

Letter to the Editor

Asthma predisposition and respiratory syncytial virus infection modulate transient receptor potential vanilloid 1 function in children's airways

To the Editor:

The transient receptor potential vanilloid 1 (TRPV₁) channel belongs to a family of proteins originally characterized in neuronal cells as a Ca²⁺-permeable, nonselective cation channels that sense physical and chemical stimuli such as noxious heat (>43°C), pH (<6.5 at 37°C), and vanilloid compounds such as capsaicin.¹ TRPV₁ 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, TRPV₁ expression increases in airway epithelium from asthmatic as compared to nonasthmatic adult donors⁶ and is upregulated by viral infections in neuroblastoma cells as well as primary and immortalized airway epithelial cells.⁷ However, age-dependent changes in TRPV₁ 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 TRPV₁ 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 TRPV₁-related Ca²⁺ changes with the specific agonist capsaicin in primary HBE from children and adults with 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 µmol/L activated TRPV₁ in a concentration-dependent fashion and with a median effective concentration of ~150 µmol/L (see Fig E1 in this article's Online Repository at www.jacionline.org). TRPV₁-dependent intracellular Ca²⁺ concentration ([Ca²⁺]_i) change was 5-fold larger in HBE from asthmatic children compared with that from age-matched nonasthmatic controls ($P < .001$) (Fig 1, A). The capsaicin effect in nonasthmatic adults was approximately twice that measured in nonasthmatic children ($P = .07$) (Fig 1, B), but it increased only 2.5-fold in asthmatic adults ($P < .001$). The average increment in TRPV₁-mediated Ca²⁺ influx was twice as large in HBE from asthmatic children than from asthmatic adults (5.9 ± 1.0 vs 2.8 ± 0.4 units; $P < .05$). We then examined the effect of RSV infection on TRPV₁ function in HBE derived from airways of children. We found that TRPV₁-dependent Ca²⁺ influx more than doubled in infected cells from both nonasthmatic ($P < .01$) and asthmatic ($P < .001$) children (Fig 1, C). Remarkably, TRPV₁-dependent [Ca²⁺]_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 (Fig 1, D). These [Ca²⁺]_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 (see Fig E2 in this article's Online Repository at www.jacionline.org). To confirm that TRPV₁ is indeed the predominant calcium channel responsible for the

increased [Ca²⁺]_i induced by capsaicin, TRPV₁ gene expression was silenced with a specific small interfering RNA (siRNA). This strategy achieved approximately 70% reduction in TRPV₁ mRNA transcripts as measured by quantitative PCR (see Fig E3 in this article's Online Repository at www.jacionline.org), and importantly, it abolished the effect of RSV infection on capsaicin-induced Ca²⁺ influx in HBE from both asthmatic children ($P < .001$) (Fig 2) and nonasthmatic controls ($P < .01$).

Our results demonstrate for the first time significant differences in TRPV₁-dependent Ca²⁺ flux in the airway epithelium that is dependent on age and asthma status. Specifically, the Ca²⁺ flux generated by stimulation of this channel is significantly greater in the bronchial epithelium of asthmatic versus nonasthmatic children. This difference can also be measured in adults, albeit its average magnitude is approximately one-half that in children. More importantly, Ca²⁺ inflow in response to TRPV₁ 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 [Ca²⁺]_i by 10-fold. In contrast, RSV has no effect on TRPV₁ function in adult HBE regardless of the asthma status of the donor. RSV effect on TRPV₁-mediated Ca²⁺ 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 with the mild upper airway symptoms typical of this infection in adult life. The larger increment in Ca²⁺ permeability observed in HBE cells from asthmatic children is likely due at least in part to upregulation of TRPV₁ rather than other Ca²⁺ channels, as shown by the finding that the increase in Ca²⁺ permeability in response to capsaicin was reduced significantly after silencing the TRPV₁ gene with a targeted siRNA. It is possible that other respiratory viruses might affect TRPV₁ differently from RSV. In particular, human rhinovirus 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 human rhinovirus is quite complex and will require a separate study.

