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Nordihydroguaiaretic acid activates hTRPA1 and modulates behavioral responses to noxious cold in mice

William John Redmond[1], Maxime Camo[1], Vanessa Mitchell[2], Christopher Walter Vaughan[2] & Mark Connor[1]

[1]Australian School of Advanced Medicine, Macquarie University, New South Wales, 2109, Australia
[2]Pain Management Research Institute, Kolling Institute, University of Sydney, New South Wales, 2065, Australia

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Correspondence
Mark Connor, Australian School of Advanced Medicine, 2 Technology Place, Macquarie University, NSW, 2109, Australia. Tel: 612-9850 2719; E-mail: mark.connor@mq.edu.au

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Abstract

Nordihydroguaiaretic acid (NDGA) is a major biologically active component of the creosote bush, *Larrea tridentate*, widely used in unregulated therapies. NDGA is a lipoxygenase inhibitor while a derivative, terameprocol, has been trialed as a chemotherapeutic agent. When investigating fatty acid activation of the human transient receptor potential cation channel subfamily A, member 1 (hTRPA1), we found that NDGA activated the channel. Here we investigate the actions of NDGA and terameprocol at hTRPA1 and the consequences of this for noxious cold sensitivity in mice. hTRPA1 was stably expressed in HEK 293 cells (HEK 293-TRPA1) and channel activity examined by measuring changes in intracellular calcium ($[Ca]_i$) using a fluorescent dye and activation of membrane currents using patch clamp electrophysiology. The effects of local NDGA and terameprocol application on acetone-induced paw flinching were examined in mice. NDGA (pEC_{50} of 5.4 ± 0.1, maximum change in fluorescence of 385 ± 30%) and terameprocol (pEC_{50} 4.5 ± 0.2, maximum 550 ± 75%) increased $[Ca]_i$ in HEK 293-hTRPA1 cells. NDGA also induced an increase in membrane conductance in HEK 293-hTRPA1 cells. These effects were prevented by the TRPA1 antagonist HC-030031, and were dependent on the presence of Cys621, Cys 641, and Cys 665 in hTRPA1. Neither NDGA nor terameprocol alone produced spontaneous pain behaviors in mice after hind paw injection, but both enhanced responses to acetone. NDGA and terameprocol are efficacious activators of TRPA1. NDGA should be used with care to probe lipoxygenase involvement in nociception while TRPA1 activity should be considered when considering use of these drugs in humans.

Abbreviations

$[Ca]_i$, intracellular calcium concentration; ANOVA, analysis of variance; CA, cinnamaldehyde; CIPN, chemotherapy-induced peripheral neuropathy; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; HBS, HEPES-buffered saline; HBSS, modified Hanks balanced salt solution; hTRPA1, human transient receptor potential ankyrin 1; I_{Ca}, voltage-gated calcium channel; I_K, potassium channel; NDGA, nordihydroguaiaretic acid; OMeNDGA, tetra-*o*-methyl nordihydroguaiaretic acid, terameprocol; RFU, relative fluorescence units; ROS, reactive oxygen species; SCLC, small cell lung cancer cells; TRPM7, transient receptor potential melastatin-like 7 channel.

Introduction

Nordihydroguaiaretic acid (NDGA) is a major pharmacologically active component of the creosote bush (*Larrea tridentata*). Creosote extracts ("Chaparral tea") have been traditionally used to treat a wide variety of conditions (Arteaga et al. 2005), and continue to be advertised extensively in unregulated environments, despite well

recognized toxicity (Sheikh et al. 1997) and the banning of NDGA from food in the United States more than 40 years ago (http://www.accessdata.fda.gov). NDGA and related molecules inhibit lipoxygenases in addition to a variety of other enzymes, and are also antioxidants (Lu et al. 2010). These properties may contribute to anti-inflammatory properties attributed to crude preparations. Synthetic derivatives of NDGA, including tetra-*o*-methyl NDGA (terameprocol, formerly M_4N or EM-1421) inhibit the activity of the transcription factor Sp1, leading to inhibition of viral replication and tumor cell growth (Chen et al. 1998; Hwu et al. 1998; Chang et al. 2004; Castro-Gamero et al. 2013).

