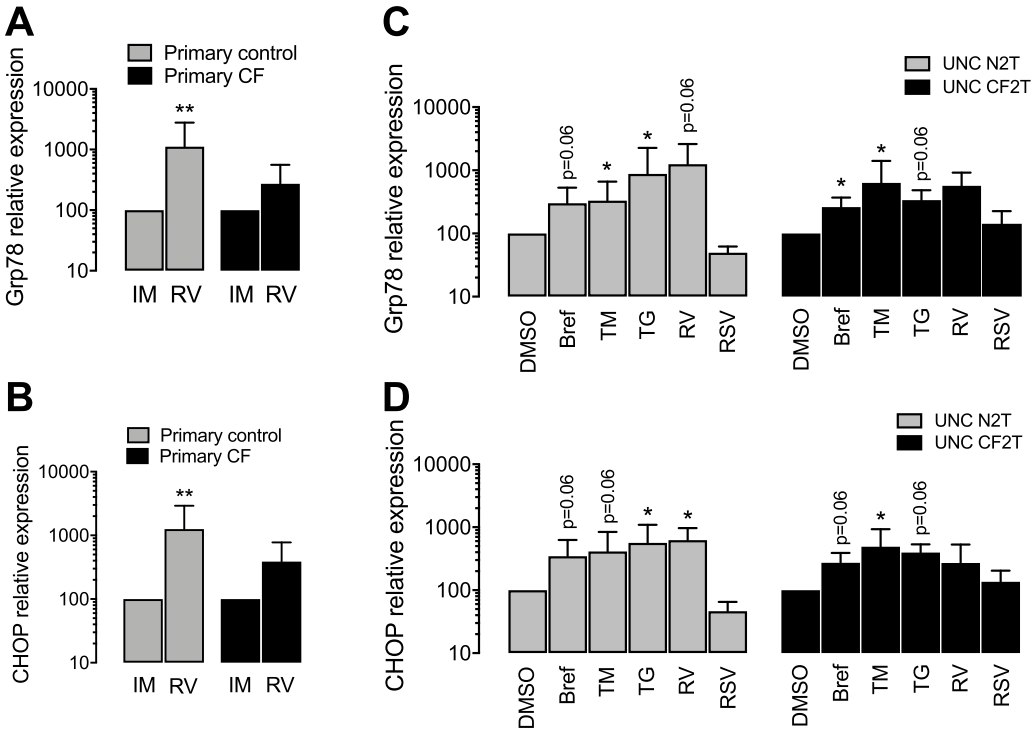


Fig. 1. Impaired ER stress response in CF cells upon RV infection. Grp78 and CHOP mRNA expression was measured by RT-qPCR at 24 h post-infection with RV in primary bronchial CF and control cells (A, B) and in UNC N2T and CF2T bronchial cells infected with RV or RSV or post-treatment with Bref (2 μg/ml), TM (5 μg/ml) and TG (100 nM) (C, D). IM: infection media, Bref: Brefeldin A, TM: Tunicamycin, TG: Thapsigargin. Data are presented as mean with SD and the results of panels A and B were generated from 12 independent control and CF donors and data in panels C and D were obtained from 3 to 6 independent experiments. *$p < 0.05$, **$p < 0.01$.

