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Volatile Emanations From *In Vitro* Airway Cells Infected With Human Rhinovirus

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Abstract

Respiratory viral infections such as human rhinovirus (HRV) can lead to substantial morbidity and mortality, especially in people with underlying lung diseases such as asthma and COPD. One proposed strategy to detect viral infections non-invasively is by volatile organic compound (VOC) assessment via analysis of exhaled breath. The epithelial cells are one of the most important cell lines affected during respiratory infections as they are the first line of pathogen defense. Efforts to discover infection-specific biomarkers can be significantly aided by understanding the VOC emanations of respiratory epithelial cells. Here we test the hypothesis that VOCs obtained from the headspace of respiratory cell culture will differentiate healthy cells from those infected with HRV. Primary human tracheobronchial cells were cultured and placed in a system designed to trap headspace VOCs. HRV-infected cells were compared to uninfected control cells. In addition, cells treated with heat-killed HRV and poly(I:C), a TLR3 agonist, were compared to controls. The headspace was sampled with solid-phase microextraction fibers and VOCs were analyzed by gas chromatography/mass spectrometry. We determined differential expression of compounds such as aliphatic alcohols, branched hydrocarbons, and dimethyl sulfide by the infected cells, VOCs previously associated with oxidative stress and bacterial infection. We saw no major differences between the killed-HRV, poly(I:C), and control cell VOCs. We postulate that these compounds may serve as biomarkers of HRV infection, and that the production of VOCs is not due to TLR3 stimulation but does require active viral replication. Our novel approach may be used for the *in vitro* study of other important respiratory viruses, and ultimately it may aid in identifying VOC biomarkers of viral infection for point-of-care diagnostics.

Keywords

Volatile organic compounds (VOC); human rhinovirus (HRV); respiratory tract infection; non-invasive diagnostics; respiratory cell culture; tracheobronchial epithelial cells (TBE); oxidative stress

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1. Introduction

Viral respiratory tract infections are a leading cause of morbidity and mortality in asthma and COPD (Varkey and Varkey, 2008). In particular, human rhinovirus (HRV) has been implicated in the initiation and intensification of asthma and as a trigger of COPD exacerbations (Grunberg and Sterk, 1999, Miller, 2010, Jacobs et al., 2013). It is well-described that the key to effective management, including avoidance of inappropriate anti-bacterial agents, is early identification of respiratory viral infections (Lee et al., 2010, Vigil et al., 2010).

The need for a non-invasive, rapid diagnostic of HRV and other viral and non-viral infections stimulates efforts to explore breath analysis as a potential diagnostic platform. To date, the complexity and low abundance of biogenic volatile organic compounds (VOCs) present serious obstacles to the wide application of breath analysis. Studies of VOCs produced by single cell lines may provide important insight into the origin of cell-specific compounds and help elucidate the impact of infection on changes in VOCs. This information may then be used to inform clinical studies assessing breath VOCs for detection of infections.

Previous studies have partially assessed host cellular changes associated with viral infection that can be used as diagnostic biomarkers. One study looked at up-regulated gene expression in peripheral blood associated with HRV, respiratory syncytial virus, and influenza infections (Zaas et al., 2009). Though the results are promising, the technology is invasive and does not reflect changes to respiratory cell VOC production specifically. Another study assessed changes in the peripheral blood cytokine profile in COPD subjects infected with HRV (Singh et al., 2010). This showed an association of HRV-infection with a skewed TH1/TH2 balance, suggesting HRV infection engenders an immunomodulatory response that leads to COPD exacerbations. Lopez-Souza et al assessed cytokine profiles in the supernatant over virus-infected airway cell cultures with an air-liquid interface design. They found increased expression of IL-1-alpha, IL-8, IP-10, and RANTES in HRV-infected airway cells (Lopez-Souza et al., 2009). Collectively, these studies are important first steps towards uncovering potential biomarkers of HRV infection in humans, though there is a paucity of studies specifically looking at VOCs emanating from infected respiratory cells. As airway cells are the primary site of many viral infections, including HRV, they represent a logical test bed to begin analyzing the complex field of VOC production with viral infection.

On a cellular level, little is known regarding VOC production in response to viral infection. Aksenov et al described VOC profile changes of human B-cell leukocytes after infection with multiple influenza virus strains and found production of VOCs associated with oxidative stress (Aksenov et al., 2014, in press). Earlier clinical studies found a subset of these VOCs in exhaled breath of patients treated with live attenuated influenza vaccine (Phillips et al., 2010), although the detailed mechanisms by which these VOCs are produced remains unclear. HRV is thought to initially trigger inflammatory effects via recognition by the epithelial pattern recognition receptor TLR3 during active replication (Wang et al., 2009, Zhu et al., 2009). Activation of TLR3 then leads to the induction of other subsequent pattern

recognition receptors such as retinoic acid inducible protein I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) (Wang et al., 2009). However, the exact subsequent inflammatory effects of TLR3-pathways stimulated by HRV are not well understood, and inflammation may result from an abnormal downstream signaling defect (Parsons et al., 2014). There have also been alternative sensing mechanisms proposed such as TLR7 and TLR8, though the data remain inconclusive as to their exact role in initiating an immune response to HRV (Morris et al., 2006). Even less clear is the role pattern recognition receptor pathways play in the generation of VOCs in virus-infected airway cells.

In this study we assessed the headspace VOC signatures of *in vitro* cultured human tracheobronchial epithelial (TBE) cells in native and HRV-infected states. Our aim was to identify specific VOCs that characterize HRV-infected TBEs which can potentially be used to diagnostically separate infected from uninfected patients. In addition, we explored one potential mechanism of VOC production by stimulating TLR3 pattern recognition receptors to determine if actively replicating virus, or the presence of dsRNA, was responsible for the observed VOC pattern seen in HRV-infected cells. Our model serves as a proof-of-concept platform that can eventually be used to detect multiple important respiratory viral infections.

