Capsaicinoids Cause Inflammation and Epithelial Cell Death through Activation of Vanilloid Receptors

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Capsaicinoids, found in less-than-lethal self-defense weapons, have been associated with respiratory failure and death in exposed animals and people. The studies described herein provide evidence for acute respiratory inflammation and damage to epithelial cells in experimental animals, and provide precise molecular mechanisms that mediate these effects using human bronchial and alveolar epithelial cells. Inhalation exposure of rats to pepper sprays (capsaicinoids) produced acute inflammation and damage to nasal, tracheal, bronchial, and alveolar cells in a dose-related manner. In vitro cytotoxicity assays demonstrated that cultured human lung cells (BEAS-2B and A549) were more susceptible to necrotic cell death than liver (HepG2) cells. Transcription of the human vanilloid receptor type-1, VR1 or TRPV1, was demonstrated by RT-PCR in all of these cells, and the relative transcript levels were correlated to cellular susceptibility. TRPV1 receptor activation was presumably responsible for cellular cytotoxicity, but prototypical functional antagonists of this receptor were cytotoxic themselves, and did not ameliorate capsaicin-induced damage. Conversely, the TRPV1 antagonist capsazepine, as well as calcium chelation by EGTA ablated cytokine (IL-6) production after capsaicin exposure. To address these seemingly contradictory results, recombinant human TRPV1 was cloned and overexpressed in BEAS-2B cells. These cells exhibited dramatically increased cellular susceptibility to capsaicinoids, measured using IL-6 production and cytotoxicity, and an apoptotic mechanism of cell death. Surprisingly, the cytotoxic effects of capsaicin in TRPV1 overexpressing cells were also not inhibited by TRPV1 antagonists or by treatments that modified extracellular calcium. Thus, capsaicin interacted with TRPV1 expressed by BEAS-2B and other airway epithelial cells to cause the calcium-dependent production of cytokines and, conversely, calcium-independent cell death. These results have demonstrated that capsaicinoids contained in pepper spray products produce airway inflammation and cause respiratory epithelial cell death. The mechanisms of these cellular responses to capsaicinoids appear to proceed via distinct cellular pathways, but both pathways are initiated by TRPV1.

Key Words: capsaicinoids; vanilloid receptors; TRPV1; cytokines; pepper sprays; inflammation; bronchiolar epithelial cells; BEAS-2B cells.
hereafter. TRPV1 was the first member of the growing family of vanilloid receptors to be characterized (Caterina and Julius, 2001; Montell et al., 2002; Szallasi, 2001) and has been described as a cell membrane-bound ligand-gated calcium channel, with high selectivity for capsaicin and other vanilloid-like compounds. TRPV1 has also been shown to be activated by acidic pH and temperatures >42°C (Caterina and Julius, 2001; Montell et al., 2002; Szallasi, 2001).

Since the discovery of TRPV1, a variety of other vanilloid receptor-like proteins (e.g., VRL-1, VRL-2, VR-OAC, SIC, TRPM8, and VR.5’sv) have been identified (Caterina et al., 1999; Delany et al., 2001; Schumacher et al., 2000). Recent data have placed the vanilloid receptors in an ever-expanding family (Montell et al., 2002) of TRP ion channels that includes not only ligand-, heat-, and pH-activated calcium channels, but receptors that are activated by cold (McKemy et al., 2002), extracellular osmolality (Liedtke et al., 2000; Strotmann et al., 2000), and cell volume (Suzuki et al., 1999). Some of these receptors (i.e., VR.5’sv and VRL-2) do not have known functions and/or agonists (Delany et al., 2001; Schumacher et al., 2000). This intriguing family of genes presents the scientific community with a cornucopia of receptors that appear to respond to an amazing variety of environmental stimuli, including noxious irritants, environmental pollutants, and temperature (Caterina and Julius, 2001).

Perhaps the most intriguing facet of the identification of the vanilloid receptor family of ion channels has been that their functions do not appear to be limited only to the perception of noxious stimuli (i.e., capsaicin, pH, or heat) through activation of nerve fibers, but that several of the vanilloid receptors (e.g., the VRL-2 and VR.5’sv receptors) are highly expressed in non-neuronal cells (Hayes et al., 2000; Inoue et al., 2002; Sanchez et al., 2001) including epithelial cells of the kidney and respiratory tissues (Delany et al., 2001; Hayes et al., 2000). To date, however, a physiological role for these receptors in non-neuronal tissues has not been established.

Previous research has demonstrated that the activation of TRPV1 expressed by cultured neurons isolated from rat dorsal root ganglia promoted cell death (Szallasi and Blumberg, 1999). The cytotoxic properties of capsaicinoids in peripheral sensory (Aδ and C-fiber) neurons have been well documented (Szallasi and Blumberg, 1999; Wood et al., 1988) and are exploited for the treatment of chronic pain (McMahon et al., 1991). The mechanism of neurotoxicity by vanilloid receptor ligands has been shown to be calcium-dependent, inhibited by capsazepine and ruthenium red, and, thus, mediated by TRPV1. The role of TRPV1 in the cytotoxicity of capsaicin in non-neuronal cell lines has also been investigated, but not fully elucidated. For example, HEK293 cells engineered to overexpress rat TRPV1 demonstrated enhanced calcium flux and cell death that was inhibited by capsazepine, ruthenium red, and by removal of calcium from the media (Caterina et al., 1997; Jordt et al., 2000). However, a variety of other cell lines, including monkey kidney (Vero; Creppy et al., 2000), human neuroblastoma (SHSY-5Y; Richeux et al., 1999), and human endothelial (ECV340; Richeux et al., 2000) were not protected from cytotoxicity by capsazepine or modulators of calcium flux unless rat TRPV1 was transfected into these cells. Similarly, human glioblastoma (A172) cells (Lee et al., 2000) were not protected by capsazepine or modulators of calcium flux, despite the apparent endogenous expression of TRPV1. Therefore, a general mechanism to explain the TRPV1-mediated cytotoxicity of capsaicin (and other vanilloid compounds) has not been established. Rather, it appears that different cell lines respond in unique manner to TRPV1-mediated signaling induced by ligand binding.