Although we have convincingly shown that TRPV₁ is the channel responsible for changes in [Ca²⁺]_i, there are some limitations to these studies. We realize that other calcium channels may contribute to the RSV-mediated increase in [Ca²⁺]_i. Of note, TRPA₁ is activated on rise in intracellular calcium⁸; therefore, activation of TRPV₁ with consequent increase in [Ca²⁺]_i may in turn activate TRPA₁ leading to a greater rise in [Ca²⁺]_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

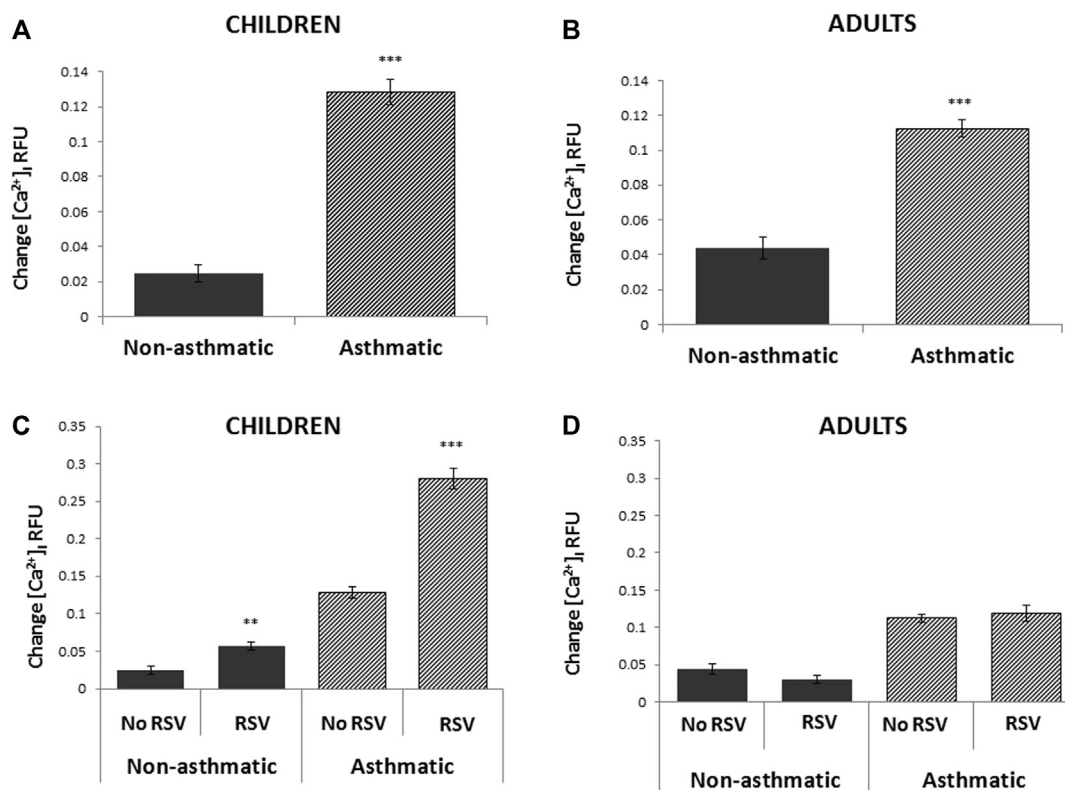


FIG 1. TRPV₁-mediated Ca²⁺ influx in HBE. **A**, [Ca²⁺]_i change induced by the specific TRPV₁ agonist capsaicin (150 μmol/L) in primary HBE was significantly larger in asthmatic versus nonasthmatic children. **B**, Difference in TRPV₁-mediated [Ca²⁺]_i between asthmatic versus nonasthmatic adults was approximately 50% less than in children. **C**, Capsaicin-induced [Ca²⁺]_i more than doubled in RSV-infected HBE (multiplicity of infection of 1 for 24 hours) from both nonasthmatic and asthmatic children. **D**, RSV infection had no significant effect on capsaicin-induced [Ca²⁺]_i in adult HBE, independently from asthma status. All experiments were repeated ≥2 times in quadruplicate. Data are expressed as mean ± SEM. ***P* < .01 and ****P* < .001 compared with noninfected controls. RFU, Relative fluorescence units.

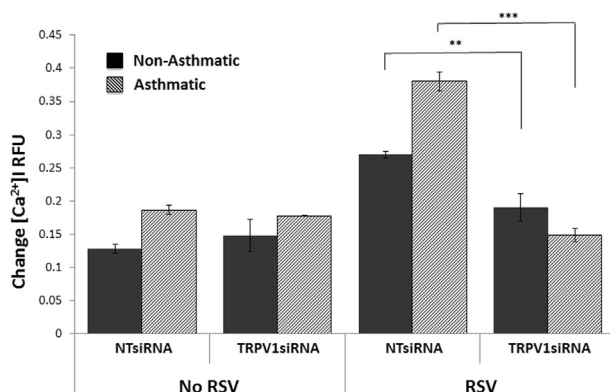


FIG 2. TRPV₁ expression was silenced with a specific siRNA (TRPV₁siRNA). This strategy achieved approximately 70% reduction in TRPV₁ mRNA transcripts as measured by quantitative PCR and abolished the effect of RSV infection on capsaicin-induced [Ca²⁺]_i in HBE from both asthmatic children and nonasthmatic controls. All experiments were repeated ≥2 times in quadruplicate. Data are expressed as mean ± SEM. ***P* < .01 and ****P* < .001 compared with controls treated with nontargeting siRNA (NTsiRNA).