2. Materials and Methods

2.1 Human respiratory cells

Human primary tracheobronchial epithelial (TBE) cells were obtained from tracheas harvested at the University of California, Davis Medical Center (Sacramento, CA) or the National Disease Research Interchange (NDRI, Philadelphia, PA). The University of California, Davis, Institutional Review Board approved all procedures involved in tissue procurement.

Preparation of the TBE cells follows the method described by Fulcher et al (Fulcher ML, 2005), and all media additives were purchased from Sigma Aldrich (St. Louis, MO). Protease-dissociated TBE cells were plated on Transwell (Corning Costar, Corning, NY) chambers (12 mm) at $1–2 \times 10^4$ cells/cm^2 in the growth medium; LHC Basal Medium (Life Technologies, Carlsbad, CA) supplemented with insulin (5 μg/ml), transferrin (10 μg/ml), epidermal growth factor (25 ng/ml), hydrocortisone (0.1 μM), triiodothyronine (0.01 μM), bovine hypothalamus extract (10 μg/ml), bovine serum albumin (0.5 mg/ml), epinephrine (0.6 μg/ml), phosphorylethanolamine (0.5 μM), ethanolamine (0.5 μM), zinc sulfate (3 μM), ferrous sulfate (1.5 μM), magnesium chloride (0.6 mM), calcium chloride (0.11 mM), and trace elements (selenium, manganese, silicone, molybdenum, vanadium, nickel sulfate, and tin).

Once TBE cultures were confluent, they were transferred to ALI (air-liquid interface) culture conditions in LHC Basal Medium/DMEM (1:1 ratio) supplemented with the additives as in the growth medium listed above, except that the epidermal growth factor was decreased to 0.5ng/mL and 30 nM ATRA was added for 7–10 days.

2.2 HRV infection

HRV 1B was kindly provided by Dr. Wai-Ming Lee (University of Wisconsin) and viral titers were determined by plaque assay as described by Duits et al (Duits et al., 2003). The virus is also available from commercial sources. A solution of phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA), with or without HRV 1B, was added to each culture chamber containing approximately 10^6 TBE cells (resulting in a multiplicity of infection (MOI) of 1). The cells were then incubated at 34°C for 1 hour then at 37°C for the time period specified in the text related to VOC sampling.

2.3 Poly(I:C) and killed HRV

To characterize if TLR3 activation is associated with VOC production in our model, we exposed TBE cultures to a synthetic analog of dsRNA, poly(I:C) (Field et al., 1972, Rider et al., 2013). Poly(I:C) was chosen because it corresponds to the transcribed ssRNA of HRV and it stimulates TLR3 but not other pattern recognition receptors such as TLR7 or TLR8. A 1 mL aliquot of 25 mcg/mL poly(I:C) (InvivoGen, San Diego, CA) was placed on each of three TBE culture wells. The wells were then placed in jars and incubated/handled as described below. Headspace VOCs were captured and analyzed at 12-, 24, and 48-hours as described below.

In addition, to further determine if VOC production was associated with the TBE cells' interaction with virus particle or with active viral replication, we exposed TBE cell cultures to heat-killed HRV. HRV 1B in PBS was heated in a 90 C water bath for 10 minutes. We have previously determined that the virus was denatured and inactivated after this exposure (data not shown). Aliquots of 100 mcl of heat-killed HRV 1B, MOI 1, were placed on each of three TBE culture wells. These were then handled and sampled as described below.

2.4 VOC collection

2.4.1 VOC headspace accumulation—60 mL clear glass jars with wide mouths (Thomas Scientific, Swedesboro, NJ) were prepared by autoclaving, and 0.045 inch thick PTFE/silicone septum screwtop lids were prepared by spraying with 70% ethanol and air drying in a laminar flow hood overnight. 5 mL of warmed air-liquid interface plus vitamin A (ALI+A) medium was added to each jar, and three individual Transwell TBE cultures were placed in the jars ensuring full contact with the medium. The lids were secured and Parafilm was placed over the jar tops to ensure no VOCs would exchange between the jar headspace and the ambient air. The jars with TBE cultures were placed in a 38°C incubator for 12 hours where VOCs were allowed to accumulate in the headspace. We selected the 12-hour time increments based on optimization studies which demonstrated the best signal:noise ratio of VOCs while maintaining the least amount of hypoxic damage.

2.4.2 VOC sampling—At 12 hour intervals, the jars were removed from the incubator and placed in a 38°C water bath. Solid phase microextraction (SPME) fibers (gray hub, divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS); Supelco, Bellefonte, PA) were introduced into the jar headspace where VOCs accumulated (see Figure 1). In addition, the headspace from jars that were i) empty and ii) contained ALI+A medium alone, were sampled to serve as background and to test if VOC signals came from these sources

(see Figure 2). The sampling outside of the incubator was necessary in order to minimize the VOC background from the incubator. We chose gray hub SPME fibers as preliminary studies showed the most complete VOC capture using these fibers in our cell culture system. VOC sampling occurred for 3 hours using an average of 2 SPME fibers in each jar, and then the SPME fibers were withdrawn and sent for analysis. Long exposure time was intended to aid in recovering greater amounts of volatile chemicals as sorption of volatiles on SPME fibers shifts equilibrium between gas-phase and dissolved compounds, as well as may lead to desorption of volatiles from the jar wall.

In the described setup, we were able to conduct VOC measurements in discrete time intervals. For cell cultures which produce high-abundance VOCs, an open system and/or continuous VOC measurements would be possible. However, the low-abundance VOCs produced in our system required that we allow the VOCs to accumulate to sufficiently high levels in order to measure them, and this precluded continuous measurement. It was imperative to maintain a sterile, contained environment to prevent cell death and to limit the introduction of extraneous contaminants during the sampling.

Following sampling, the jars were transferred from the bath to a laminar-flow hood where the lids were removed for 2 minutes in order to oxygenate the cells and purge the headspace before the next time point experiment. The lids were then replaced, Parafilm was secured over the jars, and the jars were transferred to the incubator in preparation for subsequent VOC accumulation and sampling.