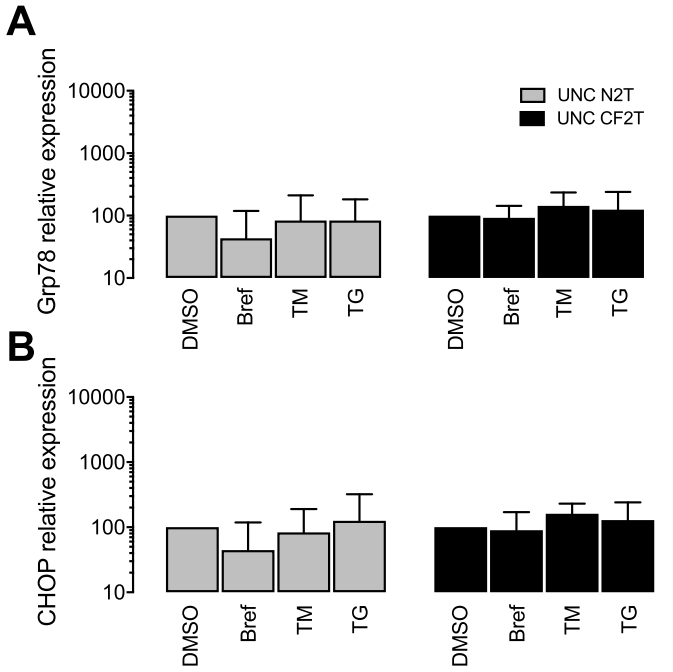


Fig. 2. Grp78 and CHOP expression upon ER stress inducers treatement in RV-infected cells. Grp78 (A) and CHOP (B) mRNA expression was measured one day post-RV infection by RT-qPCR in UNC N2T and CF2T bronchial cells pretreated 24 h with Bref (2 μg/ml), TM (5 μg/ml) and TG (100 nM). Bref: Brefeldin A, TM: Tunicamycin, TG: Thapsigargin. Data are presented as mean with SD of 6 independent experiments.

exacerbations (Flight et al., 2014; Wat et al., 2008). Therefore, novel therapeutic strategies for the management of virus-induced CF pulmonary exacerbations are certainly needed.

Numerous diseases involving elevated inflammatory response and infection are characterized by the activation of pathways involving the endoplasmic reticulum (ER). The ER is known as an important cellular component engaged in the maintenance of homeostasis and in the control of inflammation and immune response to viruses (He, 2006). Viruses cause a disruption of ER homeostasis and generate unavoidable ER stress leading to activation of the unfolded protein response (UPR) pathway, an adaptive mechanism involved in the restoration of the ER homeostasis. The chaperon immunoglobulin glucose-regulated protein 78 (Grp78) is a master regulator of the UPR and keeps the ER stress response under a repression state (Lee, 2005; Zhang and Kaufman, 2004). The UPR pathway is initiated upon binding of Grp78 to accumulate unfolded and/or misfolded proteins in the ER lumen initiating the activation of the ER stress transducers that limit protein translation, promote protein degradation, facilitate protein folding or induce cell death through the transcriptional factor C/EBP homologous protein (CHOP) (Lee, 2005; Zhang and Kaufman, 2004; Ron, 2002; Travers et al., 2000; Oyadomari and Mori, 2004). While chronic inflammation and accumulation of misfolded CF transmembrane conductance regulator (CFTR) protein in the lumen of the ER might have an impact on ER homeostasis, the exact contribution of the ER stress response and the activation of the UPR in CF is not fully understood. Indeed, several reports showed no interaction between misfolded CFTR and Grp78 suggesting that the UPR is not constitutively active in CF (Loo et al., 1998; Pind et al., 1994; Yang et al., 1993). On the other hand, other investigators demonstrated that the retention of ΔF508-CFTR in the ER is leading to constitutive ER stress and UPR activation (Kerbiriou et al., 2007; Bartoszewski et al., 2008; Martino et al., 2009). Finally, other studies revealed the absence of classical ER stress response and UPR activation in CF cells (Blohmke et al., 2012; Nanua et al., 2006; Hybiske

