

Transient Receptor Potential A1 Is a Sensory Receptor for Multiple Products of Oxidative Stress

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Transient receptor potential A1 (TRPA1) is expressed in a subset of nociceptive sensory neurons where it acts as a sensor for environmental irritants, including acrolein, and some pungent plant ingredients such as allyl isothiocyanate and cinnamaldehyde. These exogenous compounds activate TRPA1 by covalent modification of cysteine residues. We have used electrophysiological methods and measurements of intracellular calcium concentration ($[Ca^{2+}]_i$) to show that TRPA1 is activated by several classes of endogenous thiol-reactive molecules. TRPA1 was activated by hydrogen peroxide (H_2O_2 ; EC_{50} , 230 μM), by endogenously occurring alkenyl aldehydes (EC_{50} : 4-hydroxynonenal 19.9 μM , 4-oxo-nonenal 1.9 μM , 4-hydroxyhexenal 38.9 μM) and by the cyclopentenone prostaglandin, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 (15d-PG J_2 , EC_{50} : 5.6 μM). The effect of H_2O_2 was reversed by treatment with dithiothreitol indicating that H_2O_2 acts by promoting the formation of disulfide bonds whereas the actions of the alkenyl aldehydes and 15d-PG J_2 were not reversed, suggesting that these agents form Michael adducts. H_2O_2 and the naturally occurring alkenyl aldehydes and 15d-PG J_2 acted on a subset of isolated rat and mouse sensory neurons [$\sim 25\%$ of rat dorsal root ganglion (DRG) and $\sim 50\%$ of nodose ganglion neurons] to evoke a depolarizing inward current and an increase in $[Ca^{2+}]_i$ in TRPA1 expressing neurons. The abilities of H_2O_2 , alkenyl aldehydes and 15d-PG J_2 to raise $[Ca^{2+}]_i$ in mouse DRG neurons were greatly reduced in neurons from *trpa1*^{-/-} mice. Furthermore, intraplantar injection of either H_2O_2 or 15d-PG J_2 evoked a nocifensive/pain response in wild-type mice, but not in *trpa1*^{-/-} mice. These data demonstrate that multiple agents produced during episodes of oxidative stress can activate TRPA1 expressed in sensory neurons.

Key words: TRPA1; DRG; hydrogen peroxide; 4-hydroxynonenal; 15d-PG J_2 ; oxidative stress

Introduction

The transient receptor potential A1 (TRPA1) channel is a nonselective cation channel expressed by a subset of primary afferent nociceptive neurons where it acts as a sensory receptor for some pungent chemicals found in plants, including allyl isothiocyanate (from mustard and wasabi), cinnamaldehyde (from cinnamon), and allicin (in garlic). TRPA1 can also be activated by some other agents including methylsalicylate, icilin, and the environmental irritant, acrolein (Bandell et al., 2004; Jordt et al., 2004; Bautista et al., 2005, 2006) and mediates some responses to proinflammatory mediators, such as bradykinin (Bandell et al., 2004; Jordt et al., 2004; Bautista et al., 2006).

Site directed mutagenesis studies have shown that allyl isothiocyanate (AITC), cinnamaldehyde and acrolein activate TRPA1 by covalently reacting with cysteine residues in the cytoplasmic N terminus of the channel (Hinman et al., 2006; Macpherson et al., 2007a). TRPA1 can therefore act as a sensor of reactive, electrophilic chemicals. It has been unclear if there are endogenous electrophilic activators of TRPA1. To address this question, we have investigated whether endogenous thiol reactive agents activate TRPA1. For these studies we have studied the

responsiveness of heterologously expressed TRPA1 channels and sensory neurons from wild-type rats and mice and TRPA1-null mice as well as the pain behaviors of TRPA1-null mice. Our studies have focused on several thiol reactive chemicals that are produced during oxidative stress and inflammation.

Oxidative stress occurs during many pathophysiological conditions including inflammation and reperfusion after ischemia and results in the production of a range of highly reactive chemicals including hydrogen peroxide (H_2O_2), lipid peroxidation products such as 4-hydroxynonenal (4-HNE), and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 (15d-PG J_2) (Hyslop et al., 1995; Sprong et al., 1997; Chen et al., 1999; Gao et al., 2003; Uchida, 2003). H_2O_2 , which stimulates a subset of capsaicin-sensitive sensory nerves innervating the heart, lungs and gastrointestinal tract (Stahl et al., 1993; Ustinova and Schultz, 1994b; Soukhova et al., 1999; Ruan et al., 2006), is known to oxidize cysteine residues in proteins to form either cysteine sulfenic acids or disulfides (Poole et al., 2004). 4-HNE and related lipids, 4-oxo-2-nonenal (4-ONE) and 4-hydroxyhexenal (4-HHE), are highly reactive products of lipid peroxidation that contain an electrophilic α,β -unsaturated carbonyl moiety similar to that found in the TRPA1 agonist, cinnamaldehyde. These lipid peroxidation products can form adducts with lysine, histidine, and cysteine residues (Uchida, 2003). Finally cyclopentenone prostaglandins (cyPGs), produced from arachidonic acid via cyclooxygenase and prostaglandin D_2 (PGD $_2$) synthase or nonenzymatically during oxidative stress (Chen et al., 1999; Gao et al., 2003), also contain elec-

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trophilic α,β -unsaturated carbonyl moieties and are known to be thiol reactive compounds (Levonen et al., 2004).

Materials and Methods

Cell culture. Untransfected Chinese hamster ovary (CHO) cells and CHO cells expressing mouse TRPA1, mouse TRPM8, rat TRPV4, or human TRPV1 were grown in MEM- α medium supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml), L-glutamine (2 mM), and FCS (10%).

Dorsal root ganglion (DRG) and nodose ganglion neurons were prepared from adult (~200 g) male or female Wistar rats using methods described previously (Bevan and Winter, 1995). TRPA1-null mice and wild-type littermates were bred from heterozygotic mice kindly provided by Drs. Kelvin Kwan (Harvard Medical School, Boston, MA) and David Corey (Harvard Medical School, Boston, MA) (Kwan et al., 2006). DRG neurons from TRPA1-null and TRPV1-null mice and their respective wild-type littermates were prepared using the protocol used for rat neurons. The chemosensitivities of DRG and nodose neurons were investigated 18–48 h after plating the cells on laminin/poly-D-lysine-coated coverslips.

Imaging of intracellular calcium levels. CHO cells and DRG neurons were loaded with 2 μ M Fura-2 AM (Invitrogen, Carlsbad, CA) in the presence of 1 mM probenecid for ~1 h. The dye loading and the subsequent experiments were performed in a physiological saline solution containing (in mM) 140 NaCl, 5 KCl, 10 glucose, 10 HEPES, 2 CaCl₂, and 1 MgCl₂, buffered to pH 7.4 (NaOH). Compounds were applied to cells by local continuous microperfusion of solution through a fine tube placed very close to the cells being studied. Experiments were conducted at 30°C. Images of a group of cells were captured every 2 s using 340 and 380 nm excitation wavelengths with emission measured at 520 nm with a microscope based imaging system (PTI, Birmingham, NJ). Analyses of emission intensity ratios at 340 nm/380 nm excitation (R; in individual cells) were performed using the ImageMaster suite of software.