NDGA has been widely used in experimental studies of inflammation (e.g., Yoo et al. 2009; Xue et al. 2013) and its main mechanism of action appears to be through its unspecific inhibition of lipoxygenase (Salari et al. 1984; Gregus et al. 2013) and by acting as a scavenger of reactive oxygen species (ROS, Floriano-Sanchez et al. 2006; Lu et al. 2010). NDGA may also suppress tumor growth in part via its lipoxygenase inhibition and antioxidant properties (Kubow et al. 2000).

While investigating the activation of the pronociceptive ion channel transient receptor potential cation channel subfamily A (TRPA1) (Jordt et al. 2004) by arachidonic acid and potential metabolites (Redmond et al. 2014), we used NDGA as a lipoxygenase inhibitor and unexpectedly found that NDGA itself activated human TRPA1 with a potency similar to that of the widely used agonist cinnamaldehyde (CA). The discovery of TRPA1 agonist activity for NDGA and its derivative terameprocol may not only provide insight into their potential therapeutic mechanism(s) of action, but also suggest that care should be taken when attributing their biological effects as being solely mediated through enzyme inhibition, antioxidant activity or modulation of gene transcription.

Materials and Methods

Cell culture

Flp-In TRex HEK 293 (Life Technologies, Mulgrave, Victoria, Australia) stably transfected with wild-type or mutant hTRPA1 (Redmond et al. 2014) were cultivated in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U penicillin and 100 μg streptomycin mL^{-1}, hygromycin B 25 μg mL^{-1} and blasticidin S 5 μg mL^{-1}. Cells were incubated in 5% CO_2 at 37°C in a humidified atmosphere. Cells were grown in flasks with a surface area of 75 mm^2, once at optimum confluence (approximately 90%), cells were trypsinized and transferred into clear-bottomed poly-D-lysine coated 96-well plates (Corning, Castle Hill, NSW, Australia) in L15 medium supplemented with 1% fetal bovine serum, hygromycin B, and the antibiotics outlined above. The cells were plated in a volume of 100 μL and were incubated in humidified room air at 37°C overnight. Expression of the TRPA1 receptor or mutants was induced 5–8 h prior to experimentation by addition of tetracycline, 1 μg mL^{-1} to each well.

Calcium assay

Intracellular calcium ($[Ca]_i$) was measured with the calcium 5 kit from Molecular Devices (Sunnyvale, CA) using a FLEX Station 3 Microplate Reader (Molecular Devices). Hundred microliters of dye dissolved in modified Hanks balanced salt solution (HBSS) containing (in mmol L^{-1}): NaCl 140, KCl 5.33, $CaCl_2$ 1.3, $MgCl_2$ 0.5, HEPES 22, Na_2HPO_4 0.338, $NaHCO_3$ 4.17, KH_2PO_4 0.44, $MgSO_4$ 0.4, glucose 10 (pH to 7.3, osmolarity = 330 ± 5 mosmol) was loaded into each well of the plate and incubated in room temperature for 1 h at 37°C. All experiments in the Flexstation were carried out at 37°C. Calcium 5 fluorescence was measured every 2 sec ($\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 525 nm) for the duration of the experiment. Drugs were added after at least 2 min of baseline recording. In experiments where one drug addition, 50 μL of drug dissolved in HBSS was added, for two drug additions, 25 μL was added each time.