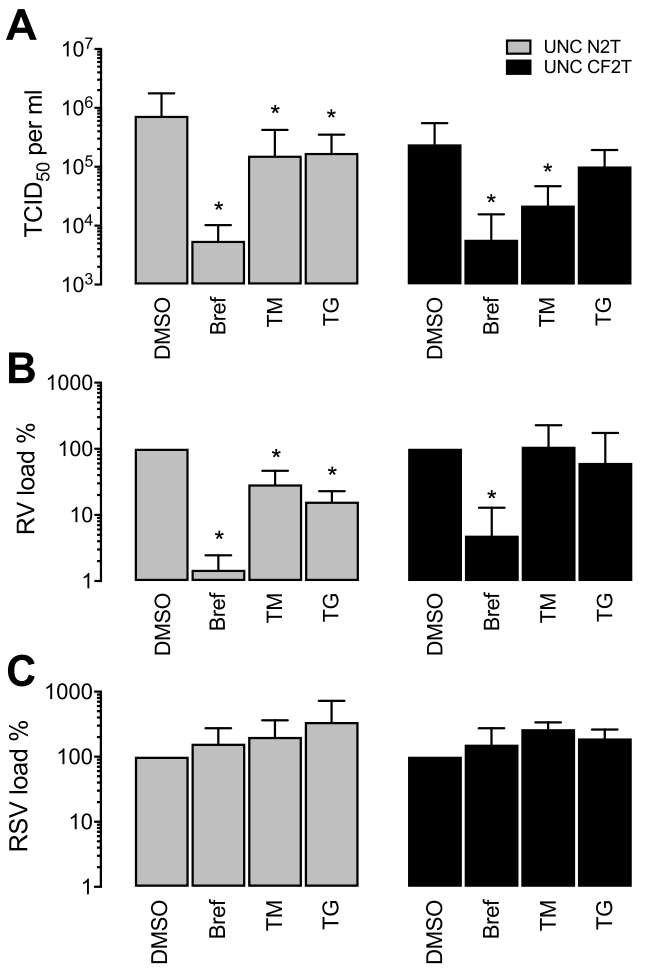


Fig. 3. Effect of ER stress-inducing drugs on respiratory virus replication in CF cells. Live RV shedding by the $TCID_{50}$ method (A) and RV (B) and RSV (C) viral RNA loads assessed by RT-qPCR were measured 24 h post-infection in UNC N2T and CF2T bronchial cells. The cells were pretreated for 24 h with Bref (2 μg/ml), TM (5 μg/ml) and TG (100 nM). Bref: Brefeldin A, TM: Tunicamycin, TG: Thapsigargin. Data are presented as mean with SD of 5–6 independent experiments. *$p < 0.05$.

et al., 2007). The discrepancy between the study results might be due to the different *in vitro* systems used and certainly requires further investigation.

We and other have showed that airway epithelial cells (AECs) isolated from CF patients have an increased susceptibility to RV infection compare to healthy subjects (Kieninger et al., 2013; Sutanto et al., 2011; Schogler et al., 2015). In addition, ER stress inducers such as Tunycamicin, Brefeldin A, or Thapsigargin have been found to inhibit the replication of a broad spectrum of viruses (Saito et al., 1996; Maheshwari et al., 1983; Pal et al., 1991; Maynell et al., 1992; Isler et al., 2005; Clavarino et al., 2012; Raekiansyah et al., 2017). Thus, by means of an *in vitro* model based on CF and healthy bronchial AECs infected with RV, we evaluated the activation of the UPR by selected ER stress inducers as an approach to control viral replication in the CF bronchial epithelium.

2. Material and methods

2.1. Study participants

Written informed consent was given by paediatric healthy volunteers, CF patients and/or caregivers participating in the study and the study was approved by the ethics committees of the Canton of Bern, Switzerland (authorization KEK/09). The clinical characteristics of the 12 healthy and CF participants were already published elsewhere (Schogler et al., 2015). The CF cells sampled where originating from CF patients with a ΔF508/ΔF508 genotype. Potential CF and healthy participants were excluded from the study upon matching one of the following criteria: bleeding tendency, therapy with anticoagulants and/or immunosuppressive agents. For the control group, steroid use within the past three months and atopy were additional exclusion criteria.

2.2. Cell culture and viruses propagation

A human bronchial epithelial cell line with a CF and a non-CF (control) phenotype was used and kindly donated by Dr. S. H. Randell (University of North Carolina, US): UNC CF2T (ΔF508/ΔF508 CFTR mutation) and UNC N2T (Fulcher et al., 2009). Cells were grown in Cnt-17 medium (CELLnTEC, Switzerland) supplemented with single quotes (CELLnTEC, Switzerland). For virus infection of UNC cells, CnT-17 medium without supplements was used as infection medium (IM). Submerged cell cultures of primary control and CF bronchial epithelial cells were grown in bronchial epithelial growth medium (BEGM, Lonza, Switzerland) as previously described (Schogler et al., 2015). Minor group RV1B stock from species A (American Type Culture Collection, US) was propagated and titrated on Ohio-HeLa cells as described (European Collection of Cell Cultures, UK) (Papi and Johnston, 1999). The RSV-A2 strain (American Type Culture Collection, US) was grown and titrated on Hep-2 cells as described (American Type Culture Collection, US) (Wong et al., 2014).