Electrophysiology. CHO cells expressing TRPA1 were studied under voltage-clamp conditions using an Axopatch 200B amplifier and pClamp 10.0 software (Molecular Devices, Sunnyvale, CA). Whole-cell recordings from CHO cells were performed at a holding potential of –60 mV, unless stated otherwise. Drugs and solutions were applied by local superfusion using a rapid solution changer (Bio-Logic, Claix, France). Borosilicate glass pipettes (2–5 M Ω , 75–80% series resistance compensation) were filled with (in mM) 140 KCl, 1 CaCl₂, 2 MgATP, 10 EGTA, and 10 HEPES buffered to pH 7.4 (KOH). The external solution was as described above for imaging of intracellular Ca²⁺ concentrations. In experiments with Ca²⁺-free external solutions, 1 mM EGTA was included and CaCl₂ was omitted. Cell-attached single-channel recordings were performed using the Ca²⁺-free external solution both in the pipette and for superfusion. Inside-out patches were superfused on the cytoplasmic side with a solution containing (in mM) 110 KCl, 10 Na₅P₃O₁₀, 1 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 EGTA, pH 7.4 (KOH). Na₅P₃O₁₀ was included to maintain channel activity in isolated patches (Kim and Cavanaugh, 2007). For inside-out patch recordings, pipettes were filled with the external solution described above for Ca²⁺-imaging experiments. All single-channel currents records were sampled at 10 kHz and filtered online at 5 kHz. The displayed single-channel records have been low pass filtered at 1 kHz. For the inside-out and cell-attached patch experiments we used higher resistance glass pipettes (8–12 M Ω) than in the whole-cell experiments.

DRG neurons were studied using a CsCl based internal solution [containing (in mM): 140 CsCl, 1 CaCl₂, 2 MgATP, 10 EGTA, and 10 HEPES, pH 7.4 (CsOH)] to block potassium currents. The external medium contained (in mM) 140 NaCl, 5 KCl, 10 glucose, 10 HEPES, 0.015 CaCl₂, and 1 MgCl₂ buffered to pH 7.4 (NaOH). The use of a reduced calcium concentration eliminated significant calcium flux through voltage-gated calcium channels and prevented sodium movements through the calcium channels, which can occur in calcium-free solutions.

The voltage sensitivity of membrane currents were investigated using either a voltage ramp protocol (1 s duration, –100 to +100 mV, CHO cells; or 2 s, –40 to +40 or +60 mV, DRG neurons) or depolarizing voltage steps up to +180 mV followed by repolarization to the holding

potential of –60 mV. Because TRPA1-mediated currents showed a rapid inactivation in calcium-containing solutions, measurements of voltage sensitivity were performed in the calcium-free solutions noted above for CHO cells and DRG neurons.

Ninety-six-well plate intracellular calcium concentration assays. In some experiments, changes in intracellular calcium ([Ca²⁺]_i) were determined in TRPA1 expressing CHO cells grown in 96-well black-walled plates (Costar, Cambridge, MA) using a Flexstation 3 (Molecular Devices). Cells were loaded with Fura 2-AM and assays were performed at 25°C. Basal emission ratios with excitation wavelengths of 340 and 380 nm were measured and changes in dye emission ratio determined at various times after compound addition.

Behavioral responses. All animal studies were performed according to the UK Home Office Animal Procedures Act (1986). Data shown are from male and female homozygote *trpa1*^{–/–} and wild-type littermates. Intraplantar injections of hydrogen peroxide (0.3% [2.2 μ mol] in saline) or 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (30 nmol [10 μ g] in 10%DMSO/saline) were used to induce and compare nociceptive/pain responses in *trpa1*^{–/–} and wild-type mice. These doses were based on dose–response relationships for hydrogen peroxide and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ determined in wild-type mice. Injections (25 μ l) were made subcutaneously into the plantar surface of one of the hind paws using a 50 μ l luer-syringe (Hamilton, Reno, NV) fitted with a 26-gauge by 3/8 inch intradermal needle. Immediately after injection, mice were placed inside a Perspex chamber and the duration of the pain-related behaviors (licking and biting or flinching and shaking of the injected paw) recorded using a digital stop-watch. Observation periods of 1 min were used and behavior recorded for up to 10 min after injection. Total pain response times over the first 3 min were used for analysis as the pain behaviors were largely confined to this period. Groups of six animals were used for each agent.

Drugs. 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂ was from Biomol (Exeter, UK). PGA₂, 9,10-dihydro-15d-PGJ₂, 4-ONE, 4-HNE, and 4-HHE were obtained from Cayman Chemical (Ann Arbor, MI). Hydrogen peroxide was from VWR International (Lutterworth, UK). All other reagents were from Sigma (Poole, UK).

Results

H₂O₂ activates TRPA1 in CHO cells

Electrophysiology

H₂O₂ evoked an inward current in TRPA1-expressing CHO cells at a holding potential of –60 mV (Fig. 1A). In contrast, concentrations of H₂O₂ up to 100 mM failed to evoke currents in untransfected CHO cells (data not shown). The time course of the current was influenced by the presence or absence of Ca²⁺ in the external medium. In Ca²⁺-free external solutions the current developed slowly and persisted in the continued presence of H₂O₂. When Ca²⁺ was subsequently added to the H₂O₂-containing solution there was a rapid increase in current amplitude, consistent with a calcium-mediated potentiation, followed by a rapid inactivation (Fig. 1A). When H₂O₂ was applied in the presence of external Ca²⁺, the current activated with a concentration-dependent latency and then inactivated. The current occurred with a short latency with high concentrations of H₂O₂ but developed after a delay of up to a minute with low concentrations (Fig. 1B). A similar waveform and calcium dependence of TRPA1 mediated currents has been noted with either AITC or cinnamaldehyde as the agonist (Nagata et al., 2005; Doerner et al., 2007).

Under physiological conditions, the cytosol usually contains millimolar concentrations of glutathione, which acts as an antioxidant. It was possible that the intracellular glutathione levels were depleted during whole-cell recording, rendering the cells more sensitive to oxidative agents. Experiments were therefore performed with 10 mM reduced glutathione in the intracellular, pipette filling solution. Inclusion of exogenous glutathione did

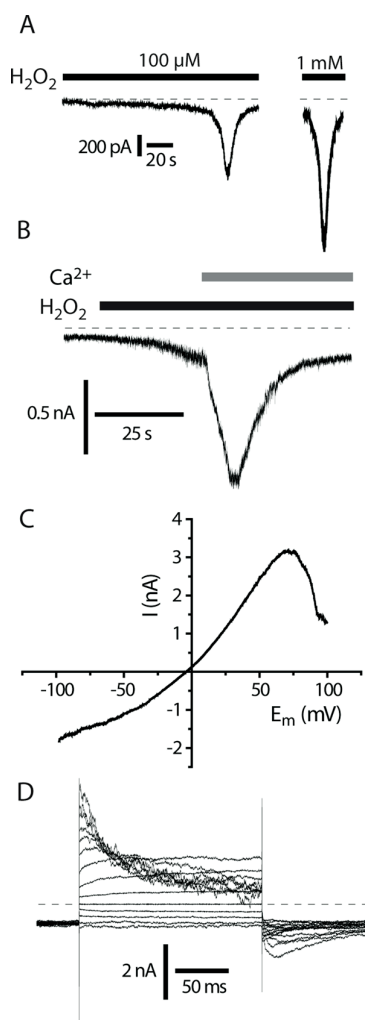


Figure 1. H_2O_2 activates TRPA1 expressed in CHO cells. **A**, H_2O_2 activates TRPA1 with a concentration-dependent latency in Ca^{2+} -containing solutions. Currents recorded in calcium-containing solution showing characteristic "threshold" with a sudden increase in membrane current. **B**, Ca^{2+} potentiates H_2O_2 -induced TRPA1 currents. Current response to 10 mM H_2O_2 in a TRPA1 CHO cell, -60 mV. Note the slow increase in current in calcium-free solution followed by a rapid current increase when Ca^{2+} (2 mM) is added. **C**, Current–voltage relationship of H_2O_2 -evoked current with 2 s voltage ramp in a TRPA1 CHO cell in calcium-free solution revealed a reduced current at positive potentials. **D**, Kinetics of H_2O_2 -evoked TRPA1 current in calcium free solution. Note the time and voltage-dependent inactivation at more positive potentials that accounts for reduced conductance seen with voltage ramp protocols (**C**) (Figs. 2C, 4C). Holding potential -60 mV with 20 mV interval steps to from -80 to $+180$ mV.

not reduce the amplitude of the H_2O_2 induced current or alter the current waveform (data not shown).

