The Rising Role of TRPA1 in Asthma

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Abstract: Asthma is an inflammatory condition of the airways triggered by exposure to allergens and/or irritants. TRPA1 is an irritant-sensing cation channel expressed in TRPV1-positive, capsaicin-sensitive chemosensory neurons that innervate various organs, including the airways. Various exogenous noxious chemicals have been described to activate TRPA1, including agents recognized to trigger and/or worsen asthma such as diisocyantes, cigarette smoke, acrolein, chlorine. During oxidative stress, a condition associated with asthma, various chemical species capable of activating TRPA1 are generated in the lungs, including reactive oxygen species (ROS), reactive nitrogen species (RNS), and several byproducts of lipid peroxidation including nitrooleic acid and 4-hydroxynonenal. Recently, a potential role of TRPA1 in mediating allergen-induced asthmatic responses has been described in ovalbumin-sensitized mice, in which genetic deletion of TRPA1 or pretreatment with a selective TRPA1 antagonist reduced leukocyte infiltration, decreased cytokine and mucus production and almost completely abolished airway hyperreactivity without affecting the immune response driven by the allergen. Moreover, two recent studies have provided strong pharmacological evidence that inhalation of TRPA1 activators, like acrolein or cinnamaldehyde, elicits cough reflexes in guinea-pigs and human volunteers. In conclusion, a compelling series of recent findings highlights the TRPA1 channel as a molecular target for a wide variety of known exogenous and endogenous inflammatory and irritating chemical agents, and suggests that TRPA1 antagonists might be taken into consideration as a novel pharmacological treatment of asthma, chronic cough and possibly other inflammatory conditions of the airways.

Keywords: TRP channel, TRPA1, HC-030031, AP18, AITC, formalin, neurogenic inflammation, asthma, respiratory, cough.

INTRODUCTION

Bronchial asthma is a respiratory disease characterized by chronic inflammation and episodes or attacks of airway narrowing. The burden from asthma in western societies has increased over the past decades, but the precise reasons for this increase remain enigmatic. Current pharmacologic treatment options for asthma are not curative [1]. In this respect, there should be a major effort to develop novel medicines that are more effective than existing drugs in facilitating the remission of the disease. To this aim, it is critical to understand the exact patho-physiological mechanisms underlying the development of asthma, in particular the complex interaction of chronic inflammation with resident lung cells such as smooth muscle cells, fibroblasts or nerve terminals of sensory neurons innervating the airways. When activated by environmental irritants, terminal varicosities of nerve fibres of primary afferents innervating the airways release their neuropeptide content, including calcitonin gene-related peptide (CGRP) and the tachykinins substance P (SP) and neurokinin A (NKA). These neuropeptides in turn may cause bronchoconstriction and various reflex responses such as cough, mucus secretion and airway inflammation [2]. This pattern of effects, referred to as neurogenic inflammation, is now well characterized in animal experimental models but has not been fully extrapolated to the humans yet.

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TRPA1, known also as ANKTM1, is a large transmembrane protein of 1119 amino acids in human (1125 in rat and mouse) with six predicted membrane-spanning domains and a single pore loop characteristic of all other TRP receptor proteins. A distinguishing feature of TRPA1 is its long N-terminal region with up to 18 predicted ankyrin repeats while most of the TRPs have zero to 8 ankyrin repeats [3]. Ankyrin repeats in TRPA1 have been implicated in protein-protein interactions. More recently it has been suggested that they provide elasticity and molecular spring properties that could be important in mechanotransduction. TRPA1 has an N-terminal EF-hand calcium-binding domain making the activation rate of TRPA1 sensitive to intracellular calcium increase, a feature that may be relevant in nociception [4, 5].

Neuronal TRPA1 expression is almost completely restricted to nocicieptive neurons of trigeminal ganglion (TG) and dorsal root ganglion (DRG) neurons that are sensitive to capsaicin and also express TRPV1 [6, 7]. In man, TRPA1 has been found also in non-sensory neurons, like spinal cord motoneurones and nerve roots, peripheral nerves, intestinal myenteric plexus neurons [8]. Outside of the peripheral nervous system TRPA1 displays a discrete expression, in particular in fibroblasts and keratinocytes, but little if any apparent expression in the CNS and in the immune system [3]. Genetic ablation of TRPA1 clearly demonstrated the role of this channel in sensing pain-producing chemicals and environmental irritants [9, 10]. In contrast, the functional role of TRPA1 present in non sensory neurons and in non neuronal cells remains to be elucidated. For example, TRPA1 was suggested to be involved in mechano-transduction in the hair cells of the

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inner ear [11]; however TRPA1 knockout mice do not appear to display hearing impediments [10]. Although low temperatures (≤ 17° C) are capable of gating TRPA1 receptors [6], TRPA1 knock out mice display normal cold sensitivity, thus challenging the concept that this receptor is required for the detection of noxious cold [13]. However, Karashima and coworkers [14] have recently reevaluated the role of TRPA1 in cold sensation, and provided several novel lines of evidence showing that TRPA1 acts as a noxious cold sensor.

