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TRPA1: Acrolein meets its target

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1. Introduction

Two papers in this issue of Toxicology and Applied Pharmacology reveal that the sensory irritant receptor, Transient Receptor Potential Ankyrin 1 (TRPA1), plays a key role in the morbidity and mortality caused by inhalation of acrolein, the major electrophile in smoke from fires, tobacco and in automobile exhaust (Conklin et al., 2016; Kurhanewicz et al., 2016). Animals deficient in TRPA1, or treated with a TRPA1 inhibitor, were resistant to acrolein-induced cardiac arrhythmia and pulmonary injury and survived severely toxic acrolein exposures at higher rates than wild-type mice. Recognizing the multiple targets and injury mechanisms previously proposed for this reactive aldehyde, these are remarkable findings pointing towards an unexpected role of chemosensory reflex and homeostatic mechanisms in toxicological injuries.

2. Acrolein: chemistry and respiratory toxicity

Very few toxicants have been studied as thoroughly as the unsaturated aldehyde, acrolein (2-propenal). Acrolein is formed when organic matter is combusted, in a burning cigarette, in vegetation and structural fires, by diesel engines or when cooking oil is heated above its smoking point. Acrolein is also an industrial product used for chemical synthesis and as a biocide. Acrolein is a highly reactive electrophile and can undergo Michael additions with thiols to form adducts with biological molecules. Due to its low boiling point and high volatility, acrolein is a substantial respiratory threat. Mainstream cigarette smoke may contain >50 ppm acrolein (Brunnemann et al., 1990). Similar levels were measured in wood or cotton smoke (Einhorn, 1975). Acrolein was determined to account for >80% of the non-cancer hazard index of cigarette smoking (Hausmann, 2012). Prolonged inhalation of acrolein or inhalation of highly concentrated vapors causes acute lung injury and edema, and can be lethal. Inhalation at sub-ppm or low ppm concentrations elicits inflammation, airway hyperreactivity, inhibition of ciliary beating in airway-lining epithelial cells and epithelial and endothelial barrier damage (Walker and Kiefer, 1966; Bein and Leikauf, 2011). Acrolein

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has also been implicated in the etiology of chronic obstructive pulmonary disease (COPD) and lung cancer and was shown to accelerate cellular senescence (Bein and Leikauf, 2011; Luo et al., 2013). Many different targets and toxicity mechanism for acrolein were identified. Acrolein depletes glutathione and other redox buffers, inhibits enzymes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), interferes with $\text{Nf}\kappa\text{B}$ signaling and other signaling pathways, reacts with metabolites and other macromolecules and damages cellular organelles, including mitochondria (Valacchi et al., 2005; LoPachin and Gavin, 2014). Acrolein also induces mucin gene transcription, leading to copious mucus production (Borchers et al., 1998), forms covalent adducts with the tumor suppressor p53, and was found to react with cellular DNA. Acrolein exposures *in vitro* and *in vivo* change the activity and levels of transcription factors, cellular adhesion molecules and cytokine receptors, and affect the proteasome and ubiquitin-guided protein degradation machinery (Bein and Leikauf, 2011). While the respiratory tract is the primary target of acrolein's toxicity, prolonged or repeated inhalation has widespread systemic effects, damaging endothelial cells and promoting atherosclerosis (Fritz and Petersen, 2013).

3. Sensory irritation by acrolein

Acrolein is also a sensory irritant that excites peripheral sensory nerves to induce pain and respiratory irritation (Kane and Alarie, 1977; Morris et al., 1999; Morris et al., 2003). Neuronal activation is immediate and occurs on a much faster timescale than the injurious chemical tissue effects of acrolein. The stinging eye pain and lachrymatory reflex caused by smoke exposures are initiated when acrolein activates trigeminal sensory nerve endings in the cornea (Weber, 1984). Due to these irritant and lachrymatory effects, acrolein was used as a tear gas in World War I (Bessac and Jordt, 2010; Rothenberg et al., 2016). Within the upper airways, acrolein stimulates trigeminal nerve endings in the nasal passages, resulting in tingling, stinging, painful sensations, sneezing and increased nasal secretions. Acrolein also triggers cough by activating vagal laryngeal sensory nerve endings. Rodent studies demonstrated that nasal trigeminal nerve activation by acrolein vapor causes respiratory depression, a reflex response decreasing respiratory rates. These acrolein-triggered acute reflex responses are thought to be defensive, expelling and inactivating a potentially injurious exposure, protecting respiratory tissue and initiating avoidance and evasive behavior (Liu et al., 2011).

