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Role of TRPA1 in Acute Cardiopulmonary Toxicity of Inhaled Acrolein

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Abstract

Acrolein is a highly toxic, volatile, unsaturated aldehyde generated during incomplete combustion as in tobacco smoke and indoor fires. Because the transient receptor potential ankyrin 1 (TRPA1) channel mediates tobacco smoke-induced lung injury, we assessed its role in high-level acrolein-induced toxicity in mice. Acrolein (100–275 ppm, 10–30 min) caused upper airway epithelial sloughing, bradypnea and oral gasping, hypothermia, cardiac depression and mortality. Male wild-type mice (WT, C57BL/6; 5–52 weeks) were significantly more sensitive to high-level acrolein than age-matched, female WT mice. Both male and female TRPA1-null mice were more sensitive to acrolein-induced mortality than age- and sex-matched WT mice. Acrolein exposure increased lung weight:body weight ratios and lung albumin and decreased plasma albumin to a greater extent in TRPA1-null than in WT mice. Lung and plasma protein-acrolein adducts were not increased in acrolein-exposed TRPA1-null mice compared with WT mice. To assess TRPA1-dependent protective mechanisms, respiratory parameters were monitored by telemetry. TRPA1-null mice had a slower onset of breathing rate suppression (‘respiratory braking’) than WT mice suggesting TRPA1 mediates this protective response. Surprisingly, WT male mice treated either with a TRPA1 antagonist (HC030031; 200 mg/kg) alone or with combined TRPA1 (100 mg/kg) and TRPV1 (capsazepine, 10 mg/kg) antagonists at 30 min post-acrolein exposure (i.e., “real world” delay in treatment) were significantly protected from acrolein-induced mortality. These data show TRPA1 protects against high-level acrolein-induced toxicity in a sex-dependent manner. Post-exposure TRPA1 antagonism also protected against acrolein-induced mortality attesting to a complex role of TRPA1 in cardiopulmonary injury.

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Graphical Abstract

Keywords

aldehydes; cardiac dysfunction; oxidative stress; pulmonary injury

INTRODUCTION

Acrolein is a volatile, unsaturated aldehyde with a long history of inducing acute toxicity. Large quantities of acrolein are synthesized annually (500,000 tons) for industrial use as an herbicide/biocide (Slimicide®, Magnacide®) to manage biological growth in waterways and oil wells and as a main precursor in acrylic acid synthesis (Ghilarducci and Tjeerdema, 1995). Moreover, in addition to being an historical chemical weapon, an industrial chemical of high toxicity and a toxic combustion product present at high levels in tobacco smoke and in fires (Stevens and Maier, 2008), acrolein also is generated endogenously during lipid peroxidation and inflammation (Anderson *et al.*, 1997). Brief, high level accidental exposure to acrolein (i.e., packaged as Magnacide®, 95% acrolein) has resulted in recorded human fatalities that occurred days after exposure despite early clinical treatment, and notably firefighters have a 10–100-fold higher risk of dying due to heart disease-related events during fire suppression than during any other activity (Kales *et al.*, 2003; Agency, 2008; Soteriades *et al.*, 2011). Despite these observations, the cause of death remains unknown.

In animal studies, brief (30 min) exposure to acrolein at levels found in enclosed and structural fires is lethal (Albin, 1962). The lethal concentration (LC₅₀) of acrolein inducing 50% mortality at 24h after a brief exposure is 175 ppm (10 min exposure) in mice (Albin,

1962). Likewise, the acrolein LC₅₀ at 24h is 150 ppm following a 30 min exposure in dogs (Albin, 1962). These data illustrate the relatively high toxicity of acrolein across different species. Recently, Leikauf et al. demonstrated that continuous exposure to 10 ppm acrolein (lacrymation level)(Beauchamp *et al.*, 1985) resulted in 100% lethality in C57BL/6J mice within 16h (Leikauf *et al.*, 2011), so longer exposures at lower acrolein levels also pose a serious threat to health, yet the mechanism of acrolein-induced lethality is poorly defined.

Life stage and sex are important modifiers of CVD risk, however, age- and sex-dependent changes in acrolein toxicity have not been investigated thoroughly. Multiple studies have shown that the very young and very old represent sensitive life stages to environmental exposures (Avdalovic *et al.*, 2012). In the young, both immune and antioxidant systems are still immature and gaining awareness (competence) of the environment, however, stem/progenitor cell numbers and activity are high indicating substantial repair potential (Thijssen *et al.*, 2006; Hoetzer *et al.*, 2007). In aged individuals, endothelial repair potential is lower and damaged tissue takes significantly longer to repair/heal (Thijssen *et al.*, 2006; Chang *et al.*, 2007). Thus, both young and old are likely more sensitive to volatile toxins such as acrolein than are healthy adults. Furthermore, because expression of many detoxification enzymes, e.g., glutathione-*S* transferases (GSTs) and cytochromes P450 (CYPs), is sex-dependent (Bammler *et al.*, 1994; Singh *et al.*, 1998; Wolbold *et al.*, 2003; Zhang *et al.*, 2011), there may be substantial sex-dependent differences in sensitivity to toxins (Singh *et al.*, 1998). Indeed in a previous study, we showed that male GSTP-null mice (oral LC₅₀ at 24h, ≈8 mg/kg) are twice as sensitive to oral acrolein as male wild-type mice (LC₅₀ at 24h, ≈16 mg/kg)(Conklin *et al.*, 2015). Because of life stage- and sex-based differences, post-exposure interventions need to account for how diversity affects outcomes.

Acrolein is a strong electrophile, and as such, it readily forms stable covalent adducts with biological nucleophiles, such as GSH and cysteine-rich proteins. Acrolein binds TRPA1 cysteines (Macpherson *et al.*, 2007), triggers opening of the cation channel and calcium entry, neuronal activation and secretion of substance P (SubP) and calcitonin gene-regulated peptide (CGRP) -- peptides that enhance pain signaling, local tissue inflammation, blood flow, vascular permeability and edema (Andrade *et al.*, 2012). Thus, acrolein-induced activation of the TRPA1 channel is linked to pain and inflammation, which may contribute to acute tobacco smoke- or acrolein-induced pulmonary (Bautista *et al.*, 2006; Andre *et al.*, 2008; Bessac and Jordt, 2008; Simon and Liedtke, 2008) and cardiovascular injury (Engel *et al.*, 2011). For example, TRPA1 appears responsible for changes in heart rate variability (HRV) in rats exposed to diesel engine exhaust (Hazari *et al.*, 2011). These data provide evidence that cardiovascular injury due to acrolein exposure (and perhaps other toxicants) likely involves TRPA1 activation. Because TRPA1 antagonists prevent acrolein-induced TRPA1 activation, edema and inflammation (Bautista *et al.*, 2006; Macpherson *et al.*, 2007; Andre *et al.*, 2008; Simon and Liedtke, 2008), evaluating TRPA1 antagonist efficacy against acrolein toxicity *in vivo* may be useful in developing treatments for victims of acute inhalation injury.