TRPA1 IN THE AIRWAYS

TRPA1 is expressed in vagal sensory nerves and in the sensory nerve fibers originating from DRG neurons innervating the airways. In particular, TRPA1, but not TRPM8, is co-expressed with TRPV1 in a subpopulation of primary afferent somatosensory neurons innervating mouse airways and containing the neuropeptides SP, NKA and CGRP [15]. The TRPA1 selective agonist cinnamaldehyde generates action potentials from bronchopulmonary C-fibers, thereby eliciting nocifensor reflex responses in vagal sensory nerves innervating mouse airways [15]. TRPA1 in the airway nerve endings is activated by pungent plant constituents such as allicin (garlic), cinnamaldehyde (cinnamon), isothiocianates (horseradish) [12, 16, 17] as well as from several volatile irritants and air pollutants such as formaldehyde [18] and acrolein [12]. More recently, various endogenous byproducts deriving from peroxidation of membrane phospholipids, such as 4-hydroxy-2-nonenal (4-HNE) and 4-oxononeal (4-ONE) were described to activate TRPA1 and, as a consequence, produce pain and neurogenic inflammation [19, 20]. Given the pathophysiological relevance of the endogenous activators of TRPA1, a role for this receptor in mediating pulmonary inflammatory processes characterized by oxidative stress can be predicted.

Among TRPA1 activators are some of the most harmful environmental/industrial irritants which, when inhaled, may cause a number of adverse reactions in the lung/airways, collectively known as RADS (reactive airways dysfunction syndrome) [21], occupational asthma [22] or sensory hyperreactivity (SHR) [23]. Therefore TRPA1 can be considered either a chemoreceptor for environmental irritants or a mediator of neurogenic inflammation responses elicited by endogenous harmful stimuli in the airways. Endogenous or exogenous TRPA1 activators in relation to asthma are discussed in the next two paragraphs and summarized in Table **1**.

ENDOGENOUS ACTIVATORS OF TRPA1 GENERATED BY OXIDATIVE STRESS

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

An imbalance between oxidants and antioxidants (oxidative stress) is associated with asthma [24], during which pulmonary oxidant burden can be increased by eosinophils, neutrophils and macrophages infiltrating the alveolar space and generating reactive oxygen species such as oxygen radicals ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hypochlorite [25, 26].

ROS, by reacting with nitric oxide (NO) which is overproduced in inflamed tissues, generate highly reactive nitrogen species (RNS), like peroxynitrite (ONOO-) and nitrogen dioxide (NO_2), and cause nitrative stress which is associated with various airway diseases including asthma [27]. Similarly to ROS, RNS directly attack unsaturated fatty acids (e.g. oleic acid) of membrane proteins, by adding NO_2 groups (nitration) to the organic acids [28], a process that ultimately leads to highly reactive electrophilic compounds generation, as nitrooleic acid that forms from nitration of oleic acid [29]. RNS have the ability to directly activate TRPA1 by oxidation of key cysteine residues within the N-terminal sequence of the channel [30]. Similarly, ROS activate TRPA1 by oxidative modification of the key cysteines of the channel (Fig. **1**). This ability of ROS has been demonstrated recently by various authors who have reported that H_2O_2 is able to produce a delayed activation of neuronal wild type or recombinant TRPA1 channel [9, 30,

Table 1. Endogenous and Exogenous TRPA1 Receptor Activators of Pathophysiological Relevance in Asthma

Compound	Source	References
Noxious cold (T ≤ 17°C) (?)	---	[6, 13, 14]
ROS (H_2O_2, $O2^{\bullet-}$)	photochemical smog neutrophils, macrophages	[30]
RNS ($ONOO^-$)	photochemical smog, automobile exhaust eosinophils, neutrophils, macrophages	[10, 31]
Hypochlorite/chlorine	macrophages, neutrophils Industrial, warfare	[10]
Formaldehyde	automobile exhaust cigarette smoke photochemical smog	[18]
Acrolein	automobile exhaust cigarette smoke photochemical smog tear gases fatty acid peroxidation	[12, 37]
Crotonaldehyde	cigarette smoke	[37]
4-hydroxy-2-nonenal	fatty acid peroxidation	[19]
4-oxononenal	fatty acid peroxidation	[20]
Nitrooleic acid	oleic acid nitration	[31]
Cyclopentenone Prostaglandins	prostaglandins` metabolism	[20, 30, 44]
Cyclopentenone Isoprostanes	isoprostanes` metabolism	[20, 30, 44]
Isocyanates/diisocynates	industrial	[43, 71]

31]. In addition, evidence has been provided showing that several other ROS such as superoxide (O_2^-), and RNS (peroxynitrite; $ONOO^-$) can activate recombinant TRPA1 [30]. H_2O_2 inhalation elicits depression of respiratory frequency in mice: a response that was significantly reduced in TRPA1 deficient animals [9]. Hypochlorite (ClO^-) is another oxidant species that has been described recently as TRPA1 activator [9]. Hypochlorite, likewise other irritants, can either be formed following inhalation of chlorine gas, or be generated endogenously by macrophages and neutrophils during oxidative burst, as a consequence of inhalation of toxic particulates. Hypochlorite can reach millimolar concentrations in inflamed tissues and contribute to cell damage [31, 32].

