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Redox Regulation of Transient Receptor Potential Channels

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Abstract

Significance: Environmental and endogenous reactive species such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and other electrophiles are not only known to exert toxic effects on organisms, but are also emerging as molecules that mediate cell signaling responses. However, the mechanisms underlying this cellular redox signaling by reactive species remains largely uncharacterized. ***Recent Advances:*** Ca^{2+}-permeable cation channels encoded by the transient receptor potential (*trp*) gene superfamily are characterized by a wide variety of activation triggers that act from outside and inside the cell. Recent studies have revealed that multiple TRP channels sense reactive species and induce diverse physiological and pathological responses, such as cell death, chemokine production, and pain transduction. TRP channels sense reactive species either indirectly through second messengers or directly *via* oxidative modification of cysteine residues. In this review, we describe the activation mechanisms and biological roles of redox-sensitive TRP channels, including TRPM2, TRPM7, TRPC5, TRPV1, and TRPA1. ***Critical Issues:*** The sensitivity of TRP channels to reactive species *in vitro* has been well characterized using molecular and pharmacological approaches. However, the precise activation mechanism(s) and *in vivo* function(s) of ROS/RNS-sensitive TRP channels remain elusive. ***Future Directions:*** Redox sensitivity of TRP channels has been shown to mediate previously unexplained biological phenomena and is involved in various pathologies. Understanding the physiological significance and activation mechanisms of TRP channel regulation by reactive species may lead to TRP channels becoming viable pharmacological targets, and modulators of these channels may offer therapeutic options for previously untreatable diseases. *Antioxid. Redox Signal*. 21, 971–986.

Introduction

THE TERM "reactive oxygen species (ROS)" collectively describes not only the oxygen (O_2) radicals, but also some nonradical derivatives of O_2, and includes the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) (45). Nitric oxide (NO) and peroxynitrite ($ONOO^-$) are known as reactive nitrogen species (RNS) (45). Production of ROS occurs naturally during respiration through the mitochondrial electron transport chain (34). ROS and RNS are also enzymatically generated by the NOX family of NADPH oxidases and NO synthases, respectively (2, 34, 79). Furthermore, ROS are generated after exposure to extracellular agents, ionizing radiation, cytotoxic drugs, and infectious agents (68, 110). Oxidative damage to DNA, lipids, and proteins causes cellular dysfunction and is a threat to the organism (45, 68). Organisms have developed defensive cellular responses and behavior to specifically detect and respond to oxidative stimuli (45, 68, 110). The phrase "oxidative stress" refers to a state of imbalance between the production of ROS/RNS and the capacity of the antioxidant defense system (68). Excessive and/or a sustained increase in ROS production have been implicated in the pathogenesis of cancer, diabetes mellitus, atherosclerosis, neurodegenerative diseases, rheumatoid arthritis, ischemia/reperfusion injury, pulmonary hypertension, and other diseases (18, 34, 150).

Apart from the role of ROS/RNS in disease, organisms have evolved to utilize ROS/RNS as signaling molecules for physiological functions. These include regulation of vascular tone, monitoring of oxygen tension to control ventilation and

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erythropoietin production, immune function, antimicrobial effects, and signal transduction (18, 34).

There are various proteins that detect the redox status of a cell. ROS activates transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (34). A complex between Kelch-like ECH-associated protein-1 and nuclear factor (erythroid-derived-2)-like-2 detects ROS in mammals, and regulates responses that protect against environmental stress (12, 31). Myofilament proteins and some protein kinases are also modulated by ROS (34, 144). RNS also regulate the activity of a diverse array of proteins, including enzymes, protein kinases, phosphatases, transcription factors, and others (51, 149). Notably, a group of calcium ion channels were also discovered to detect the redox status of the cell and to mediate cellular responses (17).

TRP proteins are the products of *trp* genes, the first of which was discovered in *Drosophila melanogaster* (103). The term "TRP" is derived from "transient receptor potential," because the *trp* gene mutant photoreceptors fail to generate the Ca^{2+}-dependent sustained phase of receptor potential and, therefore, fail to induce subsequent Ca^{2+}-dependent adaptation to light. The currently identified mammalian TRP channels are divided into six subfamilies [canonical (C), vanilloid (V), melastatin (M), polycystic kidney disease (P), mucolipin (ML), and ankyrin (A)] by the degree of homology of their protein sequences (25, 26) (Fig. 1). TRP channels have several common structural features: six transmembrane segments; a pore-forming region between the fifth and sixth transmembrane segments; cytoplasmic N- and C-terminal regions; and tetrameric subunit stoichiometry (Fig. 2). TRP proteins form cation channels that detect cellular stimuli and transduce them into electrical and chemical signals *via* changes in membrane potential and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), respectively. TRP channels are activated by (*i.e.*, sense) diverse stimuli, including receptor stimulation, heat, osmotic pressure, mechanical stress, and environmental irritants from the extracellular and intracellular milieu (25, 42, 116, 171). The TRPC homologs are receptor-activated Ca^{2+}-permeable cation channels that sense metabotropic changes after receptor stimulation, which activates and induces phospholipase C (PLC) to hydrolyze phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol-1,4,5-trisphosphate and diacylglycerol (115, 169). TRPV Ca^{2+}-permeable channels can be functionally defined as thermosensors (120). TRPV1, originally identified as the receptor for the vanilloid compound capsaicin, is responsive to heat (>43°C), proton concentration (pH <5.6), the intrinsic ligand anandamide, and

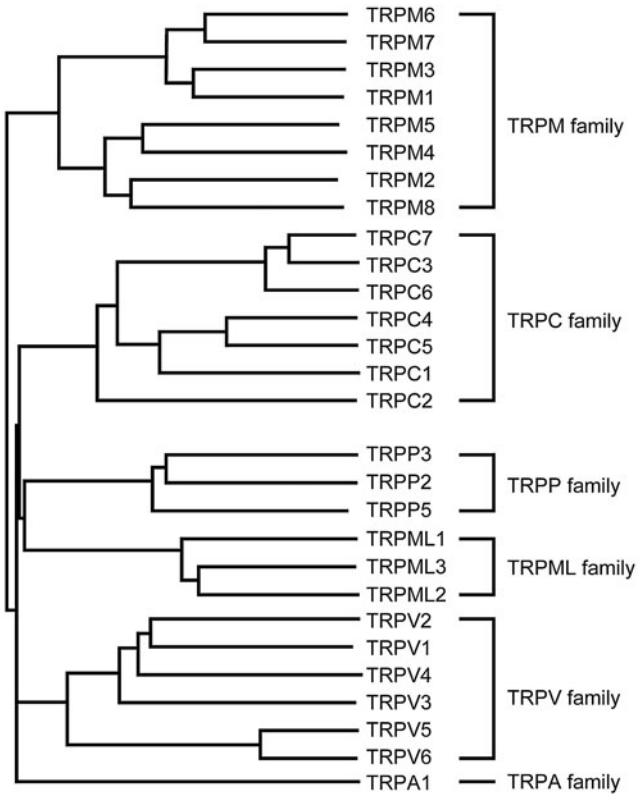

FIG. 1. Phylogenetic tree of TRP channels. Phylogenetic tree of mammalian TRP channels based on their homology. TRP, transient receptor potential.