Rodent studies revealed that acrolein activates neurons sensitive to capsaicin, the pungent ingredient in chili peppers. Sensitivity to capsaicin defines sensory C-fibers, the non-myelinated nerve fibers that signal pain (nociceptors). When animals were pre-treated with capsaicin, leading to desensitization or neuronal ablation, acrolein failed to induce respiratory depression (Morris et al., 2003). Intriguingly, many other respiratory irritants were found to depend on capsaicin-sensitive neurons to induce sensory irritation. These include chlorine gas, tear gas agents, isocyanates, formaldehyde and many other aldehydes (Morris et al., 2005). These findings suggested that capsaicin-sensitive nociceptors express receptors for reactive aldehydes and other chemical irritants. In 1997 the discovery of the neuronal capsaicin receptor was reported. This receptor, Transient Receptor Potential Vanilloid 1 (TRPV1), belongs to the Transient Receptor Potential (TRP) ion channel gene family. TRP ion channels are key integrators of sensory signals in the visual, auditory,

olfactory and somatosensory systems. In addition to pain, capsaicin induces the sensation of burning heat. Indeed, TRPV1 was found to be activated by noxious heat ($>42^{\circ}\text{C}$), serving as a sensor inducing pain to prevent thermal injuries. TRPV1 also responds to stimuli associated with injury and inflammation, including acidity, endogenous lipid-derived mediators, and activation of G-protein coupled receptors.

4. TRPA1, a receptor for acrolein in sensory neurons

In TRPV1-deficient mice, the respiratory irritation response to acrolein was retained, suggesting that the capsaicin receptor is not directly involved in the sensory detection of acrolein (Symanowicz et al., 2004). A study investigating cellular neuronal responses to unsaturated aldehydes revealed that these compounds elicited the influx of calcium ions (Inoue and Bryant, 2005). Calcium influx was sensitive to ruthenium red, a non-specific TRP channel blocker (Inoue and Bryant, 2005). Thus, while not targeting TRPV1, it was hypothesized that acrolein and other unsaturated aldehydes may activate an uncharacterized TRP ion channel in capsaicin-sensitive neurons. This TRP ion channel, TRPA1 (Transient Receptor Potential Ankyrin 1), was discovered in 2006 (Bautista et al., 2006). TRPA1 was originally characterized as the neuronal target of mustard oil (allyl isothiocyanate), the pungent compound formed in mustard and wasabi, frequently used in pain research to probe the function of sensory nerves (Jordt et al., 2004). As predicted, TRPA1 is expressed in TRPV1-positive neurons of all sensory ganglia (trigeminal, vagal and dorsal root). Acrolein activated both rodent and human TRPA1 ion channels expressed in heterologous cells (Bautista et al., 2006). Genetic deletion of the TRPA1 gene in mice eliminated acrolein-induced calcium influx in cultured sensory neurons, strongly suggesting that TRPA1 is essential for neuronal sensitivity to acrolein (Bautista et al., 2006). Subsequent studies reported a loss of sensitivity to acrolein and other unsaturated aldehydes in TRPA1-deficient mice *in vivo*. Smoke aldehydes such as crotonaldehyde and methacrolein were identified as TRPA1 agonists (Andre et al., 2008; Escalera et al., 2008). TRPA1 was found to mediate pain and cough elicited by cinnamaldehyde, the pungent unsaturated aldehyde in cinnamon (Bandell et al., 2004; Birrell et al., 2009). Painful and inflammatory terpenes containing unsaturated aldehyde moieties were found to target TRPA1 (Escalera et al., 2008). TRPA1 activation by acrolein and other unsaturated aldehydes triggers the release of pro-inflammatory neuropeptides such as CGRP and Substance P from sensory nerve endings in the lungs, trachea and larynx (Andre et al., 2008; Kichko et al., 2015). This mechanism, called neurogenic inflammation, points towards a role of TRPA1 beyond the acute and passive sensing of aldehyde exposures. Indeed, TRPA1 inhibitors were found to suppress inflammation in animal models of asthma, contact dermatitis, diabetes and other inflammatory conditions (Caceres et al., 2009). These conditions are often associated with an increased endogenous aldehyde load. Endogenous unsaturated aldehydes such as 4-hydroxynonenal (4-HNE), often contained within oxidized phospholipids and associated with asthma, and methylglyoxal, associated with diabetes, were identified as TRPA1 agonists, suggesting that chronic activity of TRPA1 may contribute to the disease etiology of these conditions (Trevisani et al., 2007; Ohkawara et al., 2012; Liu et al., 2016).

