Rhinovirus-16 increases ATP release in A549 cells without concomitant increase in production

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ABSTRACT Human rhinovirus (RV) is the most common cause of upper respiratory tract infection (URTI) and chronic airway disease exacerbation. Cough is present in 50–80% of URTI cases, accompanied by heightened airway hypersensitivity, yet no effective treatment currently exists for this infectious cough. The mechanism by which RV causes cough and airway hypersensitivity in URTI is still unknown despite recent advances in potential therapies for chronic cough.

The effect of RV-16 infection (MOI 1) on intracellular ATP stores and ATP release in A549 alveolar epithelial cells was measured.

RV-16 infection was found to significantly increase (by 50% from basal at 24 h) followed by decrease (by 50% from basal at 48 and 72 h) intracellular ATP concentrations, while increasing ATP release (from 72 h) independently of secondary stimulation. This effect was mimicked by intercellular adhesion molecule 1 receptor binding alone through ultraviolet-inactivated sham control. In addition, RV-16-infected cells became more sensitive to secondary stimulation with both hypotonic and isotonic solutions, suggestive of a hypersensitive response. These responses were not mediated via increased TRPV4 or pannexin-1 whole-cell expression as determined by Western blotting. Interestingly, the increased ATP release seen was not a result of increased mitochondrial ATP production.

Thus, this is the first report demonstrating that RV-16 infection of airway epithelial cells causes hypersensitivity by increasing ATP release. These finding provide a novel insight into the process by which viruses may cause cough and identify a potential target for treatment of viral and post-viral cough.

Rhinovirus-infected airway epithelial cells (A549) show increased ATP release with and without a secondary stimulation (mechanical or hypotonic), which may account for increased cough sensitivity seen during respiratory viral infections https://bit.ly/3eABEY9

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Introduction

Human rhinovirus (RV) is a major cause of upper respiratory tract infection (URTI). RV infections are associated with acute exacerbations of asthma and COPD [1, 2], and are thought to precipitate the development of post-viral and subsequent chronic cough [3, 4]. During URTI, vagal afferent nerves become hypersensitive to various stimuli, including tussive agents such as capsaicin [5, 6] and even inhaled saline [7]. This airway hypersensitivity in URTI has yet to be fully characterised, although it is hypothesised to contribute to the cause of cough in URTI [6–10]; however, that mechanism is also unclear.

Other groups have previously investigated the effect of human rhinovirus-16 (RV-16), and other respiratory viruses, on the expression of "cough receptors" thermo-TRP channels TRPA1, TRPV1 and TRPM8 expression in airway *in vitro* cell models [11, 12] as a means of unravelling the cause of cough in URTI, but to no avail. However, patients with chronic cough display hypersensitivity to hypotonic solutions [13]. *In vitro*, hypotonic stimulation of the thermo-TRP channel TRPV4 led to ATP release *via* pannexin-1 in the airway [14–16]. However, the role of RV-16 infection on TRPV4 expression and function is unknown.

It is now thought that ATP is a major component in causing chronic cough through stimulation of the P2X3 receptor [8, 17–19], which has been shown to be effectively blocked by specific antagonists including gefapixant (formally MK-7624, AF-219) [19]. However, peripheral ATP alone was deemed not to be the primary source of increased coughing [17]. To date, RV-14 has been shown to increase extracellular ATP levels in NCI-H292 airway epithelial cells measured at 24 and 28 h [20]. In the study presented here, ATP release from RV-16 infected A549 alveolar epithelial cells was investigated over a time course (up to 168 h post-infection) to determine whether the common cold affected ATP storage and release, and altered TRPV4 function to hypotonic stimulation.

Materials and methods

**Virus**

RV-16 was a gift from Michael Edwards from Imperial College London (UK) and was grown and titred in HeLa Ohio cells determined by 50% tissue culture infectious dose end-point (TCID$_{50}$), as previously described [21].