Capsaicinoids have also been used to study the cough reflex and neurogenic inflammation in respiratory tissues. In neurogenic inflammation, capsaicin promotes the calcium- and TRPV1-dependent release of Substance P, and other neuropeptides from neurons in the airway tissues (Veronesi et al., 1999, 2000), to stimulate inflammatory responses to potentially harmful stimuli, including particulate material. Recent work at the United States Environmental Protection Agency has demonstrated that capsaicin, particulate matter, and neuropeptides acted synergistically to promote the production of inflammatory mediators (IL-6, IL-8, and tumor necrosis factor-α [TNF-α]) by human respiratory epithelial cells: human bronchiolar epithelial cells (BEAS-2B), human lung adenocarcinoma cell line (A549), and normal human bronchiolar epithelial cells (Quay et al., 1998; Veronesi et al., 1999, 2000). Cytokine production by BEAS-2B cells was ameliorated by capsazepine and by removal of calcium from the treatment solutions (Veronesi et al., 1999, 2000). Similar cytokine responses were also observed in rats (intratracheal instillation) and humans (bronchoscope) treated with concentrated ambient particulate material (Carter et al., 1997; Lay et al., 1999). Thus, direct activation of TRPV1 in these cells by various stimuli can cause calcium-dependent cytokine production and acute respiratory inflammation.

Although these data provided evidence for the expression of functional TRPV1 in these cells, direct evidence of TRPV1 expression was not provided. Also, the influence of TRPV1 on cellular susceptibility to cytotoxicity by these substances was not investigated, despite observation in vivo that demonstrated increases in lactate dehydrogenase (LDH) activity in bronchoalveolar lavage fluid of treated animals and humans (Carter et al., 1997; Lay et al., 1999). Therefore, it seems likely that activation of vanilloid receptors, presumably TRPV1, in respiratory epithelial cells by capsaicinoids initiates the production of proinflammatory cytokines to mount a host-defense response to protect against potentially harmful inhaled cytotoxic substances including capsaicin and particulate material. Unfortunately, this response may lead to cell death.

Thus, a hypothesis is formulated that capsaicinoids, which are present in pepper spray products, induce acute inflammation and respiratory epithelial cell injury through activation of TRPV1 in rat and human respiratory tissues. Activation of TRPV1 may induce cell death through the production of cytokines that are toxic to the same cells that have produced
them; alternatively, cell death may be independent of cytokine effects. This hypothesis was addressed by nose-only inhalation exposure of rats to pepper sprays (capsaicinoids), by in vitro studies with human lung epithelial (BEAS-2B and A549) or liver cells: the human hepatoma cell line (HepG2), and by the production and characterization of a TRPV1 overexpressing human lung epithelial cell line.

MATERIALS AND METHODS

Reagents. Capsaicin (97%), nonivamide (99%), resiniferatoxin (RTX), anandamide, capsazepine, ruthenium red, EGTA, ketamine hydrochloride, and acetropomazine maleate were purchased from Sigma Chemical Corp. (St. Louis, MO). Olvanil, scutigalad, phorbol-12-myristate-13-acetate-20-homo-movanilate (PPAHV), and isovellar were purchased form Alexis Biochemicals Inc. (San Diego, CA). Cell culture media was purchased from BioSource International (Camarillo, CA). Pepper spray canisters were purchased from independent distributors and sampled as previously described (Reilly et al., 2001b,c). Briefly, pepper spray canisters were cooled to −20°C overnight in a freezer, shaken, and gently discharged into a silanized glass tube that had been previously equilibrated to −80°C using dry ice. Cooling the tubes with dry ice was necessary to prevent evaporation of the solvent during collection. The sample was immediately capped and thawed on ice. The volume sample was determined and the volatile components were permitted to evaporate at room temperature for 1 h with gentle agitation. The original sample volume was established by addition of dehydrated ethanol and the capsaicinoid concentrations were determined by LC/MS, as previously described (Reilly et al., 2001b,c).

Nose-only inhalation. Male Sprague-Dawley rats (125 g) were purchased from Charles River Laboratories (Wilmington, MA). Prior to exposure to the capsaicinoids, the rats were anesthetized by ip injection of 80 mg/kg ketamine and 5 mg/kg acetromazine. The animals were placed inside a nose-only exposure apparatus (In-Tox Products, Albuquerque, NM) and exposed (30 min) to aerosols generated from ethanolic solutions of capsaicinoids that were collected from pepper spray canisters (Reilly et al., 2001a). Aerosols were generated using a Lovelace nebulizer operated at a flow rate of approximately 0.5 l/min. The chamber flow was approximately 10 l/min and was maintained under a vacuum of −0.5 psi (approximately 26 mm Hg). Using this protocol, approximately 85% of the generated aerosol particles were between 1.7 and 0.2 μm median mass aerodynamic diameter, determined using a seven-stage cascade impactor. An estimate of the delivered dose was achieved by quantitative analysis (Reilly et al., 2001a,b) of a paper filter (0.2 μm) that collected aerosol from a sampling orifice. The sampling rate for the filter was approximately 0.5 l/min. The delivered dose was calculated using a minute volume of 0.2 l/min and an approximate deep lung deposition factor of 10% (Edward Barr, Lovelace Respiratory Research Institute, Albuquerque, NM, personal communication). Prior to each experiment, all gas flows were calibrated. The concentrations of capsaicinoids in lung and blood of rats exposed to capsaicinoids using this protocol was recently published (Reilly et al., 2002).