Electrophysiology

TRPA1 channel currents were recorded in the whole-cell configuration of the patch clamp method (Hamill et al. 1981) at room temperature. Dishes were perfused with HEPES-buffered saline (HBS) containing (in mmol L^{-1}): 140 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose (pH to 7.3, osmolarity = 330 ± 5 mosmol). Recordings were made with fire-polished borosilicate glass pipettes with resistance ranging from 2 to 3 MΩ. The internal solution contained (in mmol L^{-1}): 130 CsCl, 10 HEPES, 2 $CaCl_2$, 10 ethylene glycol tetraacetic acid, 5 MgATP (pH to 7.3, osmolarity = 285 ± 5 mosmol). Recordings were made with a HEKA EPC 10 amplifier with Patchmaster acquisition software (HEKA Elektronik, Lambrecht/Pfalz, Germany). Data were sampled at 10 kHz, filtered at 3 kHz, and recorded on hard disk for later analysis. Series resistance ranged from 3 to 10 MΩ and was compensated by at least 80% in all experiments. Leak subtraction was not used. Cells were exposed to drugs via flow pipes positioned approximately 200 μm from the cell and drugs were dissolved in HBS immediately before application. All solutions had final ethanol concentration of 0.1% v/v.

Data analysis

For measurements of drug-induced changes in calcium 5 dye fluorescence, which reflects changes in intracellular concentration ($[Ca]_i$), the response to agonists was expressed as a percentage change over the baseline averaged for the 30 sec immediately prior to drug addition. Changes produced by parallel solvent blanks were subtracted and these changes were never more than 10% of baseline. Concentration-effect data from independent experiments, each performed in duplicate or triplicate, were pooled and fit to a four-parameter logistic Hill equation to derive the EC_{50} values and Hill slope (GraphPad Prism, San Diego, CA). Results are expressed as mean ± SEM of at least 4–5 independent experiments unless otherwise stated.

Behavioral studies

Experiments were carried out on adult male C57BL/6 mice (8–10 weeks old) following the guidelines of the "NH&MRC Code of Practice for the Care and Use of Animals in Research in Australia" and with the approval of the Royal North Shore Hospital Animal Care and Ethics Committee. Mice initially weighed between 20 and 25 g and were housed in groups of three in individually ventilated cages (23 ± 1°C, humidity 70%) with environmental enrichment and free access to food and water, in a 12:12 h light–dark cycle.

Animals were allowed to acclimatize to their holding cages and the behavioral testing chambers for 2–3 days before any procedures were carried out. All testing was carried out in low level white light (<3 lux). To assess cold sensitivity, the mice were allowed to acclimatize for 20–30 min prior to testing in elevated perspex cages (15 × 10 × 10 cm) with a wire mesh floor, and 20 μL of acetone was sprayed onto the plantar surface of the left hind paw to induce evaporative cooling (Gentry et al. 2010). The number of left hind limb lifts, shakes, and licks was then counted over a 2-min period. Solutions of drugs for intraplantar injection were made up in a vehicle solution which comprised 25% dimethylsulfoxide (DMSO) and 10% Tween 80 in saline. Intraplantar injections were made in a volume of 15 μL, under brief isoflurane anesthesia (2.5% in saturated O_2, 1 mL min^{-1}) using a 30-gauge needle. Solutions of drugs for systemic injection were made up in a vehicle solution (15% DMSO in saline) and were injected intraperitoneally at a volume of 0.12 mL/10 g in lightly restrained animals.

In experiments investigating the effect of TRPA1 agonists the protocol was three predrug behavioral measurements (at 0, 15, 30 min), intraplantar agonist or vehicle injection (at 45 min), then five behavioral measurements (60, 75, 105, 135, and 165 min = 15, 30, 60, 90, and 120 min postagonist). In experiments on the effect of HC 030031 on the TRPA1 agonists the protocol was three predrug behavioral measurements (at 0, 15, 30 min), systemic injection of HC 030031 (at 45 min), two behavioral measurements (at 60, 75 min), intraplantar agonist or vehicle injection (at 90 min), three behavioral measurements (at 105, 120, and 165 min = 15, 30, 60, 90, and 120 min postagonist). Animals were euthanized at the end of the testing period. The experimenter was blinded to the agents being tested.

