





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- $\kappa$ B (NF- $\kappa$ B) 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  $\text{Ca}^{2+}$ -selective channels and are tightly regulated by  $[\text{Ca}^{2+}]_i$  (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  $\text{H}_2\text{O}_2$  (47). Accumulating evidence suggests that  $\text{H}_2\text{O}_2$ -activated  $\text{Ca}^{2+}$  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  $\text{H}_2\text{O}_2$  (47). The application of  $\text{H}_2\text{O}_2$  induces TRPM2 currents and an increase in  $[\text{Ca}^{2+}]_i$  in TRPM2-expressing HEK cells and RIN-5F rat insulinoma cells. Subsequently, substantial evidence has accumulated demonstrating the activation of TRPM2 by  $\text{H}_2\text{O}_2$  in various cell types, including TRPM2-transfected cells (175), microglia (74), hippocampal neurons (118), U937 human monocytes (180, 186), neutrophils (54), pancreatic b-cells (80), and endothelial cells (49). The  $\text{H}_2\text{O}_2$ -induced  $\text{Ca}^{2+}$  influx through TRPM2 was enhanced under intracellular  $\text{Fe}^{2+}$ -accumulated conditions and reduced by OH scavengers or an iron chelator, suggesting that the intracellular 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  $\text{Ca}^{2+}$ -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 catalyzes 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,  $\text{Ca}^{2+}$  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  $\text{H}_2\text{O}_2$ , suggesting that intracellular mediators are necessary for  $\text{H}_2\text{O}_2$ -induced TRPM2 activation (47). Initially, we showed that nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) 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  $\text{NAD}^+$  to ADPR and by the production of ADPR from mitochondria (125). Cytosolic or mitochondrial overexpression of ADPR pyrophosphatase, which degrades ADPR, suppresses  $\text{H}_2\text{O}_2$ -induced  $\text{Ca}^{2+}$  responses, confirms the involvement of ADPR in  $\text{H}_2\text{O}_2$ -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  $\text{NAD}^+$  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  $\text{H}_2\text{O}_2$ -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  $\text{H}_2\text{O}_2$ -induced  $\text{Ca}^{2+}$  response (19). More support for this mechanism comes from the observation that PARP-1 and PARG control  $\text{H}_2\text{O}_2$ -induced extracellular  $\text{Ca}^{2+}$  fluxes through TRPM2 in mouse embryonic fibroblasts (16). In inside-out patches from TRPM2-expressing *Xenopus* oocytes,  $\text{H}_2\text{O}_2$  failed to activate TRPM2 (163), suggesting that the  $\text{H}_2\text{O}_2$  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  $\text{NAD}^+$  glycohydrolases, ADPR-producing ectoenzymes such as CD38 and CD157 localized on the plasma membrane (91). CD38 and CD157 are enzymes that generate ADPR from  $\text{NAD}^+$  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 b-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  $\text{H}_2\text{O}_2$  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  $[\text{Ca}^{2+}]_i$ , enhanced cell viability, and reduced the numbers of apoptotic cells after exposure to  $\text{H}_2\text{O}_2$  (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  $\text{H}_2\text{O}_2$ -induced cell death (151).

Amyloid  $\beta$ -peptide (Ab) 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  $\text{Ca}^{2+}$  homeostasis plays a critical role in the neuropathology of AD. Incubation of cells in  $\text{Ca}^{2+}$ -free medium attenuated the direct neurotoxicity of Ab and limited their vulnerability to excitotoxicity, suggesting the involvement of  $\text{Ca}^{2+}$  influx in these processes (93). Fonfria et al. have shown that the application of Ab 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 Ab- and  $\text{H}_2\text{O}_2$ -induced striatal cell death (40). Thus, TRPM2 activity likely contributes to neuronal cell death in AD.

#### 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  $\text{Ca}^{2+}$  also plays important signaling roles in immunocytes, and increases in  $[\text{Ca}^{2+}]_i$  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  $\text{H}_2\text{O}_2$  (66) via extracellular signal-regulated kinase (Erk)-activated NF- $\kappa$ B (184). Until recently, despite evidence implicating  $\text{H}_2\text{O}_2$  and  $\text{Ca}^{2+}$  as important regulatory factors in CXCL8 production in monocytes, the molecular entities and signaling mechanisms that link  $\text{H}_2\text{O}_2$ ,  $\text{Ca}^{2+}$ , and CXCL8 production remained unclear.

In U937 monocytes,  $\text{H}_2\text{O}_2$ -induced increases in  $[\text{Ca}^{2+}]_i$  and the production of CXCL8 were significantly suppressed by TRPM2-specific siRNA (180). We found that  $\text{H}_2\text{O}_2$ -induced nuclear translocation of the NF- $\kappa$ B subunit RelA was positively regulated by Erk-mediated phosphorylation of I $\kappa$ B kinase- $\beta$  (IKK- $\beta$ ). In addition, upstream of Erk activation, TRPM2 activation by  $\text{H}_2\text{O}_2$  results in  $\text{Ca}^{2+}$  influx and consequent autophosphorylation of  $\text{Ca}^{2+}$ -sensitive proline-rich tyrosine kinase 2 (Pyk2), which amplifies Erk activation via Ras GTPase. The activation of this Pyk2/Erk/NF- $\kappa$ B pathway in response to  $\text{H}_2\text{O}_2$  was demonstrated in wild-type monocytes, but was impaired in Trpm2 KO monocytes. Thus,  $\text{H}_2\text{O}_2$ -mediated  $\text{Ca}^{2+}$  influx via TRPM2 triggers Pyk2/Ras signaling, which amplifies downstream Erk activation leading to the nuclear translocation of NF- $\kappa$ B and the production of CXCL2, the murine functional homologue of human CXCL8 (Fig. 4).