Following exposure, the animals were removed from the chamber and permitted to recover. Following the predetermined recovery periods, the animals were sacrificed by CO2-induced asphyxiation and the nasal passages, trachea, and lungs were excised. A catheter-tipped syringe was used to manually infuse approximately 250–500 μl of 10% neutral-buffered formalin into the trachea and lungs until they increased approximately two-fold in volume. This degree of inflation was sufficient to observe small airway architecture. The respiratory tissues were treated for 2–3 days in fixative prior to sample preparation and sectioning through the major bronchiolar and distal alveolar regions. Nasal samples were obtained by sectioning through the first and second palatal ridges of decalcified heads (Young, 1981), prepared by treating the heads for approximately 36 h in CalExl-decalcifying/fixing solution (Fisher Scientific, Fairlawn, NJ). The samples were sectioned, stained with hematoxylin and eosin, and evaluated using both qualitative and semiquantitative criteria (i.e., a numerical score was used to represent the frequency and/or severity of the lesions). For example, the degree of inflammation (congestion and edema) was scored 0–4 based on the size of the lesion (i.e., the entity was not present versus an entity greater than >0.5 mm). Cellular infiltrate was scored 0–4 by counting the number and type of cells observed in four different fields at 45X while epithelial loss was scored 0–4 based on the presence or absence of the lesion, its continuity, and its frequency. Necrosis, epithelial dysplasia, squamous metaplasia, inflammation (congestion and edema), epithelial loss, cellular infiltrate (lymphocytes, plasma cells, macrophages, neutrophils, and eosinophils), fibroendothelial proliferation, fibrosis, giant cells, goblet cell hyperplasia, hemorrhage, and alveolar emphysema were all evaluated by semiquantitative criteria. Lesions that appeared at a high frequency and to moderate to marked severity (a score of 1–4) in at least 50% of the treated population were identified and described below.

Cell culture. Immortalized human bronchiolar epithelial (BEAS-2B), human lung adenocarcinoma (A549), and human hepatoma (HepG2) cell lines were obtained from American Type Culture Collection (Rockville, MD). BEAS-2B cells were cultured in Lechner and LaVeek media (LHC-9), containing gluteraldehyde (33 nM) and epinephrine (2.75 μM), using plastic cell culture dishes pre-coated with LHC-basal medium containing BSA (100 μg/ml) collagen (30 μg/ml) and fibronectin (10 μg/ml) for 4 h at 37°C. A549 cells were cultured using DMEM:F12 media containing 10% FBS. HepG2 cells were cultured in Eagle’s MEM (Gibco BRL) supplemented with 1 mM sodium pyruvate, 2 mM sodium bicarbonate, and 10% FBS. All cells were maintained in 75-cm² flasks at 37°C in an air ventilated and humidified incubator maintained at 5% CO₂. Culture media was renewed every 2–3 days and cells were subcultured every 5–6 days using 0.25% trypsin.

Cytotoxicity assays. Cells were subcultured into 96-well cell culture plates at ~75% confluency and permitted to adhere for 8–12 h at 37°C. The cells were washed once with sterile phosphate-buffered saline and treated with increasing concentrations of capsaicin (0–200 μM) prepared in 100% ethanol and maintained at 0.5% v/v in the treatment solutions, or other TRPV1 ligands, in serum-free cell culture medium (minus FBS) for 24 h at 37°C. Where specified, inhibitors of TRPV1 were added 30 min prior addition of the treatment solutions. Cell viability was assessed using the Dojindo Cell Counting Kit-8 (Dojindo Laboratories, Gaithersburg, MD), according to the supplier recommendations. Cell viability was expressed as the percentage of viable cells relative to untreated cells using the absorbance at 450 nm. All experiments were performed in triplicate on three separate occasions.

ELISA assays for IL-6. BEAS-2B cells were subcultured into 24-well coated cell culture dishes at ~50% confluency and permitted to adhere for 8–12 h at 37°C. Prior to treatment, the cells were washed once with fresh media. Treatments were performed in cell culture media containing capsaicin and various modulators of TRPV1 function for 24 h at 37°C. After 24 h, the media was collected, clarified by centrifugation, and stored at −20°C until assayed for IL-6 content. To ensure consistent results, the cell viability for each well was determined using the Dojindo Cell Counting Kit-8. Samples exhibiting unusual values for cell viability were discarded. Cytokine production was assessed using commercial ELISA kits for IL-6 (R&D Systems, Minneapolis, MN) and performed as outlined by the manufacturer. All experiments were repeated on three separate occasions.

RT/PCR screening for TRPV1 expression. RT-PCR was used to assess the expression of TRPV1 mRNA in BEAS-2B, A549, and HepG2 cells. Total RNA was isolated from cultured cells (approximately 1.0 × 10⁷ cells) using the RNeasy total RNA isolation kit (Qiagen, Valencia, CA) as described by the manufacturer protocols. Total RNA was quantified using the ultraviolet-absorbance ratio (A280/A260) and 5 μg were used as a template for cDNA synthesis using Superscript II-reverse transcriptase (InVitrogen, Carlsbad, CA) and PolyT as a primer. Five μl of the first-strand synthesis reaction were used as a template for PCR. Primers specific for the published human TRPV1 sequence (Hayes et al., 2000; GenBank number XM_008512; Sense: 5'-GCCAAGAA-CATCTGGAGAAGCTGCG-3' and Antisense: 5'-CTTCTCCCAGGAGC-GACGAG-3') were used to amplify a 436 nucleotide (nt) 3'-fragment of TRPV1. β-Actin (180 nt) was also amplified by PCR as an internal control. Following an initial 2.5-min melting step, PCR was performed using a PTC-
100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA) and the following cycling program: 94°C for 1 min, 53°C for 1 min, and 72°C for 1.5 min. PCR was performed for 34 cycles and was followed by a 20-min incubation at 72°C. PCR products were resolved using a 1% Tris-ace-