For the time course experiments, comparisons of drug/vehicle treatment effects over time were made using two-way repeated measures analyses of variance (ANOVAs), with time and treatment as a within- and between-subjects factors (GraphPad Prism). When two-way ANOVAs were significant, post hoc comparisons between treatment groups at individual time points were made using the Sidak adjustment for multiple comparisons. To measure net drug effects, the postdrug measures for acetone-induced lifts/shakes/flicks were taken as the average of measurements over 15–60 min postdrug injection and compared to the preinjection baseline values (subtracted to give the change relative to baseline). Dose–response curves were constructed by fitting data to a four-parameter logistic Hill equation (GraphPad Prism). Statistical comparisons of the effect of CA and NDGA in the presence and absence of antagonists were made using two-way ANOVAs and when significant, post hoc comparisons were made using the Bonferroni adjustment for multiple comparisons.

Drugs and reagents

Drugs for in vitro experiments were dissolved in ethanol and diluted in HBS with a maximum final concentration of ethanol of 0.1%. NDGA, terameprocol, and HC 030031 were purchased from Cayman Chemical (Ann Arbor, MI). Ruthenium red was from Enzo Lifesciences (Farmingdale, NY). Ionomycin was from Ascent Scientific (Avonmouth, UK). CA was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All tissue culture reagents were from Sigma-Aldrich, Life Technologies (Mulgrave, Victoria, Australia) or InvivoGen (San Diego, CA).

Results

NDGA produced a robust elevation of $[Ca]_i$ in HEK-293 cells expressing hTRPA1, but only a small change in HEK-293-TRPA1 cells where TRPA1 expression had not been induced by tetracycline (Fig. 1). The effects of NDGA (30 μmol L^{-1}) on $[Ca]_i$ were antagonized by

