Heavy metals zinc, cadmium, and copper stimulate pulmonary sensory neurons via direct activation of TRPA1

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Gu Q, Lin R-L. Heavy metals zinc, cadmium, and copper stimulate pulmonary sensory neurons via direct activation of TRPA1. J Appl Physiol 108: 891–897, 2010. First published February 4, 2010; doi:10.1152/japplphysiol.01371.2009.—Airway exposure to zinc dust and zinc-containing ambient particulates can cause symptoms of airway irritation and inflammation, but the underlying molecular and cellular mechanisms are largely unknown. Transient receptor potential A1 (TRPA1) is selectively expressed in a subpopulation of pulmonary C-fiber afferents and has been considered as a major irritant sensor in the lung and airways. Using whole cell patch-clamp recording and Ca2 + imaging, we have demonstrated that application of ZnCl2 concentration dependently evoked inward current and Ca2 + transient in isolated vagal pulmonary sensory neurons; both responses were almost completely inhibited after pretreatment with AP18, a specific TRPA1 antagonist. In anesthetized spontaneously breathing animals, intratracheal instillation of ZnCl2 (2 mM, 25 μl) induced pronounced respiratory depression in wild-type mice, and the effect was essentially absent in TRPA1-deficient mice. In addition, our study showed that two other heavy metals, cadmium and copper, also stimulated pulmonary sensory neurons via a direct activation of TRPA1. In summary, our results suggest that activation of TRPA1 in pulmonary C-fiber sensory nerves may contribute to the respiratory toxicity induced by airway exposure to these three heavy metals.

Airway Reflex Responses such as cough and sneezing are crucial for the protection of the airways from chemical and biological challenges (10, 30). These responses are triggered by activation of peripheral sensory nerve endings in the airway lining. The majority of nerve fibers innervating the respiratory tract are bronchopulmonary C-fibers. One of the defining features of these C-fiber afferents is the functional expression of transient potential receptor vanilloid-1 (TRPV1), as evident by their exquisite sensitivity to capsaicin, the pungent ingredient in chili peppers (16, 24, 29). While these C-fibers normally express only TRPV1, all C-fibers express transient receptor potential vanilloid-1 (TRPV1), as evident by their exquisite sensitivity to capsaicin, the pungent ingredient in chili peppers (16, 24, 29). While these C-fibers normally express only TRPV1, all C-fibers express TRPA1, a novel member of the TRP family of ion channels, which is expressed by a subpopulation of TRPV1-expressing nociceptive neurons and is activated by a much broader range of molecules (e.g., 4-hydroxynonenal and 4-oxononenal) (3, 5, 8, 37). Recent studies have revealed that TRPA1 is selectively expressed by a subpopulation of TRPV1-expressing nociceptive neurons and is activated by a much broader range of molecules. These include many pungent compounds and environmental irritants such as allicin, acrolein, mustard oil, cinnamondehyde, formaldehyde, and α,β-unsaturated aldehydes, as well as a number of reactive oxygen species (e.g., hydrogen peroxide, hydroxyl, and hypochlorite) and reactive endogenous molecules (e.g., 4-hydroxynonenal and 4-oxononenal) (3, 5, 8, 11, 12, 39–42).

A recent study by Hu et al. (22) has shown that zinc excites somatosensory neurons and causes nociception in mice through a direct activation of TRPA1. Their study further demonstrates that zinc activates TRPA1 through a unique mechanism that requires zinc influx through the constitutively active TRPA1 and subsequent activation of the channel via specific intracellular cysteine and histidine residues. Whether zinc also activates TRPA1 expressed in bronchopulmonary sensory neurons, and if so how this activation contributes to the zinc exposure-induced airway irritation, and whether activation of TRPA1 represents a common mechanism for acute respiratory toxicity by other heavy metals such as cadmium and copper remain to be determined. This study was carried out to answer these questions.