Cloning and overexpression of TRPV1. The full length cDNA encoding TRPV1 was amplified by PCR from total RNA isolated from human fetal brain (Stratagene, La Jolla, CA) using pwo proofreading DNA polymerase and the following primers: Forward: 5'-CACCATGAAGAAAATGGACGGACGAC-3', containing a 5'-CAC C Koch sequence followed by the translation start-site, and Reverse: 5'-CTTCTCCCGGAAAGCGGCAGG-3'. The antisense primer was designed to amplify TRPV1, but omit the stop codon. This strategy permitted the fusion of the V5 epitope/His tag, contained within the expres-

RESULTS

Rats exposed to aerosols of capsaicinoids for 30 min (aproximately 1.0–1.2 mg/kg) exhibited a variety of lesions including infiltration of inflammatory cells, alveolar macrophage proliferation, damage to nasal, tracheal, bronchiolar, and alveolar cells, epithelial dysplasia (rounding of columnar epithelial cells), and loss of ciliated and nonciliated epithelial cells in the trachea and nasal turbinates, along with hemorrhage and congestion (Fig. 1). The most severe lesions were observed at the 24 h recovery period, although evidence of inflammation was present as early as 4 h. At 48–72 h, inflammation appeared to resolve, while altered cell morphology and cell damage in trachea and alveolar airways were still apparent.

In the upper airways, mild patchy epithelial necrosis and sloughing of cells were observed in nasal turbinate and trachea (Figs. 1A and 1C). These lesions were often accompanied by mild infiltrates of mixed inflammatory cells while some epithelial cells were more cuboidal than columnar. Mild cuboidal metaplasia was occasionally present in the epithelial cells of the bronchi and bronchioles. In some lung tissues, mild epithelial cell necrosis and sloughing were evident in terminal bronchioles (Fig. 1F). The most extensive lesions were present in the air sacs and alveoli. In general, septal walls were thickened by mild, but occasionally marked, infiltrates of mixed inflammatory cells (Fig. 1D). Moderate to marked capillary congestion with frequent mild to marked hemorrhage and occasional edema was also present in alveolar walls and spaces (Fig. 1E). In some tissues, mild to marked proliferations of foamy macrophages filled air sacs and alveoli (Fig. 1D). Granulomatous inflammation with small focal areas of necrosis was occasionally observed. Although ethanol may potentiate the effects of capsaicinoids (Trevisani et al., 2002), and ethanol vapor was probably inhaled by the animals, the control animals

Analysis of apoptosis and necrosis by flow cytometry. Differentiation between apoptosis and necrosis was assessed using the Vybrant apoptosis assay kit (Molecular Probes, Eugene, OR), as described by the manufacturer, and microscopic evaluation of cellular fluorescence. Quantitative assays for cobalt influx was achieved by treating cells (6 well plate; 1.0 × 10^6 cells) with 1.0 μM capsaicin for 10 min at 37°C in calcium- and magnesium-free Hank’s Balanced Salt Solution (HBSS) containing 2.5 mM CoCl2. After incubation, the cells were placed on ice, washed twice in HBSS, and solubilized in 0.5 mL HBSS containing 2% SDS. Cellular cobalt concentration was determined using ICP-MS, performed by the Veterinary Diagnostic Laboratory at Utah State University (Logan, UT). The use of cobalt as a measure for calcium flux through TRPV1 has previously been described by Wood et al. (1988).

Analysis of apoptosis and necrosis by flow cytometry. Differentiation between apoptosis and necrosis was assessed using the Vybrant apoptosis assay kit (Molecular Probes) containing fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide and monitoring for cellular fluorescence due to the exposure of phosphatidylserine on extracellular membrane surfaces (FITC-Annexin V binding to assess apoptosis) and nuclear staining due to loss of membrane integrity (propidium iodide staining to assess necrosis) using flow cytometry, fluorescence microscopy, and an ELISA assay measuring histone-associated DNA strand breaks. For flow cytometry and fluorescence microscopy, human bronchiolar epithelial cells (BEAS-2B) or TRPV1 overexpressing cells were treated with various concentrations of capsaicin for up to 24 h, washed once with calcium- and magnesium-free phosphate-buffered saline, harvested by trypsinization and centrifugation, and resuspended in 50 mM HEPES, pH 7.4, containing 700 mM NaCl and 12.5 mM CaCl2 (annexin binding buffer). The cells were washed once by centrifugation at 500 g for 5 min and resuspended in the same buffer. Aliquots of approximately 1 × 10^6 cells/ml were prepared, pelleted by centrifugation, and resuspended in 5 μL FITC-Annexin V (as provided in the assay kit) and 1 μL propidium iodide (100 μg/ml), and incubated at room temperature for 15 min. After 15 min, 400 μL of the HEPES buffer was added and the cells placed on ice until assayed by flow cytometry using a Becton-Dickenson FACScan™ fluorescence activated cell sorter and established methods for the analysis of Annexin V/propidium iodide staining. A total of 10,000 events (cells) were counted for each sample. The ELISA assay that was used to measure histone-associated DNA strand breaks was performed according to the manufacturer’s protocols (Roche Molecular Biochemicals).

Statistical analysis. Statistical analysis was performed using the Microsoft Excel software package. Statistical differences between samples were established using the two-sample t-test and a 95% confidence interval (p < 0.025).
that were exposed to ethanol vapors did not demonstrate respiratory lesions. Low (0.07 mg/kg) and intermediate (0.3 mg/kg) doses of capsaicinoids or pepper sprays produced very mild and moderate lesions, respectively, while higher doses (>0.8 mg/kg) produced more severe and frequent lesions. These pathologies appeared to occur in a dose/responsive manner (unpublished data, manuscript in preparation).

