Asthma Predisposition and RSV Infection Modulate TRPV$_1$ Function in Children's Airways

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To the Editor

The transient receptor potential vanilloid 1 (TRPV$_1$) channel belongs to a family of proteins originally characterized in neuronal cells as a Ca^{2+}-permeable, non-selective cation channels that sense physical and chemical stimuli such as noxious heat ($>43°C$), pH (<6.5 at $37°C$), and vanilloid compounds like capsaicin (1). TRPV$_1$ has been shown to be expressed and functional in adult human bronchial epithelial (HBE) cells, and to play a role in airway disease through increased mucus production (2), stimulation of cough reflex (3), airway smooth muscle contraction (4), and increased vascular permeability (5). Importantly, TRPV$_1$ expression increases in airway epithelium from asthmatic as compared to non-asthmatic adult donors (6) and is upregulated by viral infections in neuroblastoma cells as well as primary and immortalized airway epithelial cells (7). However, age-dependent changes in TRPV$_1$ expression and function have never been studied, nor it is known how this channel is affected by intrinsic and extrinsic factors predisposing to asthma in early life. Thus, we hypothesized that TRPV$_1$ expression, localization, and function in HBE cells is age-dependent and is modulated by intrinsic asthma predisposition and intercurrent viral respiratory infections. To test this hypothesis, we first compared TRPV$_1$ related Ca^{2+} changes with the specific agonist capsaicin in primary HBE from children and adults with

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and without asthma, and then repeated the same measurements after infection with respiratory syncytial virus (RSV).

In preliminary experiments, capsaicin concentrations ranging from 20 to 300 μM activated $TRPV_1$ in a concentration-dependent fashion and with an EC_{50} of approximately 150 μM (Figure E1). $TRPV_1$-dependent $[Ca^{2+}]_i$ change was 5-fold larger in HBE from asthmatic children compared to that from age-matched non-asthmatic controls (p<0.001; Figure 1A). The capsaicin effect in non-asthmatic adults was approximately twice that measured in non-asthmatic children (p=0.07; Figure 1B), but increased only 2.5-fold in asthmatic adults (p<0.001). The average increment in $TRPV_1$-mediated Ca^{2+} influx was twice as large in HBE from asthmatic children than from asthmatic adults (5.9 ± 1.0 vs. 2.8 ± 0.4 units; p<0.05). We then examined the effect of RSV infection on $TRPV_1$ function in HBE derived from airways of children. We found that $TRPV_1$ dependent Ca^{2+} influx more than doubled in infected cells from both non-asthmatic (p<0.01;) and asthmatic (p<0.001) children (Figure 1C). Remarkably, $TRPV_1$-dependent $[Ca^{2+}]_i$ in RSV-infected cells from asthmatic children was about 10-fold above its baseline value. In contrast, RSV infection had no significant effect on adult cells, independently of their asthma status (Figure 1D). These $[Ca^{2+}]_i$ changes roughly paralleled the efficiency of RSV replication in different HBE cells, where a heavier viral load was seen in the bronchial epithelium of asthmatic children (Figure E2). To confirm that $TRPV_1$ is indeed the predominant calcium channel responsible for the increased $[Ca^{2+}]_i$ induced by capsaicin, $TRPV_1$ gene expression was silenced with a specific siRNA. This strategy achieved approximately 70% reduction in $TRPV_1$ mRNA transcripts as measured by qPCR (Figure E3), and importantly abolished the effect of RSV infection on capsaicin-induced Ca^{2+} influx in HBE from both asthmatic children (p<0.001; Figure 2) and non-asthmatic controls (p<0.01).

Our results demonstrate for the first time significant differences in $TRPV_1$-dependent Ca^{2+} flux in the airway epithelium that is dependent on age and asthma status. Specifically, the Ca^{2+} flux generated by stimulation of this channel is significantly greater in the bronchial epithelium of asthmatic vs. non-asthmatic children. This difference can also be measured in adults, albeit its average magnitude is approximately half than in children. More importantly, Ca^{2+} inflow in response to $TRPV_1$ stimulation doubles when children's epithelium is infected by RSV – the most common lower respiratory tract pathogen in infants and young children. The combined effect of this infection in children's asthmatic cells can increase $[Ca^{2+}]_i$ by 10-fold. In contrast, RSV has no effect on $TRPV_1$ function in adult HBE regardless of the asthma status of the donor. RSV effect on $TRPV_1$-mediated Ca^{2+} inflow correlates well with viral load, and therefore might result, at least in part, from more efficient entry and/or replication of RSV in the bronchial epithelium during early life.