MATERIALS AND METHODS

Mice

Male and female C57BL/6 (wild type, WT) mice were either purchased from the Jackson Laboratory (Bar Harbor, ME) or raised in house in a barrier facility. TRPA1-null mice were obtained from Dr. S. Jordt (Duke University) in which the TRPA1 gene was “knocked out” in SV129 embryos using a single construct, and mice were bred back for 10 generations with C57BL/6 mice (Bautista *et al.*, 2006). TRPA1-null mice grow and breed normally. Mice were treated according to APS’s Guiding Principles in the Care and Use of Animals and protocols were approved by the University of Louisville IACUC.

PCR Protocol for TRPA1 Screening—PCR products were used to genotype WT and TRPA1-null mice using primers as described (Bautista *et al.*, 2006). All four primers were mixed with tail DNA, amplified using *Taq* polymerase (Promega, Madison, WI), and products run on 2% agarose gel with WT band at 296 bp and null band at 213 bp (Knowlton *et al.*, 2010).

Acrolein Exposure

Brief high-level acrolein (100–275 ppm) exposures of varying duration (10–30 min) were performed to estimate an LC₅₀. During exposures, acrolein level was monitored continuously with an in-line, calibrated photoionization detector (PID; ppbRAEPlus, Rae Industries, Sunnyvale, CA) upstream of a custom cage insert vapor delivery unit (Teague Inc.) in a standard polycarbonate cage (16”×8.75”×13.5”; ~31 l)(Suppl. Fig. 1A)(Wheat *et al.*, 2011). Acrolein (stock gas 5,000–5,100 ppm; AirLiquide, Plumsteadville, PA) was diluted first in nitrogen and then in HEPA- and charcoal-filtered air prior to entering the cage at 8–10 lpm via a cyclone-type top delivery unit that distributed air within 10 % of the mean concentration at 6 locations in the cage. The exposure protocol consisted of baseline air flow (Phase I), a 5 min ramp up (to reach 94% of target ppm; Phase II), a steady state exposure period (Phase III), and a 30 min air purge (Phase IV) to remove acrolein from the chamber before opening (Suppl. Fig. 1B). Chamber temperature was typically 20–22 °C.

LC₅₀

To estimate the lethal concentration in 50% of mice at 24h (LC₅₀, 24h), mice were exposed to a level of acrolein and mortality monitored over 24h. See **Statistics** section below for more detailed description of statistical approaches used to estimate LC₅₀, 24h.

Morbidity

BALF: Protein, Cell Counts and Flow Cytometry—Bronchoalveolar lavage fluid (BALF) was collected via intratracheal instillation in 2 fractions (after cardiac blood draw). The first fraction (1.0 mL PBS, 0.4mM EDTA) was used for cell counting via hemocytometer (diluted 1:1 with Trypan Blue Solution, 0.4%; Life Technologies, Grand Island, NY). A second fraction (1.0 mL PBS, 0.4mM EDTA) was used for total protein (colorimetric protein assay; Bio-Rad, Hercules, CA) and albumin (mouse albumin ELISA; Bethyl Laboratories, Montgomery, TX).

Lung and Plasma Biochemistry—The abundance of protein-acrolein adducts in lung homogenates and plasma was analyzed by Western blot as described before (Conklin et al., 2009). Western blots of lung homogenates were also probed for albumin using mouse albumin antibody (1:10,000, Bethyl Laboratories)(Cho, 2002, 53). Western blots were developed using ECL plus reagent (Pierce, Thermo Scientific), and band intensities were detected with an *myECL* imager (Thermo Scientific). Quantification of band intensity was performed using *myImage* analysis software (Thermo Scientific). Amido black protein stain was used as loading control (protein) where indicated. Total plasma cholesterol (Cholesterol CII Enzymatic Kit, Wako), triglycerides (L-Type TG-H Kit, Wako), protein (Bradford, Wako), albumin (bromocresol green, Wako), alanine aminotransferase (ALT, Infiniti), aspartate aminotransferase (AST, Infiniti), creatine kinase (CK, Promega) and lactate dehydrogenase (LDH, Promega) levels were measured using commercially available assay reagents and calibrated standards on a Cobas Mira Plus 5600 Autoanalyzer (Conklin et al., 2009).

Respiratory Parameters—Normal breathing rate in C57BL/6 mice is 140–160 breaths per minute but following acrolein exposure, breathing rate and depth slowed dramatically to a point where respiratory rate (most notably after mice start oral gasping) could be visually counted. To more precisely define the onset of bradypnea, respiratory rate was monitored during and after acrolein exposure in mice implanted with pressure telemeters (PA-C10, DSI). Pressure cannula was implanted intrathoracically via a ventral peritoneal incision and gently tunneled (2–3 cm) through the diaphragm into the esophagus serosa. The cannula was sutured to the body wall and the transmitter remained intraperitoneal. Pre-recordings ensured cannula patency and fidelity, and mice recovered in 1 week. Baseline respiratory rate (frequency, bpm), amplitude (mmHg, thoracic cavity pressure), and expiratory and inspiratory durations (s) were measured in mice in the exposure chamber prior to onset of acrolein exposure (5 min; Phase I), recorded throughout the exposure (5 min during ramp up, Phase II; 30 min of acrolein steady state, Phase III; 30 min air purge, Phase IV), and then intermittently over 24h post-exposure. Air control exposures were performed to assess the effect of chamber confinement or post-exposure intervention on respiratory parameters.

Core and Surface Body Temperature—Core body temperature (cBT) was recorded in C57BL/6 male mice before, during and after acrolein exposure (275 ppm, 30 min) using radiotelemetry. Mice were implanted with radiotransmitters (intraperitoneal; TA-F10, DSI) and allowed 1 week recovery. Receivers were placed under the exposure chamber and subsequently under home cages for continuous or intermittent recordings. Surface body temperature (sBT) was measured using a handheld infrared thermometer (LaCrosse Technologies, LaCrosse, WI) following manufacturer's recommendations and two criteria: 1) handheld devices were calibrated at 3 bath temperatures; and, 2) measurement of surface temperature was done midline haunch at approximately 1–2 cm from the skin surface.

Pulse Oximetry—Blood oxygen (s_pO_2) and heart (bpm) data were collected in mice via a pulse oximetry clip (right front paw, MouseSTAT, Kent Scientific) before and after air and acrolein (250 ppm, 30 min) exposures.

Vascular Function—The aorta was removed via a mid-ventral thoracotomy in sodium pentobarbital-anesthetized mice. A ‘ring’ was cut from each thoracic aorta. Rings were hung on stainless steel hooks in 15-ml water-jacketed organ baths in physiological salt solution (PSS) bubbled with 95% O₂ and 5% CO₂ at 37°C. The composition of PSS was (in mM) 130 NaCl, 4.7 KCl, 1.17MgSO₄·7H₂O, 1.18 KH₂PO₄, 14.9 NaHCO₃, 2.0 CaCl₂, and 5.0 glucose (pH 7.4). One hook was connected to an isometric strain-gauge transducer (Kent Scientific, Litchfield, CT), and the other hook was attached to a fixed support glass rod. Transducer signals were fed into an eight-channel PowerLab analog-to-digital converter and recorded on a PC running LabChart software (v.3.4.9, iWorx, Dover, NH). After 10 min without tension, rings were equilibrated to 1g of loading tension over 30 min. Aortic rings were stimulated with 100 mM K-PSS (100 mM K⁺) to test for viability, washed three times with PSS over 30 min, re-equilibrated to 1 g of resting tension, and then re-stimulated with 100 mM K⁺ followed by three bath changes and re-equilibration of aorta to 1 g tension. Rings from in vivo air or acrolein exposure were contracted with cumulative concentrations of phenylephrine (PE; 0.1 nM–10μM) and then relaxed with cumulative concentrations of ACh (0.1 nM–10μM) to determine endothelium-dependent relaxation. After a tension plateau, sodium nitroprusside (SNP; 0.1 nM–10μM) was added to determine endothelium-independent relaxation. PE-induced contractions were normalized to ring volume (mm³). Relaxations were calculated as the percent reduction of PE-induced tension. The effective concentration producing a 50% response (EC₅₀) was interpolated for all agonists (Conklin *et al.*, 2009).