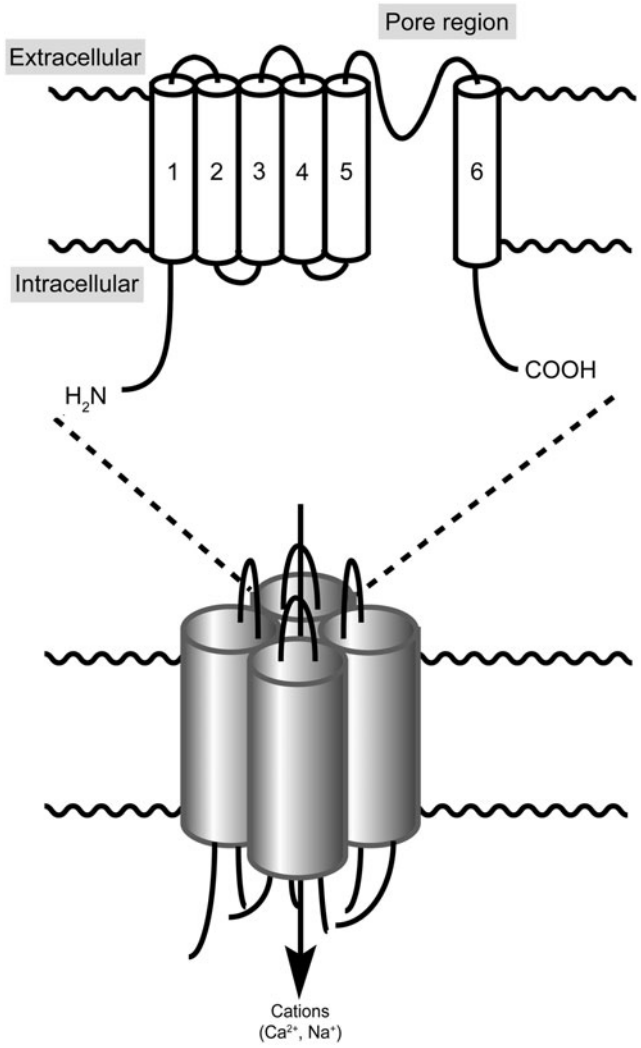

FIG. 2. Transmembrane topology of TRP channels. TRP channels have common structural features: six transmembrane segments, a pore-forming region between the fifth and sixth transmembrane segments, cytoplasmic N- and C-terminal regions, and tetrameric subunit stoichiometry.

receptor-driven PLC activity (21, 24, 162, 190). High temperature also activates TRPV2, TRPV3, and TRPV4 (120). TRPV5 and TRPV6 are distinct from other TRPVs, because they are highly Ca^{2+}-selective channels and are tightly regulated by $[Ca^{2+}]_i$ (113). The TRPM subfamily is named after melastatin (TRPM1), a tumor suppressor protein isolated in a screen for genes whose level of expression is inversely correlated with the severity of metastatic potential in a melanoma cell line (36). TRPM8 channels, in contrast to TRPV channels, are activated by low temperature and menthol (95, 122). The sole member of the TRPA subfamily, TRPA1, has a large N-terminal domain with many predicted ankyrin repeats and is activated by environmental irritant compounds and noxious cold stimuli (65, 145).

Notably, a class of TRP channels has been found to be modulated by ROS/RNS and to control various cellular processes in response. TRPM2, the first identified ROS-sensitive TRP channel, is activated by H_2O_2 (47). Accumulating evidence suggests that H_2O_2-activated Ca^{2+} influx through TRPM2 mediates several cellular responses, including cell death (47) and chemokine production in monocytes that aggravates inflammatory neutrophil infiltration (180). TRPM7 is activated by ROS/RNS and is an essential mediator of anoxic death (1). In addition to TRPM2 and TRPM7, certain members of the TRPC and TRPV subfamily, including TRPC5 and TRPV1, are activated by ROS and NO (183). Notably, the activation of TRPC5 and TRPV1 is through oxidative modification of the free sulfhydryl group of a cysteine residue (183). More recently, the activation of the TRPA1 channel has been shown to occur after oxidative cysteine modification by ROS and RNS (153). TRPA1 is also activated by environmental electrophiles and endogenous electrophilic products of oxidative stress (4, 53, 88), and it is putatively involved in pain transduction and detection of the ROS microenvironment in the airway (11). This review explores the activation mechanisms and biological significance of these TRP channels in redox-related conditions.

TRPM2

TRPM2: the first identified ROS-sensitive TRP channel

The transcripts of TRPM2 (formerly designated TRPC7 and LTRPC2) were recognized to encode a protein product bearing homology to TRP proteins (106). Notably, we have demonstrated that TRPM2 is activated by H_2O_2 (47). The application of H_2O_2 induces TRPM2 currents and an increase in $[Ca^{2+}]_i$ in TRPM2-expressing HEK cells and RIN-5F rat insulinoma cells. Subsequently, substantial evidence has accumulated demonstrating the activation of TRPM2 by H_2O_2 in various cell types, including TRPM2-transfected cells (175), microglia (74), hippocampal neurons (118), U937 human monocytes (180, 186), neutrophils (54), pancreatic β-cells (80), and endothelial cells (49). The H_2O_2-induced Ca^{2+} influx through TRPM2 was enhanced under intracellular Fe^{2+}-accumulated conditions and reduced by $^{\bullet}OH$ scavengers or an iron chelator, suggesting that the intracellular $^{\bullet}OH$ plays a key role in the activation (57). TRPM2 is also activated in response to the nitrosative stress-inducing donor *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (125).

Other studies have revealed that TRPM2 is a Ca^{2+}-permeable nonselective cation channel that is specifically gated by adenosine diphosphoribose (ADPR) (70, 76, 123, 134) and adopts a swollen, bell-shaped tetrameric conformation (92). ADPR gating of TRPM2 is made possible by a C-terminal domain, designated the NUDT9-homology (NUDT9-H) domain, which is homologous to the NUDT9 ADPR pyrophosphatase that catabolizes the conversion of ADPR into adenosine monophosphate and ribose 5-phosphate (123). The residual enzymatic activity of the purified NUDT9-H domain of TRPM2 is lower than that of NUDT9, and the binding affinity of the NUDT9-H domain to ADPR seems to be more important for the gating process of TRPM2 (77, 123–125). In addition, Ca^{2+} is a modulator of TRPM2 (28, 94, 143).

Molecular mechanisms of TRPM2 activation by ROS

In our experiments using the conventional whole-cell mode of patch clamp method, which perfuses cytoplasm with pipette solution, TRPM2 currents were not induced by H_2O_2, suggesting that intracellular mediators are necessary for H_2O_2-induced TRPM2 activation (47). Initially, we showed that nicotinamide adenine dinucleotide (NAD^+) generated on ROS stimulation can directly induce the opening of TRPM2 (47). It is now believed that this opening is mainly mediated by conversion of NAD^+ to ADPR and by the production of ADPR from mitochondria (125). Cytosolic or mitochondrial overexpression of ADPR pyrophosphatase, which degrades ADPR, suppresses H_2O_2-induced Ca^{2+} responses, confirms the involvement of ADPR in H_2O_2-induced TRPM2 activation. ADPR can be also generated in the nucleus by a pathway involving poly(ADPR) polymerase-1 (PARP-1) (139), with a similar pathway putatively functioning in mitochondria (35). In response to DNA-strand breaks arising from oxidative damage, PARP-1 uses NAD^+ as a substrate to catalyze the successive transfer of several ADPR moieties to nuclear protein acceptors (139). The resulting polymer of ADPR can interact in a selective manner with a number of protein targets that are involved in the cellular response to DNA damage and DNA metabolism. Subsequently, free ADPR is generated after the degradation of poly(ADPR) by poly(ADPR) glycohydrolase (PARG). Thus, it is hypothesized that the free ADPR which activates TRPM2 is produced by the activation of PARP-1 and PARG. Corroborating this hypothesis, three structurally distinct PARP inhibitors suppress H_2O_2-induced activation of TRPM2 (39), but electrophysiological studies have shown that these PARP inhibitors have no effect on the activation of TRPM2 by ADPR itself. Thus, the PARP inhibitors should act upstream of TRPM2. In addition, it has been reported that the PARP-deficient DT40 lymphocytes constitutively expressing TRPM2 exhibit no H_2O_2-induced Ca^{2+} response (19). More support for this mechanism comes from the observation that PARP-1 and PARG control H_2O_2-induced extracellular Ca^{2+} fluxes through TRPM2 in mouse embryonic fibroblasts (16). In inside-out patches from TRPM2-expressing *Xenopus* oocytes, H_2O_2 failed to activate TRPM2 (163), suggesting that the H_2O_2 sensitivity of TRPM2 may be dependent on the production of ADPR from both the mitochondria and nucleus (Fig. 3).