**Cells and RV infection**

A549 and HeLa Ohio cells were obtained from Public Health England (UK) and grown in DMEM, 10% heat inactivated FBS, 50 U·mL$^{-1}$ penicillin, 50 µg·mL$^{-1}$ streptomycin and 1% L-glutamine and grown at 37°C and 5% CO$_2$. Cultures of A549 cells were infected with RV-16 at a multiplicity of infection (MOI) of 1. The cultures were mock infected with ultraviolet (UV)-inactivated virus preparation, as previously described [21]. These preparations were used to determine whether direct interaction with inactive or active virus particles were responsible for the change in cellular ATP production and release.

**ATP measurement**

RV-16 infected (MOI 1) A549 cell intracellular ATP levels were measured using a commercially available kit (Abcam, Cambridge, UK), as per the manufacturer’s protocol. Measurements were assayed in duplicate, standardised to background luminescence and ATP concentration was quantified.

Extracellular ATP levels were assayed using a luminescent measurement kit (Invitrogen, MA, USA) on A549 cells, as previously described [22]. Cells infected with RV-16 were washed, incubated in Hanks' buffered salt solution (+10 mM HEPES) and allowed to equilibrate for at least 1 hour. Supernatants were sampled at regular intervals, either undisturbed (basal), following isotonic control or 33% hypotonic stimulation. Results displayed as fold change (peak) or following area under curve analysis (total).

**Western blot analysis (SDS–PAGE)**

Cells infected with RV-16 were lysed in Cell Lysis Buffer (New England Biolabs, Hertfordshire, UK) containing anti-proteases. An equal amount of protein (50 µg) was subject to Western blot analysis with antibodies raised against pannexin-1 (Santa Cruz Biotechnology, TX, USA) or TRPV4 (Abcam). Equal protein loading was confirmed by anti-tubulin staining. Membranes were imaged with LI-COR (LI-COR, NE, USA) secondary antibodies on LI-COR Odyssey-CLx. Specific bands were normalised to tubulin, quantified by densitometry and expressed as fold change from basal.

**Intracellular calcium signalling**

Receptor function was assessed using a fluorescent calcium probe, Fluo-4AM NW (Invitrogen), as per manufacture’s protocol. Cells infected with RV-16 were loaded with 2× fluo-4 direct assay reagent buffer.
(λex/em 494/506 nm) (Invitrogen) and incubated for 1 h at 37°C. Transient intracellular calcium responses were measured using Flexstation3 Molecular Devices with SoftMax Pro software. Responses were calculated as a percentage of 2 µM A23187, as previously described [23, 24].

**Mitochondrial respiration analysis**

Mitochondrial respiration was assessed using the Seahorse Extracellular Flux analyser (Agilent, CA, USA). Cells cultured on Seahorse XFp plates were infected with RV-16 and a mitochondrial stress test was performed. Lysates were salvaged and total protein was quantified using a bicinchoninic acid kit (G Biosciences, MO, USA). Respiration rates were normalised to µg protein per well.

**Statistical analysis**

Data were presented as mean (±SEM) of at least three independent experiments. Data were tested for normality using the Shapiro–Wilk test and statistical analysis was performed using one-way ANOVA followed by Dunnett’s or Sidak post hoc test for multiple comparisons to a control. Differences were considered significant at p≤0.05.

**Results**

**RV-16 infection affects intracellular ATP concentration**

Hypotonic exposure and mechanical stimulation in A549 cells cause ATP release [15, 25]. Therefore, we decided to investigate the effect of RV-16 infection on production, storage and release of ATP. First, we investigated the effect of RV-16 on total intracellular stores of ATP at both short-term (figure 1a) and long-term infection (figure 1b). Between 96 and 168 h there was no significant difference in intracellular ATP stores from basal control, including UV-inactivated sham viral control (figure 1b, p>0.05; n=4). However, there was a 50% increase in intracellular ATP concentration at 24 h (figure 1a, p=0.0034; n=4) and UV sham control (p=0.0022; n=4). Followed by a 50% decrease in intracellular ATP concentration at 48 and 72 h (p<0.0001; n=4) from basal control.