Echocardiography—Echocardiography in 2-dimensional M-mode was performed in male mice (12–16 weeks old) after air exposure (2% isoflurane-anesthetized; 24h) or post-acrolein exposure (conscious; 24h) using a VisualSonics Vevo 770. In acrolein-exposed mice, isoflurane-anesthesia resulted in rapid mortality, so these mice were conscious and held gently by the sonographer during echocardiography. Echocardiograms were analyzed using the VisualSonics Cardiac Measurement Package (v17).

Tissue/Organ Collection and Histology

At indicated times post-exposure, mice were euthanized (sodium pentobarbital, 150 mg/kg), and blood collected into EDTA-coated syringes and tubes (EDTA, 20 μL, 0.2M per 1 ml blood) for CBC performed on diluted whole blood (1:1 with DPBS; Corning, Manassas, VA; 4°C) using a Cell-Dyn 3500 (Abbott Laboratories, Abbott Park, IL) or using undiluted blood (HemaVet 950FS, Drew Scientific). Organs were weighed and snap frozen in LN₂ and stored at –80°C or fixed in neutral-buffered formalin (10% NBF). The lungs were fixed via tracheal instillation with 10% NBF at 25 cm H₂O (Hoyle *et al.*, 2010). The nose (whole rostrum) was fixed in 10% NBF followed by thorough decalcification (Corps *et al.*, 2010) with Immunocal (Decal Chemical Co., Tallman, NY), and then sectioned into thirds before embedding. Fixed materials were paraffin-embedded, sectioned (5 μm) onto glass slides and stained with H&E.

Antagonist Administration

Intervention studies were performed at typically higher acrolein levels (ranged from 225–275 ppm, 30 min). Immediately after the chamber purge (Phase IV), mice were treated with either vehicle (0.5% methyl cellulose; Sigma-Aldrich) in sterile sodium chloride solution

(0.9%; Hospira, Lake Forest, IL) or the vehicle plus treatment compound(s). The saline solution was warmed prior to methylcellulose addition, aiding in proper dissolution, and after the solution cooled and cleared, it was sterile-filtered (polyethersulfone filter, 0.2 μ m; VWR International; Radnor, PA). Intervention treatments were: TRPA1 antagonist (100–200 mg/kg bwt; HC-030031; Sigma) alone or with TRPV1 antagonist (10 mg/kg bwt; capsazepine, Sigma) suspended in vehicle and bath sonicated prior to use (10 min, RT). TRPA1 antagonist was given alone (200 mg/kg bwt; i.p., 10 μ L/g bwt) or combined (100 mg/kg) with a TRPV1 antagonist (10 mg/kg). The TRP antagonists combination is anti-inflammatory in a murine pancreatic cancer model (Schwartz *et al.*, 2011). Morbidity and mortality were monitored every half-hour for the first 4h, and then subsequently at 6, 8, 24, 48 and 72h post-exposure.

Statistics

Values are expressed as mean $\pm$ SE. The LC₅₀ was estimated by three different statistical models: probit regression model, logistic regression model, and empirical non-parametric method. Similar estimates of the LC₅₀ (+95% SE) were observed in dose (ppm $\times$ min) vs survival curves (Suppl. Fig. 2). The LC₅₀ along with additional estimates of LC₇₀ and LC₇₅ were made for power and sample size calculations for TRP antagonist intervention experiments. Comparisons between 2 groups (e.g., air vs acrolein) were done with Chi-square, Fisher's Exact test, Mann-Whitney Rank Sum or *t*-test as appropriate, wherein multiple group comparisons were done with multiple logistic regression models for mortality and one-way or two-way ANOVA with Bonferroni adjustment for continuous outcomes, e.g., CBC. *P*<0.05 was considered significant.

RESULTS

Mortality

LC₅₀—After a range finding study, the lethal concentration where 50% of exposed WT male mice died within 24 h (LC₅₀, 24h) was estimated (Suppl. Fig. 2). Although an acrolein inhalation LC₅₀, 24h of 175 ppm (10 min exposure) in mice is reported (Albin, 1962), initial studies using adult male C57BL/6 (WT) mice required both an increased level and duration of acrolein exposure to achieve an LC₅₀, 24h of 225 ppm (30 min). The probit regression model resulted in estimates of LC₅₀ (213 ppm), LC₇₀ (217 ppm) and LC₇₅ (218 ppm). Because of the steep mortality curve, however, there remained considerable uncertainty of the LC₅₀, 24h (Suppl. Fig. 2). In subsequent studies, a restricted range (210–275 ppm, 30 min) was used to assess the effects of sex, age, and genotype on acrolein-induced lethality.

Sex—Male WT mice of all age groups were significantly more sensitive to acrolein-induced mortality (Chi-square; *P*<0.001) than exposure-matched WT female mice (Table 1). Kaplan-Meier survival plot showed the majority of male mice died within 24h whereas 90% of female mice survived to 72h (Fig. 1). Female mice exposed to acrolein overall had reduced morbidity as well, e.g., more active, less hypothermia, less bradypnea, less gasping, less lacrymation and less ocular crust indicating both pulmonary and systemic protection.

Age—Overall, age of mice, unlike sex, did not affect acrolein-induced mortality at 24h (Chi-square: $P=0.234$; Table 1). Age did not affect mortality in either female (Chi-square: $P=0.251$) or male ($p=0.465$) mice.

Genotype—TRPA1-null mice were more sensitive to acrolein-induced mortality than WT mice (Table 2). There was not a gene-dose relationship observed as male TRPA1 heterozygote mice (TRPA1^{+/-}) were similarly sensitive to acrolein-induced mortality as were male WT mice (Table 2). Protection against acrolein-induced mortality observed in female WT mice was absent in TRPA1-null female mice indicating that sex-dependent protection was secondary to the presence of TRPA1 (Table 2).

Morbidity

Airway Injury—As expected, exposure to high level acrolein led to upper airway (nasal and tracheal) injury characterized by epithelial sloughing, mucus accumulation and inflammatory cell infiltration, especially in upper airways (nasal and upper trachea) (Figs. 2 & 3). The lower airways and alveolar regions were little disturbed. During and shortly after end of exposures, mice switched from rapid nasal breathing to labored, bradypneic oral breathing (gasping). Sustained oral breathing in obligate nose breathers, e.g., rodents, leads to gross accumulation of air in the proximal GI tract (e.g., stomach and SI; Fig. 2Bi,ii). Obvious GI distension was a visual indicator of severity of upper (nasal) airway injury, and nasal airway histology confirmed the severe nature of injury; Fig. 2C–E). At times after different acrolein exposure levels, the BALF was collected and measured for cell differential (flow cytometry; hemacytometer) and presence of albumin and total protein (to measure plasma leak). Analysis of BALF contents revealed significant exposure-related increases in both total cells and protein at 24h post-exposure to acrolein (e.g., Suppl. Table 1).