Materials and Methods

Animals. Young male Sprague-Dawley rats (4–6 wk old) were purchased from Harlan Laboratories (Indianapolis, IN). Male homozygote TRPA1-deficient mice (Trpa1 -/-; strain: B6;129P-Trpa1tm1Kiyw/J) and their littermates (Trpa1 +/+; strain: B6129PF2/J) were purchased from the Jackson Laboratory (Bar Harbor, ME). These mice were matched for age (12–16 wk), and the experiments were masked to the genotype. Experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Labeling Vagal Pulmonary Sensory Neurons with Dil. Cell bodies of vagal sensory nerves arising from lung and airways reside in nodose and jugular ganglia. These sensory neurons were identified by retro-
grade labeling with the fluorescent tracer 3,3'-dioctadecylindocarbocyanine (DiI), as described below. Rats or mice were anesthetized with isoflurane inhalation (1% in O$_2$) via a nose cone connected to a vaporizing machine (AB Bickford). A small midline incision was made on the ventral neck skin to expose the trachea. DiI (rats: 0.2 mg/mL, 50 μL volume; mice: 0.15 mg/mL, 20 μL) was instilled into the lungs via a 30-gauge needle inserted into the lumen of the trachea; the incision was then closed. Animals recovered undisturbed (7–10 days for rats; 5–7 days for mice) until they were euthanized for the tissue harvest and cell culture.

Isolation of nodose and jugular ganglion neurons. Animals were killed after isoflurane inhalation. Nodose and jugular ganglia in rats or nodose-jugular complex in mice were extracted under a dissecting microscope and placed in ice-cold DMEM/F12 solution. Each ganglion was desheathed, cut into small pieces, placed in the combination of 0.04% type IV collagenase and 0.02% dispase II, and incubated in 5% CO$_2$ in air at 37°C (rats: 80 min; mice: 45 min). The ganglion suspension was centrifuged (150 g, 5 min), and the supernatant was aspirated. The cell pellet was then resuspended in a modified DMEM/F12 solution (DMEM/F12 supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 μM MEM nonessential amino acids) and gently triturated with a small-bore, fire-polished Pasteur pipette. The dispersed cell suspension was centrifuged (500 g, 8 min) through a layer of 15% BSA to separate the cells from the myelin debris. The pellets were resuspended in the modified DMEM/F12 solution, plated onto poly-L-lysine-coated glass coverslips, and incubated at 37°C in 5% CO$_2$ in air. Isolated neurons were used within 48 h of culture.

Whole cell perforated patch-clamp recordings. The recording chamber with cultured cells was perfused continuously with extracellular solution (ECS; containing in mM: 136 NaCl, 5.4 KCl, 1.8 CaCl$_2$, 0.33 NaH$_2$PO$_4$, 10 glucose, and 10 HEPES, pH at 7.4). Whole cell perforated patch configuration (50 μg/ml gramicidin) was performed by using Axopatch 200B/pCLAMP 9.0 (Axon Instruments, Union City, CA). The intracellular solution contained (in mM) 92 potassium gluconate, 40 KCl, 4 NaCl, 1 CaCl$_2$, 0.5 MgCl$_2$, 10 EGTA, and 10 HEPES, pH at 7.2. The chemical stimulants were applied by a pressure-driven drug delivery system (ALA-VM8, ALA Scientific Instruments, Westbury, NY). The series resistance was usually in the range of 6–10 MΩ and was not compensated. The resting membrane potential was held at −70 mV in voltage-clamp mode. The experiments were performed at room temperature (∼22°C).

Ca$^{2+}$ imaging. Intracellular Ca$^{2+}$ was monitored using the fluorescent Ca$^{2+}$ indicator fura-2 AM. Cells were loaded with 5 μM fura-2 AM for 30 min at 37°C. The coverslip containing cells was then mounted into a chamber (0.2 ml) placed on the stage of a Zeiss fluorescence inverted microscope equipped with a variable filter wheel (Sutter Instruments, Novato, CA) and digital CCD camera (Princeton Instruments, Trenton, NJ). The recording chamber was perfused continuously with ECS or the test chemicals by a gravity-fed valve control system (VC-66CS, Warner Instruments, Hamden, CT); a complete change of bath solution occurred in 6 s. Cells were allowed to deesterify for at least 30 min before the recording when the dual images (340 and 380 nm excitation, 510 nm emission) were collected and pseudocolor ratiometric images monitored by using the software Axon Imaging Workbench (Axon Instruments), as previous described (18).