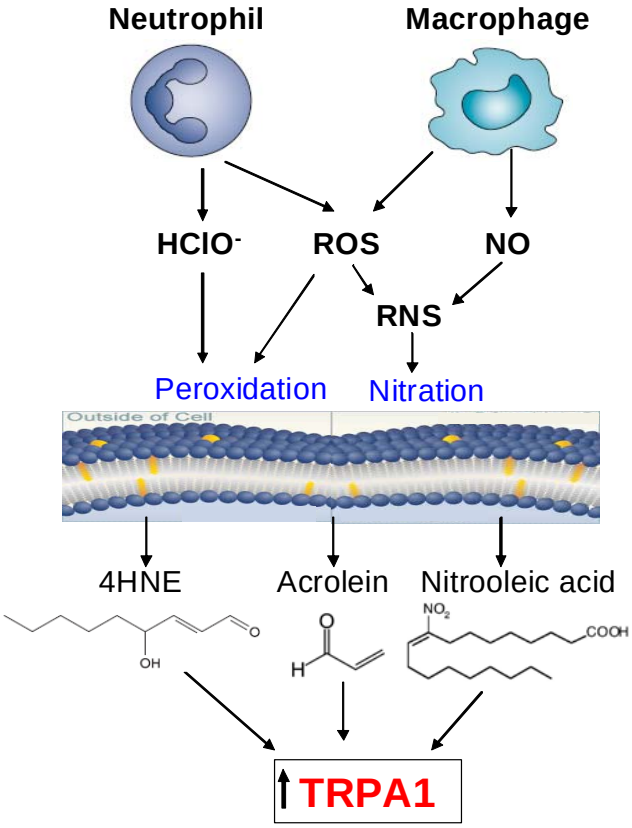

Fig. (1). ROS and RNS released by activated neutrophils and macrophages induce lipid peroxidation or nitration, respectively, and consequent generation of highly reactive compounds, including acrolein, 4HNE and Nitrooleic acid, all agents capable of activating TRPA1, and through this mechanism to elicit pain and neurogenic inflammation in the airways.

Aldehydes

Most abundant aldehydes generated from ROS-induced lipid peroxydation are malonaldehyde, 4-HNE and acrolein [33, 34]. 4-HNE is a highly reactive compound that forms from arachidonic acid, linoleic acid or their hydroperoxides, reaches high concentrations (from 1 µM to 5 mM) in pulmonary tissue, can diffuse within or even escape from the cell and attack molecular targets far from the site where it was formed [33, 35, 36]. Alkylating properties of 4-HNE depend on an electrophilic carbon atom in the structure that undergoes nucleophilic attack by cysteine, histidine or lysine residues of proteins, a process that ultimately leads to generation of stable proteic adducts. These reactions stress cells by activating intracellular signaling and also lead to cell apoptosis [34, 35]. High levels of 4-HNE were measured in lungs of patients with COPD as compared to healthy smokers [33]. Interestingly, cellular 4-HNE level and forced expiratory volume in 1 second (FEV1) in COPD patients were inversely correlated [33]. Acrolein is an α,ß-unsaturated aldehyde, which is produced endogenously from ROS-induced lipid peroxidation or forms from the metabolism of chemotherapeutic drugs like cyclophosphamide and similar antitumoral agents [34]. Nitrooleic acid, 4-HNE and acrolein have been shown to activate TRPA1, and by this mechanism to produce pain and neurogenic inflammation in the airways [12, 19, 37, 38] (Fig. **1**). TRPA1 gating by acrolein, nitrooleic acid and 4-HNE occurs through an unusual mechanism (Michael addition) involving covalent modification of cysteines and of a lysine residue within the cytoplasmic N-terminal domain of the channel [38-40]. Because capability to generate Michael adducts is virtually shared by all α,β-unsaturated aldehydes, several others of these compounds have actually been shown to activate TRPA1. An example is the endogenously-generated compound 4-oxononenal, that is even more electrophilic than 4-HNE [12, 38]. The number of compounds with a proposed pathophysiological role in airways diseases that share the ability to gate TRPA1 is rapidly increasing. Saturated aldehydes, like formaldehyde and acetaldehyde (at mM concentrations) were reported to gate TRPA1 expressed in mouse neurons and in recombinant systems, and by this mechanism elicit pain [18, 41]. However, it is unlikely that such high concentrations of acetaldehyde can be reached in the lung of asthma patients.

Cyclopentenone Prostaglandins

Cyclopentenone prostaglandins (CyPGs), namely PGA2, PGA1, and PGJ are prostaglandin (PG) metabolites that form from dehydration within the cyclopentane ring of PGE2, PGE1, and PGD2, respectively. PGJ2 undergoes further metabolism to yield Δ^{12}-PGJ2 and 15-deoxy-$\Delta^{12,14}$-PGJ2 (15-d-PGJ2). Biological actions of CyPGs do not depend on interaction with G-protein coupled prostanoid receptors, but by activation of other pathways, including nuclear receptor peroxisome proliferator-activated receptor (PPAR)-γ or IκB kinase and the DNA-binding domains of NF-κB subunits [42]. The presence of one or two highly reactive α,β-unsaturated carbonyl groups within the cyclopentenone ring, confers CyPGs unique properties, including the ability to adduct thiol-containing compounds, such as glutathione (GSH) and other thiol-containing proteins *via* Michael addition. This allows CyPGs to activate calcium influx in CHO cells expressing TRPA1 or in DRG cultured sensory neurons [31, 43, 44]. Conversely, calcium influx in DRG neurons from $trpa1^{-/-}$ mice could not be elicited by CyPGs. *In vivo* experiments with TRPA1 knock out mice demonstrated that TRPA1 was required for the pain-related behavioural responses evoked by one of the CyPGs: 15d-PGJ2 [44]. It is worth noting that PGD2, from which CyPGs of the J series are generated, is the major cyclooxygenase metabolite of arachidonic acid produced by activated mast cells in response to antigen challenge [45]. PGD2 is released in large amounts during asthmatic attacks in humans, and it has been proposed as a marker of mast cell activation in asthma [46].