2.5 VOC analysis

Following VOC collection, the gray hub SPME fibers were transported to a gas chromatography/mass spectrometry (GC/MS) for analysis. A Varian 3800 GC (VF-5ms 5% phenol/95% PDMS column), 4000 Ion Trap MS (Varian, Walnut Creek, CA) equipped with Electron Ionization (EI) source instrument was used. For analysis, each SPME fiber was inserted into the GC inlet and the adsorbed headspace chemicals were desorbed for 7 minutes 50 seconds at 250°C. The GC cycle was set as follows: 15 min hold at 5°C followed by 1°C/min oven ramp to 50°C, 5 min hold followed by 1°C/min ramp to125°C, 5°C/min ramp to 150°C and 20°C/min ramp to 230°C, for a complete run time of 149 minutes. The mass range scanned was 0–1000 Th. This protocol was optimized to allow for best resolution of individual chemical compounds produced by the TBE cells. In order to eliminate potential systematic sampling errors, the SPMEs from the virus-infected and control uninfected vials were analyzed in a random fashion.

In total 37 GC/MS profiles were collected and analyzed: 20 replicates for control and 17 for infected cells. Seventeen, 14, and 6 samples were collected for 12-, 24- and 48-hour time points correspondingly (both control and HRV-infected cells). Each peak in individual chromatograms was integrated and its area calculated using Varian MS Workstation MS Data Review software v 6.6. The peaks that corresponded to the same chemical compound, as verified by mass spectra matching, were tabulated across multiple runs.

2.6 Experimental setup

The experimental setup is detailed in Figure 2. The first experiment contained two control and two HRV-infected jars, and two SPME fibers were introduced into each jar at each time point (12- and 24-hour). Empty and ALI+A medium-only jars were sampled as well. The next three experiments used one less jar per group as the intra-group chromatograms were similar, and we omitted the baseline empty and ALI+A medium-only jars as the VOC signals from those sources were minimal. For the last two of these experiments (see Figure 2, C. & D.) we also added the 48-hour sampling time point as we demonstrated that the cells remained alive and healthy beyond 24 hours. We performed a separate experiments with the same protocol using poly(I:C) and heat-killed HRV with samples collected at 12-, 24-, and 48-hours. These independent experiments were conducted over several months.

2.7 Viability assessment

Alamar blue (Life Technologies, Carlsbad, CA) was used to assess the metabolic activity of the cells after each time point in some of the experiments. This was used as an assessment of cell viability in order to demonstrate that cell death was not responsible for any VOCs captured. A 1:10 dilution of Alamar blue:OptiMem medium was added to the apical surface of the Transwell, and OptiMem was used in the basal-lateral chamber. The Alamar blue solution was incubated with the cells for two hours at 37°C and assessed at 570 nm for a color change. Assessments were made at 1, 2, and 3-hour intervals after the initial 2 hour incubation. In addition, all cell cultures were inspected by light microscopy to assess for vacuolization and loss of mucous production, markers of cell death. See Figure 4 for a representation of the cell viability data.

2.8 Assessment for bacterial contamination

All TBE cultures were visualized at 12 hour intervals for cloudiness in the medium that would indicate microbial contamination. If the medium appeared cloudy, a small sample of the medium would be collected and stained with crystal violet to identify the contaminant. The TBE cultures were also assessed by light microscopy after 48 hours, post-VOC collection for evidence of bacterial or fungal contamination. Any indication of contamination characterized by haziness overlying the cells or in the medium would also warrant sample collection and staining to identify the contaminant.

2.9 Statistical analysis

The compiled tables of detected GC peaks were analyzed using unpaired Student's *t*-test with an alpha=0.05. In order to ensure the reproducibility of a chemical in each group (e.g. uninfected control cells at 12-hours), the presence of the chemical in >70% or <20% of the pooled samples was required to consider the chemical relevant. Peaks which did not satisfy this requirement were discarded. Peaks with unacceptably low signal:noise ratio, SPME/column/septa bleed peaks (siloxanes), and environmental contaminants were excluded as well. The peaks with statistically different abundances among control and infected groups were documented, and compounds corresponding to these peaks were identified using mass spectra matching with NIST Mass Spectral Search Software v. 2.0 with NIST 2005 and Wiley 2009 MS libraries. The matches with probability 80% or greater were assumed to be

the unknown compound; otherwise, the matching results were manually verified and the most likely candidate compound(s) were suggested as a tentative identification. The total number of detected compounds ranged from ~120 to 210 at various time points in both control and infected cells. Due to a relatively low number of compounds (see the Results section below), it was possible to manually verify the presence and identity of peaks to reduce the chance of a false discovery.

3. Results

3.1 VOC differences between control and infected cells

The GC analysis showed distinct peaks of volatile compounds emanating from the HRV-infected TBE cells as compared to control cells. Both infected and control cells had numerous VOCs which were clearly different than background VOCs (approximately 120–200 VOCs), though much fewer compounds separated the HRV-infected cells from control cells. An example of the different chromatographic patterns is shown in Figure 3. These data come from the first experiment and demonstrate and comparison of chromatograms from medium, TBE control cells, and HRV-infected TBE cells. The ALI+A medium has a low abundance VOC background as it does not contain any volatile components, and its GC patterns are similar to those seen from blank SPME fibers and the headspace from empty jars (data not shown). In this example it is notable that VOCs are either new or emitted in greater amounts after 24-hour HRV infection, and the increase in VOC output occurs in both a linear (i.e. Fig. 3, retention time 28 min) and a non-linear (Fig. 3, retention time 31.5 min) fashion. Some VOCs are emitted in lower amounts in the HRV-infection group.

After compiling the GC data from multiple individual experiments, it was possible to select VOCs that were commonly observed in all experiments using the criteria described in the “Materials and Methods, Statistical analysis” section. The list of chemicals was then reviewed, and the compounds that were differentially expressed in the control and HRV-infected groups were selected. The peaks are listed in the Table 1 along with the suggested chemical identity of these compounds (note that for some of the peaks the suggested identity is tentative). Some of the peaks, for example at R.T. 51.861 min at 12 hrs and 51.687 min at 48 hrs, based on the similarity of the EI fragmentation patterns, most likely belong to the same compound. The majority of the compounds produced by infected cells that differ from those of controls are produced at 48 hours.