Our data suggest that its role in the pathophysiology of RSV-related infant bronchiolitis and childhood asthma may be more important than in adult asthma. Also, our findings provide a suitable mechanism that may contribute to the different clinical manifestations of RSV infection in young children, in whom it frequently causes severe wheezing and cough poorly responsive to therapy with β-agonists and steroids, compared to the mild upper airway symptoms typical of this infection in adult life. The larger increment in Ca^{2+} permeability observed in HBE cells from asthmatic children is likely due at least in part to upregulation of

$TRPV_1$ rather than other Ca^{2+} channels, as shown by the finding that the increase in Ca^{2+} permeability in response to capsaicin was reduced significantly after silencing the $TRPV_1$ gene with a targeted siRNA. It is possible that other respiratory viruses might affect $TRPV_1$ differently from RSV. In particular, human rhinovirus (HRV) becomes the more common respiratory pathogen after the first years of life and is predominant in adults; however, a head to head comparison between RSV and different strains of HRV is quite complex and will require a separate study.

Although we have convincingly shown that $TRPV_1$ is the channel responsible for changes in $[Ca^{2+}]_i$, there are some limitations to these studies. We realize that other calcium channels may contribute to the RSV mediated increase in $[Ca^{2+}]_i$. Of note, $TRPA_1$ is activated upon rise in intracellular calcium (8); therefore, activation of $TRPV_1$ with consequent increase in $[Ca^{2+}]_i$ may in turn activate $TRPA_1$ leading to a greater rise in $[Ca^{2+}]_i$. We also recognize that these studies were performed *in vitro*, which may not fully recapitulate what occurs *in vivo*. Finally, studies using primary bronchial epithelial cells from deceased donors are intrinsically limited by the availability of donor cells, especially in the pediatric age group. Additionally, history of atopic status and lung function is frequently not available, as well as comorbidities and cause of death. Thus, it is possible that the age effect could be explained by other factors and future studies will need to be done using cells from identified samples from patients with documented asthma status and severity.

In conclusion, the results of this study show for the first time that lower airway epithelium from asthmatic children displays elevated basal $TRPV_1$ activity compared to non-asthmatic controls. We have also shown that RSV infection of epithelial cells from asthmatic children – but not from adults - leads to an increase in overall $TRPV_1$ activation. Pharmacological inhibition of TRPV1 may lead in the future to strategies that might reduce the impact of RSV infections in both asthmatic and non-asthmatic children.

Methods

Cells

Primary human bronchial epithelial cells derived from non-asthmatic or asthmatic Caucasian, Black, or Hispanic donors of both sexes were purchased from Lonza (Walkersville, MD). The age of pediatric donors ranged from 2 to 6 years. We chose to study this subgroup because several epidemiologic studies have shown increased risk of post-RSV wheezing by age 6, with subsequent marked decrease with age and resolution before adolescence. Cells were maintained at 37°C in 5% CO_2 using proprietary growth media and supplements provided by the vendor. The purity of cell cultures was assessed by immunocytochemistry for epithelial markers (e-cadherin; absence of fibroblast marker vimentin), visual morphology through passaging, and ability to differentiate into stratified epithelium by ciliation or goblet cells. Experiments were repeated using cells from different donors throughout the study to control for host genetics and environment (n = 3 non-asthmatic adults; 3 asthmatic adults; 4 non-asthmatic children; 3 asthmatic children). All experiments were performed on cells at passage number 2 to 5.