2.3. ER stress induction and virus infection

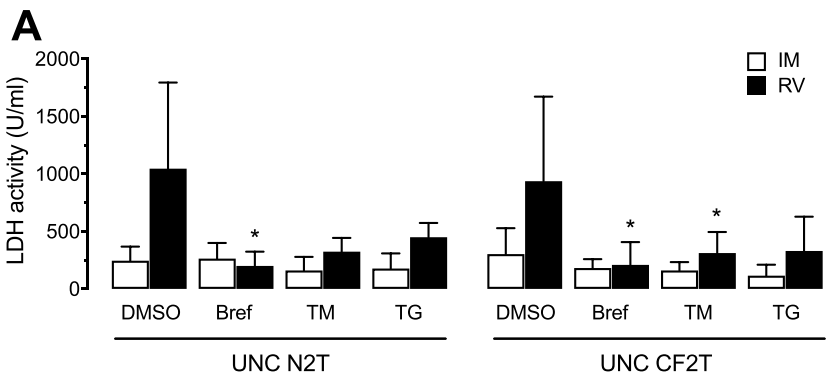
To induce ER stress and activate the UPR pathway, RV or RSV-infected and uninfected UNC and primary bronchial cells were pretreated for 24 h with Tunicamycin (TM; Sigma-Aldrich, US), Thapsigargin (TG, Sigma-Aldrich, US) or Brefeldin A (Bref, Sigma-Aldrich, US) at a final concentration of 5 μg/ml, 100 nM and 2 μg/ml, respectively. ER stress inducers were reconstituted in DMSO to a stock solution and then further diluted in culture medium to reach the final concentrations. Cells treated with medium alone (IM) was used as negative control. The highest final DMSO dilution in the culture medium was 1:200 and therefore this dilution was also added to all other conditions as a control including the negative control. After 24 h of pre-treatment, the cells were infected with RV1B or RSV-A2 with a multiplicity of infection (MOI) of 4 for 1 h. Twenty-four hours post-infection, the supernatants and cell lysates were harvested and stored at −80 °C until further analysis was performed.

2.4. RNA isolation and cDNA synthesis

Using the NucleoSpin RNA kit (Macherey-Nagel, Switzerland), total RNA isolation was performed according to the manufacturer's manual. From 200 μg of total RNA, cDNA was prepared using random primers (Thermo Fisher Scientific, US) and the Omniscript RT Kit (Qiagen, Switzerland).

2.5. Real-time PCR

Two μl of cDNA were amplified in a total of 20 μl of PCR reaction using Fast SYBR® Green Master Mix (Life Technologies, US) and specific primers (20 μM) for 18S (Schogler et al., 2015), RV (Schogler et al., 2015), RSV (Lee et al., 2010), Grp78 (Forward primer: 5′-TTTTCTTCC TGCTGGATGCTTGTCA-3`; Reverse primer: 5′-ACCAGCCCATTTCACTT GTGCTCC-3′), CHOP (Forward primer: 5′-GCACCTCCCAGAGCCCTCA CTCTCC-3`; Reverse primer: 5′-GTCTACTCCAAGCCTTCCCCCTGCG-3′). Quantitative real-time-PCR reactions were carried out on a 7500 fast Real-Time PCR System (Life Technologies, US). To quantify the mRNA