FIG. 4. TRPM2 activation by  $\text{H}_2\text{O}_2$  induces chemokine production. In monocytes,  $\text{Ca}^{2+}$  influx via  $\text{H}_2\text{O}_2$ -activated TRPM2 triggers Pyk2 phosphorylation, which amplifies Erk activation in a Ras-dependent manner. The amplified Erk activates transcription of the Cxcl2 gene by inducing nuclear translocation of the NF- $\kappa$ B subunit RelA.

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  $\text{H}_2\text{O}_2$  nor lipopolysaccharide (LPS)-induced ROS were able to induce inward currents or increases in  $[\text{Ca}^{2+}]_i$  in Trpm2 KO bone marrow-derived macrophages (32). We previously demonstrated that CXCL2 production induced by  $\text{H}_2\text{O}_2$  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

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 nonse-

lective 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

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.

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  $\text{Ca}^{2+}$ -permeable cation channel linked to PLC (117, 126). Although it is still controversial whether depletion of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  and calmodulin has been implicated in TRPC5 activation and modulation (14, 43, 119, 140), while membrane polyphosphoinositides such as  $\text{PIP}_2$  exert both stimulatory and inhibitory effects in regulating TRPC5 channel activity (165).

We have previously demonstrated that TRPC5 is directly activated by  $\text{H}_2\text{O}_2$  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 ( $>43\text{ C}$ ) (21), acidic

pH (162), and environmental irritants and endogenous algescic 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  $\text{H}_2\text{O}_2$  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  $\text{Ca}^{2+}$  influx induced by  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$ , 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  $\text{ONOO}^-$ , 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 a,b-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, thiosulfonate, and  $\alpha,\beta$ -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 ( $\text{OCl}^-$ ) (11),  $\text{H}_2\text{O}_2$  (4, 11, 136, 153), ozone ( $\text{O}_3$ ) (159), NO (101, 136, 153),  $\text{ONOO}^-$  (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 (Cys421, Cys621, Cys641, 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- $\text{NO}_2$ ) (62, 82, 167). It has been reported that 4-HNE, 4-HHE, 4-ONE, and 9-OA- $\text{NO}_2$  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- $\text{D}^{12,14}$ -prostaglandin  $\text{J}_2$  (15d-PG $\text{J}_2$ ), an endogenously generated product of the cyclooxygenase pathway with a highly reactive structure that contains  $\alpha,\beta$ -unsaturated aldehyde moieties, can also react

covalently via a Michael addition reaction with nucleophiles (168). 15d-PG $\text{J}_2$  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-PG $\text{J}_2$  demonstrated that modification of cytoplasmic N-terminal Cys621 is responsible for the incorporation of 15d-PG $\text{J}_2$  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 pinching 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  $\text{H}_2\text{O}_2$ , 15d-PG $\text{J}_2$ , 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. Quantitation 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<sub>3</sub> 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<sub>2</sub> levels in bronchopulmonary C-fibers (152). Strikingly, in vivo inhalation of H<sub>2</sub>O<sub>2</sub> and OCl<sup>-</sup> 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). André 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 a,b-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<sub>2</sub>O<sub>2</sub> (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 <sub>2</sub>	%d5-deoxy-D <sup>12,14</sup> -prostaglandin J <sub>2</sub>
4-HHE	%4-hydroxyhexenal
4-HNE	%4-hydroxynonenal
4-ONE	%4-oxononenal
9-OA-NO <sub>2</sub>	%nitrooleic acid
AD	%Alzheimer's disease
ADPR	%adenosine diphosphoribose
AITC	%allyl isothiocyanate
Ab	%amyloid b-peptide
[Ca <sup>2+</sup> ] <sub>i</sub>	%intracellular Ca <sup>2+</sup> concentration
DSS	%dextran sulfate sodium
DTT	%dithiothreitol
Erk	%extracellular signal-regulated kinase
H <sub>2</sub> O <sub>2</sub>	%hydrogen peroxide
IKK-b	%IκB kinase-b
KO	%knockout
LPS	%dipolysaccharide
NAD <sup>+</sup>	%nicotinamide adenine dinucleotide
NF-κB	%nuclear factor-κB
NMM	%N-methyl maleimide
NO	%nitric oxide
NUDT9-H	%NUDT9-homology
O <sub>2</sub>	%oxygen
O <sub>2</sub> <sup>c</sup>	%superoxide anion
O <sub>3</sub>	%ozone
OCl <sup>-</sup>	%hypochlorite
cOH	%hydroxyl radical
ONOO <sup>-</sup>	%peroxynitrite
PARP-1	%poly(ADPR) polymerase-1
PARG	%poly(ADPR) glycohydrolase
PIP <sub>2</sub>	%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