The precise molecular mechanisms by which the pepper sprays (capsaicinoids) caused inflammation and cell damage in vivo were further investigated in vitro using various cell lines derived from human lung and liver tissues. BEAS-2B cells are an SV-40-transfected, immortalized, human bronchiolar epithelial cell line that has frequently been used to study the mechanisms of airway toxicants in vitro. Similarly, A549 cells are derived from human adenocarcinoma and serve as an additional model for studying airway toxins. The A549 cells serve as a surrogate for human alveolar epithelial cells. Human hepatoma, HepG2 cells, were used to represent liver cell responses. BEAS-2B cells treated with increasing concentrations of capsaicin (0–200 μM) for 24 h exhibited a dose-dependent decrease in cell viability (Fig. 2A). The approximate LC₅₀ value was 100 μM. Approximately 80% of the decrease in cell viability was observed within 8 h (data not shown); however, a 24-h exposure period was used to ensure the complete loss of cell viability and to minimize variability. Similar decreases in cell viability were also observed for A549 and HepG2 cells (Fig. 2A). The LC₅₀ value for capsaicin in A549 and HepG2 cells was approximately 110 and 200 μM, respectively. To ensure that very brief exposures to capsaicin would also cause cell death, cells were treated for 30 min, washed extensively to remove capsaicin, and the cell viability determined 24 h later. BEAS-2B cells, treated with 100 μM capsaicin in this manner, demonstrated an approximate 40–50% loss in cell viability (data not shown). Thus, the LC₅₀ values did not significantly change even with very short exposures to capsaicin, provided that cytotoxicity was assessed 24 h after the initial treatment.

TRPV1 has been implicated as a key mediator of various cellular responses to capsaicinoids in vivo and in vitro. Therefore, we investigated the hypothesis that the relative levels of expression of this receptor in several cell lines would mirror the extent of cellular damage that was caused by exposure of the cells to capsaicin. Expression of TRPV1 in BEAS-2B,
A549, and HepG2 cells was assessed using RT-PCR and DNA sequence analysis. RT-PCR and densitometric analyses of the agarose gels were used to compare the relative abundance of TRPV1 message in BEAS-2B, A549, and HepG2 cells. In general, BEAS-2B cells expressed the highest levels of transcripts and HepG2 cells the least (Fig. 2B). These data demonstrated a correlation between TRPV1 expression and cellular susceptibility to cytotoxicity. The densitometric data for the levels of TRPV1 expression in these cells are presented in Figure 2B. Analysis of BEAS-2B cells exposed to 0, 50, and 100 μM capsaicin for 24 h and treated with FITC-annexin V and propidium iodide by flow cytometry demonstrated extensive, dose-related increases in staining by both fluorophores. These data were consistent with necrotic mechanisms of cell death (Fig. 3). These results were verified by fluorescence microscopy (cells were stained by both fluorophores) and by an ELISA assay that showed a lack of enrichment of histone-associated DNA fragments in the cells (data not shown). Necrotic mechanisms of cell death were also observed for resiniferatoxin (RTX), anandamide, and capsazepine.

Surprisingly, the cytotoxic effects of capsaicin were not ameliorated by the TRPV1 functional antagonists capsazepine and isovelleral (Jerman et al., 2000), or by modulators of calcium flux, ruthenium red, and EGTA (Table 1). These results were particularly intriguing because none of the prototypical modulators of TRP receptor function decreased susceptibility of the cells to the cytotoxicity caused by capsaicin. Unexpectedly, capsazepine and isovelleral were also toxic to BEAS-2B cells at concentrations that were much lower than capsaicin (Table 1). Although Substance P has been shown to exacerbate calcium flux into, and IL-6 production by BEAS-2B cells that were treated with capsaicin (Veronesi et al., 1999), this neuropeptide did not modulate cellular death from capsaicin exposure; a result that provided additional support for the conclusion that cell death was calcium-independent.

BEAS-2B cells treated with capsaicin also exhibited a dose-dependent increase in the production and release of IL-6, a common cytokine used to assess proinflammatory responses. Maximum induction of IL-6 (~4–7-fold) was observed at 24 h
Modulators of TRPV1 Function and Cellular Calcium Flux Do Not Ameliorate the Cytotoxic Effects of Capsaicin in BEAS-2B Bronchiolar Epithelial Cells or TRPV1 Overexpressing Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BEAS-2B Cell viability (%)</th>
<th>TRPV1 overexpressing Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions (capsaicin only)</td>
<td>51 ± 7</td>
<td>56 ± 1</td>
</tr>
<tr>
<td>Capsazepine a</td>
<td>33 ± 5*</td>
<td>58 ± 1</td>
</tr>
<tr>
<td>10 µM</td>
<td>24 ± 5*</td>
<td>31 ± 3*</td>
</tr>
<tr>
<td>30 µM</td>
<td>54 ± 8</td>
<td>53 ± 2</td>
</tr>
<tr>
<td>EGTA a</td>
<td>39 ± 5*</td>
<td>41 ± 5*</td>
</tr>
<tr>
<td>500 µM Ruthenium Red a</td>
<td>52 ± 3</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>500 µM CaCl₂</td>
<td>52 ± 9</td>
<td>ND</td>
</tr>
<tr>
<td>500 µM Substance P</td>
<td>41 ± 5</td>
<td>ND</td>
</tr>
<tr>
<td>500 µM</td>
<td>58 ± 9</td>
<td>ND</td>
</tr>
</tbody>
</table>

Note. All treatments contained capsaicin. BEAS-2B cells were treated with 100 µM capsaicin and TRPV1 overexpressing cells were treated with 1.0 µM capsaicin. ND, not determined.

a Known inhibitors of calcium flux through TRPV1.

*Significant difference versus cells treated with capsaicin only (p < 0.025).

with a concentration of 100 µM capsaicin (Fig. 4), although significant induction of IL-6 was observed in as little as 2 h with 100 µM capsaicin (data not shown). Addition of excess calcium chloride slightly increased IL-6 release (not statistically significant), while chelation of free extracellular calcium in the media (~110 µM) by EGTA, or blocking calcium flux into cells by the TRPV1 antagonist capsazepine, drastically decreased IL-6 production (Fig. 4). Therefore, these results demonstrate a requirement for calcium influx into cells through TRPV1 in order to promote IL-6 release. Release was also observed using LC₅₀ concentrations of RTX, but not anandamide or capsazepine (data not shown).