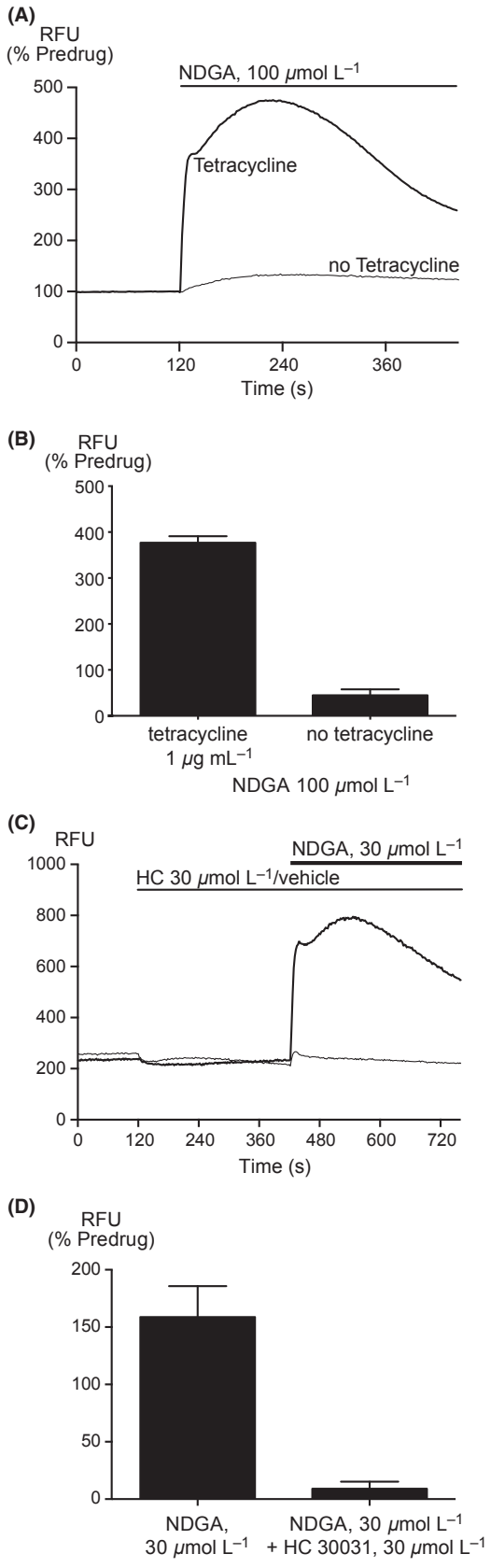

Figure 1. Nordihydroguaiaretic acid (NDGA) activates human TRPA1. Changes in intracellular calcium ($[Ca]_i$) in HEK293 cells expressing hTRPA1 were measured as outlined as in the Materials and Methods section. (A) Example traces of NDGA actions on HEK293-hTRPA1 cells where expression of hTRPA1 was or was not induced by tetracycline, addition of tetracycline produced a dramatic increase in the effects of NDGA. Traces represent the relative fluorescence units (RFU) normalized to those prior to drug addition. Data from four similar experiments are summarized in (B), with each bar representing the mean ± SEM of the maximum change in calcium 5 fluorescence. (C) Example traces of NDGA effects after preincubation of the HEK293-hTRPA1 cells with the TRPA1 antagonist HC 030031 (thin trace) or vehicle (thicker trace), traces represent unnormalized RFU. Data from six similar experiments are summarized in (D) with each bar representing the mean ± SEM of the maximum change in calcium 5 fluorescence. HC 030031 significantly inhibited the effects of NDGA ($P < 0.001$).

preincubation of the cells with the TRPA1 antagonist HC 030031 (30 μmol L^{-1}, Fig. 1). NDGA increased calcium 5 dye fluorescence with a pEC_{50} of 5.4 ± 0.1, while in parallel experiments, the prototypic TRPA1 agonist CA (CA) increased fluorescence with a pEC_{50} of 5.3 ± 0.1 ($n = 5$, Fig. 2). The maximally effective concentration of NDGA (100 μmol L^{-1}) produced a smaller change in fluorescence than a high concentration of CA (300 μmol L^{-1}, 385 ± 30% vs. 520 ± 25%, $P < 0.01$, $n = 5$).

To confirm that NDGA was activating a membrane conductance, whole-cell voltage clamp recordings were made from hTRPA1 expressing HEK 293 cells induced overnight with a low concentration of tetracycline (1 μg mL^{-1}). Whole-cell currents were elicited from a holding potential of 0 mV by repeatedly ramping the membrane potential of the cells from −80 to +80 mV over 500 msec, once every 5 sec (Redmond et al. 2014). NDGA (10 μmol L^{-1}) produced a rapid increase in membrane current measured at +80 mV (from a baseline of 150 ± 8 pA to a peak of 2.2 ± 0.1 nA, $n = 6$, Fig. 3) that was strongly attenuated by coincubation of the cells with the TRPA1 antagonist HC 030031 (30 μmol L^{-1}; control 94 ± 3 pA; after 2 min in HC 030031 110 ± 3 pA; after 3 min in HC 030031 and NDGA, 132 ± 2 pA $n = 6$, Fig. 3).

CA and other reactive electrophiles require Cys residues in the intracellular N-terminal of TRPA1 to activate the channel, but this requirement is not shared to the same degree by all TRPA1 agonists (Hinman et al. 2006; Redmond et al. 2014). NDGA elevated $[Ca]_i$ with an EC_{50} of 4.9 ± 1.7 μmol L^{-1} in cells expressing wild-type hTRPA1, and 18 ± 3 μmol L^{-1} in cells expressing hTRPA1 with Cys621, Cys 641, and Cys 665 mutated to serine (3xCys-mutant hTRPA1, $P < 0.01$, $n = 6$). The maximum elevation of $[Ca]_i$ by NDGA was significantly greater in cells expressing wild-type hTRPA1