The H_2O_2 evoked current had a reversal potential close to 0 mV (Fig. 1C) and the current–voltage relationship obtained with ramp changes in voltage showed a characteristic decreased conductance at positive (>70 mV) membrane potentials (Fig. 1C). The reduction in conductance at positive membrane potentials was caused by a time-dependent inactivation that was revealed using a voltage step protocol (Fig. 1D). In the presence of H_2O_2 the current–voltage relationship was linear over the range -80 to 0 mV. The outward current showed an initial small, time-dependent growth at more positive potentials (0 to $+60$ mV), which was associated with an instantaneous, rapidly decaying inward "tail" current (deactivation) seen when the membrane was repolarized to -60 mV. A similar time-dependent growth at positive membrane potentials and subsequent deactivation on

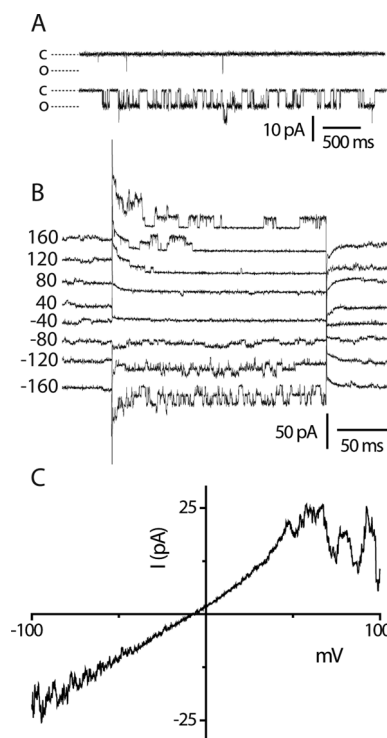


Figure 2. Single-channel activity evoked by H_2O_2 . Cell attached TRPA1 single-channel currents. **A**, Few brief openings seen in the absence of H_2O_2 (top trace), but robust channel activity elicited by 1 mM H_2O_2 (lower trace) at -100 mV. **B**, Voltage-dependent single-channel current activity showing inactivation at positive membrane potentials. Holding potential, -60 mV; traces are offset for clarity. **C**, Voltage ramp illustrating single-channel inactivation at positive membrane potentials (the trace shown is the average of 5 sweeps).

hyperpolarization has been reported for TRPV1 and TRPM8 in the absence of any added agonists as well in the presence of their respective agonists, capsaicin and menthol (Voets et al., 2004; Nilius et al., 2005). With more positive step potentials ($\geq +80$ mV), the outward current showed an additional time-dependent inactivation during the depolarizing voltage step and the inward tail current seen on repolarization to -60 mV was more complex with an initial time-dependent growth followed by a decay (deactivation) to the initial holding current (Fig. 1D). The simplest explanation for these observations is that the TRPA1 channels continue to show a time-dependent increase in open probability at the more positive membrane potentials but this is overlaid by a block of the ion channels by some unknown mechanism. The growth in the tail current on repolarization probably represents unblocking of the channels, which then close. Very similar voltage-dependent properties were seen with AITC-evoked currents (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

A voltage-dependent inactivation of the H_2O_2 -evoked currents at positive membrane potentials was also evident in H_2O_2 -evoked single-channel currents recorded in membrane attached patches (Fig. 2A–C). A single-channel chord conductance of 94 ± 3 pS was noted over the voltage range -100 to $+100$ mV. The single-channel current activity was clearly reduced at the more positive potentials, but no reduction in single-channel conductance was noted.

Measurements of $[\text{Ca}^{2+}]_i$

We used agonist-evoked changes in $[\text{Ca}^{2+}]_i$ to quantify the effects of TRPA1 ligands. H_2O_2 evoked an increase in $[\text{Ca}^{2+}]_i$ in

TRPA1-expressing CHO cells but not in untransfected CHO cells or in cells expressing TRPV1, TRPV4, or TRPM8 (data not shown). The effect of H_2O_2 was time- and concentration-dependent with larger and faster responses seen at higher H_2O_2 concentrations (Fig. 3A). Concentration–response curves obtained using changes in $[\text{Ca}^{2+}]_i$ as an index of channel activation yielded an EC_{50} value of $230 \mu\text{M}$ after 600 s exposure to H_2O_2 when the responses to each concentration had reached a plateau (Fig. 3B). The time dependence of the response is illustrated in Figure 3C where the time to half maximal response at each concentration is plotted against H_2O_2 concentration. Higher EC_{50} concentrations were calculated after shorter exposure times and an EC_{50} value of $1.2 \pm 0.4 \text{ mM}$ was estimated after 90 s exposure (Fig. 3B).

H_2O_2 can act on some proteins via the production of OH^\bullet radicals, which are generated at an accelerated rate when the intracellular concentration of iron is raised because Fe^{2+} acts as a catalyst in the Fenton reaction. We therefore examined the H_2O_2 sensitivity of TRPA1 expressing cells that had been preloaded with FeSO_4 (Fig. 3D). The EC_{50} value in Fe^{2+} -loaded cells ($53 \pm 6 \mu\text{M}$, after 90 s) was ~ 20 -fold lower than in untreated cells, which indicates that H_2O_2 exerts its effect, at least in part, by the generation of intracellular OH^\bullet (Fig. 3D). TRPA1 was also activated by $5 \mu\text{M}$ Rose Bengal, which generates another reactive oxygen species (ROS), singlet oxygen, and by the oxidizing agent chloramine T (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

H_2O_2 activates TRPA1-expressing sensory neurons

We examined whether H_2O_2 stimulated isolated sensory neurons using changes in intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) as an indicator of neuronal activation. H_2O_2 evoked a robust increase in $[\text{Ca}^{2+}]_i$ in $\sim 25\%$ of rat DRG neurons, which typically were of small diameter (20 – $25 \mu\text{m}$) (Fig. 4A). In whole-cell voltage-clamp experiments on DRG neurons, H_2O_2 evoked an inward current at -60 mV and increased membrane conductance (Fig. 4B). When the inward current was allowed to develop in solutions containing $15 \mu\text{M}$ Ca^{2+} , subsequent addition of 2 mM Ca^{2+} rapidly inactivated the current (Fig. 4B). Using the same solutions, a similar current inactivation (without the surge in current shown in Fig. 1B) was evident in TRPA1 CHO cells when 2 mM Ca^{2+} was added (data not shown). A voltage ramp protocol revealed that the H_2O_2 -evoked current had a reversal potential close to 0 mV (-6 mV), which suggested that the response was mediated by a nonselective cation channel, such as one of the TRP channels. The current–voltage relationship showed a characteristic reduction in conductance at positive membrane potentials similar to that seen for TRPA1 in CHO cells (Fig. 4C), but markedly different from the behavior of TRPV1–4 and TRPM8 mediated currents which do not exhibit any current inactivation at membrane potentials up to $+200 \text{ mV}$ (Nilius et al., 2005).