The importance of TRPV1 in cell death and proinflammatory processes was addressed by overexpression of TRPV1 in BEAS-2B cells. Human TRPV1 was cloned from fetal brain mRNA and transfected into BEAS-2B cells. Overexpression of TRPV1 in BEAS-2B cells was assessed using RT-PCR and densitometric analysis of the PCR product intensity for “normal” and TRPV1 overexpressing cells (Fig. 5A). The overexpressing cells also possessed mRNA coding for the TRPV1-V5 epitope fusion protein. Expression of this mRNA was not observed in BEAS-2B cells (Fig. 5A) or in control cells that expressed Geneticin resistance, but did not show enhanced susceptibility to cytotoxicity by capsaicin (data not shown).

Overexpression of functional TRPV1 was also determined. TRPV1 overexpressing cells (based on overexpression of mRNA), previously loaded with Fluo 4-AM, displayed increased cellular fluorescence when treated with capsaicin due to Ca²⁺-Fluo-4 complex formation. The percentage of BEAS-2B or TRPV1 overexpressing cells that exhibited fluorescence in the absence of capsaicin were less than 1% for both cell types. Upon exposure to 100 µM capsaicin (15 min), approximately 3% of the BEAS-2B cells showed increased fluorescence while approximately 61% of the TRPV1 overexpressing cells were fluorescent. Calcium influx was dose-dependent, since fluorescence was demonstrated in 7 and 18% of the overexpressing cells with 1 µM and 10 µM concentrations of capsaicin, respectively. The TRPV1 overexpressing cells also showed elevated cobalt uptake (~twofold), compared to normal BEAS-2B cells, when exposed to capsaicin. Geneticin-resistant control cells that did not show overexpression of V5 or TRPV1 mRNA were identical to BEAS-2B cells in both experiments. Further confirmation of the role of TRPV1 in these cellular responses, in either cell line, was demonstrated by the inhibition (~95%) of calcium or cobalt uptake by capsazepine.

Overexpression of TRPV1 resulted in an approximate 100-fold increase in the susceptibility to cytotoxicity (Fig. 5B). Similarly, the cytotoxicity of several other TRPV1 ligands was increased in TRPV1 overexpressing cells (Table 2). The LC₅₀ values for olvanil and RTX decreased 15-fold and 75,000-fold, respectively, although the LC₅₀ values for various other TRPV1 ligands decreased a mere 1–2-fold, suggesting that different mechanisms are involved in the cytotoxicity of these diverse compounds.

![FIG. 4. Induction and inhibition of IL-6 production by BEAS-2B cells treated with capsaicin (100 µM) or capsaicin (100 µM) plus various modulators of TRPV1 function for 24 h. Capsazepine was used at a concentration of 10 µM, EGTA at 100 µM, and CaCl₂ at 10 µM. Data represent the mean and SD of triplicate determinations for fold increases in IL-6 concentrations versus untreated cells (control values approximately 175 pg/ml). *Statistically significant difference (p < 0.025) versus untreated control cells. **Statistically significant difference (p < 0.025) from cells treated with capsaicin only.](https://academic.oup.com/toxsci/article-abstract/73/1/170/1657825)
The effects of TRPV1 overexpression in BEAS-2B cells on the proinflammatory responses to capsaicin treatment were also assessed. Overexpression of TRPV1 also caused a shift in the dose-response curve for IL-6 production and release to lower doses of capsaicin (Figure 6). Decreased IL-6 production by cells exposed to >100 μM capsaicin was presumably due to the extensive cell death. Maximum production of IL-6 (24 h treatment) by TRPV1 overexpressing cells was observed at approximately 0.5 μM capsaicin, versus approximately 100 μM for BEAS-2B cells.

The LC50 values for RTX, olvanil, and capsaicin appeared to correlate to literature data (Smart et al., 2001; Szallasi et al., 1999) on TRPV1 binding affinity (i.e., RTX > olvanil > capsaicin), determined by 3H-RTX binding, as well as the ability of the compounds to cause calcium influx through TRPV1 (i.e., RTX > olvanil > capsaicin). Flow cytometric and fluorescence microscopic analysis of TRPV1 overexpressing cells exposed to capsaicin (0, 0.5, and 1.0 μM) for 24 h demonstrated marked, dose-related increases in FITC-annexin V staining, without concomitant increases in propidium iodide staining (Fig. 7). These data were consistent with apoptotic, not necrotic, mechanisms of cell death. Similar to BEAS-2B cells, however, the cytotoxic effects of capsaicin in TRPV1 overexpressing cells were not ameliorated by functional antagonists of TRPV1, modulators of calcium flux, or extracellular calcium concentrations (Table 1).

![FIG. 5.](image_url)

**TABLE 2**

<p>| Diverse TRPV1 Ligands Exhibit Different Cytotoxic Potencies in BEAS-2B and TRPV1 Overexpressing Cells |
|-----------------------------------------|-----------------------------------------|</p>
<table>
<thead>
<tr>
<th><strong>TRPV1 ligands</strong></th>
<th><strong>BEAS-2B</strong></th>
<th><strong>TRPV1 overexpressing</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsaicin</td>
<td>100 ± 10</td>
<td>1.0 ± 0.2*</td>
</tr>
<tr>
<td>RTX</td>
<td>7.5 ± 3</td>
<td>0.0001 ± 0.00005*</td>
</tr>
<tr>
<td>Olvanil</td>
<td>0.8 ± 0.09</td>
<td>0.05 ± 0.007*</td>
</tr>
<tr>
<td>Scutigeral</td>
<td>4.5 ± 1</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>Anandamide</td>
<td>12.5 ± 3</td>
<td>10.0 ± 3</td>
</tr>
<tr>
<td>Capsazepine (antagonist)</td>
<td>25 ± 5</td>
<td>10.0 ± 2</td>
</tr>
<tr>
<td>Isovelleral (antagonist)</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.03</td>
</tr>
<tr>
<td>Ruthenium Red</td>
<td>&gt; 1000</td>
<td>ND</td>
</tr>
<tr>
<td>Substance P</td>
<td>&gt; 1000</td>
<td>ND</td>
</tr>
<tr>
<td>PPAHV</td>
<td>7.0 ± 2</td>
<td>2.5 ± 0.2</td>
</tr>
</tbody>
</table>