5. Conklin et al.: TRPA1 in the cardiopulmonary toxicity of acrolein

The study by Conklin et al. in this issue probes the role of TRPA1 in injuries elicited by exposures of mice to high levels of acrolein (100–275 ppm, 10–30 min). Exposures at these levels may potentially occur in industrial accidents or upon deliberate release of acrolein as a terrorist weapon. Acrolein caused severe injuries to the airway epithelia of the exposed mice, resulting in incapacitation due to respiratory and cardiovascular distress with a high degree of mortality. Acrolein elicited severe pulmonary edema and inflammation. The authors observed that male mice were more susceptible to acrolein's toxic effects and succumbed at higher rates to their injuries while female mice of the same strain (C57BL/6) survived at higher rates. TRPA1-deficient mice, both male and female, showed increased mortality after exposure. Acrolein-exposures depressed respiration, measured by telemetry of respiratory rates. TRPA1-deficient mice responded more slowly to acrolein exposures, displaying higher respiratory rates, suggesting that TRPA1 controls the respiratory irritation response to acrolein even at the high concentrations used by the authors (>100 ppm). Intriguingly, post exposure administration of a small molecule TRPA1 inhibitor, HC-030031, had protective effects and improved survival rates in male mice.

6. Kurhanewicz et al.: TRPA1 in acrolein-induced cardiac arrhythmia

Epidemiological studies have shown that even low level exposures to pollutants such as acrolein elicit cardiovascular distress and increase the likelihood of cardiac arrhythmia, heart failure or stroke (Perez et al., 2015). These cardiovascular events may contribute to the increased morbidity and mortality associated with air pollution.

The paper by Kurhanewicz et al. in the present issue addresses the poorly understood mechanism of these pollutant-induced cardiovascular events (Perez et al., 2015; Kurhanewicz et al., 2016). In prior pioneering studies, these authors from the Environmental Protection Agency (EPA) demonstrated that exposures of hypertensive rats to synthetic residual oil fly ash or pure acrolein vapor resulted in rapid changes in heart rate and other functional cardiovascular parameters and triggered arrhythmic episodes (Hazari et al., 2009). In a subsequent study the authors observed that administration of a pharmacological inhibitor of TRPA1 prevented (acrolein-containing) diesel exhaust-induced arrhythmia in rats, suggesting that TRPA1 activity promotes the imbalance in the autonomic regulation of cardiovascular function in response to irritant inhalation (Hazari et al., 2011). This effect was sensitive to sympathetic blockade, suggesting that acrolein, through TRPA1, affects the autonomic regulation of cardiac function. Additional studies demonstrated that a single exposure to acrolein was sufficient to dampen the vagal baroreflex in both normotensive and hypertensive rats (Hazari et al., 2014). Changes in heart performance were observed already at very low acrolein levels that did not trigger the respiratory irritation response, demonstrating the exquisite sensitivity of sensory-autonomic regulation of cardiovascular function to inhaled pollutants (Thompson et al., 2016).

In the present study Kurhanewicz et al. investigate the role of TRPA1 in the cardiovascular response of mice to acrolein (3 ppm for 3 h) or ozone (0.3 ppm for 4 h). The use of mice, requiring sophisticated surgical and technical acumen to establish reliable telemetry, allowed

comparison with responses in animals genetically deficient in TRPA1. Similar to rats, acrolein exposures increased heart rate variability in wild-type mice with a higher incidence of arrhythmic episodes. In contrast, acrolein had no such effects in TRPA1-deficient mice. Acrolein changed mechanical heart function when measured in the isolated heart 24 h after exposure revealing increased left ventricular developed pressure, an effect absent in TRPA1-deficient mice. In contrast to acrolein, ozone exposures did not cause noticeable changes in cardiac function.