Unless mentioned otherwise, both patch-clamp recording and Ca$^{2+}$ imaging analyses were performed selectively in the pulmonary sensory neurons based on the following criteria: 1) labeling with DiI as indicated by fluorescence intensity; 2) cell diameter <30 μm; and 3) response to 0.75 μM capsaicin. These neurons presumably give rise to pulmonary C-fiber afferents as proposed in our recent studies (19). Although neurons from rat nodose and jugular ganglia were isolated and studied separately, data from the neurons of these two different origins were pooled for group analysis because no difference was found between responses of the neurons obtained from these two ganglia.

Measurements of cardiorespiratory responses in anesthetized spontaneous breathing mice. Mice (21–36 g) were anesthetized with an intraperitoneal injection of α-chloralose (90 mg/kg) and urethane (1,300 mg/kg) dissolved in a 2% borax solution; supplemental doses were administered as needed to prevent eye blink, withdrawal reflexes, and fluctuations in arterial blood pressure (ABP). A short tracheal cannula was inserted after a tracheotomy, and tracheal pressure was measured via a side-port of the tracheal cannula. The right femoral artery was cannulated for recording the ABP. Mice breathed spontaneously via the tracheal cannula. Respiratory flow was measured with a heated pneumotachograph and a differential pressure transducer, and integrated to give tidal volume (VT). Respiratory frequency, VT, ABP, and heart rate were analyzed (Biocybernetics TS-100) on a breath-by-breath basis by an online computer. At the end of the experiment, the animal was euthanized by decapitation following an overdose of α-chloralose and urethane.

Chemicals. DiI was purchased from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma Chemical (St. Louis, MO). Stock solution of capsaicin (1 mM) was prepared in a vehicle of 10% Tween 80, 10% ethanol, and 80% saline. Stock solutions of AMG 9810 (1 mM) and AP18 (50 mM) were in DMSO. The stock solutions of ZnCl$_2$ (1 M), CdCl$_2$ (1 M), and CuCl$_2$ (0.5 M) were in distilled water. These stock solutions were divided into small aliquots and kept at −20°C. The solutions of these chemicals at desired concentrations were prepared daily by dilutions with ECS in vitro or isotonic saline in vivo preparations, before use. No detectable effect of the vehicles of these chemical agents was found in our preliminary experiments.

Statistical analysis. Two-way repeated-measures ANOVA was employed to evaluate the effect of airway exposure to ZnCl$_2$ on the breathing frequency in our in vivo studies. One factor of the two-way...

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**Fig. 1.** Zinc activates rat pulmonary capsaicin-sensitive neurons. A: inward currents evoked by capsaicin (1 μM, 6 s), and increasing concentrations of ZnCl$_2$ (1, 10, 30 and 100 μM, 6 s) in a single pulmonary sensory neuron. B: concentration-response relationship of the ZnCl$_2$-evoked currents in rat pulmonary capsaicin (1 μM, 2–6 s)-sensitive neurons (n = 14).
A

Cap

ZnCl₂

Cap+AMG

ZnCl₂+AMG

ZnCl₂+AP18

0.3 mA

6 s

B

Normalized current

Cap

ZnCl₂

ZnCl₂+AMG

ZnCl₂+AP18

Cap+AMG

Cap+AP18

Fig. 2. Zinc-evoked inward current is mediated through TRPA1 in rat pulmonary sensory neurons. A: experimental records illustrating the inward currents evoked by capsaicin (Cap; 1 μM, 3 s) and ZnCl₂ (30 μM, 6 s), in the absence and presence of AMG 9810 (AMG; 1 μM, 2 min), a specific TRPV1 antagonist, or AP18 (75 μM, 2 min), a specific TRPA1 antagonist, in a single neuron. B: group data showing the effects of pretreatment with AMG 9810 or AP18 on the inward currents evoked by capsaicin and ZnCl₂. *Significantly different from the corresponding control responses without AMG 9810 or AP18 (P < 0.001, n = 5).