3.2 Poly(I:C) and killed virus

The VOC spectra generated from the poly(I:C)-treated cells did not vary substantially from control TBE cells. The spectra did appear different from the HRV-infected cells (data not shown). Similarly, the heat-killed HRV-treated TBE cells also did not generate substantially different VOC spectra from the control cells (data not shown).

3.3 Bacterial contamination and viability studies

Using the methods described above, we did not identify bacterial contamination in any of the experiments.

All TBE cell cultures were assessed by light microscopy for evidence of stress (i.e. floating cells or holes in the monolayer). Cell death due to HRV infection was not anticipated as HRV generally does not cause respiratory cell death (Wark et al., 2009). The HRV-infected groups, in general, appeared to produce less of a mucous layer, but most regained that ability several days after the experiment finished. Alamar blue experiments were performed on two of the experiments and showed that all cells were i) alive and ii) had comparable activity between the control and HRV-infected groups. Figure 4 shows an example of the metabolic activity (indicated by the optical intensity measured at 570 nm wavelength) over time. Figure 4 was taken from two groups of cells at the 48-hour time point, and this is representative of all Alamar blue experiments performed.

4. Discussion

4.1 Interpretation of the main results

Individual cell lines produce distinct VOCs that may be indicative of cellular processes (Aksenov et al., 2013). The studies of VOCs produced in single cell line model systems provide important insights into the cellular processes and lay the groundwork for further diagnostic applications. The TBE cell cultures are one of the most important and interesting systems as the airway epithelium is the primary site of respiratory infections, and these cells are likely to contribute to the VOC composition of exhalate. Unfortunately, these cells are also challenging from an experimental perspective due to their low production of VOCs. As one of the main functions of the respiratory epithelium is to protect the tissues that lie beneath from pathogens, toxins, etc., the cell membrane may be more difficult to penetrate for intracellular compounds, including volatiles. Furthermore, epithelial cells form continuous sheets with very narrow intercellular space which may further prevent release of VOCs (Kojima et al., 2013).

In our experiments we overcame the challenge of low-abundance VOC production in two ways. First, by placing three ALI culture wells in each jar, we were able to increase the headspace VOC signal:noise ratio to a sufficient level. Next, we sampled the headspace for prolonged time periods using preconcentration on a sorbent, and this enabled us to increase our VOC capture. Though real-time VOC sampling will undoubtedly be necessary in a breath point-of-care diagnostic, our model is an initial platform from which we can identify important VOCs and develop future, more streamlined diagnostic systems. As stated in the “Results” section, using our experimental setup, we found that TBE cell cultures do produce cell-specific VOCs, albeit in relatively small amounts, and that these VOCs are altered by the HRV infection.

As can be seen from Table 1, the infection time course is reflected by changes in VOCs, and the most notable changes (i.e. the number of compounds differentially expressed in HRV-infected compared to control cells) occur at the prolonged infection time point of 48 hours. The specific chemicals we identified bear interpretation. Even though some of the compounds are not identified unambiguously, it is clear that the majority of the detected compounds are long chain/branched aliphatic compounds, predominantly alkanes/alkenes and alcohols. These compounds—specifically alkanes and methylated alkanes—were previously reported as biomarkers of live attenuated influenza viral vaccination in breath

(Phillips et al., 2003a, Phillips et al., 2003b, Phillips et al., 2000, Phillips et al., 2010) and they are also produced in a single cell culture (B cells) when infected with influenza A virus (Aksenov et al., 2014, in press). The production of these compounds is linked to oxidative stress during the infection (Schwarz, 1996) due to peroxidative cleavage of lipids (Phillips et al., 2000). A wide array of cellular lipids coupled with a non-specific free radical reactions would result in varying composition of produced compounds. However, the distributions of these compounds may contain useful information. Importantly, HRV is known to cause *in vitro* (as well as *in vivo*) oxidative stress (Patrick et al., 2013). Furthermore, acute asthma exacerbations and increased susceptibility to HRV effects are linked to oxidative stress (Hershenson, 2013). Thus, the production of the oxidative stress-related compounds in the present work is consistent with previous clinical studies. Though any one of these compounds may not be specific to HRV infection, per se, the distribution of them may be unique to HRV infection or to viral infection in general. To adequately study this, VOCs from many HRV replicates would need to be compared to VOCs from other viruses and from non-virus induced inflammation (i.e. bacterial infection or other inducers of oxidative stress).

An intriguing find from this study was the sulfur-containing compound dimethyl sulfide detected at the later infection stage (unique only to the 48 hour time point). This VOC has previously been identified from bacterial cultures (Schulz and Dickschat, 2007, Shestivska et al., 2012) and during bacterial infections of respiratory cells (Carroll et al., 2005, Goeminne et al., 2012). Dimethyl sulfide is likely a cleavage product of cysteine and methionine amino acids residues (del Castillo-Lozano et al., 2008) which contribute to volatile emissions during proteolysis. A similar sulfur compound, thiirane, was found to be produced by B cells when infected with influenza A virus (Aksenov et al., 2014, in press). The identification of dimethyl sulfide in our study suggests that viruses (HRV, and possibly influenza) cause protein cleavage as do bacteria. Though it is known that HRV proteases, such as picornovirus 2A and 3C proteases, mediate cleavage of necleoporin proteins that form nuclear transport pores in airway cells (Ghildyal et al., 2009), this is one of the first studies to identify volatile proteolytic products from HRV infection. We postulate that the degree to which HRV cleaves protein is likely far *less* than the cleavage from a bacteria such as *Pseudomonas*, as HRV causes much less host cellular destruction. This *differential* cleavage of proteins and subsequent release of dimethyl sulfide may form the basis of a way to differentiate between HRV and bacterial infection. We do not test this hypothesis here, though our findings do suggest that this is an important question for future research. Also, dimethyl sulfide and certain oxidized sulfur derivatives may function as cellular anti-viral compounds (Andersen et al., 1977, Aguilar et al., 2002), and low-level production of dimethyl sulfide by a respiratory cell infected with HRV may indicate natural anti-viral activity. This may, in turn, support using both the *presence* and *abundance* of dimethyl sulfide as a diagnostic biomarker of HRV activity.