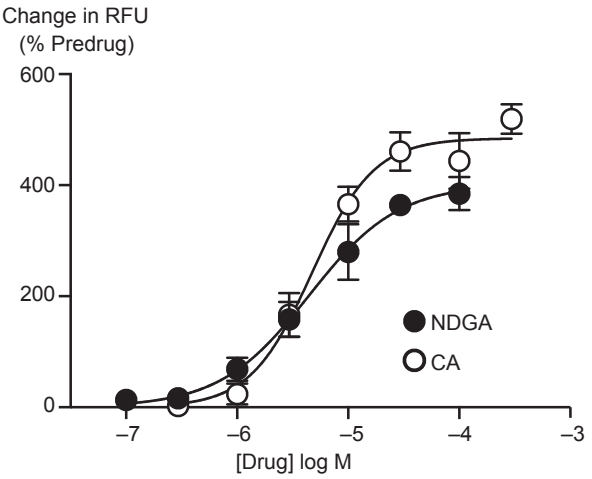

Figure 2. Nordihydroguaiaretic acid (NDGA) activates hTRPA1 with a similar potency to cinnamaldehyde (CA). Changes in intracellular calcium ($[Ca]_i$) in HEK293 cells expressing hTRPA1 were measured as outlined as in the Materials and Methods section. Concentration-effect curves for NDGA and CA were fit with a four-parameter logistic equation, each point represents the mean ± SEM of the change in fluorescence (RFU) from five experiments, each performed in duplicate or triplicate. NDGA elevated $[Ca]_i$ with an EC_{50} of 4.4 μmol L^{-1}, CA elevated $[Ca]_i$ with an EC_{50} of 4.7 μmol L^{-1}. The maximum elevation of $[Ca]_i$ by CA was significantly greater that that produced by NDGA ($P < 0.01$).

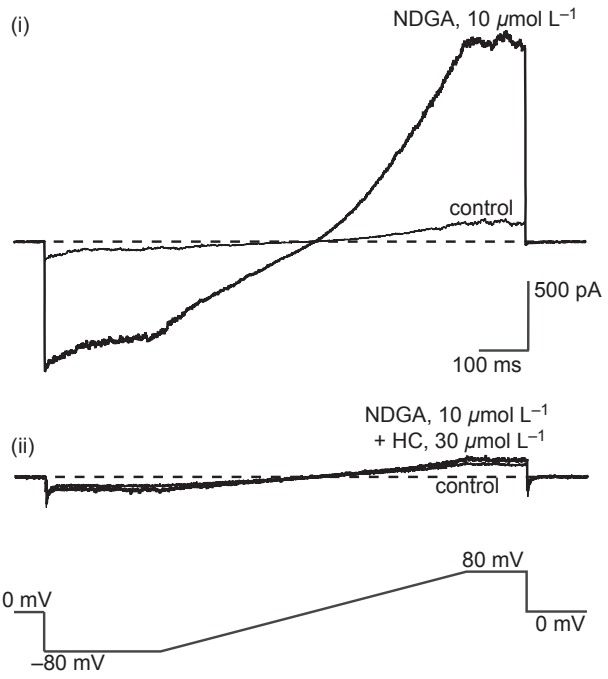

Figure 3. Nordihydroguaiaretic acid (NDGA) activates a membrane conductance in HEK 293 cells expressing hTRPA1. Whole voltage clamp recordings of membrane currents in HEK 293 cells expressing hTRPA1 were made as outlined in the Materials and Methods section. (i) Current traces from hTRPA1-expressing HEK 293 cell in control conditions (thin line) and in the presence of 10 μmol L^{-1} NDGA. The increase in current was prevented by coapplication of HC 030031 (HC), illustrated in (ii). These traces are representative of at least six cells for each condition. Cells were subject to the voltage protocol illustrated beneath the traces. Zero current is designated by the dotted line.

(365 ± 15%) than in cells expressing the 3xCys-mutant hTRPA1 (85 ± 16%, $P < 0.001$) (Fig. 4).