It remains to be determined whether CyPGs generated in asthmatic airways could be sufficient to stimulate the TRPA1.

Cyclopentenone Isoprostanes

The isoprostanes are a unique series of prostaglandin-like compounds formed *in vivo* from the free radical-catalyzed peroxidation of arachidonic acid followed by phospholipase-catalyzed release of the isoprostane moiety [47]. Importantly, unlike PG biosynthesis, generation of isoprostanes does not require activation of cyclooxygenases [48]. Isoprostanes were first recognized as convenient markers of oxidative stress [49], but their powerful effects on a variety of cell functions are now also being increasingly appreciated. Like the CyPGs, cyclopentenone IsoPs (CyIsoPs) are generated *via* dehydration of D/E-isoprostanes within the cyclopentane ring. CyIsoPs, which are isomeric to the bioactive prostaglandins PGA2 and PGJ2, contain an electrophilic α,β-unsaturated carbonyl group in their cyclopentane ring that rapidly adducts cellular thiols, including GSH and cysteins in proteins *via* Michael addition [50]. Similar to unsaturated aldehydes, both CyPGs and CyIsoPs possess the ability to activate TRPA1 in sensory neurons and to elicit pain by this mechanism [31, 43, 44]. Although formation of CyPGs has been demonstrated *in vitro* and *in vivo* from their parent PG compounds [51, 52] the levels these compounds can actually reach in tissues has not yet been described. Since unsaturated carbonyl group can rapidly react with thiol-containing proteins and peptides [48], measurement *in vivo* likely underestimates the real amount of newly formed CyPGs (e.g. 15-d-PGJ2), as well as CyIsoPs. Consequently, the issue of tissue levels of both CyPGs and CyIsoPs need to be properly investigated, in order to estimate their role in asthma and other inflammatory diseases.

ENVIRONMENTAL TRPA1 ACTIVATORS AND AIRWAY DISEASES

Cigarette Smoke

Cigarette smoke can trigger acute symptoms in patients with asthma, and exposure to cigarette smoke is strongly correlated with asthma severity and neutrophil infiltration [53]. *In vitro* and *in vivo* experimental models support these conclusions [54, 55]. Passive inhalation of tobacco smoke by smoke-sensitive subjects, like asthmatics, may increase bronchial hyperreactivity [56]. Moreover, recent evidence points out that smoking may confer a degree of corticosteroid resistance in asthma and that smoking cessation may at least partially restore corticosteroid responsiveness in asthmatic ex-smokers [57]. In rodents, inhaled cigarette smoke causes neurogenic plasma protein leakage mediated by the neuropeptides SP and NKA, released from capsaicin-sensitive sensory nerves, which activate tachykinin NK1 receptors in postcapillary venules to cause edema [2]. Neurogenic response to cigarette smoke in the airways is abolished in capsaicin-pretreated rats in which SP-containing C-fiber nociceptors in the respiratory tract had degenerated [58]. However, plasma protein extravasation elicited by cigarette smoke is not mediated through TRPV1 as it is not counteracted by the selective TRPV1 receptor antagonist capsazepine [59]. Hence, the mechanism through which cigarette smoke evokes neurogenic inflammation remained unknown until recently, when it was shown by Andrè and coworkers [37] that it depends on TRPA1 receptor activation. These authors showed that either cigarette smoke aqueous extract (CSE) or the unsaturated aldehydes crotonaldehyde and acrolein, which are abundant in the CSE [54] (Fig. **2**), mobilized calcium in cultured guinea-pig jugular ganglion neurons, in dorsal root ganglion sensory neurons and in TRPA1-transfected HEK293 cells. In addition, CSE was able to contract guinea-pig isolated bronchi, as did acrolein. All these responses were abolished by a TRPA1 selective antagonist (HC-030031) or by the aldehyde scavenger glutathione, but not by the TRPV1 antagonist, capsazepine, or by various ROS scavengers. Intratracheal instillation of CSE elicited tracheal plasma extravasation in wild-type, but not in TRPA1-deficient mice [37]. On the basis of these data, it can be concluded that airway neurogenic inflammation evoked by cigarette smoke in rodents is mediated through TRPA1 stimulation, and the α,ß-unsaturated aldehydes contained in cigarette smoke are the main causative agents.