*Note. ND, not determined. *Represents a significant difference versus BEAS-2B cells (p < 0.025).
DISCUSSION

Capsaicinoids have been known for centuries to produce severe irritation, coughing, and respiratory inflammation in experimental animals and man. The use of pepper extracts for the production of pepper spray self-defense weaponry has utilized this dramatic acute response to incapacitate attackers and uncooperative suspects. Although vast literature exists concerning the responsiveness of many different types of cells to capsaicinoids, including respiratory epithelial and neuronal cells, the precise molecular mechanisms that governed the physiological and pathological responses to capsaicinoids have not been identified or characterized. With the recent cloning of TRPV1, and several other members of the TRP/vanilloid receptor family of ion channel receptors, scientists have begun to understand the molecular basis for these diverse physiological responses produced by capsaicin and other TRPV1 agonists (Gunthorpe et al., 2002). The studies presented here have demonstrated that acute inhalation of capsaicinoids, in the form of pepper sprays, by nose-only administration to rats, produced acute inflammation, moderate epithelial cell dysplasia, and necrosis in the upper and lower respiratory tract. However, the most severe lesions were present in the terminal bronchioles and alveoli where the capsaicinoids produced marked inflammation, multifocal macrophage proliferation, bronchiolar and alveolar epithelial cell injury, and mild to marked vascular congestion with septal and alveolar hemorrhage. In order to elucidate the molecular mechanisms responsible for these pathological effects, and to predict the potential effects of capsaicinoids in humans, we utilized immortalized human lung epithelial cells to evaluate cellular death/cytotoxicity and proinflammatory cytokine production.

The doses used for animal inhalation experiments (approximately 1.0–1.2 mg/kg in a 30-min exposure) are similar to doses that humans would receive during a 5–10 s exposure of pepper spray. Pepper sprays contain approximately 1 to 32 μg total capsaicinoids per μl of condensed spray, depending upon the product and formulation (Reilly et al., 2001a,b). If one assumes that the pepper spray canisters contain between 5 and 50 ml of condensed spray (unpublished observations), then each canister would contain approximately 5 mg to 1.6 g total capsaicinoids. Therefore, it is reasonable to predict that a dose of 1 mg/kg (or more) could be inhaled by people exposed to large amounts of pepper spray. Our previous research (Reilly et al., 2002) has shown that the concentrations of capsaicinoids in the blood of rats exposed to 0.57 mg/kg (approximately half of the dose used in the current studies) were as high as 125 ng/ml in blood and 174 ng/mg for lung tissues. Extrapolation of these values to the dose used in the current studies (1.0–1.2 mg/kg) would predict a concentration of approximately 1 μM (vide supra), which happens to be the LC₅₀ of capsaicin in the TRPV1 overexpressing cell line. Concentrations much higher than 1 μM (concentration in blood) would be expected at the site of delivery (particle deposition) in respiratory cells after an inhalation exposure. Thus, it is also reasonable to assume that humans exposed to pepper sprays could have nasal, tracheal, bronchiolar, and/or alveolar capsaicinoid exposures similar to the concentrations that elicited cellular death and/or cytokine release in these cell culture studies, even if the subject did not receive a total dose of 1 mg/kg. Since capsaicinoids can produce significant cell death and IL-6 production in very short time periods (0.5–2 h), it is also reasonable to predict that inhaled doses of pepper sprays in humans could cause adverse respiratory inflammatory responses similar to those characterized in this study.

Recent work at the USEPA has demonstrated a key role for TRPV1 in mediating inflammatory responses to capsaicin and various forms of airborne particulate material in airway tissues (Veronesi et al., 1999, 2000). Given these data, and the knowledge that TRPV1 and other TRP receptors are expressed in respiratory tissues, including the trachea, bronchi, and alveoli,
we investigated the role of TRPV1 in these pathologies by the use of human airway and hepatic cell lines. The use of RT/PCR techniques confirmed the expression of TRPV1 mRNA transcripts in human lung epithelial (A549 and BEAS-2B) and liver (HepG2) cells. Capsaicin-induced cell death was greater in the two lung epithelial cell lines than the liver hepatoma cell line. Furthermore, the relative rank order of susceptibility to cytotoxicity by capsaicinoids correlated to the relative levels of TRPV1 transcripts in the three cell lines. These data suggested that TRPV1 may be a key mediator of the cytotoxic effects of capsaicin in these cells.

The mechanism of cell death in BEAS-2B cells was shown to be necrosis, not apoptosis. Surprisingly, several experimental variables designed to block cell death, including removal and chelation of calcium and the use of functional antagonists to TRPV1, were ineffective in ameliorating cell death. In addition, other TRPV1 ligands, both prototypic agonists such as RTX, olvanil, and anandamide, as well as prototypic antagonists such as capsazepine and isovelleral, were also cytotoxic at concentrations less than capsaicin. Thus, ligand binding to TRPV1 appears to trigger key cytotoxic responses that are independent of calcium flux into the cell through TRPV1 (as required for IL-6 production).