Another potential source of free ADPR is the NAD^+ glycohydrolases, ADPR-producing ectoenzymes such as CD38 and CD157 localized on the plasma membrane (91). CD38 and CD157 are enzymes that generate ADPR from NAD^+ by their extracellular domains. CD38 could potentially contribute to the signaling events of TRPM2-mediated ion fluxes, although

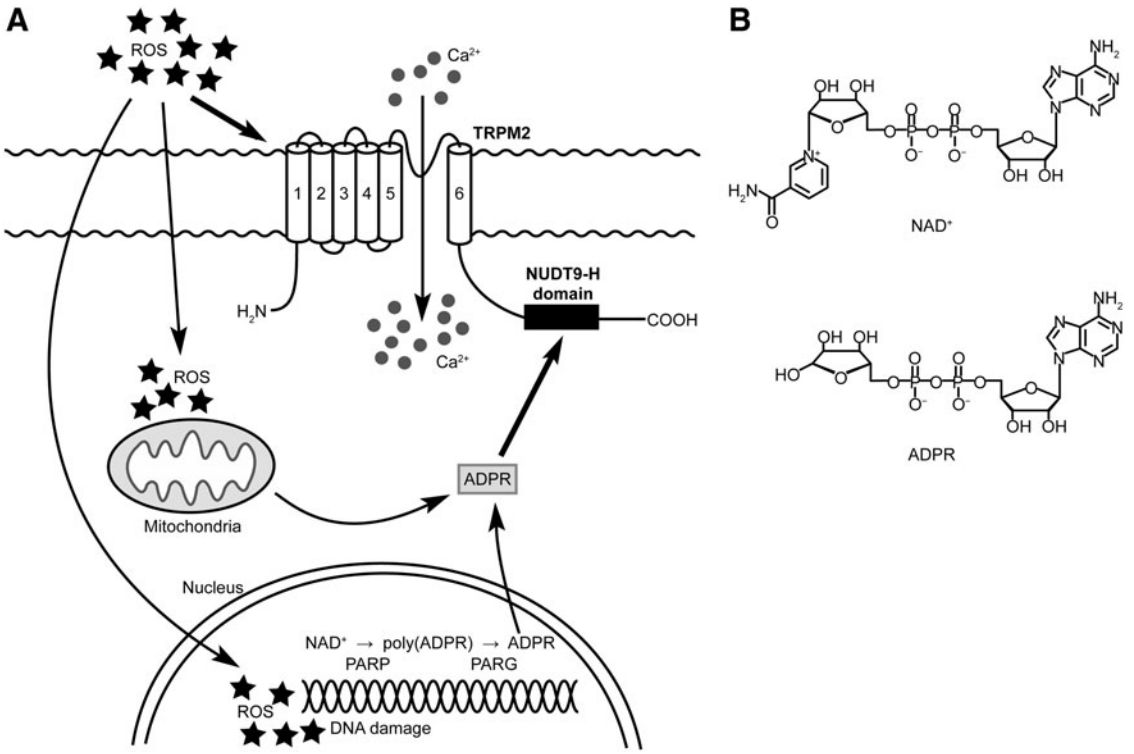

FIG. 3. Possible pathways for the activation of TRPM2 by ROS. (A) H_2O_2 mobilizes ADPR from the mitochondria and nucleus through PARP-1/PARG pathway, which then activates TRPM2. TRPM2 may also be gated directly by H_2O_2. **(B)** Chemical structures of NAD^+ and ADPR. ADPR, adenosine diphosphoribose; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; NAD^+, nicotinamide adenine dinucleotide.

if and how ADPR generated extracellularly by CD38 enters the cell to reach the intracellular ADPR binding domain of TRPM2 has yet to be determined.

Contradicting the hypothesis that H_2O_2 activates TRPM2 indirectly through ADPR, lines of evidence suggest that TRPM2 can be gated directly by H_2O_2 (Fig. 3). For instance, a splice variant of TRPM2, a deletion mutant lacking amino acids 1292–1325 in the C-terminal of NUDT9-H domain and thus displaying decreased affinity for ADPR, is not activated by the intracellular application of ADPR, but responds to H_2O_2 (175). H_2O_2 also triggers single-channel activity in excised membrane patches of TRPM2-transfected CHO cells (111, 112). This suggests that H_2O_2 may activate TRPM2 through a mechanism independent of ADPR. It is suggested that H_2O_2 acts as a direct stimulus for TRPM2 activation, as it can initiate the release of ADPR from the mitochondria and at the same time, functions as a potentiating cofactor of ADPR (71). Methodological disparities such as culturing conditions, cell types, and drug application are likely responsible for the conflicting reports on TRPM2 activation by H_2O_2 (111). Further investigation is necessary to elucidate the ROS-dependent activation mechanisms of TRPM2.

Cell death and TRPM2

On the basis of major association of ROS with cell death, we have previously proposed that TRPM2 mediates cell death (47). Since the first report of the involvement of TRPM2 in cell death, a substantial body of evidence obtained from various independent groups has supported this idea (97). TRPM2-expressing HEK cells are susceptible to cell death triggered by exposure to H_2O_2 through the elevation of $[Ca^{2+}]_i$ (47). Co-expression of TRPM2 and the dominant-negative TRPM2 splice variant (in which the four C-terminal transmembrane domains and the entire C terminus are deleted) suppressed H_2O_2-induced Ca^{2+} influx through TRPM2, reduced susceptibility to cell death, and blocked the onset of apoptosis in HEK cells (185). The role of endogenous TRPM2 in H_2O_2-induced cell death was revealed in various cell types, including cultured striatal cells (40), cultured cerebral cortical neurons (67), myocytes (182), RIN-5F insulinoma cells (47), U937 monocytic cells (186), and an endothelial cell line (147). A TRPM2-specific antisense oligonucleotide significantly suppressed Ca^{2+} influx and cell death induced by H_2O_2 in RIN-5F cells (47). Interestingly, PARP inhibitors protect rat striatal cells from H_2O_2-induced cell death (40), while PARP-1 and PARG were found to control Ca^{2+} fluxes through TRPM2 in a cell death signaling pathway induced by H_2O_2 in mouse embryonic fibroblasts (16). In addition, Lange *et al.* reported that TRPM2 functions as a Ca^{2+}-release channel activated by intracellular ADPR in a lysosomal compartment in addition to its role as a plasma membrane channel, and both functions of TRPM2 were shown to be critically linked to H_2O_2-induced pancreatic β-cell death using short-interfering RNA (siRNA) and *Trpm2* knockout (KO) mice techniques (80).

Consistent with the hypothesis that activation of TRPM2 results in cell death, TRPM2 appears to regulate caspase signaling. Most apoptotic pathways converge on the family of caspases (15). Caspase-8 plays a central role in the extrinsic cell death pathways involving transmembrane receptor-

mediated interactions, whereas caspase-9 is an important component in the intrinsic cell death pathway. Caspases-6, -7, and -3 are downstream of caspase-8 and caspase-9, with caspase-3 ultimately executing cell apoptosis. In the U937 cell line, in which H_2O_2 activates caspases-8, -9, -3 and -7 and dramatically up-regulates the cleavage of PARP protein, down-regulation of endogenous TRPM2 by RNA interference inhibited the rise in $[Ca^{2+}]_i$, enhanced cell viability, and reduced the numbers of apoptotic cells after exposure to H_2O_2 (186). Other groups also showed that TRPM2 activity stimulated the activation of caspases-8, -9, -6, and -3 (16, 147). These results link TRPM2 to the activation of caspases in H_2O_2-induced cell death (151).