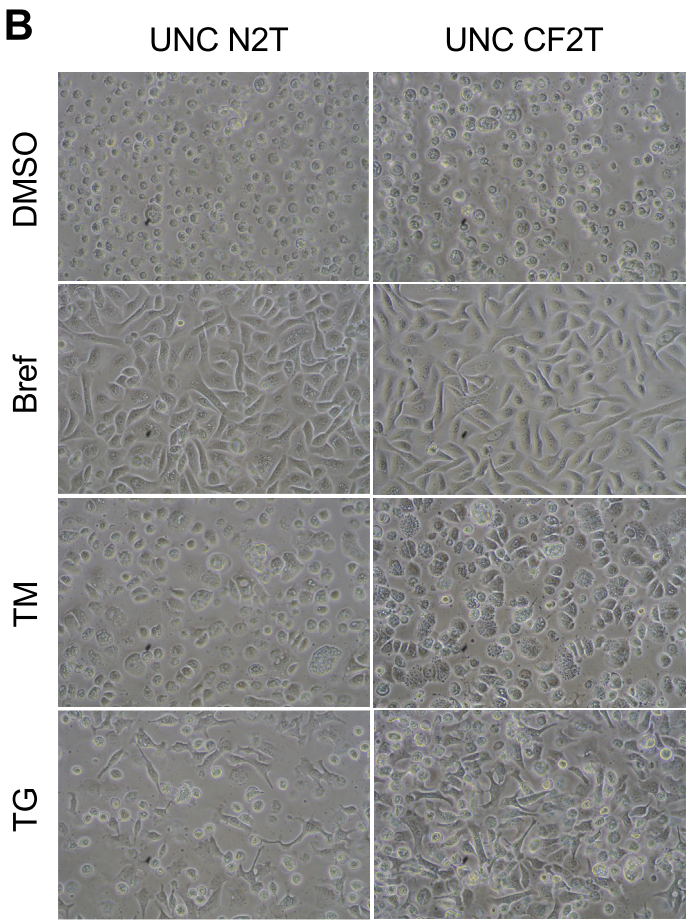


Fig. 4. LDH activity and microscopic evaluation of CF and control bronchial cells upon treatment with ER stress-inducing drugs. LDH activity (A) was assessed in supernatants after ER stress induction in UNC N2T and CF2T bronchial cells at 24 h post-infection with RV. Cells were pretreated for 24 h with Bref (2 μg/ml), TM (5 μg/ml) and TG (100 nM). Micrographs of the RV-infected UNC N2T and CF2T bronchial cell layer with and without ER stress inducers were taken with a conventional light microscope 24 h after RV infection. Bref: Brefeldin A, TM: Tunicamycin, TG: Thapsigargin. Data are presented as mean with SD of 6 independent experiments. *$p < 0.05$.

expression of endogenous genes, the ΔΔCt method was used and the results were expressed as relative expression to DMSO or IM controls. Gene expressions were normalized to the housekeeping gene 18S rRNA.

2.6. Measurement of live virus release

Culture supernatants of RV-infected cells were serially diluted in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, US) supplemented with 4% fetal calf serum (FCS; BioConcept, US) and subsequently titrated on Ohio-HeLa cells to define the 50% tissue culture infective dose ($TCID_{50}$) per ml of RV using the Spearman-Karber method (Gielen et al., 2010; Lennette et al., 1995).

2.7. Cytotoxicity

Cell death was measured by assessing lactate dehydrogenate (LDH) activity in the culture supernatants of UNC cells as previously described (Barlow et al., 2005).

2.8. Statistical analysis

To perform statistical analysis, the GraphPad Prism 7 software (GraphPad Software Inc., US) was used. Data are presented as mean and SD and the comparison of two groups were analysed using the non-parametric, paired Wilcoxon test. Multiple comparison was done with a one-way ANOVA and the Tukey post-hoc test. A p value < 0.05 was considered statistically significant.