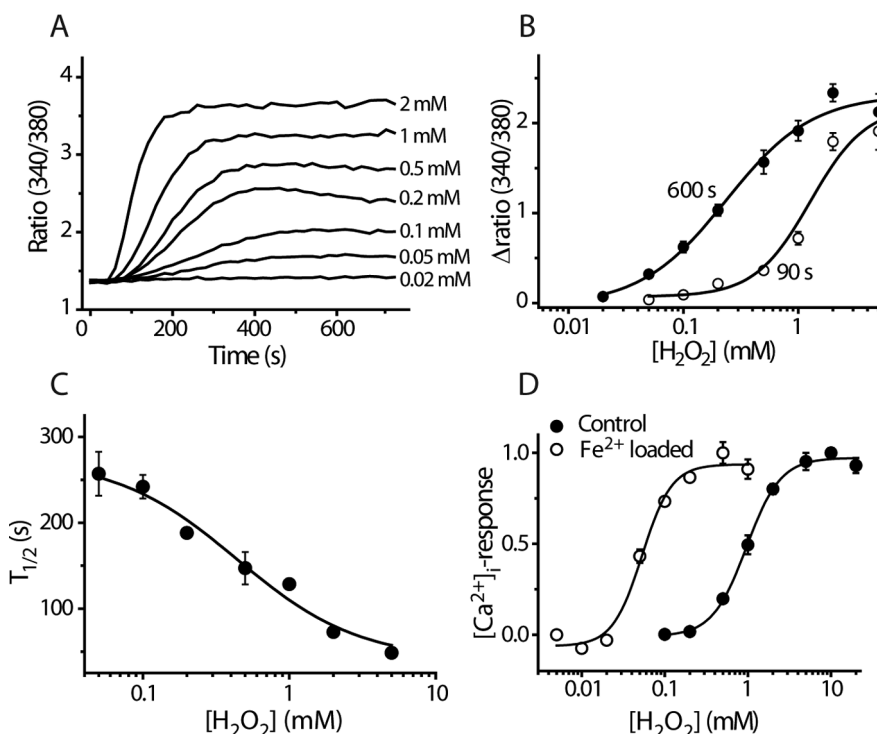


Figure 3. Concentration-dependent effect of H_2O_2 on TRPA1. **A**, Concentration-dependent time course of $[\text{Ca}^{2+}]_i$ -responses to stimulation with H_2O_2 in CHO cells expressing TRPA1. Traces are mean ratios from quadruplicate wells. **B**, Concentration–response curves constructed from the experiment shown in (**A**) 90 and 600 s after addition of H_2O_2 (mean \pm SEM). **C**, The time required for half-maximal activation ($T_{1/2}$) is concentration dependent (data points are mean \pm SEM of 4 measurements). **D**, Fe^{2+} potentiates the effect of H_2O_2 , suggesting that H_2O_2 acts via intracellular production of hydroxyl radicals. Concentration–response curves for H_2O_2 -evoked increase in $[\text{Ca}^{2+}]_i$ in normal and Fe^{2+} -loaded TRPA1 cells are shown. Fe^{2+} -loaded cells were incubated with $100 \mu\text{M}$ FeSO_4 for 1 h and then washed so that no extracellular FeSO_4 was present during the experiment (mean \pm SEM; $n = 4$).

To gain an insight into the molecular identity of the H_2O_2 -activated channels in the native cells, we used changes in $[\text{Ca}^{2+}]_i$ to examine the responses of rat DRG and nodose neurons to a sequence of agonists that activate different TRP channels and differentiate subpopulations of DRG neurons. In initial experiments, we found no correspondence between H_2O_2 - and menthol-sensitivity ($100 \mu\text{M}$), which was expected given the relatively low percentage ($\sim 8\%$) of menthol sensitive DRG neurons in DRG cultures (Andersson et al., 2007). Therefore, for most experiments we used AITC and capsaicin to activate TRPA1 and TRPV1, respectively. Any cell responding to a given stimulus with a $[\text{Ca}^{2+}]_i$ -increase of at least 15% of the response to a subsequent challenge with 50 mM KCl, was considered “positive.” There was a striking correspondence between H_2O_2 - and AITC-sensitive neurons (Fig. 4E). This was evident from the very similar percentage of DRG neurons responding to these agents (H_2O_2 , 25.1% ; AITC, 24.4% in DRG neurons; H_2O_2 , 49.8% ; AITC, 54.0% in nodose neurons) and from investigations of the chemosensitivities of individual neurons. All the H_2O_2 -sensitive DRG neurons responded to AITC and treatment with AITC always occluded the response to a subsequent challenge with H_2O_2 . As TRPA1 is expressed in a subpopulation of TRPV1 neurons we found neurons that responded to both H_2O_2 and capsaicin but $\sim 50\%$ of capsaicin-sensitive DRG neurons and $\sim 30\%$ of capsaicin-sensitive nodose neurons did not respond to H_2O_2 (Fig. 4D,E). Furthermore, the proportion of H_2O_2 -sensitive DRG neurons was unchanged in $\text{trpv1}^{-/-}$ mice (31.8% , 113 of 355).