Amyloid β-peptide (Aβ) is the main component of the plaques that characterize Alzheimer's disease (AD) and may induce neuronal death through mechanisms which involve oxidative stress (181). Accumulating evidence suggests that a failure of Ca^{2+} homeostasis plays a critical role in the neuropathology of AD. Incubation of cells in Ca^{2+}-free medium attenuated the direct neurotoxicity of Aβ and limited their vulnerability to excitotoxicity, suggesting the involvement of Ca^{2+} influx in these processes (93). Fonfria *et al.* have shown that the application of Aβ led to the generation of ROS in cultures of rat striatal neurons, and used data from siRNA experiments targeting TRPM2 to assert that the activation of TRPM2, functionally expressed in primary cultures of rat striatum, contributes to Aβ- and H_2O_2-induced striatal cell death (40). Thus, TRPM2 activity likely contributes to neuronal cell death in AD.

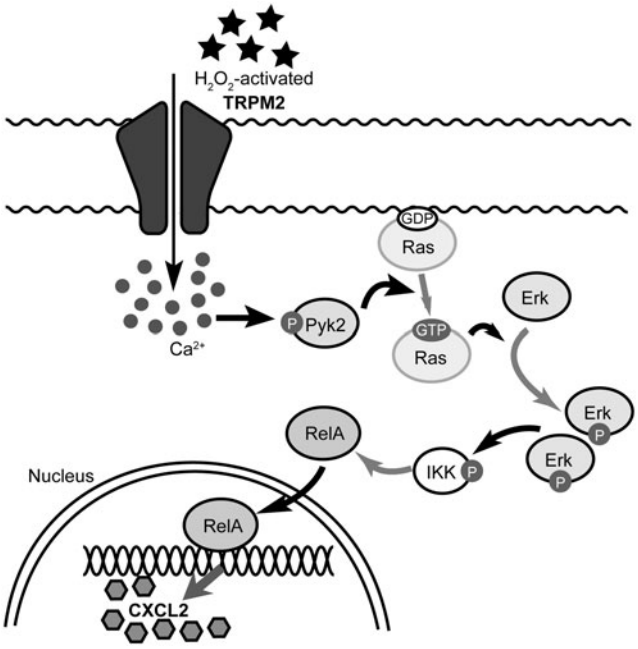

FIG. 4. TRPM2 activation by H_2O_2 induces chemokine production. In monocytes, Ca^{2+} influx via H_2O_2-activated TRPM2 triggers Pyk2 phosphorylation, which amplifies Erk activation in an Ras-dependent manner. The amplified Erk activates transcription of the C*xcl2* gene by inducing nuclear translocation of the NF-κB subunit RelA.

Inflammation and TRPM2

At sites of inflammation, ROS are secreted from immunocytes and epithelial cells (34, 50), such that a large amount of ROS is present at these sites. Intracellular Ca^{2+} also plays important signaling roles in immunocytes, and increases in $[Ca^{2+}]_i$ are important for the production of certain cytokines, including interleukin-2 in T cells (38) and CXCL8 in U937 human monocytic cells (170, 176). CXCL8 is a key chemotactic cytokine mediating the recruitment of neutrophils migrating into inflammation sites (87). In human monocytes, production of CXCL8 is induced by H_2O_2 (66) *via* extracellular signal-regulated kinase (Erk)-activated NF-κB (184). Until recently, despite evidence implicating H_2O_2 and Ca^{2+} as important regulatory factors in CXCL8 production in monocytes, the molecular entities and signaling mechanisms that link H_2O_2, Ca^{2+}, and CXCL8 production remained unclear.

In U937 monocytes, H_2O_2-induced increases in $[Ca^{2+}]_i$ and the production of CXCL8 were significantly suppressed by TRPM2-specific siRNA (180). We found that H_2O_2-induced nuclear translocation of the NF-κB subunit RelA was positively regulated by Erk-mediated phosphorylation of IκB kinase-β (IKK-β). In addition, upstream of Erk activation, TRPM2 activation by H_2O_2 results in Ca^{2+} influx and consequent autophosphorylation of Ca^{2+}-sensitive proline-rich tyrosine kinase 2 (Pyk2), which amplifies Erk activation *via* Ras GTPase. The activation of this Pyk2/Erk/NF-κB pathway in response to H_2O_2 was demonstrated in wild-type monocytes, but was impaired in *Trpm2* KO monocytes. Thus, H_2O_2-mediated Ca^{2+} influx *via* TRPM2 triggers Pyk2/Ras signaling, which amplifies downstream Erk activation leading to the nuclear translocation of NF-κB and the production CXCL2, the murine functional homologue of human CXCL8 (Fig. 4).

We characterized the *Trpm2* KO mouse phenotype in a dextran sulfate sodium (DSS)-induced model of ulcerative colitis that was associated with ROS production (72, 180). As expected, the *Trpm2* KO mice were largely protected from DSS-mediated colitis. Histological assessments revealed profound inflammation and tissue destruction characterized by mucosal ulceration, serosa destruction, epithelial injury, and inflammatory cell infiltration in the colon of wild-type mice. In *Trpm2* KO mice, the severity of the DSS-induced colitis was dramatically reduced, although epithelial injury was still observed in the colon. Expression of CXCL2 and the number of recruited neutrophils were severely diminished in the colon tissue of DSS-treated *Trpm2* KO mice. The number of macrophages and other inflammatory cells infiltrating into the colon was indistinguishable between wild-type and *Trpm2* KO mice. These results suggest that the diminished expression of CXCL2 in monocytes/macrophages induced by ROS contributes to the paucity of neutrophil accumulation in the colon of DSS-treated *Trpm2* KO mice. Thus, TRPM2-mediated chemokine production in monocytes/macrophages is an important mechanism in the progressive severity of DSS-induced ulcerative colitis (Fig. 5). Recently, Di *et al.* also showed that neither H_2O_2 nor lipopolysaccharide (LPS)-induced ROS were able to induce inward currents or increases in $[Ca^{2+}]_i$ in *Trpm2* KO bone marrow-derived macrophages (32). We previously demonstrated that CXCL2 production induced by H_2O_2 and LPS is diminished in *Trpm2* KO monocytes (180). Conversely, Di *et al.* showed that LPS challenge caused greater release of CXCL2 and inflammatory responses in lung in *Trpm2* KO mice than in wild-type mice, suggesting that TRPM2 activation by ROS may reduce rather than enhance the production of CXCL2 and the inflammatory