**Basal ATP release is increased during rhinovirus infection**

To clarify the decline in ATP concentration seen during early infection time points, ATP release in response to RV-16 infection was investigated. We assayed extracellular supernatants for ATP concentration in RV-16-infected A549 cells basally (without any further stimulation).

Over a 72-h period there was no significant change in peak ATP release basally (figure 2a, p>0.05; n=3). However, all other time points displayed a time-dependent increase in ATP release. Area under the curve (AUC) analysis revealed a significant increase in total ATP release at 72 h when compared to basal control (figure 2b, p=0.0185; n=3), all other time points were not significant (p>0.05; n=3).

When measuring the effect of RV-16 at MOI 1 over a long-term infection (96–168 h), no significant change in unstimulated peak ATP release could be seen (figure 2c, p>0.05; n=3), despite a 7–12-fold increase seen in all conditions. However, once again AUC analysis revealed a significant increase in total ATP release from 120 to 168 h including UV-inactivated viral sham control (figure 2d, 120 h p=0.03,
RV-16 infection increases ATP release following secondary stimulation

To determine whether the cells became more sensitive to mechano-sensation, a stimulatory response caused by the physical addition of solution to cells creating a mechanical force during RV-16 infection, the effect of isotonic control of A549 cells was investigated. During short-term infections with RV-16 there was no significant change in peak ATP release following isotonic addition at any time point (figure 3a, p>0.05; n=3). However, AUC analysis identified significant increases in total ATP release at 24 and 72 h (figure 3b, p = 0.0233 and p = 0.0464, respectively; n=3) when compared to uninfected isotonic control. There was no significant difference in ATP release at either 3 h, 48 h or UV control (p > 0.05; n=3).

Over long-term infection with RV-16, isotonic control induced a general trend of increased peak ATP release from 96 to 168 h, including UV control. Only 168 h was deemed significant with a 9.5-fold increase in ATP release from basal control (figure 3c, p = 0.0204; n=3). Following the application of AUC analysis, there was no significant change in total ATP release at 96 and 168 h following isotonic control (figure 3d, p > 0.05; n=3). However, there was a significant increase in ATP release at 120 h, 144 h and UV control (figure 3d, p = 0.0097, p = 0.0005 and p = 0.0012, respectively; n=3).

Finally, we looked at the effect of hypotonic stimulation on RV-16 infected A549 cells. Once again, despite a general trend of increased peak ATP release over short-term infection when compared to uninfected hypotonic control, only 72 h was significant with a 7.38-fold increase in ATP release (figure 3c, p = 0.0185; n=3 and p = 0.0012, respectively; n=3). Importantly, incubation with UV-inactivated viral
FIGURE 3 Extracellular ATP release significantly increased during rhinovirus (RV)-16 infection when exposed to secondary stimulation compared to basal. Isotonic (ISO) stimulation (a–d). Hypotonic (HT) stimulation (e–h). Peak ATP release shown as fold change from basal (a, c, e and g). Area under the curve (AUC) analysis determined over a 5-min period (b, d, f and h). Results are displayed as mean±SEM of three independent experiments. Statistical analysis was carried out using multiple comparison one-way ANOVA followed by Dunnett’s post hoc test. NS: not statistically significant. b–d) (ISO) *p<0.04, **p<0.0097, ***p=0.0005. e–h) (HT) *p<0.04, **p<0.01, ***p<0.0001, n=3.
sham control induced a 2.9-fold increase in ATP release compared to uninfected hypotonic control (p=0.0394; n=3), suggesting an intercellular adhesion molecule (ICAM)-mediated response. Following AUC analysis, compared to the infected hypotonic control, all time points and UV control were significant (figure 3, 120 h p=0.0011, 144 h p=0.0035, 168 h p<0.0001, UV p=0.0049; n=3), except 96 h (figure 3h, p=0.2466; n=3).