Lung Injury and Biochemistry—Because epithelium injury was present at multiple levels of acrolein and to address the obvious TRPA1-dependence of mortality, mice were exposed to acrolein (250 ppm, 30 min) followed by 30 min of air exposure before collecting lungs and blood. Lung weight:body weight ratios were significantly increased in TRPA1-null mice and WT male mice but not in WT female mice (Fig. 4A). Plasma extravasation was apparent by detecting both significantly lower plasma albumin levels (Fig. 4B) and greater levels of albumin in lungs of TRPA1-null mice exposed to acrolein (Fig. 4C). Notably, WT female mice appeared little affected by high level acrolein exposure at this early time point. To understand this disparity, the level of protein-acrolein adducts, an expected marker of overall acrolein exposure (Conklin *et al.*, 2009; Wheat *et al.*, 2011), was measured in lung lysates and plasma. Surprisingly, protein-acrolein adducts were not increased in the lungs of acrolein-exposed mice (Fig. 4Di). In fact, a few protein bands were decreased significantly (or nearly so) in lungs of acrolein-exposed male (i.e., 45, 25 kDa; Fig. 4Dii) and female TRPA1-null mice (i.e., 45, 37, 25 kDa; Fig. 4Diii), which perhaps suggests that TRPA1-null mice had less lung exposure to acrolein than WT mice. Similarly, acrolein exposure did not increase plasma protein-acrolein adducts in any group (Fig. 4E).

Respiratory Parameters—To more precisely define acrolein-induced changes in pulmonary injury, respiratory parameters were monitored before, during and after acrolein

exposure by telemetry. Changes in respiratory parameters are presented as raw group data in Fig. 5 and as a % of pre-exposure baseline data in Suppl. Fig. 3.

Breathing Rate (bpm): Basal breathing rates (Phase I) were similar among all groups (Fig. 5A; Suppl. Fig. 3A). Compared with WT, TRPA1-null mice had a significantly delayed response to acrolein exposure during the 5 min ramp up (Phase II) indicating a TRPA1-dependent suppression of breathing rate was operative at lower acrolein levels. All groups, though, had significantly depressed breathing rates during both the steady state acrolein exposure (250 ppm, 30 min; Phase III) and subsequent air purge (Phase IV) phases compared with Phase I. Breathing rate remained suppressed even after Phase IV as confirmed by visual counting (Suppl. Fig. 4). Three of 4 female WT mice (with transmitters) survived to 24h, whereas all other mice with transmitters died before 24h (Suppl. Fig. 4).

Amplitude (mmHg): Basal amplitudes (Phase I) were similar amongst all groups by telemetry (Fig. 5B; Suppl. Fig. 3B). As amplitude reflects breathing effort (but not flow), it was notable that WT mice (female and male) rapidly increased amplitude upon onset of acrolein exposure (in Phase II) whereas this effect was delayed in TRPA1-null mice (Fig. 5B; Suppl. Fig. 3B). Amplitude in male TRPA1-null mice remained elevated in Phase III, whereas it decreased to near baseline levels in the other three groups. TRPA1-null female mice had increased amplitude near the end of Phase III and it remained elevated as in TRPA1-null male mice throughout most of Phase IV (purge).

Inspiratory and Expiratory Time (s): Basal inspiratory and expiratory times (Phase I) were similar across groups (Fig. 5C,D; Suppl. Fig. 3C,D). As noted for breathing rate and amplitude, inspiratory and expiratory times rapidly increased in WT mice upon exposure to acrolein (Phase II) with TRPA1-null mice lagging. As breathing rate decreased with acrolein, inspiratory or expiratory (or both) time increased. Two distinct patterns emerged: 1) TRPA1-null females had the slowest increase in inspiratory time and the greatest increase in Phase IV; and, 2) WT male mice had the greatest increase in expiratory time especially at the end of the air purge (Phase IV; Fig. 5C,D; Suppl. Fig. 3C,D).

Systemic Toxicity—Blood and plasma parameters were significantly altered after acrolein exposure in a concentration- and time-dependent manner (Tables 4 & 5; Suppl. Tables 2 & 3). Plasma albumin and total protein were both decreased by 25% in TRPA1-null mice (both sexes) exposed to acrolein (250 ppm, 30 min) at 30 min postexposure (Fig. 4B; Table 5). Acrolein-induced plasma fluid extravasation was reflected in blood hemoconcentration, i.e., increased RBC, hematocrit and Hb concentration (Fig. 6A,B; Table 4). Rapid fluid and albumin accumulation in the lungs likely led to the drop in both plasma albumin and total protein (Fig. 4A–C; Table 5), and indicated that acrolein severely compromised cardiopulmonary vascular barrier function to a greater extent in TRPA1-null than in WT mice. Hepatic (ALT, AST), heart (CK) and non-specific cell (LDH) toxicity enzyme levels were all significantly increased in TRPA1-null mice (Table 5). Surprisingly, female WT but not male WT mice exposed to acrolein had significantly increased levels of ALT, CK and LDH (Table 5). It should be noted, however, that changes in the levels of these enzymes in WT mice exposed to acrolein were not statistically different compared with air control group

(or female WT levels) due to a combined higher absolute baseline and greater variability (Table 5). Acrolein exposure also significantly decreased platelet (Plt) count, perhaps reflective of increased coagulation, in TRPA1-null mice (both sexes) but not in WT mice (Fig. 6C,D).

Cardiovascular Toxicity

Pulse Oximetry and Heart Rate—The depressed breathing rate observed during acrolein exposure (Fig. 5A; Table 3) prompted measurements of blood oxygen saturation (SpO₂) before and after exposure. Using pulse oximetry, SpO₂ and heart rate (HR) were measured before and 30 min after acrolein exposure (Table 4). Baseline SpO₂ and HR measurements were highly variable in conscious mice, yet acrolein significantly decreased SpO₂ in TRPA1-null ($-22\pm 3\%$; $n=7$) and WT ($-14\pm 5\%$; $n=8$) male mice but not in female mice (Table 4).

Aorta Function—Because vascular leak was dramatic after exposure, vascular function was measured post-exposure in isolated aorta of male WT and TRPA1-null mice after air or acrolein (250 ppm, 30 min) exposure. Concentration response curves to PE and ACh revealed no changes in efficacy or sensitivity of contraction or relaxations, respectively, between acrolein-exposed and matched air control group (data not shown). Sensitivity to ACh (an endothelium-dependent agonist) was modestly increased in acrolein-exposed WT compared with matched air control (WT ACh EC₅₀ [nM]: air, 552 ± 164 , $n=5$; acrolein, 136 ± 24 , $n=4$; $P=0.06$). This shift in sensitivity mirrored an increased relaxation stimulated by 10 μ M SNP (NO donor and endothelium-independent agonist: WT SNP, % relaxation: air, 66.5 ± 4.2 , $n=5$; acrolein, 81.1 ± 3.2 , $n=4$; $P<0.05$). These vascular effects of acrolein exposure were not observed in aorta of male TRPA1-null mice suggesting possible TRPA1-dependence.