ANOVA was the effect of ZnCl₂ or vehicle instillation; the other factor was the time course after the instillation. When the ANOVA showed a significant interaction, pairwise comparisons were made with a post hoc analysis (Fisher’s least significant difference). Student’s paired t-test was used in our in vitro studies to evaluate the effect of antagonist treatment (API8 or AMG 9810). A P value of <0.05 was considered significant for all tests. Data are means ± SE.

RESULTS

Zinc evokes inward current in rat pulmonary sensory neurons through activation of TRPA1. Whole cell perforated patch-clamp recording was performed in the neurons isolated from rat nodose and jugular ganglia and identified by retrograde labeling with Dil. In 14 of 25 (56%) capsaicin (1 μM, 2–6 s)-sensitive neurons, ZnCl₂ (1–100 μM, 6 s) concentration dependently evoked an inward current (Fig. 1). None of the capsaicin-insensitive neurons tested (n = 8) responded to 30 μM ZnCl₂. The inward current evoked by ZnCl₂ was almost completely inhibited by API8 (75 μM, 2 min) (P < 0.001, n = 5), a specific TRPA1 antagonist (35), whereas it was not affected by AMG 9810 (1 μM, 2 min) (P = 0.92, n = 5), a selective TRPV1 antagonist (15) (paired t-test) (Fig. 2). In contrast, capsaicin-evoked inward current in these neurons was completely blocked by AMG 9810 (P < 0.001, n = 5), as what we have reported previously (19), but not significantly affected by AP18 (P = 0.37, n = 5) (paired t-test).

Zinc evokes Ca²⁺ transient by activating TRPA1 in mouse pulmonary sensory neurons. Fura-2-based ratiometric Ca²⁺ imaging was carried out in pulmonary sensory neurons isolated from Trpa1+/− mice. As shown in Fig. 3, application of ZnCl₂ (1, 10 and 30 μM, 30 s) evoked Ca²⁺ transient in a subset of capsaicin (1 μM, 30 s)-sensitive neurons (41/63 = 65.1%). This Ca²⁺ transient was almost completely abolished after pretreatment with AP18 (75 μM, 5 min) (P < 0.005, n = 13; paired t-test) (Fig. 4), indicating the involvement of TRPA1 activation by ZnCl₂.

Airway exposure to zinc evokes respiratory irritation in Trpa1+/− but not Trpa1−/− mice. In anesthetized, spontaneously breathing Trpa1+/− mice, intratracheal instillation of 25 μl isotonic saline, the vehicle for ZnCl₂, induced a slight and transient decrease in respiratory frequency (Fig. 5). Intratracheal instillation of ZnCl₂ (2 mM, 25 μl) induced a dramatic depression in respiratory rate, resulting from the airway sensory irritation as observed by previous investigators during airway exposure to many respiratory irritants, including TRPA1 and TRPV1 agonists in mice (11, 33, 40). The effect of ZnCl₂ took place immediately after the instillation and lasted <10 min (P < 0.01, n = 6; 2-way ANOVA); for example, the respiratory frequency was 163.9 ± 8.1 breaths/min at basal and 112.4 ± 6.7, 137.5 ± 7.9, and 164.1 ± 10.6 breaths/min at 2, 5, and 10 min after the ZnCl₂ instillation, respectively (Fig. 5).
In sharp contrast, airway exposure to the same dose of ZnCl₂ (2 mM, 25 μl; intratracheally) in Trpa1⁻/⁻ mice did not induce any significant change in respiratory frequency at any time points after the instillation compared with that after instillation of the same volume of saline control (P > 0.05, n = 6; 2-way ANOVA) (Fig. 6).

**DISCUSSION**

In the present study, we demonstrate that Zn²⁺ concentration dependently stimulates both rat and mouse vagal pulmonary sensory neurons through a direct activation of TRPA1. In addition, our data showed that airway exposure to Zn²⁺ evoked a reflex respiratory depression in Trpa1⁺⁺/⁺⁺ but not Trpa1⁻/-⁻ mice. Our data further showed that the effect of Zn²⁺ on pulmonary sensory neurons was mimicked by Cd²⁺ and Cu²⁺, two other heavy metal ions.