**Ratiometric analysis of intracellular ATP and extracellular ATP release**

To contextualise, quantified extracellular release (ExR) and intracellular ATP concentration ([ATP]) ratiometric analysis of short (figure 4a) and long-term infection was performed (figure 4b). A ratio was determined for ExR (isotonic and hypotonic conditions) to intracellular basal [ATP], for each time point over the 168-h time course.

At 72 h, there was a significant increase in the ratio of unstimulated ExR to intracellular [ATP] when compared to basal (figure 4a, p=0.0001; n=3). At 48 and 72 h, there was a significant increase in the ratio of isotonic-induced ExR to intracellular [ATP] when compared to basal (p=0.0001; n=3). Finally, at 72 h, there was a significant increase in the ratio of hypotonic induced ExR to intracellular [ATP] when compared to basal (p=0.0041; n=3). No other short-term infection time points in any condition had significant change in their ratio of extra/intracellular ATP release (p>0.05).

During long-term infection with RV-16 there was a significant increase at 144 and 168 h in the ratio of unstimulated ExR to intracellular [ATP] when compared to basal (figure 4b, p=0.0003 and p=0.016, respectively; n=3). There was no significant change at any time point in the ratio of isotonic-induced ExR to intracellular [ATP] (p>0.05; n=3). Finally, all time points and UV sham infection control had significant increases in the ratio of hypotonic induced ExR to intracellular [ATP] when compared to basal (96 h p=0.0125, 120 h p=0.0004, 144 h p=0.0001, 168 h p<0.0001 and UV p=0.0313; n=3).

**Rhinovirus infection has no effect on global pannexin-1 or TRPV4 protein expression**

To determine whether the increased ATP release seen was a result of increased pannexin-1 or TRPV4 expression, Western blot analysis was performed on whole-cell lysates isolated from A549 cells infected with RV-16 at MOI 1, with and without isotonic or hypotonic stimuli. Lysates were probed for global expression of pannexin-1, TRPV4 and loading control \(\alpha\)-tubulin.

Surprisingly, our data indicated that endogenous expression of either pannexin-1 or TRPV4 was not affected by RV-16 infection at any time point (figure 5a p>0.05; n=5, B p>0.05; n=3, C p>0.05; n=5, D p>0.05; n=3) or isotonic or hypotonic conditions (data not shown). Loading control \(\alpha\)-tubulin expression remained constant across the infection time course. However, expression does not correlate to function. Therefore, TRPV4 function during RV-16 infection was investigated to see if altered TRPV4 function explained the heightened response to hypotonic stimulation and the resultant ATP release seen here. The

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**FIGURE 4** Ratio of ATP release to ATP stores significantly increased as time of infection with rhinovirus (RV)-16 increased. Ratio analysis of sampled supernatant from basal, isotonic and hypotonic sampling experiments (figures 2 and 3) against total intracellular ATP concentration (figure 1) of RV-16 infected, UV-inactivated sham viral control (UV) or uninfected (basal) A549 cells. Results displayed as means±SEM of three independent experiments. One-way ANOVA followed by Dunnett’s post hoc statistical analysis was carried out, or paired t-test (basal versus 144, basal versus 168, HT versus 96). UV: ultraviolet; ABU: arbitrary units; HT: hypotonic; ISO: Isotonic; Intra/extra: intercellular/extracellular ratio. *: p<0.03, **: p=0.0041, ***: p<0.0004, ****: p<0.00001, n=3.
data showed that there was no statistical difference in TRPV4 response to specific agonist GSK1016790A at any time point over the 168-h time course (p>0.05; n=3; data not shown).