Echocardiography—Because brief acrolein (250 ppm, 10 min) exposure elevated plasma CK levels (Table 5), echocardiography was done in WT male mice to examine potential cardiotoxicity. Acrolein induced significant cardiac depression characterized by diminished cardiac output (-60%) due both to reduced left ventricle chamber volume during diastole (decreased relaxation) and systole and to bradycardia at 24h post-acrolein exposure (225 ppm, 30 min)(Suppl. Table 4). Although these changes led to a mathematically increased ejection fraction (EF; $>99\%$), the decrement in cardiac output was severe. Collectively, these data indicate that acute acrolein exposure led to significant cardiac depression and toxicity with limited change in aortic function.

Body Temperature (BT, °C)

Acrolein rapidly and significantly decreased surface BT (sBT) in a level-dependent manner (Suppl. Fig. 5A). Core BT (cBT) measured by telemetry also decreased rapidly upon acrolein exposure and remained depressed throughout air purge (Phase IV)(Suppl. Fig. 5B). Although mortality occurred in mice with low/near ambient sBT (Suppl. Fig. 5A), the rate of sBT drop following exposure (i.e., in first 90 min) did not predict subsequent mortality, and the absolute change in sBT (°C) was not TRPA1-dependent (°C: Male WT = -4.4 ± 0.2 ; TRPA1-null = -4.1 ± 0.4 ; Female WT = -5.2 ± 0.7 ; TRPA1-null = -5.2 ± 0.2 ; $n=3-5$ per

group). Surface BT partially recovered 24h after exposure in surviving mice (mostly female mice).

TRP Antagonist Intervention

We tested the potential beneficial effects of a TRPA1 antagonist alone or in combination with a TRPV1 antagonist because these antagonists have been demonstrated to prevent TRPA1 activation and injury in multiple pathological settings including tracheal edema, tracheal contractile hyper-reactivity and in prevention of pancreatic pain and inflammation (Andre *et al.*, 2008; Schwartz *et al.*, 2011). We hypothesized that inhaled acrolein (as well as endogenously formed aldehydes) could activate TRPA1 receptors post-exposure, and thus, continued TRPA1 activation may contribute to nociception (pain), increased vascular permeability, tissue edema and upper airway inflammation, congestion and cardiac dysfunction. For TRPA1 antagonist intervention, mice received HC-030031, a TRPA1 antagonist (200 mg/kg bwt, ip), at 30 min after the end of an acrolein exposure (225 ppm, 30 min). In 2 separate consecutive trials, mice receiving HC-030031 treatment were significantly protected from acrolein-induced lethality (100 % survival at 24h; 0 dead of 10 exposed) whereas only 40% of vehicle-treated WT mice survived to 24h (i.e., 6 of 10 died, 60% mortality)(Table 6). Similarly, to test whether a combined intervention could enhance protection against acrolein, HC-030031 (100 mg/kg) was combined with TRPV1 antagonist (capsazepine, 10 mg/kg, ip) and given 30 min after the end of an acrolein exposure (275 ppm, 30 min). Similar to TRPA1 antagonist alone, combined TRP antagonist treatment (in 4 separate trials; III-VI) significantly protected acrolein-exposed mice (i.e., mortality; body temperature) over 72h post-exposure (Table 6; Suppl. Table 6). Because TRP antagonists improved survival, a return toward baseline body temperature was observed in TRP-treated (surviving) mice. Combined TRP antagonists injected into naïve mice had no effect on breathing rate (Suppl. Fig. 5) or surface body temperature (Suppl. Fig. 6) within 24h.

DISCUSSION

Because of the worldwide threat of catastrophic events, e.g., terrorist attacks, structural fires, wildfires and occupational exposures, it is expected that large numbers of civilians will be exposed acutely to high levels of combustion-derived aldehydes, such as acrolein. Thus, there is a real need to prepare for these inevitable situations, and understanding the determinants of morbidity and mortality of inhaled toxicants is a first step in developing rational interventions to reduce harm. Notably we identified two major determinants of inhaled acrolein-induced morbidity and mortality in mice that likely bear importantly on the future development of appropriate post-exposure interventions. First, we observed that TRPA1 is an important pre- and post-exposure determinant of acrolein-induced mortality, i.e., TRPA1-null mice are more sensitive than WT mice, and TRPA1 antagonist injection post-acrolein exposure reduces acrolein-induced mortality. Second, we show that female WT mice (regardless of age) are significantly protected from acrolein-induced mortality compared with male WT mice. Female protection, however, is lost in TRPA1-null females. These data indicate TRPA1 plays both context-specific and sex-dependent roles in high-level acrolein-induced cardiopulmonary toxicity and lethality.

TRPA1 is a promiscuous sensory channel/receptor and it is activated by a variety of environmental and endogenous noxious compounds including the α,β -unsaturated aldehydes, acrolein (propenal), crotonaldehyde (butenal) and 4-hydroxy-*trans*-2-nonenal (4HNE)(Bessac *et al.*, 2008). TRPA1 is in the pulmonary airways and it plays a role in the protective physiological maneuver called “respiratory braking” elicited by inhaled toxins (Alarie, 1973). Thus, a primary function of TRPA1 is to limit pulmonary exposure (as well as systemic) to inhaled toxins. The increased sensitivity of TRPA1-null mice to acrolein-induced morbidity and mortality (shown herein) supports this general function. Thus, it is not surprising that we observed a significant delay in onset of acrolein-induced respiratory braking in TRPA1-null mice (see Fig. 5; Table 3) accompanied by greater pulmonary (and systemic) injury (see Fig. 4A,B; Tables 4&5). Yet, curiously, we did not observe an increase in lung protein-acrolein adducts in TRPA1-null mice as predicted (see Fig. 5), however, the delayed onset of respiratory braking in TRPA1-null mice is relatively short (1–2 min) and ultimately TRPA1-null mice decrease respiratory rate to a degree present in WT mice by the end of Phase II (Fig. 5A). Perhaps more indicative of pulmonary distress, however, was the robust increase in amplitude observed in TRPA1-null mice but not in WT mice (Fig. 5B). This metric reflects ‘breathing effort’ and during acrolein exposure, it increased up to 7-times more than baseline in TRPA1-null male mice (Suppl. Fig. 3). Although we do not know the exact cause of this increased effort, a significant rapid loss of blood platelets in TRPA1-null mice (Table 4; Fig. 6C,D) suggests that pulmonary (nasal and/or lung) vascular congestion due to excessive thrombosis may occur in TRPA1-null mice. Previously, we have shown that low level acrolein inhalation (5 ppm, 360 min) induces platelet activation in male WT mice (Sithu *et al.*, 2010). Furthermore, our pulse oximetry data supports that oxygen exchange was most compromised in male TRPA1-null mice. Nonetheless, a detailed histopathological study is needed to document these specific events.