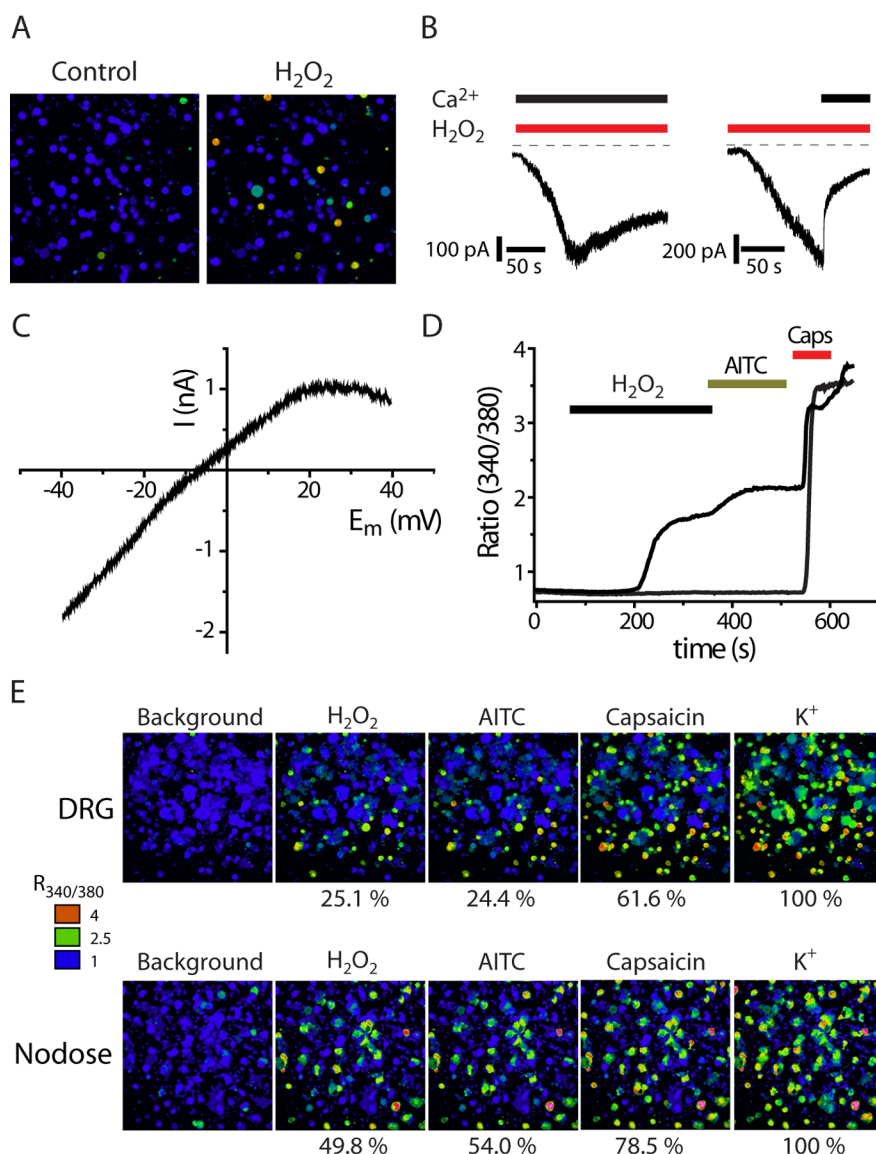


Figure 4. H₂O₂ activates TRPA1-containing DRG and nodose neurons. **A**, Pseudocolored images illustrating [Ca²⁺]_i responses evoked by H₂O₂ (5 mM) measured with Fura-2 in rat DRG neurons. **B**, Currents evoked by H₂O₂ (1 mM) in DRG neurons in the presence (left) and absence (right) of extracellular calcium (at a holding potential of −60 mV). The current rapidly inactivated when Ca²⁺ was applied after an initial current had developed in Ca²⁺-free conditions. **C**, Current–voltage plot for the H₂O₂ response in a DRG neuron generated by a slow 2 s voltage ramp in Ca²⁺-free solution. Note the characteristic reduced conductance at positive membrane potential. **D**, Change in [Ca²⁺]_i (340/380 ratio) of typical DRG neurons in response to sequential applications of H₂O₂, AITC, and capsaicin (Caps) showing H₂O₂-sensitive and H₂O₂-insensitive TRPV1 expressing neurons. **E**, [Ca²⁺]_i responses in DRG (top sequence) and nodose neurons (bottom sequence) to sequential application of H₂O₂ (5 mM), AITC (50 μM), and capsaicin (1 μM). All neurons in the culture were identified by the [Ca²⁺]_i increase elicited by application of 50 mM K⁺. H₂O₂, AITC and capsaicin stimulated a larger proportion of neurons dissociated from nodose than dorsal root ganglia. The number of neurons tested in each group was between 363 and 744.

compared with wild-type mice (30.1%, 134 of 445), ruling out TRPV1 as a mediator of DRG H₂O₂ responses.

TRPA1 is activated by products of lipid peroxidation

Oxidative stress generates other reactive chemicals, including products of lipid peroxidation, some of which have an electrophilic α,β-unsaturated carbonyl moiety like that found in the TRPA1 agonist, cinnamaldehyde. We therefore examined the effects on TRPA1 of three major lipids produced during episodes of oxidative stress (Uchida, 2003): 4-HNE, 4-HHE, and 4-ONE.

In calcium imaging experiments, 4-HNE (30 μM) and 4-ONE

(3 μM) increased [Ca²⁺]_i in ~25% of DRG neurons, similar to the percentage that responded to AITC or H₂O₂. Approximately 50% of the capsaicin-sensitive neurons responded to 4-HNE and 4-ONE and there was an exact correspondence between the 4-HNE/4-ONE responsive and AITC sensitive neurons (Fig. 5A). This pattern suggests that TRPA1 mediates the response to 4-HNE and 4-ONE in DRG neurons.

To examine the effects of 4-HNE on TRPA1 further, we studied its effect on heterologously expressed channels. 4-HNE evoked membrane currents and a robust [Ca²⁺]_i increase in TRPA1 CHO cells, but not in untransfected cells or in TRPV1 expressing CHO cells (data not shown). The characteristics of the TRPA1-evoked currents were similar, although not identical, to those found with AITC or H₂O₂ activation (Fig. 5B). A relatively sustained current developed after several seconds delay in a Ca²⁺-free external solution. Addition of Ca²⁺ evoked a sudden decrease in current, not seen with the other agonists, followed by a transient increase in current and rapid inactivation.

In DRG neurons, application of 4-HNE evoked a sustained inward current in solutions containing 15 μM Ca²⁺. Furthermore, the 4-HNE current showed a voltage-dependent inactivation at positive membrane potentials as shown by the current responses to a voltage ramp in TRPA1 expressing CHO cells and DRG neurons (Fig. 5B,C). These results are consistent with the independent previous reports by two other groups that 4-HNE activates TRPA1 (Macpherson et al., 2007b; Trevisani et al., 2007).

All three lipid peroxidation products tested (4-HHE, 4-HNE, and 4-ONE) evoked increases in [Ca²⁺]_i in TRPA1-expressing CHO cells (Fig. 6A). Concentration–response curves for the agonist induced increases in [Ca²⁺]_i (Fig. 6A) revealed that 4-HHE and 4-HNE were approximately equipotent (EC₅₀ values: 4-HNE, 19.9 ± 2.7 μM; 4-HHE, 39.9 ± 12.0 μM) whereas 4-ONE was more potent (EC₅₀ value, 1.9 ± 0.7 μM). These EC₅₀ values are consistent with the greater thiol re-

activity of 4-ONE (Lin et al., 2005).

Cyclopentenone prostaglandins are TRPA1 agonists

15d-PGJ₂ is a major cyclopentenone prostaglandin produced nonenzymatically by dehydration of the labile prostaglandin, PGD₂. The electrophilic 15d-PGJ₂ is thiol reactive, induces intracellular production of reactive oxygen species and contributes to oxidative stress (Kondo et al., 2001; Levonen et al., 2004). In calcium-imaging experiments, we found that 20 μM 15d-PGJ₂ elicited an increase in [Ca²⁺]_i in AITC-sensitive DRG neurons (Fig. 5A). To investigate whether 15d-PGJ₂ could activate

TRPA1, we examined the effects of 15d-PGJ₂ on TRPA1 CHO cells. 15d-PGJ₂ evoked inward currents and an increase in $[Ca^{2+}]_i$ in TRPA1 CHO cells (Fig. 5D), but had no effect on either TRPV1 or untransfected CHO cells (data not shown). The effect of 15d-PGJ₂ was concentration dependent with an EC₅₀ value of $5.6 \pm 1.1 \mu M$ calculated from increases in $[Ca^{2+}]_i$ (Fig. 6A). Importantly, the structurally related, but chemically less-reactive analogs 9,10-dihydro-15d-PGJ₂ and PGA₂ failed to elicit any significant $[Ca^{2+}]_i$ -increase at concentrations $< 50 \mu M$ (data not shown).

Reversibility of TRPA1 activation by DTT

Reactive oxygen species and the lipid activators of TRPA1 are able to modify cysteine, lysine, and histidine residues. H₂O₂ may promote the formation of disulfide bonds between cysteine residues, whereas the lipid activators are likely to form Michael adducts and may cross-link vicinal reactive groups. We therefore examined whether the effects of these agonists could be reversed by dithiothreitol (DTT) (1 mM), which will reduce disulfide bonds but is unable to hydrolyze Michael adducts. DTT reversed the effects of H₂O₂, but not the responses to 4-HNE or 15d-PGJ₂ (Fig. 6B).

Effect of H₂O₂, 4-ONE, and 15d-PGJ₂ on TRPA1 in isolated patches

Compounds with electrophilic or oxidative properties are likely to modify the activity of many cellular proteins and processes in addition to TRPA1. To examine the possibility that the novel endogenous ligands activate TRPA1 by interacting with cytoplasmic proteins, we investigated whether H₂O₂, 4-ONE, and 15d-PGJ₂ were able to activate TRPA1 in isolated inside-out membrane patches. As shown in Figure 7, application of H₂O₂ (Fig. 7A), 4-ONE (Fig. 7B), and 15d-PGJ₂ (Fig. 7C) opened TRPA1 channels in isolated inside-out patches, consistent with a membrane-delimited site of action that is not dependent on cytosolic mechanisms. In some patches, we noted that TRPA1 could be repeatedly activated and inactivated by H₂O₂ and DTT, respectively, further suggesting a direct action of these compounds on the channel (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

Effects of oxidative stress products on DRG neurons from *trpa1*^{-/-} mice

The correspondence between AITC-sensitivity and sensitivities to H₂O₂, 4-HNE, 4-ONE and 15d-PGJ₂ in DRG neurons and the abilities of these agents to activate TRPA1 in CHO cells is consis-