As any viral infection is a dynamic process, it is reasonable to assume that a cell's response to infection changes as infection progresses. Though the exact mechanisms by which VOCs are produced and released are not always clear, it is likely that they are products of biochemical reactions that occur during an infection of a host cell (or during any cellular

perturbation, for that matter). As these biochemical reactions change during the course of an infection, the VOCs from these reactions would change as well. This is probably why the VOCs we identified from HRV-infected cells were different at the separate time points. These findings reflect the natural course of an HRV viral infection and support the concept of using VOCs to identify not only if an HRV infection exists, but the actual time since HRV infection occurred. In light of our data, we favor the 48 hour time point as the most diagnostically useful for two notable reasons. First, 48 hours represents the average time that people become symptomatic with an HRV infection. If we could encourage at-risk patients, such as those with severe asthma, to contact a physician at the first symptom of a viral infection, 48 hours post-infection would be the time (i.e. clinical relevance). Next, we observed the most *abundant* and the most *interesting* virus-related compounds at 48 hours. In particular, dimethyl sulfide may be a key molecule to help differentiate between bacterial infections (produced in relatively large amounts), HRV and other viral infections (produced in minor amounts), and non-infectious causes of upper respiratory tract inflammation (not produced). The clinical utility of this biomarker needs to be tested in future studies. Other compounds observed in this study, specifically oxidative stress products, may also be informative. For example, the distribution of produced VOCs in this study is different from that for the influenza infection (Aksenov et al., 2014, in press), which may bear phenomenologic significance (Wood et al., 2006). Future studies are needed to correlate the distributions of oxidative stress products with the underlying cause. At the same time, these compounds are rather non-specific, and may come from a multitude of sources. For example, acetic acid may arise through various pathways and its presence is only indicative of an oxidative environment during later stages of HRV infection. In this regard, compounds that may be directly attributed to a specific source, such as dimethyl sulfide, are of greater interest as potential biomarkers.

Our approach has several strengths. It is one of the first models, to our knowledge, that shows an epithelial cells' VOC response to HRV infection. As the respiratory epithelium is one of the first sites of HRV infection and replication, it is important to know which VOCs, and in what abundances, are released from these cells. This is essential to the body of work required to appreciate the origin of VOCs from human tissues, especially in pathologic states. Clearly more studies are needed to further our findings.

Our study demonstrates that respiratory epithelial cells emit VOCs both at baseline and in response to HRV infection in relatively low abundance. By comparison, in a separate study we conducted experiments of VOC production by B-lymphocytes (Aksenov et al., 2012) which, under similar conditions, produce a much greater amount of VOCs both in number and abundance. In addition to the specific properties of TBE cells discussed above such as low permeability of cell membrane, this difference may be due, in part, to the mucous layer produced by the epithelial cells. Such layers would present a barrier to the intracellularly-produced VOCs, although this still needs to be established.

As the human host response to HRV infection is complex and involves both the respiratory epithelium and other immunologic cells (innate and adaptive), our results suggest that due to low levels of VOC production, the contribution of epithelial cells to the total VOC output on a systemic level may be much less significant than for other cells. This is an important

implication of this study. Future *in vitro* studies should focus on non-epithelial cell lines including lymphocytes, neutrophils, and macrophages. In addition, there is evidence that T-cells potentiate TBE cell activity in response to HRV (Jornot et al., 2011), and therefore co-culturing of epithelial cells and lymphocytes using our approach is an important next step. Determining the VOC profiles of infected cells and comparing the results to clinical studies may help to understand the origin of exhaled VOCs and advance the field of breath analysis.

Clinical applications of the information gained from this study are several-fold. Validation studies may reveal single or a small set of VOCs which correlate well with HRV infection in human airway cells. This would form a basis for *in vivo* validation of non-invasively collected biomarkers. This would also enable early recognition of HRV infection which could translate to improved assessment of viral triggers for asthma and COPD exacerbations, improved epidemiologic surveillance of HRV, and a sparing of inappropriate anti-bacterial agents used for viral infections. In fact, even if the pattern of oxidative stress-related VOCs simply separates viral from bacterial infections (but cannot distinguish between different viruses), this information alone may help curb the overuse of antibacterial agents for upper respiratory tract infections (Linder, 2013). Furthermore, clinical studies designed to correlate levels of excreted dimethyl sulfide and other sulfur-containing compounds with the specific type of inflammation (viral, bacterial, allergic, etc.) may permit the development of non-invasive diagnostic tools to help triage patients.

4.2 Interpretation of Poly(I:C) and heat-killed HRV data

As TLR3 is one pathogen recognition receptor involved in viral detection, we assessed if triggering a TLR3 pathway yielded similar VOCs to those seen in live HRV-infected cells. In both experiments with the poly(I:C) and heat-inactivated virus, the VOC production did not differ significantly compared to control cells. Since poly(I:C) mimics double-stranded viral products, the lack of similar response to poly(I:C) suggests that the VOC signals produced by HRV infection occur prior to the conversion of HRV ssRNA to double-stranded RNA forms that are recognized by TLR3. We also assessed VOC production resulting from contact of TBE cells with heat-inactivated virus. The lack of response to heat-inactivated virus suggests that VOC production depends on live virus and not on inactive virus binding to the cell surface. This was expected, as formation of proteolysis-related compounds would not occur without virus activity and proliferation. Taken together, this indicates that VOC production in HRV-infected TBE cells *does not* depend on TLR3 signaling, and *does* require actively replicating virus. Whether this is a reflection of virus uncoating, viral transcription, or viral translation is unclear at this time. Because virus uncoating is presumed to occur in heat-inactivated virus as well, thus triggering TLR7 and TLR8, our data suggest that the observed VOC production after viral infection is more likely due to pathways independent of typical pathogen (virus)-associated molecular pattern (PAMP) signaling. As the majority of the VOCs observed in this study are presumed to result from oxidative stress, the triggering of the TLR3 pathway does not appear to result in activation of concomitant processes.