The idea that TRPA1 influences overall pulmonary exposure to toxicants is well supported. For example, Ha *et al.* (2015) show that menthol exposure (present in tobacco smoke and electronic cigarette aerosol) activates a TRPM8-mediated (M = melastatin) inhibition of the TRPA1 receptor leading to greater nicotine (and perhaps acrolein) exposure (Ha *et al.*, 2015). Capsaicin-sensitive fibers containing both TRPA1 and TRPV1 receptors are implicated in respiratory braking in rodents in response to high level acrolein (350 ppm) exposure (Lee *et al.*, 1992). Lee *et al.*, characterized acrolein-induced “braking” as vagus nerve-mediated and capsaicin- and cold-sensitive (Lee *et al.*, 1992). They conclude that acrolein stimulates airway C-fibers and rapidly adapting irritant receptors and that the reflexive apnea (and bradypnea) is mediated by C-fibers. The specific pulmonary location of these protective sensory TRPA1 receptors is unclear, yet it is likely these are present in the nasal epithelium as part of a trigeminal sensory apparatus. Studies of TRPA1-dependent changes in nasal histopathology of obligate nose breathers after acrolein exposure likely will provide clues that relate nasal and lung exposure to overall injury.

Female mice of all age groups tested (5–52 weeks) survive to a greater extent after acrolein exposure than age- and exposure-matched male mice, however, female-dependent protection against acrolein is abolished in TRPA1-null females indicating a sex-dependent role of TRPA1. Similarly, female mice are more sensitive to pain-inducing stimuli than male mice (Patil *et al.*, 2013a) and this sensitivity is under the control of prolactin.

receptor regulates TRPA1, TRPV1 and TRPM8 expression in sensory fibers (Patil *et al.*, 2013b). Perhaps, if female mice have more TRPA1 than males in pulmonary sensory fibers, we would expect to see a more robust “respiratory braking” (or another maneuver) to reduce acrolein exposure. In fact, female WT mice decrease breathing rate within the first minute of exposure whereas males are slightly delayed (Table 3). Perhaps this subtle difference explains female-dependent protection and why it is abolished to a similar degree in male and female TRPA1-null mice. On the other hand, having more TRPA1 in the lungs theoretically may lead to more pulmonary inflammation, edema, cough and airway hyperreactivity (Polverino *et al.*, 2012). In fact, women are at greater risk of chronic cough than are men although this may be due to increased TRPV1 expression as well (Groneberg *et al.*, 2004; Polverino *et al.*, 2012). The exact mechanism of female-dependent protection in mice will need to be investigated in future studies. Perhaps female-dependent protection is in part a function of acrolein metabolism as well because significant protection against oral and inhaled acrolein exposure is present in male WT vs male glutathione-*S*-transferase P (GSTP)-null mice (Conklin *et al.*, 2009; Conklin *et al.*, 2015).

TRPA1 is widely distributed in the periphery and its activation by tissue injury contributes to the local inflammatory response to increase blood flow, vascular permeability, leukocyte extravasation and pain (nociception) (Trevisani *et al.*, 2007; Schwartz *et al.*, 2011). So, we tested whether post-exposure TRPA1 antagonist intervention (and in combination with TRPV1 antagonist) could attenuate morbidity and mortality after acrolein inhalation. In our model, early intervention (30 min after end of exposure) with the TRPA1 antagonist, HC-030031 (alone), or in combination with a TRPV1 antagonist (capsazepine) provides measurable protection against mortality of inhaled acrolein. The systemic benefit of the combined TRPA1+V1 antagonist treatment (as used herein) also is described in a model of pancreatic cancer pain (Schwartz *et al.*, 2011). Nonetheless, the specific mechanism and site of protection of TRPA1 or combined TRPA1+TRPV1 antagonism remain to be elucidated although it does not appear to be due to stimulation of an increased breathing rate or inhibition of the well-known, robust rodent ‘hypothermic response’ (Gordon *et al.*, 1988) or hyperthermia (see Suppl. Figs. 6 & 7). Because the TRPA1 antagonist was administered intraperitoneally, we infer the treatment primarily targeted pulmonary vascular TRPA1 more so than other sites, e.g., airway sensory fiber or CNS TRPA1 (see **Graphical Abstract**). This is, of course, contradicted by the high level of morbidity and mortality observed in TRPA1-null mice indicative of worsened pulmonary vascular leak. As above, TRPA1 is thought to mediate the untoward pulmonary injury (e.g., edema, hypercontractility of airway smooth muscle) of tobacco smoke exposure as recapitulated by exposure of isolated trachea to unsaturated aldehydes such as cigarette smoke extract (CSE), acrolein, crotonaldehyde or cinnamaldehyde (Facchinetti *et al.*, 2007; Andre *et al.*, 2008; Simon and Liedtke, 2008). More research using novel tools such as animals with selective TRPA1 gene deletion in sensory fibers or vascular endothelium, will be required to address the specific mechanism of how TRPA1 antagonism protects post-acrolein.

This study has some important limitations. First, rodents are obligate nose breathers, and thus, these animals struggle to breathe when forced into oral (mouth) breathing, which confounds assessment of morbidity and mortality after extreme nasal congestion.

Nonetheless, female mice appear protected against nasal congestion (e.g., preserved breathing parameters, less albumin in lungs, less hemoconcentration), so the nature of this female protection likely reflects a relevant endogenous protective pulmonary mechanism. As our primary goal is to develop a post-exposure intervention, we did not pursue whether blockade of TRPA1 *pre-acrolein* would worsen outcomes in WT females (we expect it would). Instead, we used mice deficient in TRPA1, and, targeted transgenic mice may have compensatory mechanisms that dramatically alter exposure-related injury in an unpredicted way. As far as we know, however, there is no such evidence for such an underlying compensatory change in TRPA1-null mice (Bautista *et al.*, 2006). Third, we did not test whether TRPA1 antagonism had protective yet nonspecific effects (we expect it does not). Despite these model-based caveats, our primary observations of TRPA1- and sex-dependent toxicity of inhaled (high level) acrolein represent important “stepping-stones” to developing rational and effective post-acrolein exposure interventions. A TRPA1-targeted post-exposure therapy may be useful in treating victims of smoke inhalation including first responders and fire fighters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- TRPA1 protects mice against toxicity and mortality of inhaled high-level acrolein
- TRPA1 protection against inhaled high-level acrolein is sex-dependent in mice
- Age (5–52 weeks old) was not a determinant of acrolein-induced mortality in mice
- TRPA1 antagonist is protective after inhaled high-level acrolein in male mice

Figure 1.

Kaplan-Meier survival curves in male and female mice following acute exposure to high-level acrolein. n, total number of mice per group (combined from 2 separate trials). *, $P < 0.05$; significant difference from male WT (Chi-square).

Figure 2.

Gross pulmonary and microscopic nasal airway histopathology post-exposure to high-level acrolein. **A)** Formalin-fixed lungs of acrolein-exposed male and female mice. Note: Black-dashed lines represent approximate location of segments used for microscopic histology shown in Fig. 3. **Bi ii)** Accumulated gas in upper GI tract of acrolein-exposed mice at 24h. Proximal nasal histopathology of **Ci,ii**, air- and **Di,ii**, acrolein-exposed male mice indicating epithelial injury and mucus accumulation (pink-stained aggregates; black arrows; 100–400× mag.) at 24h. Proximal nasal histopathology of **Ei**, air- and **Eii**, acrolein-exposed male mice

indicating epithelial injury, mucus accumulation and edema (black arrows; 40x mag.) at 1 h post-acrolein (250 ppm) exposure.

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Figure 3. Pulmonary histopathology at 24h post-exposure to high-level acrolein. **A)** Histopathology (40x mag.) of **i,ii**, upper, **iii,iv**, mid- and **v,vi**, lower trachea, and **vii,viii**, lung parenchyma/alveolar regions from air- and acrolein-exposed (210 ppm, 30min) male mice at 24h post-exposure. **B)** Histopathology (100x mag.) of **i**, air and **ii–iv**, acrolein-exposed upper trachea of male mice showing acrolein level-dependent epithelial layer injury and sloughing.