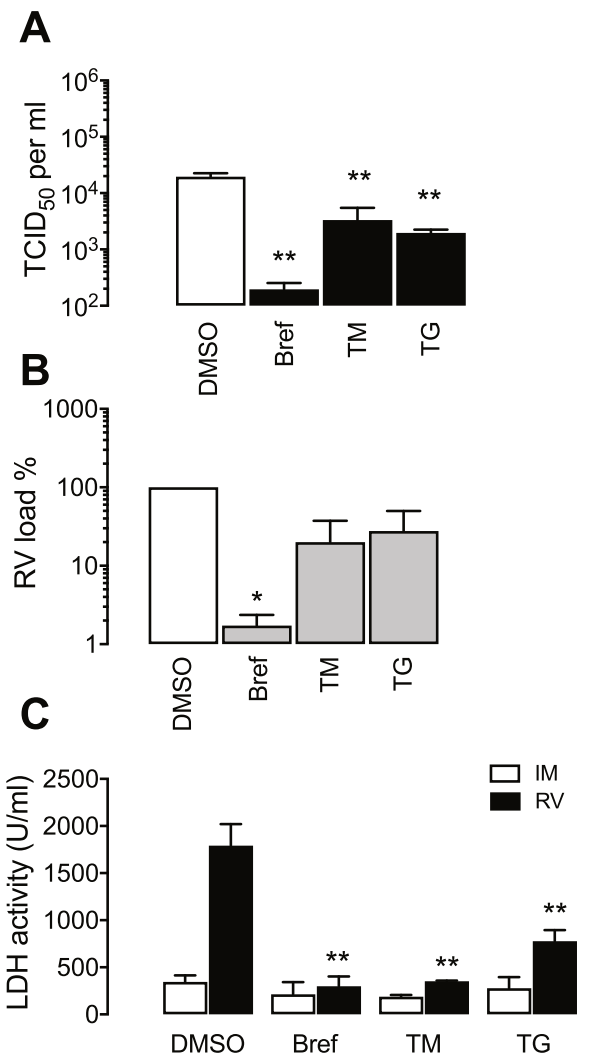


Fig. 5. Virus replication upon treatment with ER stress-inducing drugs in primary CF cells. Primary CF bronchial cells were pretreated for 24 h with Bref (2 μg/ml), TM (5 μg/ml) and TG (100 nM). Next, Live RV shedding measured by the $TCID_{50}$ method (A), RV RNA loads assessed by RT-qPCR (B) and LDH activity (C) were measured at 24 h post-infection. Bref: Brefeldin A, TM: Tunicamycin, TG: Thapsigargin. Data are presented as mean with SD of 2–3 donors. *$p < 0.05$, **$p < 0.01$.

3. Results

3.1. Impaired RV-induced ER stress response in primary CF bronchial epithelial cells

In order to determine if CF cells are able to mount an effective ER stress response during virus infection, we analysed the activation of the UPR during RV infection through the measurement of Grp78 and CHOP expression in infected primary CF and control bronchial cells. Grp78 is highly expressed in the ER and is commonly used as an ER stress marker. Also, The UPR pathway protects the cell during viral infection by modulating the capacity and the load of the ER and by triggering cell death if the stress is too high. Thus, we selected the pro-apoptotic CHOP level as an additional UPR marker. Indeed, the baseline expression of CHOP is low and its induction can be measured by RT-qPCR (Harding et al., 2000). We found a significant increase of the Grp78 (11.1-fold, $p < 0.01$) and CHOP (12.6-fold, $p < 0.01$) expression in primary control cells upon infection compared to uninfected cells pointing to the activation of the UPR, whereas in RV-infected CF cells no significant induction of Grp78 (2.7-fold) and CHOP (3.8-fold) was observed in comparison to uninfected cells. This suggests a diminished UPR activation (Fig. 1A and B) in primary CF cells upon RV infection.

3.2. CF bronchial epithelial cells mount an ER stress response upon pharmacological stimulation

We observed a similar reduced Grp78 induction in CF cells upon RV infection when using the UNC N2T (12.5-fold, $p = 0.06$) and UNC CF2T (5.7-fold) cell lines (Fig. 1C). Additionally, after RV infection, diminished CHOP levels were found in UNC CF2T (2.6-fold) in comparison to UNC N2T (6.3-fold, $p < 0.05$) cells was found (Fig. 1D). Thus, we decided to continue our investigation by using the UNC cell line as a surrogate of the primary cells which are more difficult to obtain. Contrary to RV infection, we didn't observe an induction of Grp78 and CHOP when infecting CF and control UNC cells with RSV, suggesting a RV-specific effect in CF cells (Fig. 1C and D). In order to determine if CF cells can mount a normal ER stress response with classical ER stress inducers, we induced the UPR pathway by treating uninfected control and CF UNC cells with Brefeldin A, Tunycamicin, and Thapsigargin. With all the drugs tested, we observed both in control and CF cells a significant or a trend towards an upregulation of Grp78 (Fig. 1C, $p < 0.05$) and CHOP (Fig. 1D, $p < 0.05$), suggesting that CF cells are able to mount an ER stress response following stimulation with commonly used ER stress inducers.

3.3. Impaired RV-induced UPR in CF bronchial epithelial cells can be restored by ER stress inducers treatment

In order to determine if the impaired RV-induced UPR activation in CF cells can be overcome and be restored by chemical ER stress inducers, we assessed the mRNA expression of Grp78 and CHOP in RV-infected CF and control UNC cells upon pretreatment with the ER stress inducers for 24 h. We observed that Grp78 (Fig. 2A) and CHOP (Fig. 2B) expression reached similar levels in RV-infected CF and control UNC cells upon treatment with the ER stress inducers suggesting a restoration of the UPR response in CF cells.