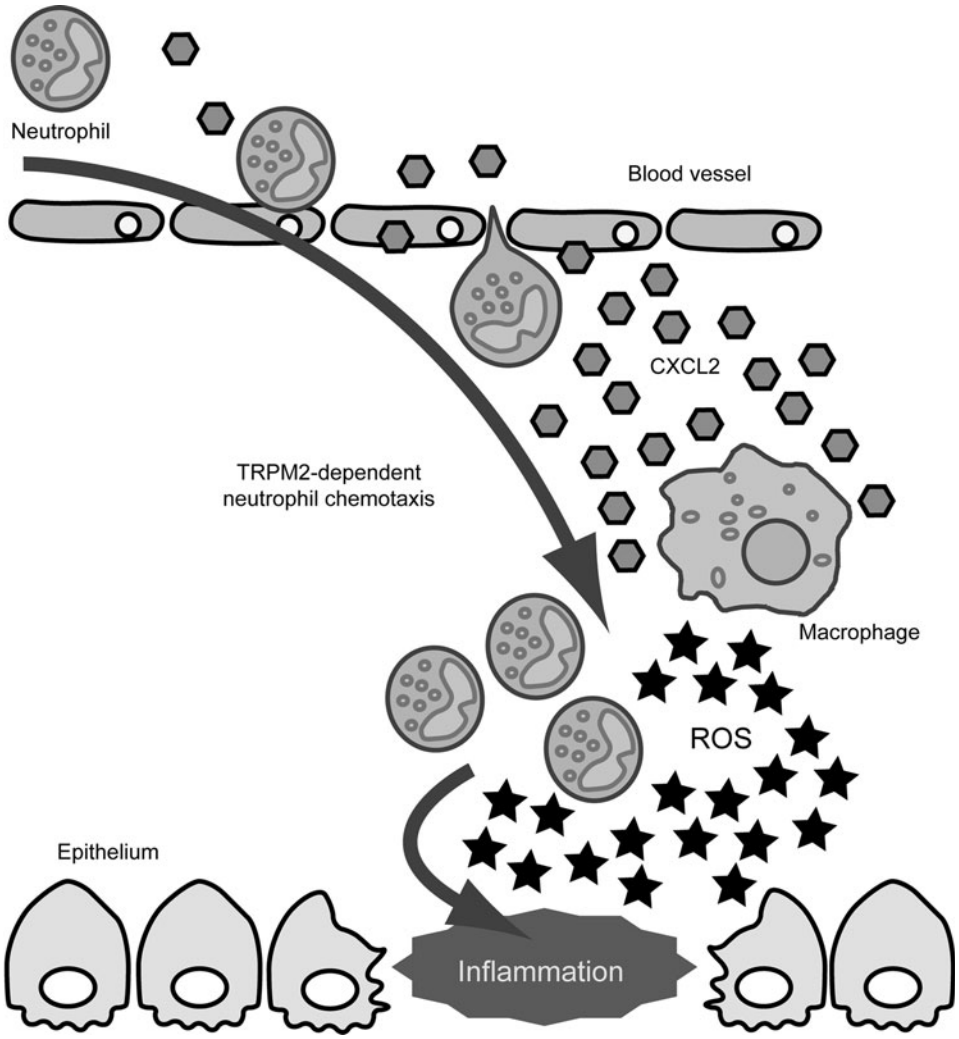

FIG. 5. ROS-induced TRPM2-dependent chemokine production in monocytes/macrophages aggravates inflammatory neutrophil infiltration. ROS, including H_2O_2, generated and released from various cells at inflamed sites, activate TRPM2 channels in monocytes/macrophages. CXCL2-induced neutrophil accumulation at the inflamed sites plays an important role in the pathogenesis of ulcerative colitis.

response (32). In conclusion, since the roles of ROS-sensitive TRPM2 in inflammatory diseases are controversial, it will be important to delineate and clarify these differences in the pathological phenotype of *Trpm2* KO mice.

TRPM7

Anoxic cell death and TRPM7

TRPM7, a divalent cation channel permeable to Mg^{2+} and Ca^{2+} and containing a C-terminal serine/threonine kinase domain, is a widely expressed member of the TRPM ion channel subfamily (102, 105, 131, 138). TRPM7 currents are inhibited by Mg^{2+} and Zn^{2+}, and activated by low levels of MgATP (73, 105). Targeted deletion of TRPM7 in DT-40 B cells is lethal. These cells exhibited Mg^{2+} deficiency, growth arrest, and death within 24 h unless rescued by increased levels of extracellular Mg^{2+} (105). These studies, along with others in which TRPM7 expression was down-regulated (46, 48), demonstrate that precise regulation of TRPM7 expression is necessary for cell viability and growth.

ROS and RNS can serve as activators of cation conductance through TRPM7, contributing to anoxic neuronal death (1). It has been proposed that during oxygen and glucose deprivation of primary cortical neurons, a Ca^{2+}-permeable nonselective cation conductance mediated by TRPM7 is activated by ROS/RNS and is primarily responsible for neuronal death. This is supported by the observation that ROS/RNS are able to enhance the TRPM7-mediated inward current in TRPM7-transfected HEK293 cells. In addition, in primary neurons, it appears that the electrophysiological properties of currents activated by oxygen- and glucose deprivation, including currents enhanced by low Mg^{2+} and inhibited by high Mg^{2+}, are characteristic of TRPM7 and are not shared by TRPM2. Suppression of TRPM7 expression in primary cortical neurons blocked TRPM7 currents, Ca^{2+} influx, and ROS production, protecting cells from anoxic cell death. However, siRNA targeted against TRPM7 also reduced TRPM2 levels (1), suggesting that expression of TRPM2 and TRPM7 are codependent. In summary, although it is difficult to definitively distinguish the roles of TRPM2 and TRPM7 in anoxic injury, both TRPM7 and TRPM2 channels appear to be important in oxidative stress-induced cell death (100). Interestingly, the proposed role of TRPM7 in anoxic cell death was confirmed *in vivo* (146). The suppression of TRPM7 in rats by intrahippocampal injections of viral vectors bearing small hairpin RNA specific for TRPM7 made neurons resistant to ischemic death and preserved neuronal function and performance for hippocampus-dependent learning tasks after brain

ischemia. This suggests that TRPM7 can be selectively targeted to prevent ischemic brain damage.

TRPC5

Direct modulation of TRPC5 via *NO oxidative modification of cysteine residues*

TRPC5 was originally cloned from mouse brain and functionally identified as a receptor-activated Ca^{2+}-permeable cation channel linked to PLC (117, 126). Although it is still controversial whether depletion of Ca^{2+} stores can activate TRPC5, a number of proteins and factors have been shown to act as direct triggers and modulators of TRPC5 channel activation (63, 104). For example, binding of intracellular Ca^{2+} and calmodulin has been implicated in TRPC5 activation and modulation (14, 43, 119, 140), while membrane polyphosphoinositides such as PIP_2 exert both stimulatory and inhibitory effects in regulating TRPC5 channel activity (165).

We have previously demonstrated that TRPC5 is directly activated by H_2O_2 and the NO donor, *S*-nitroso-*N*-acetyl-DL-penicillamine, *via* cysteine modification (154, 183). In endothelial cells, native TRPC5 is likely to be activated by NO generated by endothelial-type NO synthase (183). Redox modification of cysteine residue sulfhydryl groups has emerged as an important elementary step in the signal transduction cascades that underlie many physiological responses (12, 135). NO-activated TRPC5 channels were significantly but not entirely suppressed by ascorbate, which reduces *S*-nitrosothiols (but not disulfides) to thiols. However, dithiothreitol (DTT), which reduces both *S*-nitrosothiols and disulfides to thiols, fully suppressed NO-activated TRPC5 channel activity. Thus, both nitrosylation and disulfide bond formation are likely to be involved in NO-induced TRPC5 activation. Our functional assays employing cysteine mutants have shown that cysteine residues accessible from the cytoplasm (namely Cys553 and nearby Cys558 on the N-terminal side of the putative pore-forming region between the fifth and sixth transmembrane domains) are essential for NO-induced mouse TRPC5 activation (Fig. 6). In an *S*-nitrosylation assay (59), *S*-nitrosylation was abolished by mutation of Cys553, but was unaffected by mutation of Cys558. Our data suggest that the TRPC5 channel is opened *via S*-nitrosylation of Cys553 and a subsequent nucleophilic attack on nitrosylated Cys553 by the free sulfhydryl group of Cys558 to form a disulfide bond that stabilizes the open state. However, the NO sensitivity of TRPC5 channels was refuted by another group (177). It has been reported that TRPC5 may form a disulfide bridge between Cys553 and Cys558, and is activated by the reducing agent DTT and by extracellular reduced thioredoxin, both of which cleave the disulfide bridge (179). Thus, it is possible that the sensitivity of TRPC5 to both NO and redox status is dependent on culturing conditions, drug application, cell density during measurements, and/or other experimental conditions, which may affect the redox sensitivity of TRPC5, levels of antioxidants, or other molecular and cellular redox states.