Figure 4.

Lung and plasma biochemistry 30 min after acute high-level acrolein exposure (250 ppm, 30 min). **A)** Lung weight:body weight (BW) ratios, **B)** plasma albumin and **C)** lung albumin in male and female WT and TRPA1-null mice. Western blots **(i)** and quantification **(ii,iii)** of protein-acrolein adducts in **D)** lung and **E)** plasma in **ii**, male and **iii**, female mice. Protein-acrolein adducts normalized to amido black are presented as fold changes relative to WT male air-exposed control group. Values are mean $\pm$ SE. n=4–5 mice per group in **A,C–E** and n=7–9 mice per group in **B**. *, significant difference ($P<0.05$) between acrolein and matched air control; +, $0.10>P>0.05$ between acrolein and matched air control.

Figure 5.

Respiratory parameters before, during and after acute exposure to acrolein. Acrolein level was monitored continuously using an inline photoionization detector (PID; see Suppl. Fig. 1A). Respiratory parameters were continuously recorded before (Phase I), during 5 min ramp up (Phase II), in steady state (Phase III) and after (30 min purge, Phase IV) acrolein exposure (275 ppm, 30 min) by telemetry via an implanted pressure cannula (PA-C10; DSI) burrowed into the esophageal serosal layer within the thoracic cavity. **A**, Breathing rate (breaths per min, bpm) decreased rapidly upon exposure to acrolein (Phase II; in WT faster than in TRPA1-null mice) and remained suppressed during and after exposure (Phases III and IV). **B**, Amplitude ('breathing effort'; mmHg) increased rapidly upon exposure to acrolein (Phase II; transiently in WT faster than in TRPA1-null mice) but remained elevated during and after exposure in TRPA1-null mice only (Phases III and IV). **C** & **D**, Inspiratory and expiratory times (s), respectively, were increased by onset of acrolein exposure (Phase II) and remained elevated. Points are mean $\pm$ SE of 1 min recording. (n) = number of mice.

Figure 6. Select blood parameters at 30 min after acute high-level acrolein exposure (250 ppm, 30 min). Hemoglobin (Hb) and platelet (Plt) levels in **A, C**) female and **B, D**) male WT and TRPA1-null mice. Values are mean $\pm$ SE. (n) = number of mice. *, significant difference ($P < 0.05$) between acrolein and matched air control.

Table 1

Acrolein inhalation-induced mortality in C57BL/6 (wild-type, WT) mice.

C57BL/6 Mice (Age range, weeks)	Sex	Exposed (n)	Died (n)	Mortality (%)
Adult (12–20)	Male	23	11	47.8
	Female	15	1	6.6 [*]
Youth (5–6)	Male	18	12	66.7
	Female	18	2	11.1 [*]
Aged (>52)	Male	15	9	60.0
	Female	24	6	25.0 [*]

Mice were exposed to acrolein (210–250 ppm) for 30 min followed by 30 min of air to purge chamber. Mice were returned to home cages with food and water and monitored up to 8h and then again at 24h for mortality. n = number of mice.

^{*} P<0.05; significant difference from age-matched male group.

Table 2

Acrolein-induced mortality: TRPA1- and sex-dependence.

TRPA1 Mice (genotype)	Sex	Exposed (n)	Dead (n)	Mortality (%)
TRPA1 ^{+/+} (WT)	Male	5	1	20
	Female	2	0	0
TRPA1 ^{+/-} (heterozygote)	Male	8	1	12.5
	Female	4	0	0
TRPA1 ^{-/-} (null)	Male	9	9	100 [*]
	Female	6	5	83.3 [*]

Mice were exposed to acrolein (210 ppm) for 30 min followed by 30 min of air to purge chamber. Mice were returned to home cages with food and water and monitored every h for 8h and then again at 24h for mortality. (n) = number of mice.

^{*} P<0.05; significant difference from other sex-matched genotypes.

Note: Due to the low numbers of exposed female WT and heterozygote mice, these 2 groups were summed and then compared statistically with female TRPA1-null group.

Table 3

Respiratory parameters in wild type (WT) and TRPA1-null mice by telemetry.

Parameter		Breathing Rate (bpm)									
Grp	Phase	I	II (1 min)	II (2 min)	II (3 min)	II (4 min)	II (5 min)	III	IV		
WT female		136.5±4.4	95.4±14.5 *	83.1±20.9 *	75.4±28.8 *	80.3±23.8 *	84.4±21.2 *	84.1±8.1 *	60.9±7.7 *		
Null female		144.5±11.6	157.8±14.8	144.8±24.8	125.5±13.9 *	86.8±18.8 *	85.8±13.8 *	54.8±6.2 *	41.4±4.1 *		
WT male		154.9±8.6	125.2±23.5 +	66.5±7.6 *	64.4±8.9 *	57.8±16.6 *	56.1±9.9 *	58.8±5.2 *	41.5±7 *		
Null male		167.4±6.6	160±7.4	132.1±17.9 *	130.9±17.6 *	101.7±16 *	86.7±16.4 *	59.3±7 *	49.9±9.1 *		
Parameter		Amplitude (mm Hg)									
WT female		12.5±1.5	35.5±12.8 *	20.5±5.2	19.5±11.8	9.4±3.8	11.4±5.8	12.5±2.1	15.5±3.4		
Null female		8.5±2.7	8.2±2.8	9.7±3	12.1±4.5	16.2±8.4	17.1±7.1	18.3±6.7	27.8±14 *		
WT male		11.1±2.0	20.6±3.2	15±4.6	17.5±5.7	16±9.4	13.4±3.4	12.5±2	11.3±2.3		
Null male		13±3.4	13.7±3.1	12.4±3	11±2.1	23±6.3	29.4±7.5 *	30.9±6.5 *	32.9±10.5 *		
Parameter		Inspiratory Time (s)									
WT female		0.3±0.0	0.6±0.1	0.6±0.1	0.7±0.2 *	0.5±0.1	0.6±0.2	0.5±0.1	0.6±0.1		
Null female		0.3±0.0	0.3±0.0	0.3±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.6±0.1	1.2±0.3 *		
WT male		0.3±0.0	0.4±0.1	0.5±0.1	0.8±0.4 *	0.6±0.3	0.6±0.2	0.6±0.1	0.6±0.1		
Null male		0.3±0.0	0.3±0.0	0.3±0.0	0.4±0.1	0.7±0.2 *	0.6±0.1 +	0.7±0.2 *	0.8±0.1 *		
Parameter		Expiratory Time (s)									
WT female		0.1±0.0	0.3±0.1	0.2±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.4±0.1 *	0.5±0.0 *		
Null female		0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.3±0.1	0.5±0.1 *	0.6±0.0 *		
WT male		0.1±0.0	0.1±0.0	0.3±0.0	0.4±0.1 *	0.4±0.1 *	0.4±0.1 *	0.5±0.1 *	0.9±0.3 *		
Null male		0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.3±0.1	0.5±0.1 *	0.7±0.2 *		

Values are means±SE. Abbr.: Grp, group; bpm, breaths per minute; Phase I is 5 min pre-exposure; Phase II (1 min), II (2 min), II (3 min), II (4 min), and II (5 min) are the first, second, third, fourth and fifth minute of acrolein exposure (i.e., "Ramp Up" Phase); Phase III is 30 min of acrolein exposure at target level; Phase IV is 30 min purge of exposure chamber with filtered room air. Number of mice: WT female=4; Null female=4; WT male=5; Null male=6.