7. Mechanisms of TRPA1-mediated toxicological injuries by acrolein

The results by Conklin et al. and Kurhanewicz et al. provide genetic and pharmacological evidence for a single target of acrolein, TRPA1, being responsible for the local and systemic toxicological injuries following exposures to both low and high acrolein levels. Acknowledging the many different targets proposed for acrolein in the past, this new finding is of great significance for understanding the mechanism of acrolein toxicity (Fig. 1).

Conklin et al. demonstrated that pharmacological inhibition of TRPA1 rescued mice from mortality following severe exposures. While high level exposures to acrolein are rare, acrolein was used as a warfare agent and may be diverted as a weapon again, or released by accident. TRPA1 inhibitors, currently in development for the treatment of pain and asthma, may be effective mechanism-based countermeasures. However, for further validation, testing of TRPA1 inhibitors in non-rodent species is essential. As observed by Conklin et al., acrolein caused gasping and severe respiratory distress in mice, probably resulting from hypoxia due to nasal obstruction in these animals that are obligate nasal breathers. TRPA1 inhibitors may relieve nasal obstruction thereby allowing sufficient respiration to prevent severe hypoxia. Whether TRPA1 inhibitors can counteract respiratory damage and improve recovery in humans and other large species capable of breathing through nose and mouth remains to be established.

While it was known that acrolein causes acute neuronally-mediated TRPA1-mediated respiratory irritation and pain, Kurhanewicz et al. clearly demonstrate that TRPA1 is also essential for the acrolein-induced systemic dysregulation of cardiovascular function causing heart rate variability and arrhythmia. These effects may compound the highly injurious tissue effects of inhaled acrolein in the respiratory system occurring at high concentrations as demonstrated by Conklin et al. The severe pain and irritation of high-level exposures incapacitates and disorients the victim leading to more extended exposures and injuries while suffering from cardiovascular distress. TRPA1, by triggering acute neurogenic inflammation, may contribute to local respiratory tissue injury promoting extravasation and edema formation and recruitment of neutrophils and other pro-inflammatory cell types that, if uncontrolled, may cause further tissue damage. In fact, neutrophils produce a wide range of chemical species known to activate TRPA1, including reactive oxygen species and hypochlorite (Panasenکو et al., 2013). It is possible that the chronically oxidizing and electrophilic milieu in the lung continuously activates TRPA1-expressing sensory nerves and leads to prolonged sensory-autonomic reflex dysregulation of cardiovascular function. TRPA1 activation is also known to lead to vasodilation through local release of neuropeptides, an effect that may occur when acrolein's systemic levels increase, first within

the pulmonary vasculature, and then systemically (Jordt et al., 2004; Bautista et al., 2005; Pozsgai et al., 2010).

Acrolein, similar to other electrophilic agonists, likely activates TRPA1 through covalent modification of the TRPA1 protein that leads to increased open probability of the ion channel that conducts cations, leading to neuronal depolarization and calcium ion influx into sensory nerve endings. High-level exposures may cause receptor desensitization, leaving neurons insensitive to subsequent exposures. It is currently unknown how quickly covalently modified TRPA1 channel complexes are internalized, recycled or replaced with new TRPA1 complexes capable to re-establish sensory capacity. Excessive influx of calcium ions into sensory nerve endings damages nerves and may result in peripheral neurodegeneration. As such, acrolein exposures may cause neuropathic injuries in airway-innervating nerves. Together, these molecular and cellular mechanisms may result in permanent changes of cardiovascular control by sensory nerves due to acrolein exposure. Acrolein-induced neuropathic mechanisms may also contribute to chronic cough and airway hyperreactivity, symptoms frequently observed after inhalation of acrolein-containing exposures.