TRPV1

Modulation of TRPV1 by ROS/RNS via *oxidative modification of cysteine residues*

The nonselective cation channel TRPV1 is activated by various noxious stimuli, including heat (>43°C) (21), acidic pH (162), and environmental irritants and endogenous algesic substances, including capsaicin (21), camphor (178), 12-hydroperoxyeicosatetraenoic acid (56, 141), bradykinin (127), and anandamide (190). TRPV1 is expressed in primary afferent nociceptors of dorsal root ganglia, trigeminal ganglia, nodose ganglia (21, 162), the central nervous system, and non-neuronal tissues (22, 98). Importantly, TRPV1 can detect and integrate sub-threshold stimuli owing to its sensitivity to multimodal stimuli. For example, capsaicin- and heat-induced activation of TRPV1 is enhanced by mild acidosis and inflammatory agents involved in the activation of PLC signaling (3, 24, 61, 127, 162).

In addition to these stimuli, TRPV1 was also found to be sensitized and activated by oxidative stress. We found that TRPV1 channels heterologously expressed in HEK cells are activated by both H_2O_2 and NO (183). An alignment of amino-acid sequences surrounding Cys553 and Cys558 of TRPC5 (which confer its redox sensitivity) with counterpart sequences in TRPV1 shows cysteine residues conserved on the N-terminal side of the putative pore-forming region (Cys616 and Cys621 in rat) located between the fifth and sixth transmembrane domains. Mutation of these cysteines reduces the Ca^{2+} influx induced by H_2O_2 and NO, suggesting that TRPV1 redox sensitivity is conferred by these cysteine residues (183). However, this view has been challenged by Miyamoto *et al.*, who reported that the double cysteine mutant C616W:C621S shows normal NO-induced calcium responses when normalized against capsaicin responses (101). Other groups have reported that both oxidizing agents (such as H_2O_2, diamide, and chloramine-T) and reducing agents (*i.e.*, DTT) sensitize rat TRPV1 and amplify its response to heat and capsaicin, with Cys621 identified as a key residue in its sensitization by a reducing agent (148, 172). In avian TRPV1, which is capsaicin insensitive, sensitivity to oxidation was attributed to multiple cysteine residues on the N- and C-terminal domains (23). It is generally believed that formation of a disulfide bond(s) is the molecular mechanism underpinning oxidative sensitization/activation. A biochemical study has demonstrated that oxidation of TRPV1 induces C-terminal dimerization *via* disulfide bond formation (173), but there is currently no direct evidence corroborating this hypothesis *in vivo*.

Recently, it has been suggested that the activity of neuronal NO synthase contributes to the activation of TRPV1. Ito *et al.* reported that mechanical overload-induced muscle hypertrophy is triggered by the activation of TRPV1 localized in sarcoplasmic reticulum by NO and $ONOO^-$, which are downstream products of neuronal NO synthase (58).

TRPV1 is not activated by all oxidizing agents. Intriguingly, oxidation of TRPV1 by some oxidizing agents, such as the antiseptic thimerosal or copper-*o*-phenanthroline, suppresses TRPV1 activation (64, 164). The effect of thimerosal was blocked by the application of DTT and by point mutation of Cys621, suggesting that oxidative modification of cysteine residues was involved in the mechanism of suppression (64). In contrast, the effect of copper-*o*-phenanthroline seems to be independent of cysteine residues: mutation (individually and collectively) of three putative cysteine residues located on the extracellular membrane leaflet could not preclude copper-*o*-phenanthroline from effectively blocking TRPV1 activation by heat, protons, and capsaicin (164).

TRPV1 also shows sensitivity to pungent compounds from onion and garlic, such as allicin (89), through covalent