3.4. ER stress inducers have RV-specific antiviral activity in CF bronchial epithelial cells

To evaluate the possible antiviral effect of ER stress inducers in CF cells, we pre-treated the CF and control UNC cells with Brefeldin A, Tunycamicin, and Thapsigargin for 24 h and then challenged the cells with RV. We found a significant reduction of virus shedding in both control ($p < 0.05$) and CF ($p < 0.05$) cells upon treatment with ER stress-inducing drugs (Fig. 3A). A particularly strong antiviral effect was observed with Brefeldin A which decreased RV shedding by 2 orders of magnitude (Fig. 3A, $p < 0.05$). Further, the intracellular RV load was also significantly reduced upon pre-treatment in both control (Fig. 3B, $p < 0.05$) and CF (Fig. 3B, $p < 0.05$) cells. In line with the lack of UPR pathway induction upon RSV infection of CF bronchial cells (Fig. 1C and D), we did not observed an antiviral activity of ER stress inducers when using RSV as infection model (Fig. 3C).

3.5. Reduced RV-mediated cytopathic effect upon UPR activation in CF bronchial epithelial cells

Since cell death is a direct cause of RV-induced cytopathic effect, LDH quantification in the supernatants of treated and untreated control and CF UNC cells prior and after infection was performed. We found similar LDH activities between ER stress inducers-treated and untreated cells in both control and CF cells suggesting that the drugs do not promote cell cytotoxicity (Fig. 4A, IM). We observed significantly lower LDH activities upon treatment with the drugs, particularly with Brefeldin A, in RV-infected control ($p < 0.05$) and CF ($p < 0.05$) cells

compared to untreated RV-infected cells (Fig. 4A). Also, micrographs of the cultures showed evidence of a cytoprotective effect of Brefeldin A in infected control and CF cells compared to untreated RV-infected cells (Fig. 4B).

3.6. Primary CF bronchial cells showed a similar reduced RV replication upon ER-stress inducing drugs treatment

Further, to confirm our findings in UNC CF cell lines, we measured RV replication in primary CF bronchial epithelial cells after treatment with ER stress-inducing drugs. Particularly with Brefeldin A and to a lower extent with Tunycamicin and Thapsigargin, we observed a significant reduced viral replication in treated CF cells at the level of live virus release (Fig. 5A, $p < 0.01$) and viral loads (Fig. 5B, $p < 0.05$). Similar to the CF cell line data, we observed an inhibition of RV-induced cytotoxicity by ER stress inducers in primary bronchial CF cells (Fig. 5C, $p < 0.01$).

4. Discussion

Viral infections of the respiratory tract are a major cause of morbidity and mortality in CF patients and are presented with limited treatment options (Wat et al., 2008; Asner et al., 2012). To evaluate the contribution of the UPR in the control of respiratory virus infection in CF, we studied the effect of ER stress-induced UPR activation on RV replication in CF airway epithelial cells. We found impaired induction of the UPR pathway in CF bronchial cells following RV infection. The impaired UPR in CF cells upon RV infection was restored by ER stress-inducing drugs to the levels of control cells and the viral replication and shedding was reduced in both CF and control cells upon drug treatment. As a consequence of the inhibition of RV replication, RV-triggered cell death was reduced in both CF and control cells by ER stress-inducing drugs.