Photochemical Smog

Air pollution has become one of the most important problems of urban areas in which about 50% of the world's population now lives. Photochemical smog is a complex mixture of harmful chemicals that forms when UV rays of sunlight hit various pollutants in the air. Photo-oxidation of hydrocarbons and nitric oxides, both coming from automobile exhausts, power plants, and industrial and commercial establishments, ultimately forms ozone at ground level. Hazardous components of photochemical smog are volatile organic compounds, including acrolein and other aldehydes released from petrol combustion, solvents, paints and pesticides [60]. It is well known that asthmatic subjects are more sensitive than healthy individuals to air pollutants such as traffic emissions and photochemical smog components. It has also been demonstrated that exposure to a mix of allergens and irritants may induce the development phase of the disease [61]. Given that many photochemical air components, including acrolein and other oxidant species are TRPA1 activators, a causal role of this channel in neurogenic inflammation associated with asthma may be hypothesised.

Chlorine

Chlorine is a dangerous gas, employed during World War I and other conflicts as a warfare agent, currently in use for disinfection and as chemical reagent in the industry. Inhalation of chlorine or reactive chlorine products frequently occurs in industrial or domestic accidents, causing respiratory adverse effects and tissue injury (e.g. pulmonary oedema, restrictive lung disease, and obstructive disease), all symptoms that are treated in the environmental and occupational health practices [62]. Chlorine inhalation elicits nasal responses and nasal congestion in man, particularly in subjects suffering from allergic rhinitis, even at concentrations that are not perceived as irritating or painful [63, 64]. As previously mentioned, Bessac and coworkers [9] have recently shown that hypochlorite (that is the oxidative mediator of chlorine) increases the influx of calcium into cultured mouse trigeminal and nodose ganglion neurons innervating the upper and lower airways, respectively. The effect of hypochlorite was absent in TRPA1-KO mice. Hypochlorite was shown to activate cloned human and

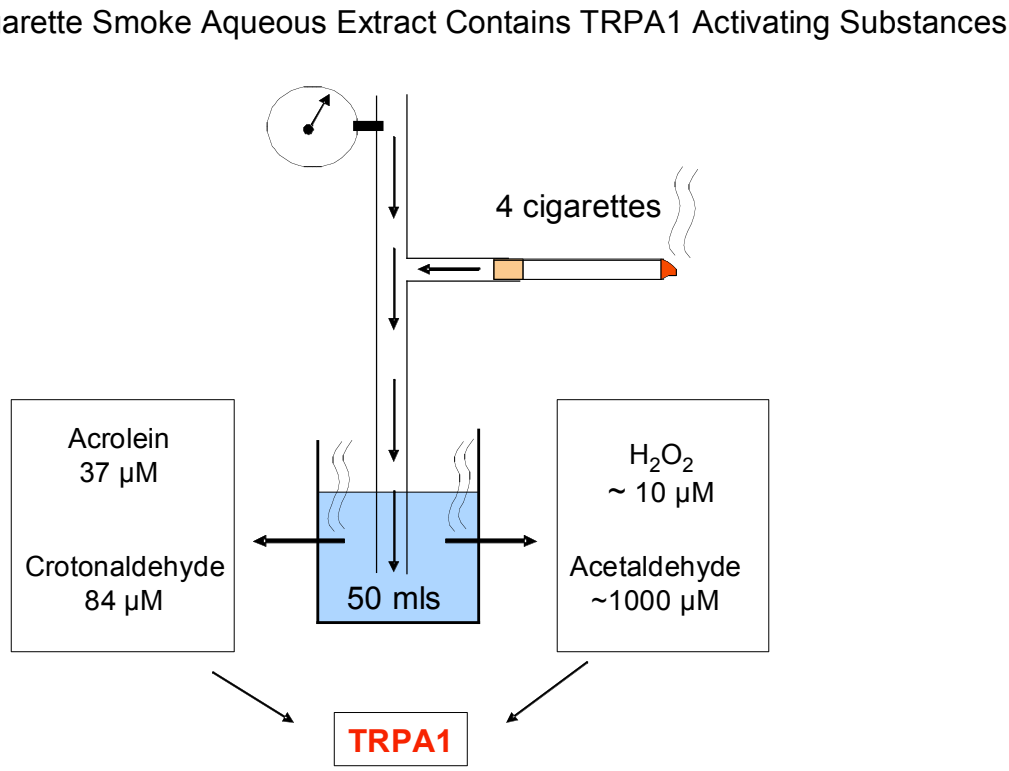

Fig. (2). Schematic representation of aqueous cigarette smoke extract (CSE) generation from the combustion of cigarettes (in the example: Marlboro Red, 12 mg tar, 0.9 mg nicotine) bubbled through cell culture medium. In CSE are contained about 5000 constituents including unsaturated and saturated aldehydes at concentrations sufficient to activate TRPA1. Data shown in the figure are from reference [54].

murine TRPA1 channels expressed in HEK293 cells. Finally, unlike wild-type mice, TRPA1-deficient mice do not undergo a decline in respiratory function following hypochlorite exposure, demonstrating that TRPA1 is actually targeted by hypochlorite/chlorine to elicit respiratory adverse effects [9].