Looking forward, identification of HRV infection-specific VOCs coupled with an understanding of biochemical pathways that produce those VOCs will shed an important

light on several components of this research. Though we did not find that TLR3 stimulation lead to similar VOC production compared with HRV infection, there are many other pathways to explore. These include, but are not limited to, the other important pattern recognition receptor pathways TLR7 and TLR8. The benefits of this line of study are multiple. First, an understanding of how VOCs are produced, beyond nitric oxide, will provide a deeper understanding of how cells react to stress (e.g. inflammation, infection, hypoxia, etc.). This will augment our understanding of phenomena such as apoptosis, autophagy, and potentially malignancy. Next, by attaining further knowledge of cellular metabolic mechanisms, we may gain insight into the VOCs we see. For example, at this juncture it is largely unclear if VOCs are metabolic byproducts or key ingredients of biochemical reactions. Also, though some correlative VOC studies may be able to show *if* a VOC is increased in a disease state, they often cannot answer *why*. An understanding of the pathways that generate VOCs will help answer this important question. Last, an understanding of metabolic pathways that generate several VOCs will surely improve our focus and potentially lead to a refined VOC classification system. This will improve our diagnostic capabilities.

4.3 Limitations

As this study is proof-of-concept and *in vitro*, there are several limitations. First, the different VOCs we see between control and HRV-infected cells may not be reproducible *in vivo*. Many potential confounders exist when sampling VOCs from humans, including oropharyngeal, gastrointestinal, and systemically-derived VOCs (Solga and Risby, 2013). These may obscure VOCs from virus-infected cells. Also, though *in vitro* studies are important to understand a number of biologic processes, they often do not represent the complex biologic interactions present *in vivo*. Clearly, a large number of samples (e.g. breath or breath condensate) will need to come from many humans with viral respiratory tract infections to determine which VOCs are unique for specific viruses and stable under multiple physiologic conditions. Next, we used a single virus subtype, HRV 1B. As several HRV subtypes exist, each with differing levels of pathogenicity (Jacobs et al., 2013, Cox et al., 2013), one can expect distinct groups of VOCs to correlate with different subtypes. Future studies will need to assess this concept. Though we attempted to determine if the VOCs from our model derived from a TLR3-associated pathway or were a response to cellular contact with viral particles (heat-killed HRV), the results presented do not elucidate any particular mechanism for VOC production. In fact, the true mechanism for VOC production is likely complex and involves multiple pathways. Many more studies are needed to link VOC production to specific cellular pathways.

5. Conclusion

The experiments described here demonstrate that HRV 1B-infected TBE cells emit distinct VOCs compared to control cells in a model VOC collection system. The VOCs identified were reproducible over three independent experiments at different time points separated by several weeks, both for controls and infected cells. Thus, the differentially expressed VOCs are believed to represent products of inflammation. It is clear from our results that the main contribution to the produced VOCs comes from oxidative stress, a process commonly

associated with a viral infection. The most interesting VOC we identified was dimethyl sulfide, and this is likely due to the HRV virus causing low-level host cell proteolysis. Though this process also occurs during bacterial infections (both in *in vivo* and *in vitro* cell cultures), the liberation of dimethyl sulfide is likely lower in HRV infection compared to bacterial infection. This differential emanation of dimethyl sulfide may have potential as a diagnostic biomarker of HRV infection. The VOCs do not appear to result from cell death as viability experiments showed metabolically-active cells during and after the experiments, and there was no evidence of bacterial contamination. It is unclear which cellular pathways are responsible for VOC emanation as this study was limited in its exploration of the TLR-3 signaling pathway. Future studies will need to examine VOCs emanating from different HRV species, focus on biochemical pathway analysis, and move to understanding VOC production in human breath following HRV-infection. Our results indicate that the contribution of volatiles from epithelial cells may be minor compared to other cells in a systemic VOC profile.

Our approach holds promise as a useful tool to identify VOCs from multiple respiratory viral infections. This may lead to a practical, non-invasive, and rapid ability to diagnose viral respiratory infections in humans. By identifying unique VOC metabolites we hope to permit early detection of respiratory virus infection based on easily-obtained VOCs. This will enable up-front, aggressive treatments—such as bronchodilators, anti-inflammatory agents, and available anti-viral agents—which may reduce asthma and/or COPD exacerbation severity and improve long term control. Also, further studies will identify VOCs which may mark or participate in TBE-virus interactions, thereby enhancing our understanding of these processes.

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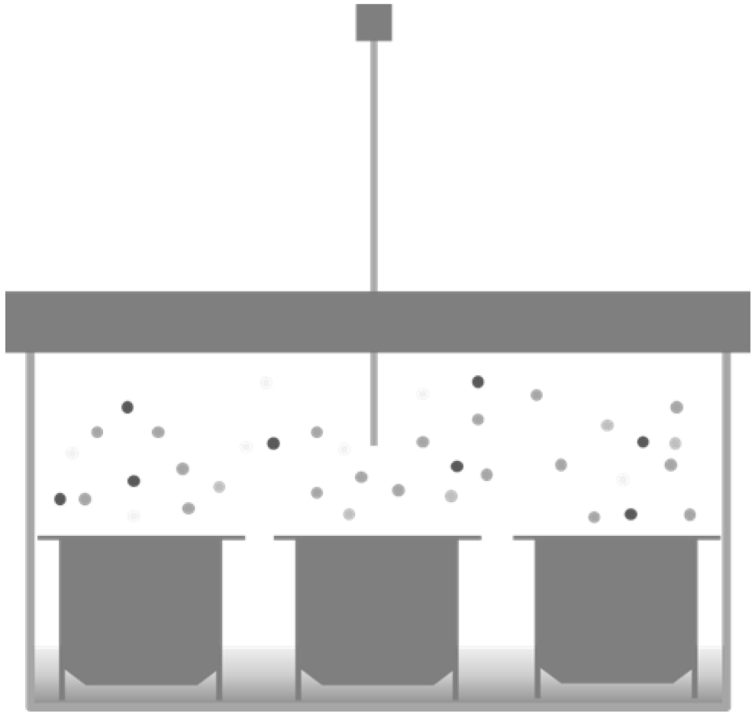


Figure 1.