Virus

In order to easily verify active viral replication in our cells, we used a recombinant RSV A_2 strain expressing the red fluorescent protein (RFP) gene (rrRSV), which was kindly provided by Dr. Mark Peeples (Nationwide Children's Hospital, Columbus, OH) and Dr. Peter Collins (National Institutes of Health, Bethesda, MD) and was propagated using HEp-2 cells grown at 37°C/5%CO$_2$ in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% each of Glutamax, penicillin/ streptomycin, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Cells at approximately 50% confluence were inoculated with 3 ml per dish of virus stock diluted 1/10 with heat-inactivated FBS. After incubation for 2 h at 37°C, the inoculum was removed and replaced with 25 ml of fresh medium. The medium was replaced again 2 days later, and the virus was harvested after 1 to 2 additional days of incubation, at which point all cells appeared bright red when viewed under a fluorescent microscope. To harvest the virus, infected cells were scraped from the plate, separated with a pipette, mixed at medium speed, and pelleted by centrifugation at 1,200 g for 5 min. The supernatant was collected and cell debris removed by centrifugation at 9,500 g for 20 min in a centrifuge refrigerated at 4°C. One-ml aliquots of the supernatant were snap-frozen in liquid nitrogen and stored at −70°C until use. The final titer was determined with a modified plaque-forming unit assay.

To measure viral load in infected HBE, total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) and 25 ng per reaction was used for quantitative PCR (qPCR) analysis using the iTaq Universal Probes One-Step Kit (BioRad, Hercules, CA) and the CFX Connect real-time cycler (BioRad). Fluorescein amidite (FAM)-labeled RSV primer/probe set designed to detect the nucleocapsid gene and the positive control template were purchased from PrimerDesign (Chandler's Ford, United Kingdom). Ten-fold serial dilutions of cRNA transcripts corresponding to copy numbers 10^1 to 10^6 were used to build the standard curve. C_q values were plotted on the standard curve and copy number was calculated automatically with the CFX Manager software (BioRad). Each sample was tested in triplicate, and the arithmetic mean of the measurements was used as the sample copy number. Samples were considered negative if C_q values exceeded 38 cycles.

Measurement of intracellular calcium

The calcium response to TRPV$_1$ channel stimulation with capsaicin was measured using the Fluorogenic Imaging Plate Reader (FLIPR) assay (Molecular Devices, Sunnyvale, CA) in 80–90% confluent HBE grown in black-walled, clear-bottom Costar 96-well plates. Cells were loaded with Calcium-6 dye for 3 h and incubated at 37°C/5%CO$_2$, according to manufacturer's instructions. Plates were read in a Flexstation-3 reader, and fluorescence was measured over 3 min at 7-s intervals upon addition of ionomycin (0.5–1.0 μM in dimethyl sulfoxide; DMSO, Sigma-Aldrich, St. Louis, MO), capsaicin (150 μM in DMSO; Sigma-Aldrich), or vehicle. The ratio of maximum-minimum fluorescence/ionomycin fluorescence was used to calculate changes in intracellular Ca^{2+}. For selected experiments measuring release of Ca^{2+} from intracellular stores, HBE were incubated overnight with or without RSV, then loaded with Calcium-6 dye in Hanks Balanced Salt Solution (HBSS) containing HEPES either with or without Ca^{2+} for 3 h. All samples were measured in quadruplicate and experiments were repeated a minimum of 3 times.

Gene silencing

Exponentially growing bronchial epithelial cells were transfected with SMARTpool ON-TARGET plus (GE Dharmacon, Lafayette, CO) small interfering (si)RNA directed against TRPV$_1$ (50 nM) using Lipofectamine®2000 (Life Technologies, Carlsbad, CA), or ON-TARGET plus non-targeting pool siRNAs (50 nM). After 6 h transfection, the medium was replaced with fresh culture medium. Cells were harvested 48 h later and analyzed for knockdown of mRNA expression by qPCR.

Statistical analysis

All data are expressed as mean ± SEM. Multiple comparisons were performed by ANOVA followed by post-hoc analysis with the Dunnett's test using the software GraphPad Prism version 5.0 (La Jolla, CA). P values less than 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

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TRPV$_1$	Transient receptor potential vanilloid 1
RSV	Respiratory syncytial virus
HBE	Human bronchial epithelial
LRTI	Lower respiratory tract infection
siRNA	Small interfering RNA
[Ca^{2+}]$_i$	Intracellular Ca^{2+} concentration

References

1. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature. 1997; 389:816–24. [PubMed: 9349813]

2. Yu H, Li Q, Zhou X, Kolosov VP, Perelman JM. Transient receptor potential vanilloid 1 receptors mediate acid-induced mucin secretion via Ca2+ influx in human airway epithelial cells. J Biochem Mol Toxicol. 2012; 26:179–86. [PubMed: 22566028]