The sex difference in acrolein-induced mortality is intriguing and warrants further investigations. While it is possible that sex-specific regulation of TRPA1 expression is responsible for the observed difference, other sexually dimorphic mechanisms may play a role such as the differential production of antioxidant defense systems, protective enzymes, cytokines and other mediators. Increased male susceptibility has been reported for other types of lung injury, including hypoxic lung injury where female mice of the same strain (C57BL/6) displayed higher recovery rates (Lingappan et al., 2013). Differences in acrolein-induced mortality also exist between inbred mouse strains, however, the *Trpa1* gene was absent in the list of polymorphic genes linked to strain-specific acrolein susceptibility (Leikauf et al., 2011).

Increased sensitivity to pulmonary injuries extends to humans. Analysis of clinical data revealed that male patients are at higher risk for ARDS-induced mortality (Moss and Mannino, 2002). The reason for the higher susceptibility of males remains to be explored. Investigating and engaging these sexually dimorphic mechanisms may yield additional therapeutic strategies to counteract acrolein toxicity in males.

8. TRPA1: one receptor to sense them all?

In addition to acrolein, TRPA1 is sensitive to many other chemical exposure threats, including mixtures containing several chemically reactive components. Smoke from tobacco or fires, as well as diesel exhaust, contain additional reactive saturated and unsaturated aldehydes. Besides the volatile constituents, smoke and diesel exhaust particulates were observed to activate TRPA1 (Deering-Rice et al., 2011; Shapiro et al., 2013). Thus, while acrolein levels may oftentimes be lower in air pollution than the concentrations used in the present studies, environmental exposures may contain additional TRPA1-activating stimuli sufficient to elicit significant cardiovascular and respiratory distress. Kurhanewicz et al. reported that ozone, at 0.3 ppm, failed to elicit TRPA1-mediated cardiovascular effects in mice. This ozone concentration may be too low to activate TRPA1 in airway sensory

neurons of mice. Ozone levels in smog in large cities can exceed 0.3 ppm. Photochemical oxidation in smog produces secondary organic aerosols that likely contain additional TRPA1-activating pollutants (Doyle et al., 2007). These may include terpenes and their reaction products, aldehydes and particulates (Lin et al., 2013). In combination, these pollutants may activate TRPA1 during exposures to photochemical smog and may increase the incidence of respiratory distress and adverse cardiovascular events.

While environmental regulations and policies aim to reduce air pollution and associated health effects, pharmacological intervention, potentially involving TRPA1 inhibitors, may represent a complementary approach to reduce the probability of life-threatening complications during episodes of high air pollution. This strategy might be especially beneficial in patients affected by chronic respiratory and cardiovascular conditions such as COPD, asthma and hypertension (Caceres et al., 2009; Balakrishna et al., 2014; De Logu et al., 2016). The work by Kurhanewicz et al. is exemplary for EPA-supported environmental health research with far-reaching implications for other research areas such as the development of countermeasures for the national defense against chemical terrorism and against injuries due to chemical industrial and transportation accidents. The reduction in mortality by TRPA1 inhibitor treatment observed by Conklin et al. in acrolein-exposed mice may, in part, be the result of diminished TRPA1-dependent dysregulation of cardiovascular function discovered by Kurhanewicz et al.

TRPA1 is activated by many other lethal chemical threats, including chlorine gas, currently used as a chemical weapon by the Syrian government, and methyl isocyanate (MIC), the toxicant released in the 1984 Bhopal industrial disaster in which >3700 exposure victims perished and many thousands more were injured (Dhara and Dhara, 2002; Svendsen et al., 2012; Padley, 2016). Exposures to these agents have effects similar to acrolein exposures, including irritation and severe pain, blepharospasm, choking, pulmonary edema, and cardiovascular damage that can result in circulatory collapse and death. The studies by Conklin et al. and Kurhanewicz et al. strongly suggest that TRPA1 inhibitors may be of benefit to counteract exposure injuries caused by chlorine, MIC and the many other electrophilic and oxidizing TRPA1 agonists.