Volatile organic compound (VOC) collection system. Three Transwell culture wells are placed in a jar with the basal ends in contact with ALI+A medium. VOCs accumulate in the headspace (represented by small dots). The top is sealed to prevent VOC-ambient air exchange. A solid-phase microextraction (SPME) fiber (top) is introduced through the lid septum into the headspace to sample the VOCs. *ALI+A*, air-liquid interface plus vitamin A

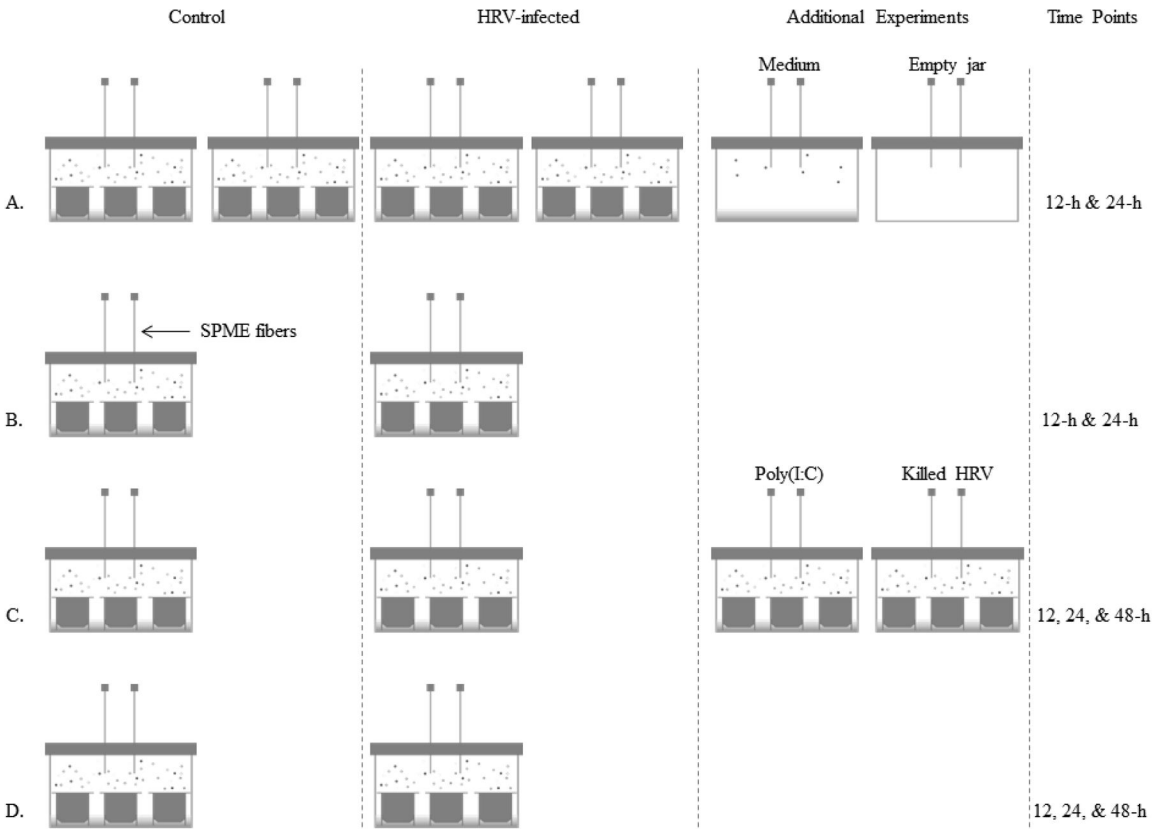


Figure 2.

Experimental setup. Four main experiments were performed, A through D. Two additional experiments were also performed, third column, "Additional Experiments": A. medium and empty jar; and, C. Poly(I:C) and heat-killed HRV. Each jar contains three air-liquid interface cultures and 5 mL of ALI+A medium (except in A, additional experiments). Each jar was sampled with two SPME fibers (arrow, noted) at each time point, designated in the last column, "Time Points." The major groups studied were Control and HRV-infected cells, first two columns, respectively. Experiments B, C & D contain one less jar in each main group because the VOC spectra within each group (e.g. the control group) were identical and therefore did not warrant replication. The 48-h time points were added to the last two experiments, C & D, as the cells were found to be alive and healthy after the first two experiments, A & B. *HRV*, human rhinovirus; *SPME*, solid-phase microextraction; *ALI+A*, air-liquid interface plus vitamin A.

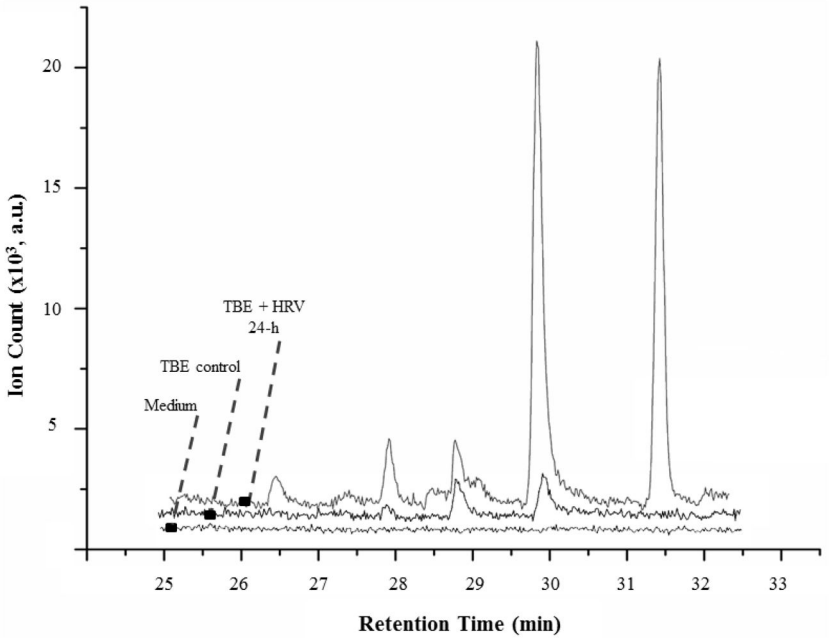


Figure 3.