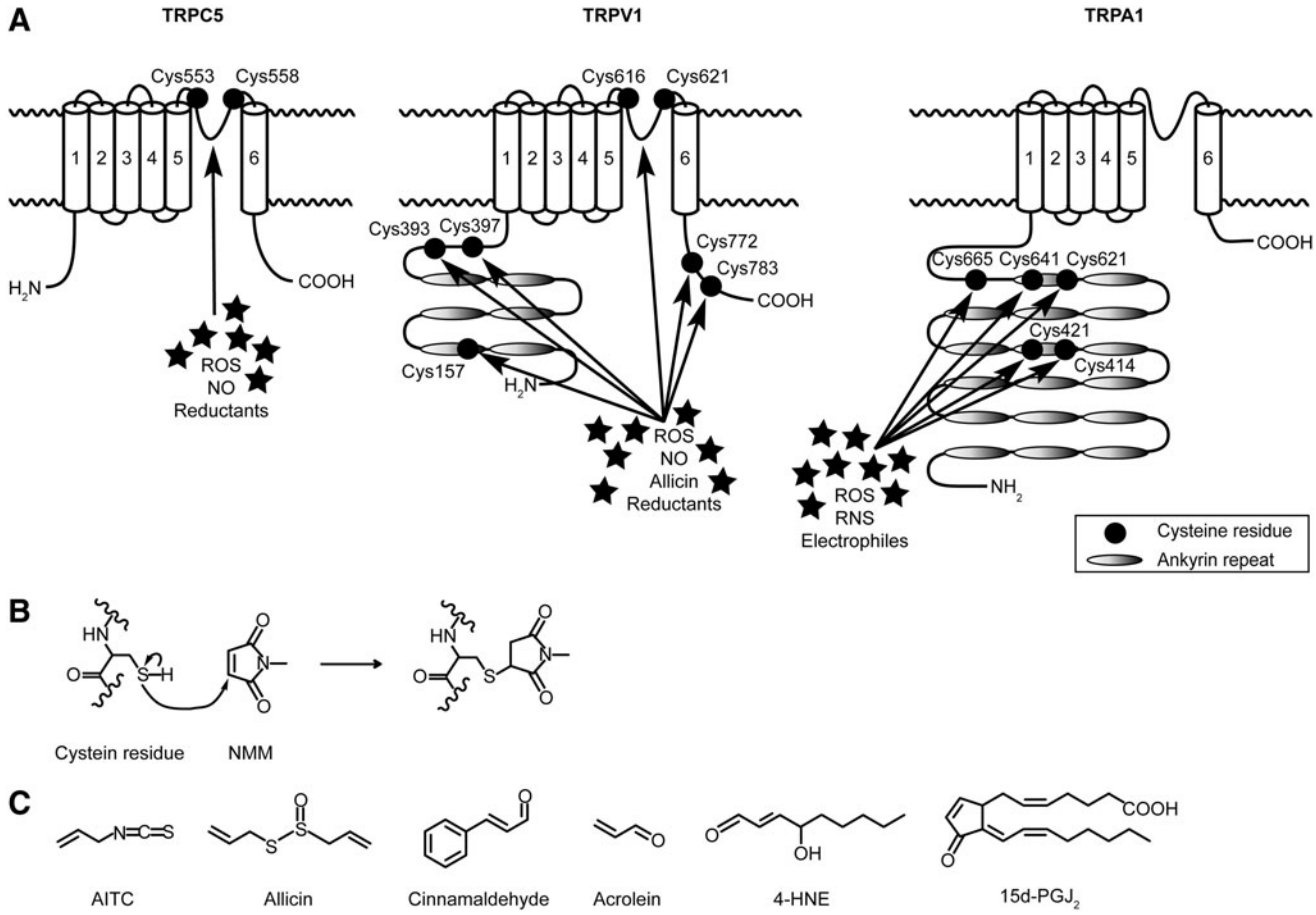

FIG. 6. Redox regulation of TRPC5, TRPV1, and TRPA1 through direct modification of cysteine residues. (A) ROS/RNS, electrophiles, and reductants regulate TRPC5, TRPV1, and TRPA1. TRPC5 is regulated through the modification of specific cysteine residues in the pore region (mouse Cys553 and Cys558). TRPV1 is regulated through the modification of specific cysteine residues in the pore region (rat Cys616 and Cys621) and the cytoplasmic N- and C-terminal region (rat Cys157, chicken Cys393, Cys397, Cys772, and Cys783) (23). TRPA1 is regulated through the modification of multiple cysteine residues in the cytoplasmic N-terminal region (human Cys414, Cys421, Cys621, Cys641, and Cys665) (41). **(B)** The chemical mechanism underlying the action of NMM, one of α,β-unsaturated aldehydes, on cysteine sulfhydryls (9, 53). **(C)** Chemical structures of major agonists of TRPA1 (8, 62, 88, 153).

modification of a single cysteine residue (Cys157) located in the N-terminal region, which contains six ankyrin repeats (84, 133). This target cysteine is different from those for oxidizing and reducing agents described earlier, suggesting that TRPV1 harbors multiple modifiable cysteines which could mediate channel activation in an agent-specific manner (Fig. 6).

Research on TRPV1 sensitivity to oxidants is so far limited to molecular and pharmacological characterization. The molecular mechanism of oxidation-induced TRPV1 activation appears to involve cysteine modification, but further studies are necessary to prove the involvement of disulfide bonds and to delineate the complex and nonuniform activation effects of different oxidizing and reducing agents. More importantly, the physiological and pathological roles of TRPV1 sensitivity to the redox state *in vivo* have yet to be characterized. However, tissue injury and inflammation results in abundant production of ROS/RNS, which may enhance the pain response through the sensitization of TRPV1 (101). Ultimately, the use of knock-in mutants of Cys621 or other oxidation-insensitive cysteines will be necessary to explore the physiological role of TRPV1 redox sensitivity.

TRPA1

Modulation of TRPA1 by ROS/RNS and various electrophiles via *oxidative modification of cysteine residues*

TRPA1 is the only member of the TRPA sub-branch of the TRP gene superfamily in mammals that is characterized by a large number (between 12 and 17) of N-terminal ankyrin repeats (29, 41, 114). TRPA1 was first cloned from a fibroblast cell line (60), and its expression *in vivo* is largely restricted to a subset of nociceptive C-fiber nerves, including the somatosensory and vagal nerves (7, 107, 109, 145). TRPA1 was initially identified as a cold-sensitive ion channel in a small subset of sensory neurons (145), but subsequent pharmacological experiments have revealed it is also the sensory neuronal receptor for exogenous pungent compounds such as allyl isothiocyanate (AITC) from mustard oil and wasabi (7, 65), cinnamaldehyde from cinnamon (7), and allicin from onion and garlic (89). TRPA1 is also activated by Ca^{2+} (33, 189), receptor stimulation (7, 30, 137, 174), cannabinoids (65), caffeine (108), nicotine (155), and heavy metals (44, 55).

AITC, allicin, and cinnamaldehyde are isothiocyanate, thiosulfinate, and α,β-unsaturated aldehyde compounds, respectively. These compounds are susceptible to attack by the sulfhydryl groups of cysteine residues *via* nucleophilic substitution or Michael addition (53, 135). Related exogenous compounds such as acrolein, formalin, *N*-methyl maleimide, and tear gases also activate TRPA1 (7, 8, 10, 53, 81, 88, 96, 128, 132, 187, 188). Systematic mutation of TRPA1 cysteine residues identified three neighboring cysteines within the cytoplasmic N-terminus on human TRPA1 (Cys621, Cys641, and Cys665), simultaneous mutation of which decreases channel activation by several cysteine-modifying reagents (53). Mass spectrometry and site-directed mutagenesis independently implicated three cysteines in mouse TRPA1 (Cys415, Cys422, and Cys622, conserved in the human homolog as Cys414, Cys421, and Cys621) as the target sites for AITC and cinnamaldehyde (88) (Fig. 6). Thus, TRPA1 is a redox-mediated receptor for environmental electrophilic compounds.

We and others have demonstrated that TRPA1 is also modified *via* oxidative cysteine modification by ROS and RNS. TRPA1 is activated by ROS/RNS such as hypochlorite (OCl^-) (11), H_2O_2 (4, 11, 136, 153), ozone (O_3) (159), NO (101, 136, 153), $ONOO^-$ (136), and the ROS generated by ultraviolet light (52). In these reports, functional characterization of site-directed cysteine mutants of TRPA1 collectively demonstrated that modifications of cytoplasmic N-terminal-specific cysteine residues (Csy421, Cys621, Cya641, and Cys665 in human TRPA1) are the primary targets of these reactive species.

ROS and RNS can react with unsaturated fatty acids, causing production of highly reactive electrophilic compounds, including 4-hydroxynonenal (4-HNE), 4-hydroxyhexenal (4-HHE), 4-oxononenal (4-ONE), and nitrooleic acid (9-OA-NO_2) (62, 82, 167). It has been reported that 4-HNE, 4-HHE, 4-ONE, and 9-OA-NO_2 activate TRPA1 channels through oxidative modification of the same cysteine residues previously identified by (4, 53, 156, 158, 166). In addition to lipid peroxidation products, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), an endogenously generated product of the cyclooxygenase pathway with a highly reactive structure that contains α,β-unsaturated aldehyde moieties, can also react covalently *via* a Michael addition reaction with nucleophiles (168). 15d-PGJ_2 also activates TRPA1 in heterologous systems and endogenous TRPA1 in dissociated sensory neurons (4, 90, 153, 161). Functional characterization of site-directed cysteine mutants of TRPA1 in combination with labeling experiments using biotinylated 15d-PGJ_2 demonstrated that modification of cytoplasmic N-terminal Cys621 is responsible for the incorporation of 15d-PGJ_2 into TRPA1 (153). Taken together, we can surmise that endogenous electrophilic products activate TRPA1 channels.

It has been demonstrated that TRP channels such as TRPC5, TRPV1, and TRPA1 are sensitive to cysteine oxidation. In order to quantitate their cysteine oxidation sensitivity, we have systematically compared the responses of redox-sensitive TRP channels with a congeneric series of reactive disulfides, which show different electron acceptor (oxidation) capacity indicated as redox potential obtained using rotating disc electrode voltammetry (152). Strikingly, among the TRPs tested, only TRPA1 responded to those reactive disulfides with the least oxidative capacity, suggesting that TRPA1 possesses the highest oxidation sensitivity (Fig. 7).

TRPA1 in detecting noxious stimuli and the airway ROS microenvironment

The detection of noxious stimuli elicits nociceptive pain. Similar to capsaicin, the application of AITC produces an acute noxious response, followed by neurogenic inflammation and robust hypersensitivity to thermal and mechanical stimuli (8). Behavioral responses such as licking and flinching were abolished in *Trpa1* KO mice, whereas wild-type mice showed characteristically robust behavior (8, 78). Unlike wild-type mice, *Trpa1* KO mice also showed no hypersensitivity to thermal and mechanical stimuli after mustard-oil treatment (8). TRPA1 mediates pain responses to not only AITC but also formalin and other compounds (37, 96, 121). *Trpa1* KO mice have been used to show that an intraplantar injection of H_2O_2, 15d-PGJ_2, or 4-HNE induces pain sensation by activating TRPA1 (4, 11, 27, 166). Thus, TRPA1 is a redox-mediated nociceptive receptor for exogenous and endogenous electrophiles.