Isocyanates/Diisocyanates

Isocyanates/Diisocyanates are the leading cause of occupational asthma, the most commonly reported lung disease associated with the workplace (Fig. **3**) [64]. Isocyanates are a group of small molecular weight chemical compounds used for the production of fungicides, pesticides and polymers. Compounds bearing two isocyanate groups (diisocyanates), as toluene diisocyanate, are manufactured for reaction with polyols in the production of polyurethanes that are the constituents of various products such as paints and foams. Clinical studies have implicated the immune system in the pathogenesis of occupational asthma as sensitization to low-molecular weight chemicals, including isocyanates. Asthma is considered to result from a response of the immune system to haptens conjugated with endogenous proteins [65]. However, inhalation of isocyanates/diisocynates can cause immediate respiratory symptoms like cough, nose and throat irritation, dyspnea and chest tightness and late asthmatic reactions as increase in airway responsiveness to methacoline and increased number of neutrophils, eosinophils and albumin in bronchoalveolar lavage fluid [66-68]. Much evidence has been accumulating since long time that toluene diisocyanate targets capsaicin-sensitive sensory neurons to produce bronchocostriction, sneezing and watery rhinorrhea in guinea-pigs [69, 70], but the molecular mechanism of toluene diisocyanate action has remained undetermined until recently. Taylor-Clark and coworkers [43] demonstrated that TRPA1 is necessary and sufficient to mediate toluene diisocyanate evoked-respiratory effects. Indeed, it was shown that toluene diisocyanate selectively activates TRPA1 in heterologous expression systems and in trigeminal sensory neurons from wild-type, but not TRPA1-deficient mice. *In vivo*, toluene diisocyanate caused a decrease in breathing rate suggesting a respiratory sensory irritation; a reflex response that was absent in TRPA1-deficient mice [43]. Other industrial isocyanates, including methyl isocyanate, a precursor in pesticide production that was the major causative agent of the environmental disaster in Bhopal, India, produce their noxious effects in the respiratory system *via* TRPA1 receptor activation [71].

INFLAMMATORY MEDIATORS AND TRPA1 SENSITIZATION

Nerve Growth Factor

A large variety of inflammatory autacoids are generated in the airways of asthma patients which are capable of sensitizing sensory neuron terminals by increasing TRPV1 and/or TRPA1 receptor expression or by more directly activating these channels. Nerve growth factor (NGF), a neurotrophin required for survival of newborn rat dorsal root ganglia, can be released from mast cells during asthma exacerbations [72]. NGF is known to augment TRPA1 expression in DRG neurons through the activation of the p38

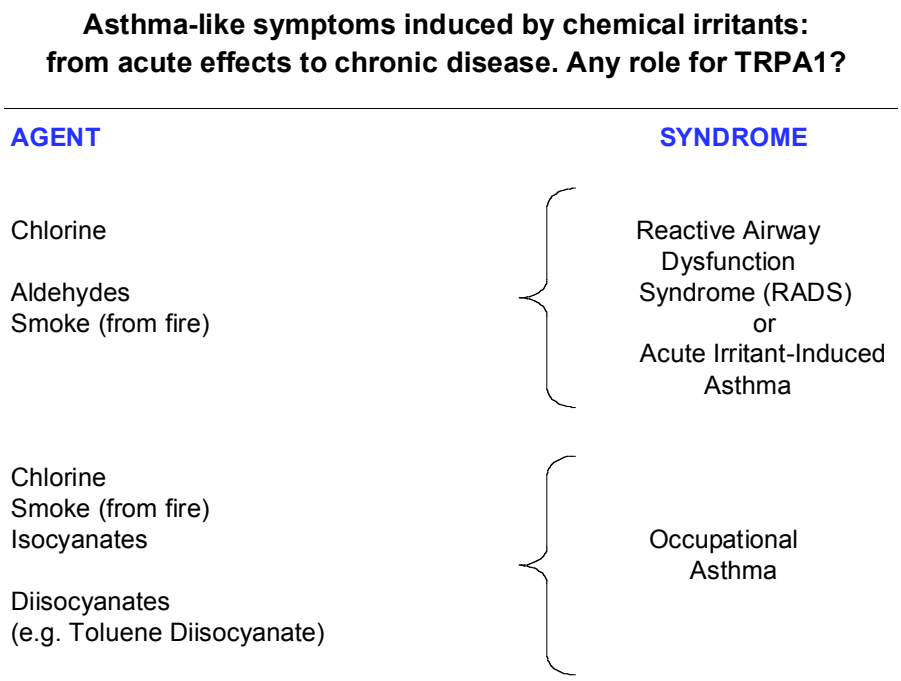

Fig. (3). Diverse chemical irritants known to induce acute inflammatory reactions of the airways and also more prolonged asthma-like symptoms are TRPA1 activators. It is conceivable that TRPA1 might play a role not only in mediating acute effects, but also in the pathogenesis of RADS and occupational asthma.

MAPK pathway, whereas administration of anti-nerve growth factor (anti-NGF) or a p38 MAPK inhibitor decreases the induction of TRPA1 and suppresses inflammation- and nerve injury-induced cold hyperalgesia [73].