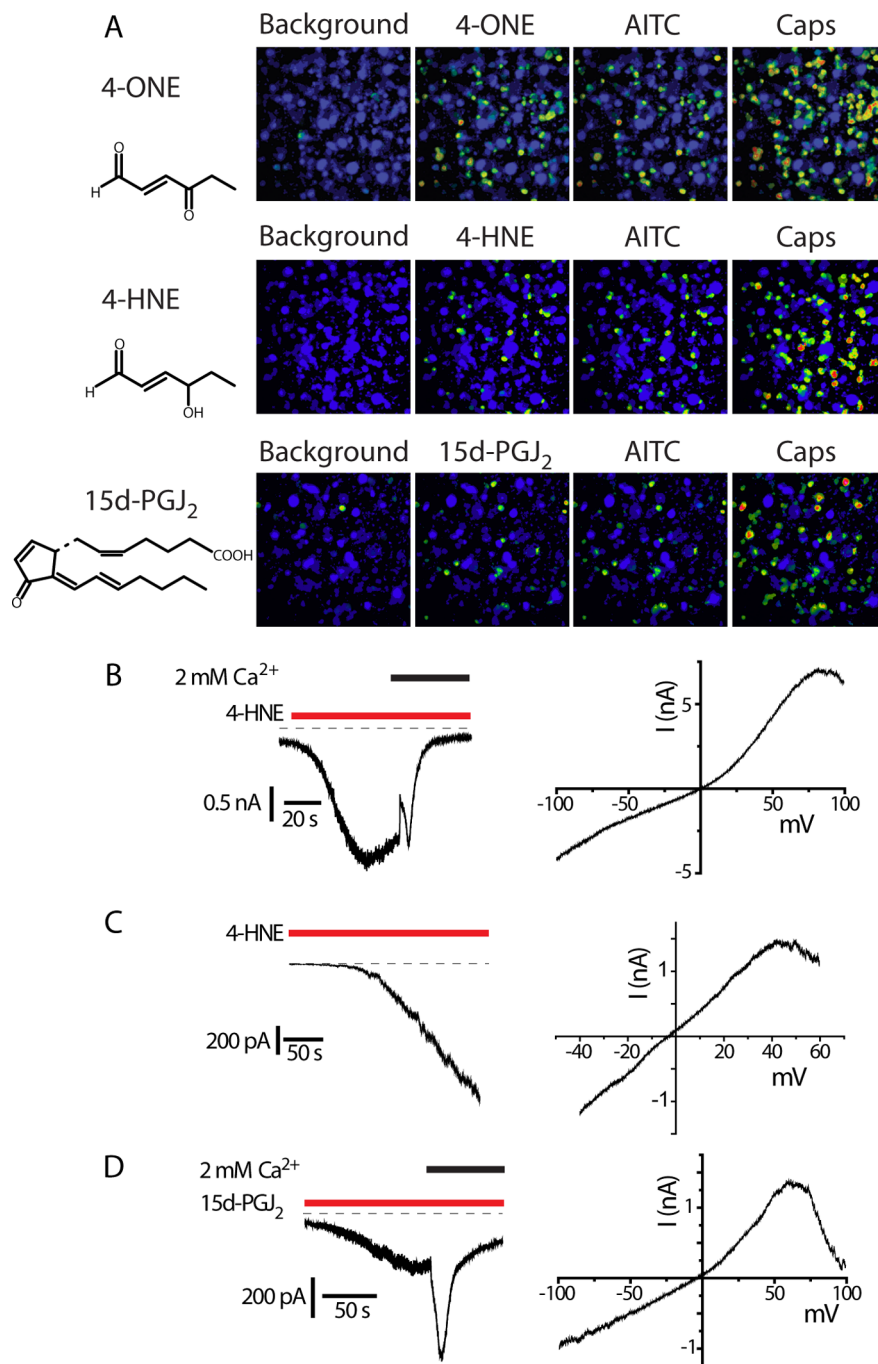


Figure 5. Lipid peroxidation products and 15d-PGJ₂ are TRPA1 agonists. **A**, $[Ca^{2+}]_i$ increases evoked by 4-ONE (30 μM), 4-HNE (30 μM), and 15d-PGJ₂ (20 μM) in DRG neurons. Sequential applications of AITC and capsaicin (Caps) show that 4-ONE, 4-HNE, and 15d-PGJ₂ activate the same subset of TRPV1-expressing, capsaicin-sensitive neurons as AITC. **B**, Left, 4-HNE-evoked current in a TRPA1 CHO cell initially in calcium-free solution. Admission of calcium led to rapid inactivation. Right, Current-voltage relationship of 4-HNE-evoked current. **C**, Left, 4-HNE-evoked current in a DRG neuron (external solution containing 15 μM Ca²⁺, -60 mV). Right, Current-voltage relationship of the 4-HNE-evoked current in the same neuron. **D**, Time course (left) and voltage-dependent kinetics (right) for 15d-PGJ₂-evoked TRPA1 current in a CHO cell. Right, Current-voltage relationship of 15d-PGJ₂-evoked current.

tent with TRPA1 acting as the neuronal sensor. To confirm this hypothesis we examined the responses of DRG neurons from wild-type and TRPA1-null allele mutant mice using calcium imaging. The results of these experiments are shown in Table 1. The percentage of responsive DRG neurons from wild-type mice was very similar to the percentages seen for rat DRG neurons. First, we confirmed the loss of TRPA1 in the *trpa1*^{-/-} DRG neurons

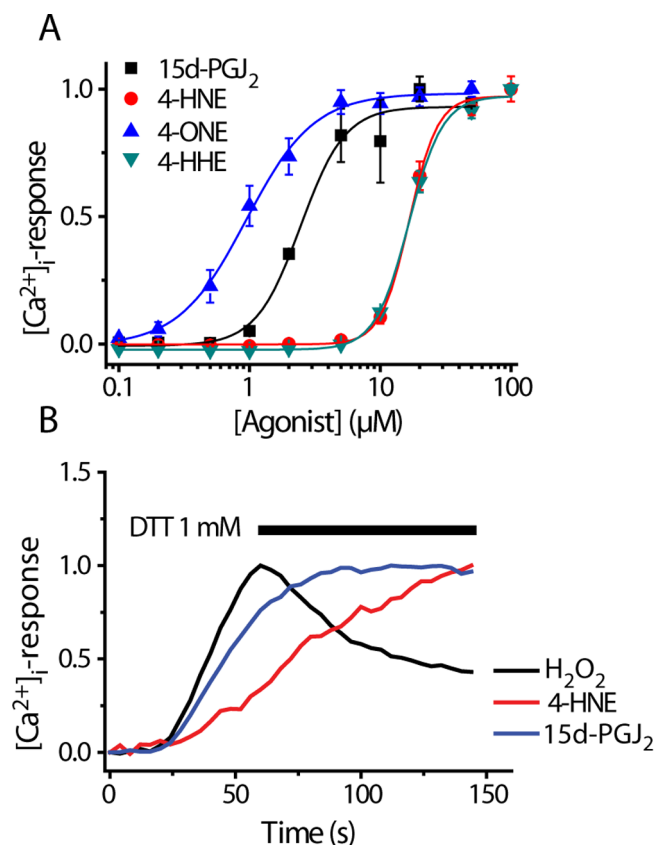


Figure 6. Concentration dependence of lipid mediators and dithiothreitol sensitivity. **A**, Concentration–response curves for 4-ONE, 4-HNE, 4-HHE, and 15d-PGJ₂ in TRPA1 CHO cells (mean \pm SEM, $n = 4$, representative of at least 3 experiments). **B**, Application of DTT reverse [Ca²⁺]_i-responses induced by application of H₂O₂, but not 4-HNE or 15d-PGJ₂.

using AITC as an agonist. Robust responses were noted in neurons from wild-type mice but responses to AITC were largely absent in *trpa1*^{−/−} mouse neurons as previously described (Kwan et al., 2006; Macpherson et al., 2007b). Similarly, the responses to H₂O₂, 4-HNE, 4-ONE, and 15d-PGJ₂ were essentially eliminated in the DRG neurons from knock-out mice. When present, the residual responses in *trpa1*^{−/−} neurons were usually slow in onset, small and showed oscillating changes in [Ca²⁺]_i, unlike the sustained responses in wild-type neurons.