Representative chromatograms of control and HRV-infected TBE cells compared to medium background. The chromatograms are slightly offset for clarity, and this example is from retention time 25 to 32.5 min. Note that the medium (ALI+A) gives little signal, as represented by the low ion count. In this example there is a significant up-regulation of VOC production after infection. *TBE*, tracheobronchial epithelial cells; *HRV*, human rhinovirus; *VOC*, volatile organic compound; *ALI+A*, air-liquid interface plus vitamin A

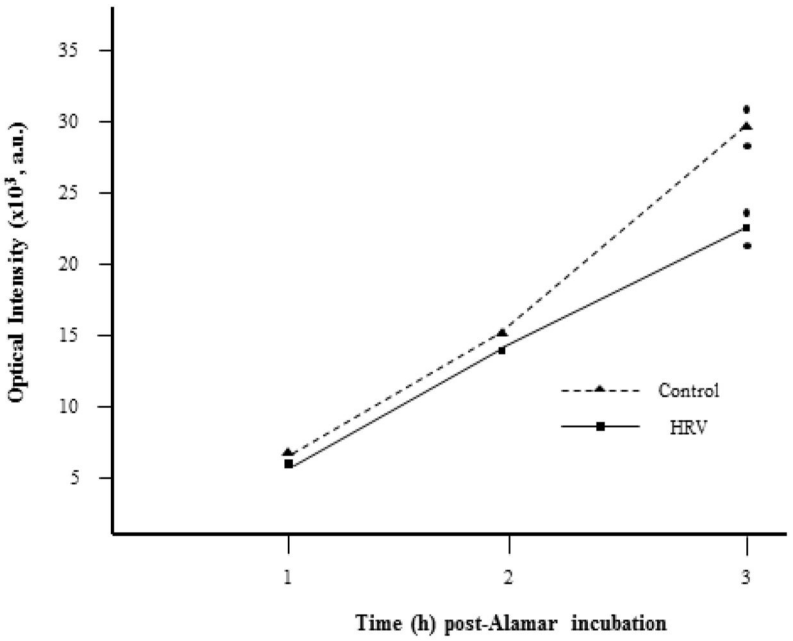
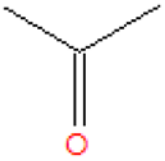


Figure 4.

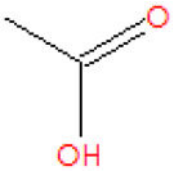
Representative Alamar blue measure of TBE cell viability. Both HRV-infected and control cells were assessed at three 1-hour time points after 2 hours incubation with Alamar blue. Optical intensity of the supernatant liquid was measured against a standard 570 nm wavelength. Increasing intensity correlates with cellular metabolic activity, which is a surrogate for viability. As seen, the control (dashed) and HRV-infected (solid) TBE cells have similar increases in optical intensity indicating that both are alive. Error boundaries (dots) are shown around the 3-hour time point, and these are similar for the other time points. This experiment was done on cells at 48-hours post-placement in the jars, and Alamar experiments on 12 and 24-hour cells provided similar results. *HRV*, human rhinovirus; *TBE*, tracheobronchial epithelial cells

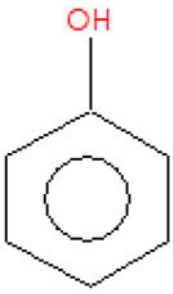
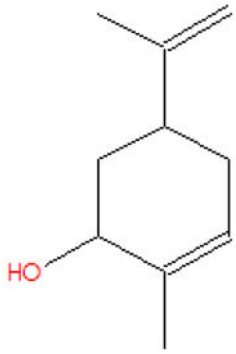
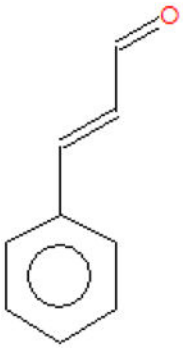
Table 1

Compounds differentially expressed between control and HRV-infected TBE cells at different infection time points.

12-hour infection		
Peak (R.T.)	**Proposed Structure**	**Other Possible Structure(s)**
1.690*	 Acetone	Oxygenated small molecule
29.934*	Organic ester, likely aromatic	
51.861*	Not identified (likely an aliphatic hydrocarbon)	
11.244*	Aliphatic compound	Isomer/homologue
124.614*	Aliphatic compound, e.g. E-7-tetradecenol	Isomer/homologue

24-hour infection		
Peak (R.T.)	**Proposed Structure**	**Other Possible Structure(s)**
50.955*	Not identified (likely an aliphatic hydrocarbon)	
71.855*	Hydrocarbon, e.g. 2,3,4-trimethyl-hexane	Isomer/homologue

48-hour infection		
Peak (R.T.)	**Proposed Structure**	**Other Possible Structure(s)**
2.624	 Dimethyl sulfide	
5.702	 Acetic acid	
51.687*	Not identified (likely an aliphatic hydrocarbon)	

48-hour infection		
Peak (R.T.)	**Proposed Structure**	**Other Possible Structure(s)**
59.847	 Phenol	
68.499*	Hydrocarbon, e.g. 2,3,4-trimethyl-2-pentene	Isomer/homologue
72.156*	Aliphatic alcohol, e.g. 2-propyl-1-heptanol	Isomer/homologue
74.064*	Aliphatic alcohol, e.g. 2-butyl-1-octanol	Isomer/homologue
75.964*	 2-methyl-5-(1-methylethenyl)-2-cyclohexen-1-ol	Isomer/homologue
94.805	 3-phenyl-2-propenal	

* Suggested chemical identities for these peaks are tentative, as described in the “Materials and Methods” section.