3. Groneberg DA, Niimi A, Dinh QT, Cosio B, Hew M, Fischer A, et al. Increased expression of transient receptor potential vanilloid-1 in airway nerves of chronic cough. Am J Respir Crit Care Med. 2004; 170:1276–80. [PubMed: 15447941]

4. Lin RL, Hayes D Jr, Lee LY. Bronchoconstriction induced by hyperventilation with humidified hot air: role of TRPV1-expressing airway afferents. J Appl Physiol. 2009; 106:1917–24. [PubMed: 19299576]

5. Hu DE, Easton AS, Fraser PA. TRPV1 activation results in disruption of the blood-brain barrier in the rat. Br J Pharmacol. 2005; 146:576–84. [PubMed: 16056236]

6. McGarvey LP, Butler CA, Stokesberry S, Polley L, McQuaid S, Abdullah H, et al. Increased expression of bronchial epithelial transient receptor potential vanilloid 1 channels in patients with severe asthma. J Allergy Clin Immunol. 2014; 133:704–12. [PubMed: 24210884]

7. Omar S, Clarke R, Abdullah H, Brady C, Corry J, Winter H, et al. Respiratory virus infection up-regulates TRPV1, TRPA1 and ASICS3 receptors on airway cells. PLoS One. 2017; 12:e0171681. [PubMed: 28187208]

8. Cavanaugh EJ, Simkin D, Kim D. Activation of transient receptor potential A1 channels by mustard oil, tetrahydrocannabinol and Ca2+ reveals different functional channel states. Neuroscience. 2008; 154(4):1467–76. [PubMed: 18515013]

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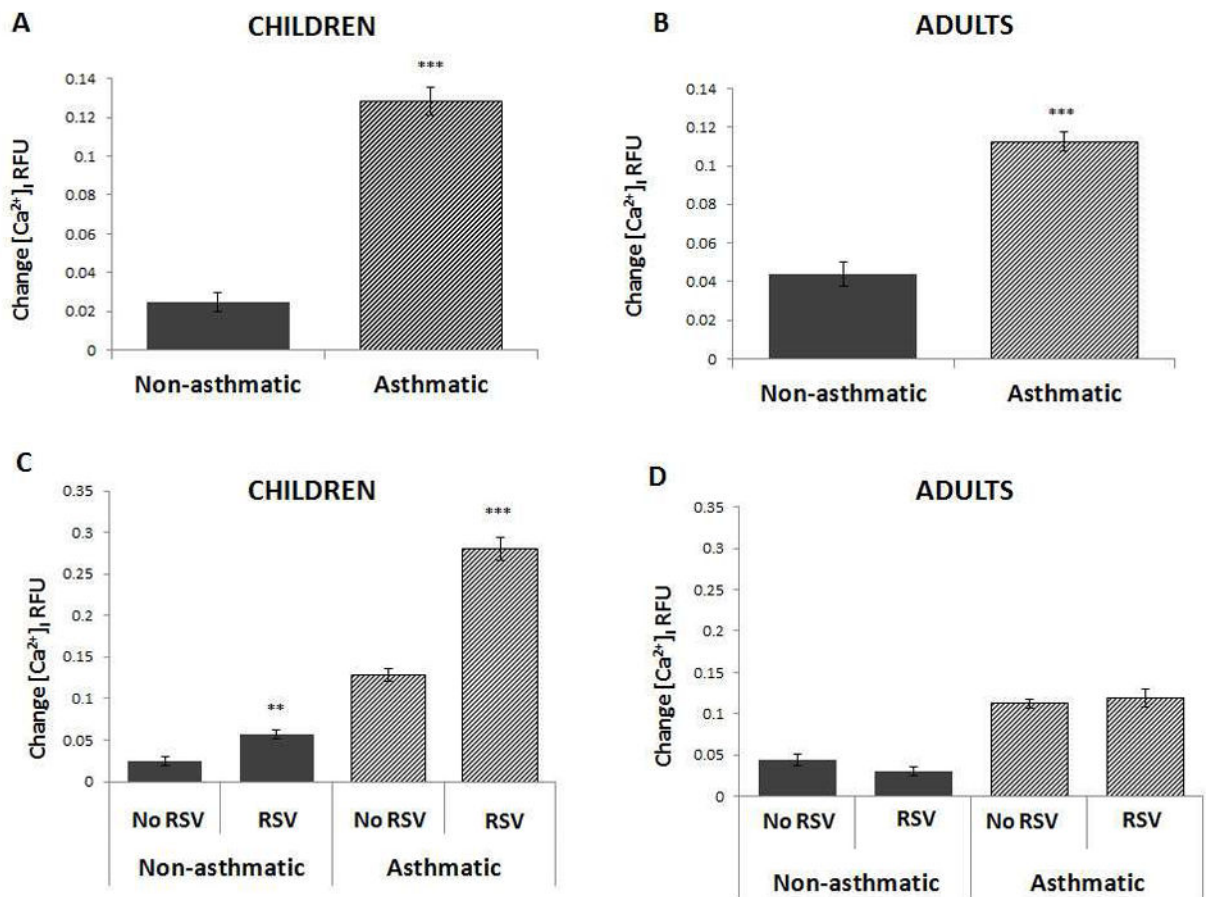
Figure 1. TRPV$_1$-mediated Ca^{2+} influx in HBE