In order to elucidate the mechanisms that produced cellular toxicities, we cloned the human TRPV1 cDNA and overexpressed this receptor in BEAS-2B cells. These engineered cells expressed much higher levels of TRPV1 transcripts than the parent cell line as well as much higher capsaicin-induced ion flux that was ameliorated by capsazepine. Additional “control” cell lines were also produced in these studies. These cell lines showed stable incorporation of the Geneticin resistance expression cassette, but did not overexpress mRNA for TRPV1 or the TRPV1-V5 fusion protein. In addition, these cells did not exhibit functional increases in ion flux (cobalt and calcium) in the presence of capsaicin. The lack of TRPV1 overexpression, despite expression of Geneticin resistance, was likely due to the incorporation of the TRPV1 expression cassette into a silent portion of the genome or from interruption of the gene during recombination. However, the cells that did overexpress TRPV1 were dramatically more susceptible to capsaicin-induced cell death (approximately 100-fold) than either normal BEAS-2B or other control cell lines. Several other TRPV1 ligands were also evaluated for enhanced cytotoxicity, but only RTX and olvanil caused marked increases in toxicity in the overexpressing cells. Surprisingly, cytotoxicity in the overexpressing cells was, again, not blocked by TRPV1 antagonists or dependent on calcium flux from extracellular media.

Unexpectedly, the overexpressing cells were killed by capsaicin through apoptotic, not necrotic, mechanisms. A shift in the mechanism of cell death may indicate that TRPV1-mediated cellular injury in the TRPV1 overexpressing cells was truly occurring through programmed cell death mediated by TRPV1. However, the cytotoxicity observed in the parent cell line by a necrotic mechanism may have been the result of a composite response of several biochemical targets. We speculate that other vanilloid receptors (e.g., VRL-2, VR.5’sv, TRPM8, VRL-1, etc.), or vanilloid receptors comprised of mixed populations of vanilloid receptor family protein subunits—TRPV1 and others exist as tetramers that appear to form in response to agonist exposure (Delany et al., 2001; Kedei et al., 2001; Kuzhikandathil et al., 2001; Schumacher et al., 2000)—may be activated by these structurally diverse xenobiotics. These mixed receptors may also participate in the regulation of the responses that were observed in these studies. For example, activation of these mixed receptors or other vanilloid receptors may contribute to both pro- and antiapoptotic responses in BEAS-2B cells, with anti-apoptotic responses dominating. The net result could be necrotic cell death produced primarily from non-TRPV1-mediated processes. However, overexpression of TRPV1 may “dilute” these alternate targets for cytotoxicity, either by altering the subunit composition of mixed receptor complexes, or by increasing the density of homogenous TRPV1 tetramers, such that TRPV1-mediated proapoptotic signals dominate the cellular responses to capsaicin exposure. Regardless of the mechanism, these data reinforced the concept that cell death in lung epithelial cells treated with capsaicin was calcium-independent, but related to TRPV1 expression. These results also demonstrated that the TRPV1 overexpressing cells are a valuable tool for differentiating cytotoxicities (and proinflammatory responses) that are truly mediated by TRPV1 (as observed for capsaicin, RTX, and olvanil) versus toxicities that occur as a result of other processes that are probably independent of TRPV1 binding (e.g., capsazepine, anandamide, and others).

Another significant finding was that the TRPV1 overexpressing cells were also more responsive to proinflammatory stimuli. IL-6 production by “normal” BEAS-2B cells increased dramatically (4.5–7-fold) in the presence of 100 μM capsaicin. Increases in IL-6 production were also observed with 7.5 μM RTX (~twofold), but not in the presence of 12.5 μM anandamide or 25 μM capsazepine. The TRPV1 overexpressing cells doubled IL-6 production in response to capsaicin concentrations that were approximately 100–200-fold lower than the concentrations that produced this response in the parent cell line. Interestingly, the only ligands that increased cytokine production in BEAS-2B cells were the same ligands that exhibited enhanced cytotoxicity in the TRPV1 overexpressing cells. Cytokine production by these cells was also inhibited by capsazepine and EGTA. These data confirmed the vital role of TRPV1 and calcium flux through TRPV1 in the induction of cytokine production by lung epithelial cells that are exposed to capsaicin.

From these data, we conclude that TRPV1 activation mediated cell death and cytokine production by BEAS-2B cells treated with capsaicin and other selected TRPV1 ligands. However, these data may also suggest that the cytotoxicity observed in normal BEAS-2B cells treated with capsaicin and other TRPV1 ligands that do not exhibit enhanced cytotoxicity in TRPV1 overexpressing cells was probably caused by interaction with additional biochemical targets. Although we have not
identified the alternate biochemical targets that mediated the cytotoxicity of these xenobiotics, one hypothesis is that other members of the TRP receptor superfamily, whose function has not been evaluated in human lung epithelial cells, may mediate these responses. These hypotheses may help explain the apparent discrepancies between the cytotoxicity and cytokine data, and the inability of capsazepine to block cytotoxicity, as well as provide insight into the unique nature of the cytotoxicity of capsaicin in BEAS-2B, and possibly other lung cells. For example, it may be possible that heterogeneous receptor complexes that mediate cell death are activated by capsaicin, but are not inhibited by capsazepine. Similarly, alternate receptor complexes (probably homogeneous TRPV1 tetrameric complexes) that mediate cytokine production may be activated by capsaicin and inhibited by capsazepine. Studies to characterize the other vanilloid receptors, and the potential functional significance of heteromeric complexes of vanilloid receptors, are underway in our laboratory.

In summary, these studies demonstrated that capsaicinoids produced acute pulmonary inflammation and respiratory cell injury in experimental animals and in human lung epithelial cells. These pathologies and toxicities appeared to occur through activation of TRPV1, and possibly other related vanilloid receptor proteins, through complex processes that appear, at least in part, to be mediated by unique and separate calcium-dependent and calcium-independent mechanisms. Thus, the cytotoxic and proinflammatory response mechanisms emerge as distinct processes in human lung epithelial cells that are mediated, at least in part, by TRPV1. These studies provide a fascinating foray into the precise molecular mechanisms that control respiratory responsiveness to a large number of environmental irritants, including pepper sprays and possibly other respiratory irritants and toxicants such as ambient particulate matter. Additional characterization of other TRP/vanilloid receptor proteins that may also be expressed in respiratory epithelial cells should help clarify the relative contributions made by the plethora of vanilloid receptors that control airway responsiveness to various environmental stimuli.

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