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 TRPV₁ activity when compared with that of nonasthmatic controls. We have also shown that RSV infection of epithelial cells from asthmatic children—but not from adults—leads to an increase in overall TRPV₁ activation. Pharmacological inhibition of TRPV₁ may lead in the future to strategies that might reduce the impact of RSV infections in both asthmatic and nonasthmatic children.

This article is dedicated to the memory of Dr Mark E. Lauer, whose diligence and passion for science is left imprinted on all those lucky enough to have met and worked with him. We are indebted to Dr Mark Peeples (Nationwide Children's Hospital Research Institute, Columbus, Ohio) and Dr Peter Collins (National Institutes of Health, Bethesda, Md) for providing the original batch of the red fluorescent protein-expressing RSV virus.

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METHODS

Cells

Primary human bronchial epithelial cells derived from nonasthmatic 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% CO₂ 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 nonasthmatic adults; 3 asthmatic adults; 4 nonasthmatic children; 3 asthmatic children). All experiments were performed on cells at passage numbers 2 to 5.

Virus

To easily verify active viral replication in our cells, we used a recombinant RSV A₂ strain expressing the red fluorescent protein gene (rrRSV), which was kindly provided by Dr Mark Peeples (Nationwide Children's Hospital, Columbus, Ohio) and Dr Peter Collins (National Institutes of Health, Bethesda, Md) and was propagated using human epidermoid cancer cells grown at 37°C/5% CO₂ in Eagle minimum essential medium supplemented with 10% FBS and 1% each of Glutamax (Life Technologies, Carlsbad, Calif), penicillin/streptomycin, and 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 hours 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 1200g for 5 minutes. The supernatant was collected and cell debris removed by centrifugation at 9500g for 20 minutes in a centrifuge refrigerated at 4°C. One-milliliter 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, Calif) and 25 ng per reaction was used for quantitative PCR (qPCR) analysis using the iTaq Universal Probes One-Step

Kit (BioRad, Hercules, Calif) and the CFX Connect real-time cyler (BioRad). Fluorescein amidite-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 complementary RNA transcripts corresponding to copy numbers 10¹ to 10⁶ 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 TRPV₁ channel stimulation with capsaicin was measured using the Fluorogenic Imaging Plate Reader assay (Molecular Devices, Sunnyvale, Calif) in 80% to 90% confluent HBE grown in black-walled, clear-bottom Costar 96-well plates. Cells were loaded with Calcium-6 dye (Molecular Devices) for 3 hours and incubated at 37°C/5% CO₂, according to manufacturer's instructions. Plates were read in a Flexstation-3 reader (Molecular Devices), and fluorescence was measured over 3 minutes at 7-second intervals on addition of ionomycin (0.5-1.0 μmol/L in dimethyl sulfoxide; Sigma-Aldrich, St Louis, Mo), capsaicin (150 μmol/L in dimethyl sulfoxide; Sigma-Aldrich), or vehicle. The ratio of maximum-minimum fluorescence/ionomycin fluorescence was used to calculate changes in intracellular Ca²⁺. For selected experiments measuring release of Ca²⁺ from intracellular stores, HBE were incubated overnight with or without RSV, then loaded with Calcium-6 dye in HBSS containing HEPES either with or without Ca²⁺ for 3 hours. 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, Colo) siRNA directed against TRPV₁ (50 nmol/L) using Lipofectamine 2000 (Life Technologies, Carlsbad, Calif), or ON-TARGET plus nontargeting pool siRNAs (50 nmol/L). After 6 hours of transfection, the medium was replaced with fresh culture medium. Cells were harvested 48 hours later and analyzed for knock-down 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 test using the software GraphPad Prism version 5.0 (La Jolla, Calif). *P* values of <.05 were considered statistically significant.