* P<0.05; significant difference from other matched Phase I (air control) period.

Table 4

Complete blood count (CBC) and pulse oximetry in WT and TRPA1-null mice 30 min after acrolein inhalation exposure (250 ppm, 30 min).

Group (N)	WT Female (4-8)		TRPA1-null Female (4-10)		WT Male (4)		TRPA1-null Male (4)		
	Rx	Air	Acrolein	Air	Acrolein	Air	Acrolein	Air	
CBC									
WBC		0.97±0.11	1.6±0.11	0.86±0.09	1.21±0.19	1.88±0.30 [#]	2.26±0.23	1.05±0.05	1.85±0.13
Neutr		29±4	27±2	28±3	29±3	58±1 [#]	33±2 [*]	40±6	41 ±3
Lymph		68±4	62±8	68±2	68±3	37±2	64±1 [*]	57±6	55±3
Mono		2±0	3±0	4±1	3±0	3±1	3±1	1±0	3±0 [*]
Eo		0.3±0.0	0.6±0.5	0.4±0.2	0.2±0.1	0.8±0.2	0.1 ±0.1	0.5±0.2	0.1 ±0.1
RBC		8.7±0.4	7.2±0.6	8.4±0.1	8.4±0.7	8.4±0.3	8.8±0.7	8.9±0.3	10.1 ±0.5 [*]
Hb		11.8±0.6	10.8±0.8	10.7±0.2	12.7±1.2 [*]	10.6±0.2	11.4±0.6	11.8±0.1	13.2±0.8 [*]
HCt		39.7±2.2	32.1 ±2.8	36.7±0.4	37.3±3.3	39.8±1.0	41.5±3.2	42.3±1.4 [#]	47.4±2.5 [#]
MCV		45.4±0.7	44.8±0.3	44.0±0.4	44.6±0.2	47.3±0.7	47.2±0.1	39.9±7.5	46.8±0.3
MCH		13.6±0.2	15.1 ±0.2	12.9±0.1	15.1 ±0.2 [*]	12.5±0.2	12.9±0.2 [#]	13.2±0.3	13.0±0.2 [#]
MCHC		29.8±0.2	33.6±0.6	29.2±0.5	33.9±0.2	26.5±0.1	27.4±0.4 [#]	27.8±0.7	27.9±0.2 [#]
RDW		17.4±0.3	16.2±0.1	18.1 ±0.2	17.2±0.2	16.6±0.3	15.8±0.1	16.6±0.3	16.1±0.1
PLT		607±20	511±38	698±30	473±35 [*]	679±46	571±33	721±50	556±20 [*]
SpO ₂ ^a		81±12	70±12	78±5	82±5	93±3	79±5 [*]	88±3	67±5 [*]
HR ^a		568±51	441±54	575±45	461±48	602±57	442±48 ⁺	588±62	480±63

Values are means±SE. Abbr: N, number of mice; WBC, white blood cell count, $\times 10^3$ per μ L; Neut, Neutrophils; Lymph, Lymphocytes; Mono, Monocytes; Eo, Eosinophils; % of WBC; Basophils were not detected; RBC, red blood cells, $\times 10^6$ per μ L; Hb, hemoglobin, g/dL; Hct, hematocrit, %; MCV, mean corpuscular volume, fL; MCH, mean cellular hemoglobin, pg/cell; MCHC, mean corpuscular hemoglobin concentration, g/dL; RDW, RBC distribution width, %; PLT, platelets, $\times 10^3$ per μ L; SpO₂, oxygen saturation, %; HR, heart rate, bpm;

^a parameters measured pre- (Air) and post-acrolein (Acrolein) exposure by pulse oximeter (n=7-9; paired F-test).

^{*} P<0.05, significant difference from matched air control group;

[#] P<0.05, significant difference between male and female of the same exposure and genotype;

⁺ 0.10>P>0.05, from matched air control group.

Table 5

Plasma parameters in WT and TRPA1-null mice measured 1h after acrolein (250 ppm, 30 min) exposure.

Factor	Rx (N)	WT Female			TRPA1-null Female			WT Male			TRPA1-null Male		
		Air (7)	Acro (7)	Acro (7)	Air (9)	Acro (9)	Acro (7-9)	Air (8-9)	Acro (8)	Air (7)	Acro (6-7)	Air (7)	Acro (6-7)
Cholesterol ¹		65±5	83±9		68±2	73±6		88±13	102±7	85±2	80±11		
Triglycerides ¹		39±4	63±4 *		29±1	48±3 *		56±5 #	76±5 *	41±2 #	64±3 #		
Albumin ²		2.54±0.08	2.52±0.17		2.68±0.09	1.98±0.16 *		2.96±0.21	2.57±0.22	2.73±0.11	1.81±0.19 *		
Total Protein ²		3.20±0.19	3.35±0.08		3.63±0.11	2.73±0.07 *		3.96±0.07	3.60±0.20	3.86±0.12	2.64±0.30 *		
ALT ³		24±2	34±2 *		34±2	69±8 *		41±6 #	52±5 #	36±3	126±30 #		
AST ³		64±7	103±18		86±7	187±25 *		90±15	126±19	80±8	264±58 *		
CK ³		161±22	445±103 *		198±25	871±159 *		499±86	725±153	380±61	1629±298 #		
LDH ³		197±14	268±21 *		233±14	455±35 *		337±47	356±55	258±37	636±73 #		

Values are means±SE. Units:

¹ mg/dl;

² g/dl;

³ U/L;

Abbr: N, number of mice; Acro, acrolein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; LDH, lactate dehydrogenase;

* P<0.05 vs matched air control group;

P<0.05, significant difference between male and female of the same exposure and genotype.

Table 6

TRPA1 and TRPA1+TRPV1 antagonist intervention efficacy in male WT mice exposed to high-level inhaled acrolein.

Trial	Vehicle	% Survival	Intervention	% Survival
I	2/5	40	5/5	100
II	3/5	60	5/5	100
I & II	5/10	50	10/10	100 [*]
III	2/5	40	4/5	80
IV	1/5	20	2/5	40
V	2/5	40	4/5	80
VI	1/5	20	2/5	40
III, IV, V & VI^a	6/20	30	12/20	60 [*]

Intervention was a single bolus given at 30 min post-exposure of HC-030031 compound (200 mg/kg bwt, ip; 10 µl/g bwt) in Vehicle (0.5% methylcellulose). In **Trials I-II**, mice were exposed to 225 ppm acrolein for 30 min after which morbidity (i.e., bradypnea, oral breathing, hypothermia) and mortality were monitored over 24h.

^a**Trials III-VI**, mice exposed to 275 ppm acrolein (30 min) received combined TRPA1 and TRPV1 antagonists (HC-030031, 100 mg/kg bwt; Capsazepine, 10 mg/kg bwt, ip) 30 min after end of acrolein (Phase IV; purge) with mortality recorded out to 72h.

* P<0.05; significant difference with Vehicle group.