Bradykinin

Bradykinin (BK) is a major pro-inflammatory endogenous nonapeptide capable of eliciting cough and bronchocostriction *via* B2 bradykinin receptor stimulation. BK is elevated in bronchoalveolar lavage fluid (BAL) and serum of asthmatics and rhinitis patients [74, 75]. BK was shown to sensitize TRPV1 by activating various intracellular mechanisms, including protein kinase C (PKC)-ε [76, 77], displacement of PIP2 from TRPV1 binding [78] and 12- / 5-lipoxygenase metabolites production [79]. Bradykinin evokes B2 receptor-mediated action potential discharge in bronchopulmonary vagal afferent nerve fibres, exclusively targeting capsaicin-sensitive C-fibres [80]. Interestingly, BK maintains its efficacy in activating vagal afferents also in C-fibres from TRPV1$^{-/-}$ mice, although the response is significantly less persistent than in TRPV1$^{+/+}$ C-fibres, thus suggesting that BK sensitizing activity on sensory neurons does not occur solely through TRPV1. Indeed, it has been shown that BK activates cells (CHO or DRG neurons) in which both TRPA1 and B2 receptors are co-expressed. BK injected into the hindpaw of TRPA1-deficient mice failed to elicit mechanical or thermal hyperalgesia thus demonstrating that TRPA1 is necessary, at least in part, for BK pro-algesic activity [12]. These observations are consistent with a model in which TRPA1 is activated by BK in two ways: through PLC-mediated increases in intracellular calcium and *via* calcium influx through TRPV1 [12].

Protease-Activated Receptor 2

Protease-activated receptor 2 (PAR-2), is a ubiquitous receptor expressed by various pulmonary cells and by capsaicin-sensitive sensory neurons, and is activated through cleavage of its extracellular domains by proteases like trypsin and mast cell tryptase [81]. PAR-2 stimulation contributes to neurogenic inflammatory responses in the airways, such as exaggerated allergic reactions [82], bronchoconstriction, plasma protein extravasation [83]. It has been shown that PAR-2 enhances TRPV1-mediated effects [84, 85]. PKC and cAMP-dependent PKA pathways, that are activated by bradykinin and PAR-2 leading to sensitization of TRPV1, also play a role in TRPA1 sensitization operated by bradykinin and PAR-2, as shown by Dai *et al*. [85] and Wang *et al*. [86].

TRPA1 AND COUGH

Chronic cough represents a major medical need, and at present its treatment is unsatisfactory, mainly because of the poor understanding of the underlying mechanisms of cough generation associated to a variety of different diseases [87]. Atopic and occupational asthma, cough variant asthma and the closely related corticosteroid responsive cough syndromes, eosinophilic bronchitis and atopic cough, are common causes of chronic cough [87].

In most cases the trigger that causes the cough is uncertain, however, treatment of underlying inflammatory conditions and removal of potential triggers is a therapeutic option worthy of consideration, particularly in cases of occupational exposure to known sensitizers. In a recent study [88], it has been shown that inhalation of different TRPA1 stimulants, including the selective agonists allyl

isothiocyanate and cinnamaldehyde, provokes a dose-dependent and robust tussive response in guinea pigs. Strong pharmacological evidence that TRPA1 is actually the mediator of the tussive effects produced by these compounds was achieved from the observation that their tussive effects were prevented by (+)camphor and gentamicin, two compounds that can act as antagonists for TRPA1 channel subtype [89] and by the selective TRPA1 antagonist, HC-030031 [18], whereas the TRPV1 antagonist, capsazepine [90] was without effect. In the same study [88] the authors showed that a significant proportion (~ 50 %) of the tussive response evoked in guinea-pigs by inhalation of cigarette smoke was inhibited by ruthenium red, (+)camphor and HC-030031. A more recent study confirms the data of Andrè and colleagues [88] and extends these findings in humans by showing that the TRPA1 agonist cinnamaldehyde elicits a dose-dependent cough in healthy volunteers [91]. In view of these recent findings it can be inferred that cough caused by cigarette smoke may be triggered by some of its components such as ROS, acrolein and crotonaldehyde, which are capable of activating TRPA1 [12, 31, 37]. It is worth considering that activated mast cells and increased concentrations of mast cell products are common to conditions associated with cough [92]. Moreover, mast cells are known to release not only tussive mediators but also neutrotrophic factors (e.g. NGF) with may enhance TRPA1 expression and consequently heighten TRPA1-mediated cough reflex. In sum, TRPA1 should be taken into consideration as a rising target for novel antitussive drugs, in light of the recent finding that TRPA1 stimulation elicits cough and that TRPA1 is a receptor promiscuously activated by a wide range of stimuli, including several major environmental irritants as well as endogenously-generated byproducts of oxidative stress and by major inflammatory mediators.