RV-16 infection does not change overall ATP production rates

Finally, to determine whether the increased ATP release we identified during RV-16 infection was due to viral hijacking of cellular ATP production rates, we carried out a mitochondrial stress test. The data provided read outs for ATP-linked, nonmitochondrial respiration, proton leak and ATP production rates. Surprisingly, infection with RV-16 did not significantly affect the ATP production rates at any time point or UV sham control when compared to uninfected basal control (figure 6a and b, p>0.05). Furthermore, the data demonstrate that during short-term infection (24–72 h) ATP-linked respiration and proton leak was not affected by RV-16 infection (figure 6c, p>0.05). However, nonmitochondrial respiration was significantly increased (35% versus 69%) when incubated with UV-inactivated viral sham control (p=0.0299) when compared to basal control. In contrast, during long-term infection (96–168 h) there was no significant change in ATP-linked respiration, nonmitochondrial respiration or proton leak at any time point or UV sham control when compared to basal (figure 6d, p>0.05).

**Discussion**

The common cold is a major cause of morbidity in society and it has been estimated that, in the United Kingdom, 120 million episodes occur each year [26], with children each accounting for 7–10 infections annually. The societal impact in terms of education, working days lost and morbidity is enormous. While this condition can be caused by a myriad of viruses, RV is a major culprit. The illness typically starts with a sore throat, rhinitis and nasal congestion, followed by cough in approximately half of all URTIs. Cough generally starts 2–4 days after the initial prodrome. However, it is reported as the most troublesome
symptom and may persist long after resolution of other symptoms leading to a condition known as post-viral cough [27]. Unfortunately, little is still known of the mechanism(s) by which RV causes cough.

Airway hypersensitivity seen in viral infection was initially thought to be caused by thermo-TRP channels, TRPA1 and TRPV1 [8, 17, 18]. However, clinical trials have failed to show the importance of TRPA1 and TRPV1 in causing cough, particularly in chronic cough conditions [28]. Recent clinical trials, that have shifted focus to ATP as the culprit in causing cough and have shown potentially life-changing results through inhibition of the ATP-sensitive P2X3 receptor [19]. Despite these advances, we are still no closer to defining the mechanism of cough during URTI. We and others believe that ATP/TRPV4 may underpin the mechanism of viral cough during URTI.

Here we show that, no change in global TRPV4 expression or function was detected following RV-16 infection. TRPA1 and TRPV1 global expression and function was also unchanged (data not shown). This is in contrast to Abdullah et al. [11] who saw that RV-16 infection led to increased TRPA1, TRPV1 and TRPM8 expression, which may be due to differences in cell models used. We therefore sought to investigate whether altered ATP release in vitro could account for increased cough sensitivity seen during in vivo URTI. To investigate the effects of the common cold virus (RV-16) on ATP release, we used airway alveolar epithelial A549 cells infected with RV-16 for up to 168 h (7 days), a model commonly used for RV infections [21, 29]. Analysis of global protein expression, ATP release and mitochondrial function was undertaken to determine the role that RV-16 exerts on these essential cellular mechanisms. We have shown, for the first time, through the course of this research that RV-16 causes increased basal ATP release, and increased ATP release following stimulation with hypotonic solution and after the addition of nonstimulating, but mechano-activating, isotonic control. RV-16-infected A549 cells may become more mechano-sensitive under relatively innocuous stimulation the longer infection continues, with the largest impact becoming apparent between 96 and 168 h (4–7 days), thus mimicking the clinical course of disease. During the early stages of RV-16 infection (24 h post-infection), total intracellular ATP concentration was increased, followed by a large significant reduction (48–72 h post-infection) coinciding with increased extracellular release. We also show that this effect was mimicked by ICAM-1 receptor binding alone through our UV-inactivated sham control. Furthermore, we identified for the first time that RV-16
infection causes increased ATP release irrespective of further stimulation. This supports previous studies that ATP and P2X3 signalling are the molecular underpinning of chronic, and potentially viral, cough [19]. It is also in agreement with SHISHIKURA and colleague’s [20] work, which showed that RV-14 increased extracellular ATP levels in a model of viral-induced COPD exacerbations. These findings also correlate with the incubation period of in vivo rhinoviral infection and the onset of a symptomatic cough.