H₂O₂ and 15d-PGJ₂ produce pain-related behavior through activation of TRPA1

We examined whether TRPA1 is required for pain responses elicited by H₂O₂ and 15d-PGJ₂ *in vivo*. Intraplantar injections of either compound evoked dose-dependent pain related behaviors in wild-type mice (licking, biting, flinching or shaking of the injected paw) that lasted for at least 3 min after injection (Fig. 8A,B). Doses that evoked reproducible robust responses were used to test the effects on *trpa1*^{−/−} mice and wild-type littermates. Injections of either 2.2 μmol of H₂O₂ or 32 nmol of 15d-PGJ₂ (in 25 μl) induced marked nocifensive behaviors in wild-type mice that were greatly reduced or absent in *trpa1*^{−/−} mice (Fig. 8C,D).

Discussion

Our results demonstrate that reactive oxygen species, alkenyl aldehydes and 15d-PGJ₂, which are generated during oxidative stress, activate TRPA1 in sensory neurons. All the agents acti-

vated TRPA1 expressed in CHO cells and evoked increases in [Ca²⁺]_i in ~50% of capsaicin sensitive DRG neurons. There was a close correspondence between the sensitivity of individual sensory neurons to the known TRPA1 agonist, AITC, and responsiveness to either H₂O₂, 4-HNE, 4-ONE, or 15d-PGJ₂. Importantly, the responses to H₂O₂, 4-HNE, 4-ONE, and 15d-PGJ₂ were almost absent in DRG neurons from *trpa1*^{−/−} mice. Furthermore, our *in vivo* experiments with *trpa1*^{−/−} mice demonstrated that TRPA1 was required for the pain-related behavioral responses evoked by H₂O₂ and 15d-PGJ₂.

Several studies have suggested that TRPV1 mediates the sensory neuron responses to H₂O₂ (Schultz and Ustinova, 1998; Ruan et al., 2005, 2006; Yoshida et al., 2006). However, the mismatch between H₂O₂- and capsaicin-sensitivity and the loss of H₂O₂ responses in *trpa1*^{−/−} mice indicate that TRPV1 is not primarily responsible for H₂O₂ activation of sensory nerve fibers. Unlike Yoshida et al. (2006), we found no evidence for direct activation of TRPV1 by H₂O₂ in our experiments. Furthermore, we observed no loss of H₂O₂ responses in DRG neurons from mice lacking TRPV1 ruling out the possibility that responses to H₂O₂ are mediated by a splice variant of TRPV1.

TRPA1 activation by H₂O₂ is likely to be an important pathway for neuronal stimulation *in vivo*. Single-unit recording from afferent nerve fibers innervating the heart showed that ~50% of capsaicin sensitive units were activated by H₂O₂ via production of OH[•] (Ustinova and Schultz, 1994). This corresponds well with our findings that a similar percentage of isolated, capsaicin sensitive DRG neurons were activated by H₂O₂ in calcium imaging experiments and that activation was, at least in part, mediated by OH[•] radicals.

The finding that H₂O₂, 4-HNE, or 15d-PGJ₂ activated TRPA1 in isolated membrane patches is consistent with direct chemical modification of TRPA1, although we cannot rule out effects on closely associated interacting proteins. Six cysteine residues located in the N-terminal segment have been identified as potential sites for oxidation and activation of TRPA1 by exogenous electrophilic reagents (AITC, cinnamaldehyde), whereas other molecular mechanisms appear to operate for agonists such as icilin and THC (Hinman et al., 2006; Macpherson et al., 2007a). DTT, which can reduce disulphide bonds but does not affect Michael adducts (Macpherson et al., 2007a), did not affect the responses to 4-HNE or 15d-PGJ₂, suggesting that these lipid mediators form covalent adducts with TRPA1. This conclusion is consistent with reports that the actions of 15d-PGJ₂ on Kelch-like ECH-associated protein 1 (KEAP1) and peroxisome proliferator-activated receptor-γ (PPAR-γ) are exerted by Michael additions to redox-sensitive cysteine thiols and that 4-HNE and 4-ONE form adducts with cysteine residues (Shibata et al., 2003; Levenon et al., 2004; Sayre et al., 2006). The finding that DTT reversed the effects of H₂O₂ indicates the formation of disulfide bonds between vicinal cysteine residues. Although actions on cysteine residues in TRPA1 are therefore likely, it is premature to conclude that all the effects reported in this study result exclusively from cysteine modifications. Hinman et al. (2006) showed that one lysine residue (K708) also influenced the activity of AITC. The alkenyl aldehydes studied here preferentially react with cysteine residues (Petersen and Doorn, 2004), but can also form Michael adducts with histidine, and 4-ONE has been reported to form a Schiff base with ε-amino groups of lysine residues (Lin et al., 2005). In TRPA1 CHO cells the waveform of the membrane currents evoked by increasing the extracellular Ca²⁺ concentration from Ca²⁺-free to 2 mM Ca²⁺ were agonist dependent. The current suddenly increased and then inactivated with H₂O₂, 15d-

PGJ₂, or AITC as the agonist, whereas with 4-HNE, the current showed a sudden decrease before increasing and then inactivating. These findings suggest some agonist-dependent differences in the interactions with TRPA1. Identification of the residues and mechanisms responsible for TRPA1 activation by H₂O₂, alkenyl aldehydes and 15d-PGJ₂ remains to be explored.

Our studies show that H₂O₂ and 15d-PGJ₂ activate sensory neurons *in vivo* to evoke pain responses. The responses were almost completely absent in *trpa1*^{−/−} mice, demonstrating that sensory stimulation by these agents was mediated by TRPA1. These results complement the previous finding that 4-HNE evokes pain responses by activating TRPA1 (Trevisani et al., 2007). The concentrations of H₂O₂, 4-HNE, and 15d-PGJ₂ required to stimulate TRPA1 and pain responses are higher than those that normally occur in tissues, but all the agents are produced at much higher concentrations during periods of oxidative stress.

NADPH oxidase-derived ROS, such as H₂O₂, have been proposed to play a role in local cell signaling by affecting the function of various kinases, phosphatases, phospholipases, and transcription factors, at low submicromolar concentrations (Lambeth, 2004; Bedard and Krause, 2007). ROS occur at higher concentrations in conditions of oxidative stress when their production exceeds the antioxidant activity of the cell. H₂O₂ concentrations of 10–100 μ M have been measured in situations of physiological stress such as inflammation and reperfusion after ischemia (Hyslop et al., 1995; Sprong et al., 1997; Stone and Yang, 2006), which is similar to concentrations that activate TRPA1 (50–100 μ M). The intracellular H₂O₂ concentration is probably 7–10 times lower than the extracellularly applied concentration (Stone and Yang, 2006), suggesting that TRPA1 can be activated when the intracellular H₂O₂ concentration reaches tens of micromolar. TRPA1 activation may be mediated directly by H₂O₂, but the finding that the potency of H₂O₂ was increased by 20-fold in Fe²⁺-loaded cells indicates that the effect is mediated, at least in part, by OH[•] produced from H₂O₂ by the Fenton reaction. Our data fit well with the observations that cardiac reperfusion after a brief experimental ischemia generates reactive oxygen species (O'Neill et al., 1996) and activates H₂O₂-sensitive sensory nerves. This activation is inhibited by deferoxamine pretreatment consistent with an effect mediated by OH[•] radicals (Ustinova and Schultz, 1994a; Huang et al., 1995b).

TRPA1 was activated by several naturally occurring alkenyl aldehydes produced by lipid peroxidation (4-HNE, 4-ONE, and 4-HHE). The concentrations of 4-HNE (EC₅₀, 19.9 μ M) and 4-ONE (EC₅₀, 1.9 μ M) required to activate TRPA1 are within the concentration range of 10 μ M to >100 μ M attained during oxidative stress (Esterbauer et al., 1991; Sayre et al., 2006). These electrophilic chemicals form adducts with many cellular molecules and the presence of these adducts is often used as an index of oxidative stress and damage. The finding that 4-HNE evokes pain responses by activating TRPA1 *in vivo* (Trevisani et al., 2007) supports a role for alkenyl aldehydes in signaling potentially damaging conditions of oxidative stress.