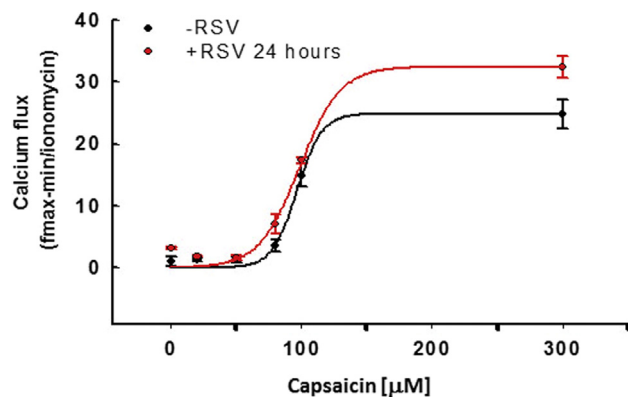


FIG E1. Dose-dependent effect of capsaicin on TRPV₁-mediated Ca²⁺ influx. Change in [Ca²⁺]_i following stimulation with the specific TRPV₁ agonist capsaicin was measured in HBE after 24 hours' incubation with RSV at multiplicity of infection of 1 or sterile medium. Capsaicin concentrations ranging from 20 to 300 μmol/L activated TRPV₁ in a concentration-dependent fashion and with a median effective concentration of approximately 150 μmol/L. [Ca²⁺]_i was measured using Calcium-6 dye and expressed in relative fluorescence units. All experiments were repeated ≥2 times in quadruplicate. Data are expressed as mean ± SEM.

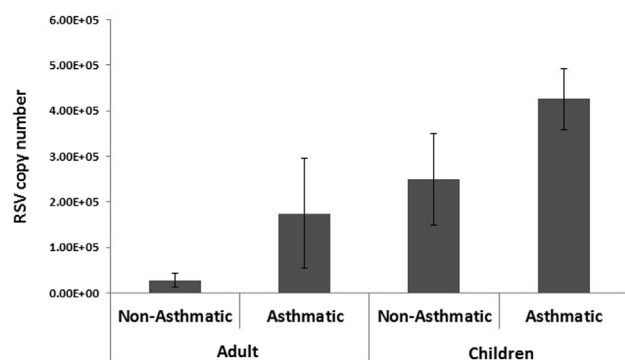


FIG E2. Efficiency of RSV replication as a function of age or asthma status. Viral load measured by qPCR in HBE from adult or children with or without asthma after 24 hours' incubation with RSV at multiplicity of infection of 1. RSV replication in the bronchial epithelium was dependent on asthma predisposition and age, being more efficient in asthmatic children than in nonasthmatic children and more efficient in nonasthmatic children than in asthmatic adults. All experiments were repeated ≥ 2 times in quadruplicate. Data are expressed as mean $\pm$ SEM.

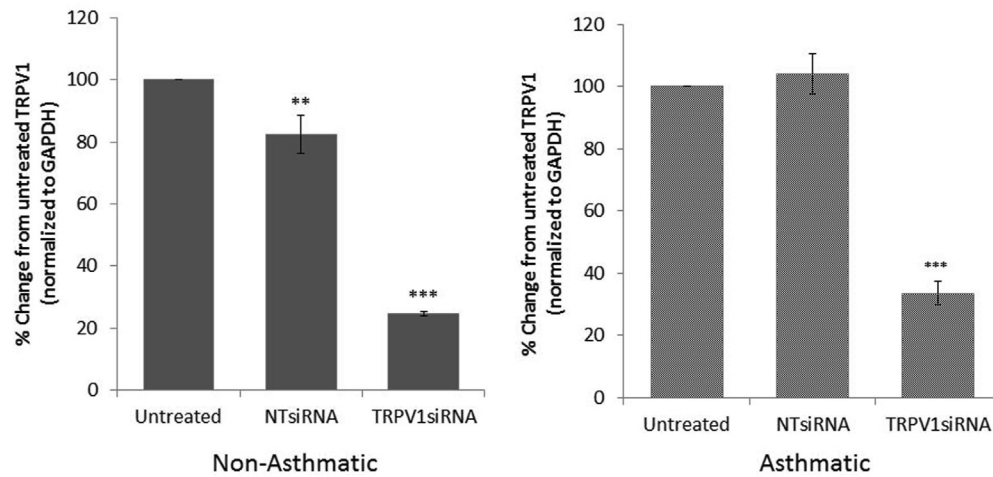


FIG E3. Efficiency of TRPV₁ gene silencing. Primary HBE from nonasthmatic or asthmatic donors were transiently transfected with NTsiRNA or 50 nmol/L TRPV₁-specific siRNA for 48 hours and then assessed for TRPV₁ mRNA expression by qPCR. All experiments were repeated ≥ 2 times in triplicate. Data are expressed as mean $\pm$ SEM. ** $P < .01$ and *** $P < .001$ compared with untreated controls ($n = 2$ per group). *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.