Ratiometric analysis between intracellular ATP and extracellular ATP release identified a significantly larger ratio of ATP release from 72 h post-infection, irrespective of stimulation type. This trend continued throughout the 168-h time course. Despite these findings, we found no significant change in pannexin-1 protein expression at any point in the infection.

Previous studies have shown that RV-B14 is able to directly influence the cellular metabolism of HeLa and fibroblast cells causing an anabolic reprogramming of host metabolism [30]. However, the use of nonairway cells and a nonphysiological MOI (3.5–5) means we cannot assume this same reprogramming response occurs in the airway during an URTI in vivo. Therefore, we carried out mitochondrial stress tests on A549 cell line infected with RV-16. Consistent with published literature [30], A549 cells displayed a minor glycolytic shift in phenotype during RV-16 infection following mitochondrial stress; however, this was not significant (data not shown). We had expected to see a larger shift in phenotype and increased ATP production from basal as indicated by our extracellular ATP sampling and intracellular ATP concentration experiments. However, given the well-characterised Warburg effect seen in cancerous cell lines, including A549 [31], we cannot assume that RV infection is not able to induce a stronger and more significant glycolytic shift in phenotype. There was no discernible difference from basal at any infection point for ATP-linked respiration, or proton leak. Although, we did identify a general trend of increased proton leak during early infection, not seen by late infection. We hypothesise that this is a result of increased reactive oxygen species production, a feature previously reported in RV infection [32, 33], due to an inducible proton leak [33, 34]. Furthermore, there was increased nonmitochondrial respiration in cells stimulated with UV-inactivated viral sham control. Collectively, these data suggest, and we hypothesise that at 48 and 72 h, a reduction in total intracellular ATP concentration, and an increase in ATP release without concurrent increased production is caused by a metabolic pressure elsewhere in the cell, probably a result of ICAM-1 stimulation (which will still be induced by the UV-inactivated viral sham control) and/or viral replication and early immune signalling pathways such as MAP kinase and NF-κB pathways, within the cells, enough to increase cellular metabolism. This would require further work to confirm, not under the scope of the work discussed here.

There are several limitations of this study, including our sampling method, duration of infection and cell line choice. We are aware that studying the effect of RV-16 infection long term without the presence of an aiding immune system makes our in vitro model not physiological; a tissue model would have been preferential. Our sampling method, while not ideal, has been used and published previously and we aimed to minimise any negative effect of our sampling, so we are confident in our data. Finally, the common cold, in particular RV, causes symptoms predominately in the upper respiratory tract, whereas our cell line of choice, alveolar epithelial A549 cells, is from the lower respiratory tract. However, RV infections are associated with frequent acute exacerbation of airway disease and research has shown the presence of pathogens including RV, thus whether these represent commensals of the microbiome of the upper and lower respiratory tract is unknown [35, 36]. We also believe that any effect that RV infection exerts in the airway would not be localised to the upper or lower respiratory tract. A prime example is that our data indicated ATP release. This ATP would move freely through the respiratory tract and likely cause hypersensitive airways precipitating a cough reflex, further spreading ATP throughout the airway.

The data presented here give an unprecedented insight into the aetiology of viral and post-viral cough. To date, research in the field of cough has largely focussed on the importance of ATP-sensitive P2X3 receptors and pharmacological inhibition of vagal sensitivity within the airway. However, the data presented here lead us to conclude that infection of RV-16 causes increased extracellular ATP release in the airway without a concomitant increase in ATP production and thus accounts for the airway hypersensitivity seen with viral cough. Thus, ATP release is important in post-viral cough and may be a target for the treatment of viral and post-viral cough.

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