The expression of TRPA1 in bronchopulmonary C-fibers has been recently demonstrated (33), and this receptor has been considered a major irritant sensor in the lung and airways because of its sensitivity to a broad range of chemical stimuli (10). In the present study, our data showed that Zn²⁺ concentration dependently evoked an inward current in 56% (14/25) of rat capsaicin-sensitive neurons and in 55% (17/31) of capsaicin-sensitive or 35% (17/48) of all vagal pulmonary sensory neurons isolated from Trpa1⁺⁺/⁺⁺ mice. The inward current or Ca²⁺ transient evoked by Zn²⁺ in neurons from these two animal species was almost completely inhibited by the pretreatment with AP18, a specific TRPA1 antagonist, indicating the involvement of TRPA1 activation. Our results are in consistent with the previous findings from other investigators that TRPA1 is coexpressed with TRPV1 in a subset of nociceptors (3, 11, 25, 33, 37).

**Fig. 5.** Airway exposure to zinc induces respiratory depression in anesthetized spontaneous breathing Trpa1⁺⁺/⁺⁺ mice. A–D, experimental records illustrating the breathing pattern before (basal) and 2, 5, and 10 min after intratracheal instillation of ZnCl₂ (2 mM, 25 μl) in a Trpa1⁺⁺/⁺⁺ mouse (27.3 g). Vt, tidal volume (+: inspiratory volume; −: expiratory volume). E: group data showing the changes in respiratory frequency [breaths/min (bpm)] after instillation of ZnCl₂ or its vehicle (isotonic saline) in Trpa1⁺⁺/⁺⁺ mice. *Significantly different from the corresponding vehicle control; †significantly different from the corresponding basal value (P < 0.01, n = 6).
Our study showed that airway instillation of ZnCl₂ (2 mM, 25 µl) induced a drastic decrease in respiratory rate in Trpa1−/− mice. We argue that this central reflex response is most likely due to the activation of TRPA1 expressed in the bronchopulmonary C-fiber afferents. Our rationale for this prediction is as the following.

1) Many respiratory irritants including TRPA1 (e.g., cinnamaldehyde, toluene diisocyanate, chlorine, hydrogen peroxide) and TRPV1 (e.g., capsaicin) agonists have been reported to evoke respiratory depression in several strains of mouse (e.g., BALB/c, C57BL/6J, Swiss Webster), via a nocisensor reflex initiated by bronchopulmonary C-fibers stimulation (2, 11, 33, 40).

2) Our Ca²⁺ imaging and patch-clamp studies demonstrated that ZnCl₂ activated a subpopulation of capsaicin-sensitive (but not any capsaicin-insensitive) lung-specific vagal sensory neurons that presumably give rise to pulmonary C-fiber afferents (19); the responses were almost completely prevented by AP18, a specific TRPA1 antagonist.

3) So far, the only organs where TRPA1 mRNA and protein expression has been reliably detected are two specialized in sensation: the peripheral ganglia that contain nociceptive neurons (including dorsal root, trigeminal, and nodose ganglia) and the mechanosensory epithelia of the inner ear (14).

4) Our results showed that the reflex respiratory depression evoked by zinc instillation was essentially abolished by genetic deletion of TRPA1, despite that heavy metals including zinc are known to have toxic effects on a variety of tissues other than sensory nerves in the respiratory tract (7, 32, 34).

Occupational exposure to high levels of zinc could happen in numerous industrial processes such as welding, galvanizing, and manufacturing of alloys, pigments, and pesticides (34). Zinc can also enter the environment and become a common component of particulate air pollution. Indeed, zinc is detected in relatively higher levels than most metals in many ambient air samples (1, 13, 20, 26), and its level in soluble form has been reported to be directly related to the acute respiratory toxicity of many atmospheric particulate samples or extracts (1, 44). It has long been known that airway overexposure to zinc can cause symptoms of airway irritation and inflammation, including cough, dyspnea, mucus secretion, and airway hyperreactivity, and in severe cases, zinc fume fever (17, 21, 34). The zinc-induced respiratory toxicity is known to be mimicked by excessive inhalation of some other heavy metals such as cadmium and copper (9, 31, 34). However, the molecular and cellular mechanisms underlying the respiratory toxic effects of zinc and other heavy metals are not well understood.