During virus infections, the ER machinery is exploited for the synthesis of new viral particles. Rapid accumulation of viral proteins in the ER leads to increased amount of unfolded or misfolded proteins in the lumen of the ER and to perturbation of ER homeostasis. This generates ER stress and activates the adaptive UPR to alleviate adverse effects and decrease viral protein load in the ER (Jheng et al., 2014). Our results demonstrated that RV infection in control cells increased ER stress markers, indicative of a disrupted ER homeostasis, and initiated an adaptive UPR. However, upon RV infection, CF cells failed to elicit a proper ER stress response characterized by a diminished expression of Grp78 and CHOP. However, we observed a similar UPR activation in uninfected CF and control bronchial cells upon treatment with ER stress-inducers Brefeldin A, Tunicamycin and Thapsigargin suggesting normal UPR activation in CF cells. Our observation was confirmed by Kerbiriou et al. who showed that the ER stress response with Thapsigargin or Tunicamycin was not different between CFTR and ΔF508-CFTR expressing cells (Kerbiriou et al., 2007). Interestingly, we showed a restored UPR activation in RV-infected CF cells upon pre-treatment with ER stress-inducing drugs to a comparable level as in control cells. Chemical ER stress induction may therefore overcome the trend towards RV-induced impaired UPR activation in CF cells. The factors leading to a diminished UPR activation in CF cells upon RV infection are so far unclear. It is conceivable that an atypical association between virulence factors, mutated ΔF508-CFTR and Grp78 suppresses the UPR pathway in CF bronchial cells. In line with this hypothesis, de Jong et al. showed that the RV non-structural replication protein 2B is retained in the ER leading to a disturbance of the calcium homeostasis and subsequent inhibition of protein trafficking through the Golgi complex (de Jong et al., 2008).

By means of different mechanisms such as inhibition of translation or protein degradation, the UPR pathway activation is a system that could help controlling virus replication (He, 2006). Indeed, several studies show an association between ER stress activation and replication of several viruses including picornaviruses (Tardif et al., 2005; Jordan et al., 2002; Su et al., 2002; Jheng et al., 2010; Zhu et al., 2013; Zhang et al., 2010). In line with this hypothesis, we observed a remarkably strong decrease of viral replication in both cell lines treated with Brefeldin A which induced up to a 100 times decreased virus shedding in the supernatants. On the other hand, Tunicamycin and Thapsigargin had a significant anti-RV activity in control cells at the level of virus shedding and intracellular viral loads and a limited anti-RV activity in CF cells. Also, both cell lines showed a reduced level of viral RNA upon treatment with Brefeldin A. In addition, an antiviral activity of ER stress inducers was also demonstrated with a more physiological system based on primary CF bronchial epithelial cells infected with RV. In line with our findings, it has been recently reported that Brefeldin A has inhibitory effects on replication of picornaviruses (Cuconati et al., 1998; Gazina et al., 2002). Further, Tunicamycin shows antiviral effects on herpes simplex virus (Maheshwari et al., 1983). Also, a published study of our group confirmed antiviral effects of Azithromycin, a macrolide antibiotic related to Brefeldin A, suggesting that macrolide antibiotics in general have a strong antiviral effect in CF cells (Schogler et al., 2015). Interestingly, we could show that the lack of UPR activation in CF is RV-specific. Indeed, RSV infection didn't induce significantly the expression of Grp78 and CHOP in control and CF cells, and virus replication was not reduced after treatment with pharmacological ER stress inducers in neither CF nor control UNC cells. The lack of antiviral activity of ER stress inducers during RSV infection might be due to the diverse life cycle of RV and RSV but further investigation is required to define precisely the mechanism of the antiviral properties of ER stress inducers in the CF bronchial epithelium.

The reduced LDH activity of cells upon treatments confirms that the antiviral effects are not due to cytotoxic effects of the drugs. The observed cell morphology upon treatments do not indicate adverse effects of the drugs. In fact, activation of the UPR pathway in CF and control cells by ER stress-inducing drugs had cytoprotective properties in RV-infected cells as shown by decreased levels of LDH activity. Since RV infection induced significant cell death *in vitro*, the reduced LDH levels might be explained by the antiviral effect of drug treatment (Kieninger et al., 2012).

In conclusion, we demonstrated that ER stress inducers have anti-RV properties and protect cells from RV-induced cell death and this reduced viral replication is not due to cytotoxicity of ER stress-inducing drugs. Because of the limited therapeutic options and the high prevalence of RV in pulmonary exacerbation in CF patients it is essential to further dissect the UPR pathway and its contribution in RV-associated morbidity, which ultimately will help in the development of new therapeutic strategies.

Author contributions

Conception and design: AS, IN, AG, MPA. Acquisition of data: AS, OC, MB, BIOE, MPA. Analysis and interpretation: AS, TG, MPA. Drafting the manuscript for important intellectual content: all authors. Final approval of the manuscript: all authors.

Conflicts of interest

None of the authors has a conflict of interest to report.

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