9. Implications for toxicological testing

The findings by Conklin et al. and Kurhanewicz et al. have important implications for toxicological testing programs such as the National Toxicology Program (NTP) of the United States and similar programs elsewhere. Based on recommendations from the Tox21 (Toxicology in the 21st century) initiative, programs such as NTP are migrating to quantitative structure-activity relationship (QSAR) modeling approaches and cell based assays to examine and predict the potential tissue and systemic toxicities of new chemicals to be used in food, consumer and personal care products, clothing, prescription drugs, cleaning products, agriculture and many other applications (Tsakovska et al., 2008). The current cell-based models used in these screens focus on genotoxicity, metabolic toxicity and other types of cellular damage. They are poor predictors of the sensory irritation potential of a given chemical and its capacity to initiate subsequent organ and system pathologies. Similar limitations apply to organotypic cultures or organ-on-a-chip models since they do

not contain sensory nerve endings that detect irritants, release neuropeptides and initiate reflexes and other systemic responses that affect organ and system health. A remedy for this situation would be to add TRPA1 activation assays to the testing repertoire, with TRPA1 expressed in heterologous cells and calcium ion influx measured with fluorescent indicators in response to a given chemical (Lehmann et al., 2016). Such assays should become standards similar to the hERG/Kv11.1 test that predicts the potential for a drug development candidate to cause cardiac arrhythmia by affecting the function of a cardiac potassium channel (Moller, 2010). Other chemosensory TRP ion channels recommended to be included in toxicological screens are TRPV1, the capsaicin receptors, and TRPV4, due to their key roles in irritation and pain, reflex initiation and control of endothelial and epithelial barriers. While the electrophilic properties of a given chemical may be predictive of TRPA1 agonist activity, some electrophiles actually inhibit TRPA1 (Chen et al., 2008; Xiao et al., 2008). TRPA1 is also activated by many non-electrophilic chemicals, including natural products such as menthol, carvacrol and sesquiterpene irritants (Escalera et al., 2008). Finally, some chemicals that do not activate TRPA1 *in vitro* are metabolically converted into TRPA1 agonists *in vivo*, eliciting TRPA1-dependent sensory irritation and other toxicological effects. Examples include styrene and naphthalene that require Cytochrome P450 activity to cause TRPA1-dependent respiratory irritation in mice *in vivo* (Lanosa et al., 2010), and chemotherapy drugs such as cyclophosphamide and ifosfamide. The latter are metabolized forming acrolein as a by-product that, if not scavenged, damages the bladder to cause hemorrhagic cystitis and TRPA1-dependent visceral pain (Conklin et al., 2009; Pereira et al., 2013). The present studies by Conklin et al. and Kurhanewicz et al. further demonstrate the complexity of both the organ-specific and systemic toxicological effects of sensory irritants mediated by TRP ion channels through sensory neurons and, potentially, local cells. Thus, the RD₅₀, the exposure concentration leading to a 50% decline in respiratory rate for a given irritant determined *in vivo*, remains an important toxicological parameter to identify and rank respiratory irritants and predict their toxicological effects in the respiratory and cardiovascular system (Alarie, 1973; Kuwabara et al., 2007).

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Fig. 1. Acrolein causes cardiopulmonary effects through the activation of TRPA1 ion channels

Sources of environmental acrolein are diesel exhaust, cigarette smoke, burning of organic matter, industrial sources or overheated cooking oil. At the molecular level, acrolein reacts with N-terminal amino acid residues of TRPA1 channels in sensory nerve endings, triggering nerve excitation and calcium influx, followed by activation of intracellular inflammatory signaling involving ATP release and NF- κ B pathways.

Acrolein activates trigeminal sensory nerve endings in the cornea and upper airways, resulting in eye irritation, the lachrymatory reflex, tingling, sneezing, painful and burning

sensations, and increased nasal secretions. Activation of vagal laryngeal sensory nerve endings in the throat triggers the cough reflex and the sensations of coarseness and burning pain. TRPA1 activation stimulates the neuronal release of the pro-inflammatory neuropeptides, Substance P (SP) and Calcitonin Gene-Related Peptide (CGRP), among others. These peptides increase mucus production from goblet cells (blue) and submucosal glands and increase the contractility of airway smooth muscle cells increasing airway resistance. The release of CGRP acts on pulmonary vasculature resulting in increased vasodilation and permeability leading to plasma extravasation, edema and neutrophil infiltration. In addition to pulmonary effects, acrolein also causes cardiac arrhythmia through increased excitability of sensory nerves and autonomic imbalance.

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