TRPA1 IN EXPERIMENTAL ALLERGEN-INDUCED ASTHMA

Atopic asthma is an allergic condition involving a humoral immune response (IgE) against specific inhaled allergen. However asthma-like symptoms and asthma-like syndromes can be triggered by exposure to airborne chemicals present in photochemical smog or by accidental inhalation in the workplace of irritant compounds. As described in the previous paragraphs, accidental inhalation of these substances has been reported to various symptoms, including cough, wheezing, chest tightness, dyspnoea and heightened sensitivity to different chemical and physical stimuli. Chronic asthmatic conditions, known as RADS [21, 93] or occupational asthma [22] (Fig. **3**) may outlast the short-lived exposure to the irritant agent by months or years [94]. Remarkably, the majority of the substances which cause RADS, are TRPA1 activators (Fig. **3**). Although the pathway(s) leading from acute to chronic respiratory symptoms caused by these irritants needs clarification, the possibility that TRPA1 plays a key role not only in mediating acute neurogenic inflammatory responses but perhaps in the perpetuation of the inflammatory response is conceivable. This hypothesis has received strong support recently by a study showing that genetic deletion of TRPA1 drastically attenuates inflammation and hyperreactivity in a mouse model of asthma [95]. In particular TRPA1-deficent mice backcrossed into the C57BL/6 background and sensitized to ovalbumin (OVA), showed reduced airway infiltration by eosinophils and other inflammatory leukocytes compared to wild-type littermates. This was accompanied by a reduction in inflammatory T-helper 2 cytokines such as IL-5 and IL-13 and pro-inflammatory chemoattractants eotaxin and RANTES. Even more striking was the nearly complete absence of airway hyperreactivity in TRPA1 knock out mice upon OVA challenge [95]. Administration of the TRPA1 selective antagonist HC-030031 during OVA airway challenge protected mice from developing the inflammatory responses similarly to genetic deletion of TRPA1 [95]. Either pharmacological blockade or genetic ablation of TRPA1 did not affect OVA-reactive IgE levels in serum indicating that the systemic response to allergen immunization is not affected by interfering with TRPA1 function. Because mRNA transcripts in leukocytes and airway tissues were nearly undetectable, it was suggested that OVA-activated TRPA1-mediated effects are essentially neurogenic [95].

Although animal models are widely utilized for studying the immunopathology underlying asthma, the majority of them are based on artificial triggers to induce airway disease such as in the OVA-sensitized animals. Several newly developed highly specific anti-inflammatory drugs found very effective in murine asthma models failed in humans [96]. Therefore, translating into humans the finding that TRPA1 plays a role in a murine model of allergic asthma may have limitation and need to be strengthened with studies involving other asthma and inflammatory airways models. Thus, future studies aimed to establish the mechanisms through which TRPA1 mediates neurogenic responses in the airways, leukocyte recruitment and airway hyperreactivity will be necessary for clearly defining TRPA1 role in asthma.

PHARMACOLOGICAL ANTAGONISTS OF TRPA1

The number of antagonists described so far is small, especially when considering the large number of identified TRPA1 agonists. Until recently there were no selective TRPA1 antagonists available and four nonselective blockers were used in the past as experimental tools: gentamicin, ruthenium red, gadolinium and amiloride. These antagonists displayed IC_{50}s in the low micromolar range towards heterologously expressed TRPA1 channels *in vitro* but were not very effective *in vivo* and also blocked other channel types [3]. A major improvement in the field occurred with the appearance of the selective TRPA1 antagonist HC-030031 [97, 98]. Although *in vitro* potency of HC-030031 is in the low micromolar range, selectivity of the compound was proven against other TRP channels [97] and *in vivo* the molecule proved effective in inhibiting TRPA1-mediated responses in various experimental models including bradykinin-induced mechanical allodynia, formalin-induced flinching, carrageenan-induced paw oedema and mechanical hyperalgesia after CFA or spinal nerve ligation and more recently, airway inflammation and hyperresponsivness in OVA-sensitized mice [95, 97].

Another TRPA1 receptor-selective antagonist, AP-18, a close structural analogue of cinnamaldehyde, is effective at reversing CFA-induced mechanical hyperalgesia when injected locally into the paw in mice [99]. Two recent

patents describing AP-18 related antagonists have been deposited by Abbott Laboratories [100, 101]. In September 2008, at the IBC's 2008 ACT Conference in San Diego, CA, data were presented about GRC-17266 (undisclosed structure), a compound synthesized by Glenmark Pharmaceuticals with a claimed IC_{50} value around 70 nM.

Given the rising interest of TRPA1 in several pathophysiological conditions, it can be anticipated that in the near future the number of new antagonists will increase sharply [98]. Because genetic ablation of TRPA1 does not appear to cause major phenotypical alteration and does not affect overall viability and reproductive capabilities in mice [9, 12] it may be speculated that future pharmacological therapies based on the use of highly selective TRPA1 receptor antagonists should not be accompanied by major side effects.

CONCLUSIONS

Robust evidence has been accumulating at a very impressive pace indicating that TRPA1, expressed on peripheral endings of primary afferent neurons, is activated by several environmental as well as endogenous irritants in the airways. Recent data showing the essential role of TRPA1 in inflammation and bronchial hyperreactivity in a murine asthma model strengthen the concept that TRPA1 could be a key integrator between the immune system and airways nerve terminals in the perpetuation of the inflammatory response to allergens. Moreover, two studies have provided strong pharmacological evidence that inhalation of TRPA1 receptor stimulants elicits cough reflex in guinea-pigs and human volunteers. Thus, targeting TRPA1 may allow direct inhibition of sensory nerve activity thereby suppressing peripheral and central sensory neuron reflexes and thus limiting neurogenic inflammatory responses. Finally, present knowledge on TRPA1 role in the airways suggests that TRPA1 antagonists might be taken into consideration for a novel pharmacological treatment of asthma, chronic cough and possibly other inflammatory conditions of the airways, including COPD and RADS.

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