A. [Ca^{2+}]$_i$ change induced by the specific TRPV$_1$ agonist capsaicin (150 μM) in primary HBE was significantly larger in asthmatic vs. non-asthmatic children. **B.** Difference in TRPV$_1$-mediated [Ca^{2+}]$_i$ between asthmatic vs. non-asthmatic adults was approximately 50% than in children. **C.** Capsaicin-induced [Ca^{2+}]$_i$ more than doubled in RSV-infected HBE (MOI of 1 for 24 h) from both non-asthmatic and asthmatic children. **D.** RSV infection had no significant effect on capsaicin-induced [Ca^{2+}]$_i$ in adult HBE, independently from asthma status. All experiments were repeated 2 times in quadruplicate. Data are expressed as mean ± SEM. **P<0.01, ***P<0.001 compared to non-infected controls.
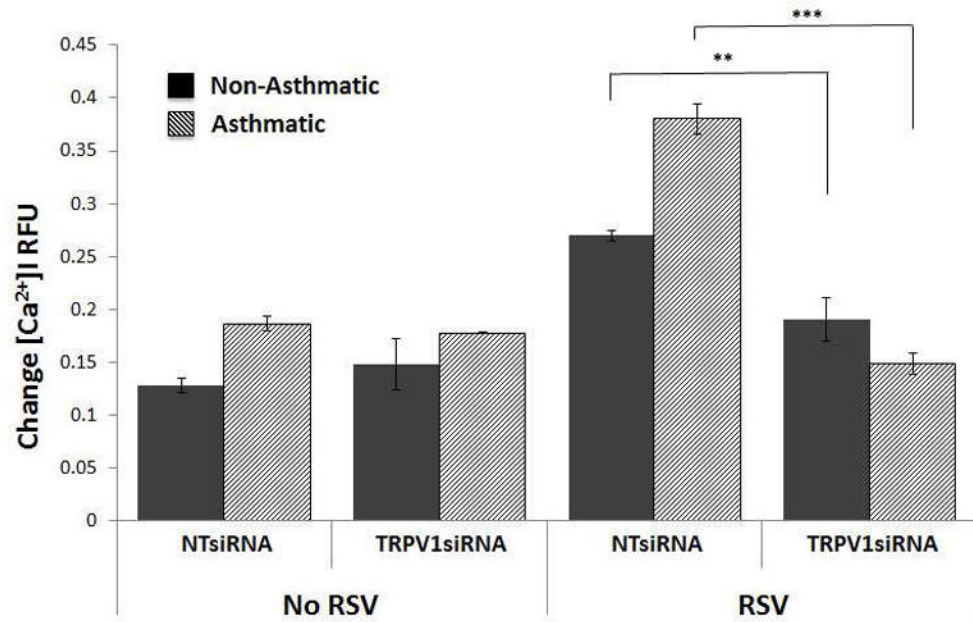
Figure 2.
TRPV$_1$ expression was silenced with a specific small interfering RNA (TRPV$_1$siRNA). This strategy achieved approximately 70% reduction in TRPV$_1$ mRNA transcripts as measured by qPCR and abolished the effect of RSV infection on capsaicin-induced [Ca^{2+}]$_i$ in HBE from both asthmatic children and non-asthmatic controls. All experiments were repeated 2 times in quadruplicate. Data are expressed as mean ± SEM. **P<0.01, ***P<0.001 compared to controls treated with non-targeting siRNA (NTsiRNA).