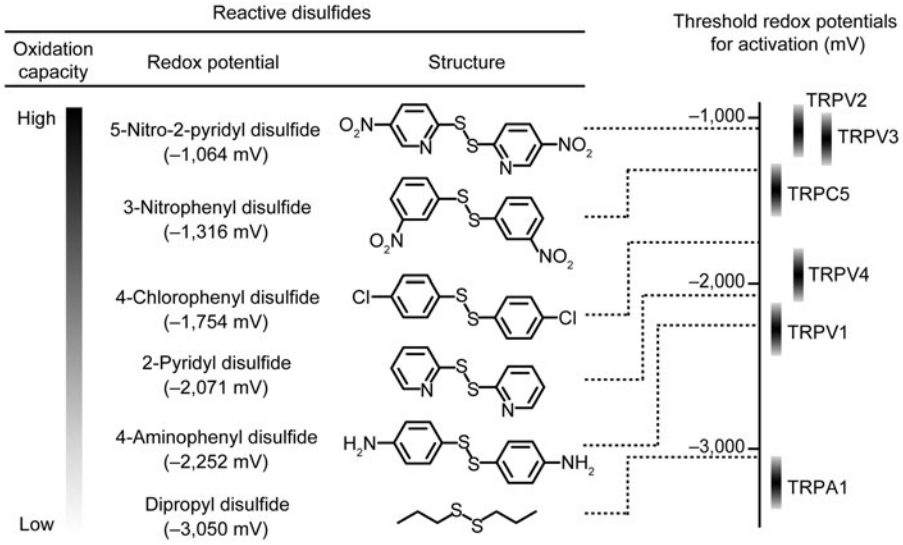

FIG. 7. Quantification of oxidation sensitivity of TRP channels. The threshold redox potentials for activation of respective TRP channels by reactive disulfides (152).

The respiratory tract can be subjected to substantial oxidative stress, both in the form of inhaled irritants and pollutants and during chronic inflammatory diseases (9, 160). Bronchopulmonary C-fibers of vagal nerves innervate the airways and play a critical role in the detection of the airway microenvironment (75). AITC, cinnamaldehyde, and O_3 activate dissociated vagal neurons and bronchopulmonary C-fibers in a TRPA1-dependent manner (109, 158, 159). Recently, our systematic evaluation of the oxidation sensitivity of TRP cation channels using reactive disulfides with different electrophilicity revealed the capability of TRPA1 to sense O_2 levels in bronchopulmonary C-fibers (152). Strikingly, *in vivo* inhalation of H_2O_2 and OCl^- given as an aerosol evoked a decrease in respiratory rate and an increase in end expiratory pause, but these responses were abolished in *Trpa1* KO mice (11). In addition, exposing the airways to AITC or toluene diisocyanate evokes a decrease in respiratory rate in wild-type but not *Trpa1* KO mice (157). In guinea pigs, several electrophiles (*e.g.*, cinnamaldehyde and acrolein) cause a cough that could be inhibited by a TRPA1 antagonist (6, 13). Taken together, these findings implicate TRPA1 expressed in vagal afferents as being at the frontline of the defense against pulmonary ROS and electrophilic compound challenges (152), with its activation leading to modulation of protective reflexes.

The role of ROS-modulated TRPA1 signaling in the airway afferents has been studied in relation to cigarette smoke. In rats, cigarette smoke-induced neuronal responses in capsaicin-sensitive vagal and superior laryngeal afferents were mediated through an ROS-dependent mechanism involving the activation of TRPA1 and TRPV1 receptors (83, 85, 130). Andrè *et al.* showed that cigarette smoke-induced neurogenic airway inflammatory responses, which consist mainly of bronchoconstriction and plasma protein extravasation, may be the result of TRPA1 activation by crotonaldehyde and acrolein, two α,β-unsaturated aldehydes present in cigarette smoke (5, 142). Another important role for TRPA1 has been suggested from a mouse model of allergic asthma (20). However, both cigarette smoke and inflammation can modulate airway nociceptor function independently of ROS, so further analyses are necessary to determine whether ROS-mediated TRPA1 activation induced by smoke and inflammation is physiologically relevant (160).

Concluding Remarks

A subclass of TRP channels is activated by reactive oxygen and nitrogen species and other electrophiles either directly through oxidative cysteine modification or indirectly *via* the downstream products of ROS generation. TRP channel cysteine residues susceptible to oxidation may have specific activation ranges, and it is conceivable that these modified cysteines control the activity of TRP channels under widely differing cellular redox conditions in order to gate the channel appropriately. Although many of the reports cited in this article name cysteine oxidation as the activation mechanism, it is almost certain that other factors also play a role. Future studies should investigate not only cysteine but also methionine and lysine residues as potential oxidation targets, because these are also prone to modification by endogenous oxidants. Indeed, methionine in TRPM2 is thought to play a role in sensing H_2O_2 (69), and a lysine residue in TRPA1 was identified as a target of electrophiles (53). For TRPC5 and TRPV1, both cysteine oxidation and reduction play roles in channel sensitization (172, 179). This sensitization function of reductive agents is an exciting phenomenon that is currently unexplored.

Redox-sensitive TRP channels are ubiquitous, widely distributed, and participate in ROS-dependent cellular functions, including cell death, chemokine production, and ROS detection, but novel roles are emerging for redox-sensitive TRP channels in ischemia/reperfusion, neurodegeneration, mental illness, vascular hyperpermeability, and itch sensation (49, 54, 86, 99, 111, 129). Considering the ubiquitous nature of TRP channels and ROS/RNS production, it is conceivable that TRP channel redox sensitivity may also participate in other unidentified biological phenomena. Thus, characterization of the *in vivo* functions of ROS-sensitive TRP channels in physiological and pathological conditions is a fertile field for exploration.

Redox sensitivity of TRP channels is now known to mediate previously unexplained biological phenomena, and is involved in various pathological states. A better understanding of the activation mechanisms and physiological significance of TRP channel regulation by reactive species may position TRP channels as significant pharmacological targets, which may realize the prospect of discovery of highly novel therapeutics for the management of previously untreatable diseases.

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Abbreviations Used

15d-PGJ_2 = 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
4-HHE = 4-hydroxyhexenal
4-HNE = 4-hydroxynonenal
4-ONE = 4-oxononenal
9-OA-NO_2 = nitrooleic acid
AD = Alzheimer's disease
ADPR = adenosine diphosphoribose
AITC = allyl isothiocyanate
Aβ = amyloid β-peptide
$[Ca^{2+}]_i$ = intracellular Ca^{2+} concentration
DSS = dextran sulfate sodium
DTT = dithiothreitol
Erk = extracellular signal-regulated kinase
H_2O_2 = hydrogen peroxide
IKK-β = IκB kinase-β
KO = knockout
LPS = lipopolysaccharide
NAD^+ = nicotinamide adenine dinucleotide
NF-κB = nuclear factor-κB
NMM = *N*-methyl maleimide
NO = nitric oxide
NUDT9-H = NUDT9-homology
O_2 = oxygen
$O_2^{\bullet -}$ = superoxide anion
O_3 = ozone
OCl^- = hypochlorite
$^{\bullet}OH$ = hydroxyl radical
$ONOO^-$ = peroxynitrite
PARP-1 = poly(ADPR) polymerase-1
PARG = poly(ADPR) glycohydrolase
PIP_2 = phosphatidylinositol-4,5-bisphosphate
PLC = phospholipase C
Pyk2 = proline-rich tyrosine kinase 2
RNS = reactive nitrogen species
ROS = reactive oxygen species
siRNA = short-interfering RNA
TRP = transient receptor potential