Cyclopentenone prostaglandins of the A and J series are produced *in vivo* by dehydration of the pentane ring of the prostaglandins, PGE₂ and PGD₂. The parent prostaglandins are usually

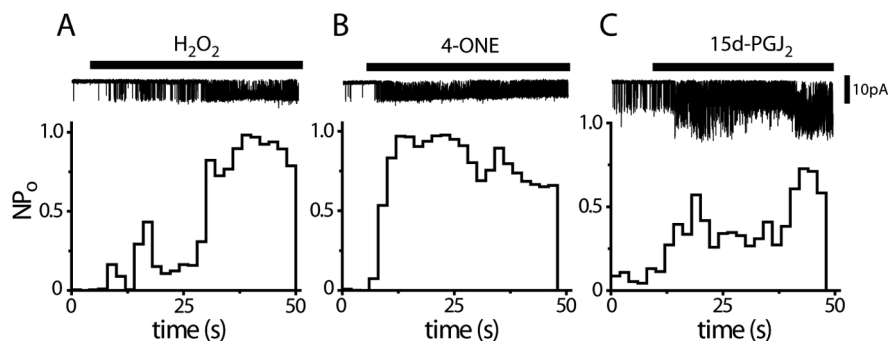


Figure 7. H₂O₂, 4-ONE and 15d-PGJ₂ activate TRPA1 in a membrane-delimited manner. **A–C**, Application of 5 mM H₂O₂ (**A**), 5 μ M 4-ONE (**B**) and 20 μ M 15d-PGJ₂ (**C**) evoke single-channel activity in excised inside-out patches (−100 mV, each trace is representative of at least 3 experiments).

produced enzymatically by the cyclooxygenase pathway. In conditions of oxidative stress, the *cis*-isomers of PGE₂ and PGD₂ (iso-PGE₂ and iso-PGD₂) are also generated nonenzymatically *in vivo* at high concentrations as products of free radical-induced peroxidation of arachidonoyl lipids (Chen et al., 1999; Gao et al., 2003). These isoprostanes are unstable and undergo epimerization to form PGE₂ and PGD₂, which in turn leads to an increased production of PGA₂ and PGJ₂ and their dehydration products, including 15d-PGJ₂ (Gao et al., 2003). At low concentrations, 15d-PGJ₂ can exert an anti-inflammatory effect and protect against oxidative stress by activation of PPAR γ and KEAP1 (Lan-dar et al., 2006; Lin et al., 2006; Ou et al., 2006; Napimoga et al., 2008), but at higher concentrations, the reactive cyclopentenone prostaglandins can cause tissue damage (Koh et al., 2005; Musiek et al., 2007). Low micromolar concentrations of 15d-PGJ₂ activated TRPA1. The estimated EC₅₀ value for TRPA1 agonism (5.6 μ M) compares well with the concentrations of 15d-PGJ₂ usually used to activate PPAR γ (Forman et al., 1995; Kliewer et al., 1995).

Our studies show that TRPA1 can be activated by several different agents produced during conditions of oxidative stress. We propose that these chemicals activate TRPA1 and stimulate sensory neurons to elicit pain and to promote immediate protective responses either by local release of neuropeptides from the peripheral sensory nerve terminals or by sympathetically and vagally mediated neuronal reflexes. For example, activation of cardiac afferents evokes the symptoms of angina pectoris and reflex changes in blood pressure and heart rate (see e.g., Huang et al., 1995a). Similarly ROS activation of afferents innervating the airways causes a reflex increase in respiratory rate and bronchial vasodilation that increases airway blood flow (Soukhova et al., 1999; Ruan et al., 2006).

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Table 1. Comparison of $[Ca^{2+}]$ responses in DRG neurons from *trpa1*^{+/+} and *trpa1*^{-/-} mice

Agonist	<i>trpa1</i> ^{+/+}		<i>trpa1</i> ^{-/-}	
	Responding neurons ^a	Response amplitude (% of K ⁺) ^b	Responding neurons ^a	Response amplitude (% of K ⁺) ^b
AITC	29% (133 of 466)	86%	2% (4 of 181)	21%
H ₂ O ₂	33% (91 of 274)	70%	3% (12 of 403)	31%
4-ONE	31% (97 of 310)	89%	7% (27 of 364)	34%
4-HNE	28% (98 of 356)	61%	1% (4 of 469)	34%
15d-PGJ ₂	32% (96 of 304)	63%	10% (15 of 148)	47%

^aNeurons responding with a $[Ca^{2+}]$ increase of at least 15% of the response to a subsequent challenge with 50 mM KCl.

^b $[Ca^{2+}]$ increase expressed as percentage of the response to a challenge with 50 mM KCl. The weak residual $[Ca^{2+}]$ responses in DRG neurons from *trpa1*^{-/-} mice were qualitatively different from the responses seen in neurons from wild-type mice and showed delayed, oscillating signals. Similar weak responses in *trpa1*^{-/-} DRG neurons were noted for AITC in our study and by Kwan et al. (2006). These evoked changes in $[Ca^{2+}]$ probably represent actions of the reactive compounds on other cellular mechanisms.

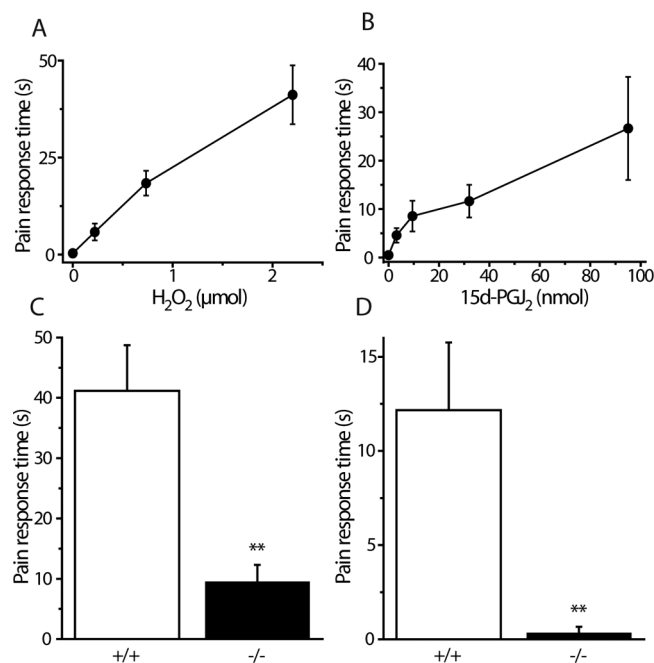


Figure 8. H₂O₂ and 15d-PGJ₂ induce pain-related behavior *in vivo* by activating TRPA1. **A, B**, Duration of nocifensive (licking/flinching) behavior in wild-type mice evoked by intraplantar hindpaw injections of H₂O₂ in saline (**A**) or 15d-PGJ₂ in saline containing 10% DMSO (**B**). The injection volume was 25 μl. Pain-related behavior was recorded over 5 min (mean ± SEM, *n* = 6 for each group). **C, D**, Wild-type (+/+) and TRPA1-deficient mice (-/-) were injected in the hind paw with H₂O₂ (**C**) (2.2 μmol/25 μl) or 15d-PGJ₂ (**D**) (32 nmol/25 μl). Pain-related behavior (licking, biting, flinching, or shaking of the injected paw) was recorded for 3 min after injection. The nocifensive responses induced by H₂O₂ and 15d-PGJ₂ were dramatically reduced or absent in mice lacking TRPA1 (mean ± SEM; *n* = 6 in each group; ***p* < 0.01).

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