Fig. 6. Reflex respiratory depression evoked by zinc exposure is absent in Trpa1−/− mice. A–D: experimental records illustrating the breathing pattern before (basal) and 2, 5, and 10 min after intratracheal instillation of ZnCl₂ (2 mM, 25 µl) in an anesthetized spontaneous breathing Trpa1−/− mouse (28.7 g). E: group data showing no significant change in respiratory frequency at any time points after instillation of ZnCl₂ compared with that after its vehicle control (P > 0.05, n = 6).

Fig. 7. Cadmium and copper stimulate pulmonary sensory neurons from Trpa1−/− mice via activation of TRPA1. A: inward currents evoked by capsaicin (1 µM, 2 s), ZnCl₂ (30 µM, 6 s), and increasing concentration of CdCl₂ (10, 30, and 100 µM, 6 s) in a single neuron. The CdCl₂ (30 µM, 6 s)-evoked current was reversibly inhibited by AP18 (75 µM, 2 min). B: group data showing the concentration response to CdCl₂ (n = 17) and its inhibition by AP18 (n = 6). C: inward currents evoked by ZnCl₂ (30 µM, 6 s), and increasing concentration of CuCl₂ (10, 30, and 100 µM, 6 s) without or with AP18 pretreatment (75 µM, 2 min). D: group data showing the concentration response to CuCl₂ (n = 7) and its inhibition by AP18 (n = 5). *Significantly different (P < 0.05) from the corresponding control responses without AP18.
these metal ions are largely unknown (7). TRPA1 has recently been suggested as a potential mediator of heavy metal toxicity by two groups of investigators, based on their independent findings that intracellular Zn$^{2+}$ activates TRPA1 in somatic nociceptors or in heterologous cells (4, 22). Our data from the present study indicate that Zn$^{2+}$ appears indeed to stimulate bronchopulmonary C-fiber sensory nerves via activation of TRPA1, and the subsequent reflex responses may therefore, at least in part, account for the respiratory toxic effect of this metal. In addition, our finding that the effect of Zn$^{2+}$ was mimicked by two other heavy metal ions, Cd$^{2+}$ and Cu$^{2+}$, suggests that activation of TRPA1 in bronchopulmonary sensory nerves may represent a common mechanism underlying heavy metal-induced respiratory toxicity. It is worthy noting that, other than many industrial processes (welding, smelting, automobile emissions, manufacturing of alloys and pigments, etc.) considered as sources of air-borne cadmium (23, 34), cigarette smoking is another major source of cadmium exposure due to the propensity of the Nicotiana species to concentrate cadmium independent of soil-cadmium content (36). Interestingly, many other components of tobacco smoke such as nicotine and unsaturated aldehyde have also been reported recently as TRPA1 agonists (5, 38).

Activation of bronchopulmonary C-fibers is known to cause airway irritation, cough, bronchoconstriction, neurogenic inflammation, shortness in breadth, mucus secretion, protein extravasation, etc. (28, 30, 43). These responses are mediated by both central reflex pathways and by local axon-reflex mechanisms involving the release of neuropeptides from sensory endings. While these respiratory reflexes and sensations are believed to contribute to airway protection, eliminating inhaled irritants, and promoting healing and recovery, excessive reflex responses can lead to debilitating respiratory symptoms that command medical attention and care (10, 28). Results from our present study suggest that TRPA1 may represent a promising pharmacological target for heavy metal toxicity, a problem that is especially relevant to individuals suffering from chronic airway conditions. However, the translational potential of TRPA1 antagonism should be evaluated cautiously before the detailed mechanistic role of TRPA1 in heavy metal inhalation-induced airway inflammation, injury, and hypersensitivity is further elucidated.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