Tetra-*o*-methyl-NDGA (OMeNDGA, terameprocol) is an analog of NDGA being developed as a chemotherapeutic agent. Terameprocol also produced a concentration-dependent increase in calcium 5 fluorescence in cells expressing hTRPA1, with a pEC_{50} of 4.5 ± 0.2 and a maximum change in fluorescence of 550 ± 75% (300 μmol L^{-1}, Fig. 5). The effects of terameprocol (30 μmol L^{-1}) were antagonized by preincubation of cells with HC 030031 (Fig. 5, $P < 0.001$, $n = 6$).

In C57BL/6 mice intraplantar injection of NDGA and CA at doses of up to 300 and 1000 nmol, respectively, had no effect on spontaneous hind paw movement and produced no overt behavioral effects. At doses of 3000 nmol and above, CA produced whole body responses that were relatively delayed in onset and included decreased locomotion and shaking/shivering.

When acetone was sprayed onto the plantar surface of the hind paw before drug injection, it produced on average of 2.0 ± 0.1 localized hind limb responses (hind paw lifts/flinches/licks) which lasted an average of 7.3 ± 0.3 sec. Intraplantar injection of vehicle did not produce a change in the number of localized hind limb responses to acetone (Fig. 6A and B). Intraplantar injection of CA produced an increase in the number of localized hind limb responses to acetone which peaked at 15–30 min postinjection and returned to baseline levels within 60 min (Fig. 6A). The increase hind limb acetone responses produced by intraplantar CA was significantly greater than that produced by intraplantar vehicle at the 100 nmol ($P < 0.001$ at 15 min), 300 nmol ($P < 0.001$, 0.01 at 15, 30 min), and 1000 nmol doses ($P < 0.0001$ at 15, 30 min). The increase in the number of localized hind limb responses displayed dose dependence, with an EC_{50} of 60 ± 4 nmol (Fig. 6A and C).

Intraplantar injection of NDGA also produced an increase in the number of localized hind limb responses to acetone which peaked at 15–30 min postinjection and gradually returned toward baseline levels (Fig. 6B). The increase hind limb acetone responses produced by intraplantar NDGA was significantly greater than that following intraplantar vehicle at the 3 nmol ($P < 0.0001$ at 15 min), 10 nmol ($P < 0.0001$ at 15 min), 30 nmol ($P < 0.0001$, 0.01 at 15, 30 min), 100 nmol ($P < 0.0001$, 0.05 at 15, 30 min), and 300 nmol doses ($P < 0.0001$, 0.0001, 0.0001, 0.01 at 15, 30, 60 min). The increase in

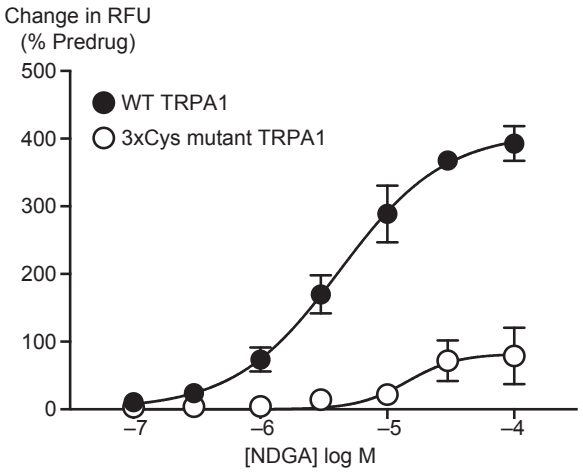

Figure 4. Nordihydroguaiaretic acid (NDGA) activation of hTRPA1 is strongly dependent on conserved Cys residues in the intracellular N-terminus. Changes in intracellular calcium ($[Ca]_i$) in HEK293 cells expressing hTRPA1 and mutant hTRPA1 where Cys 621, Cys 641, and Cys 665 were mutated to Ser (3xCys hTRPA1 mutant) were measured as outlined as in the Materials and Methods section. Concentration-effect curves for NDGA were fit with a four-parameter logistic equation, each point represents the mean ± SEM of the change in fluorescence (RFU) from six experiments, each performed in duplicate or triplicate. In cells expressing wild-type hTRPA1, NDGA elevated $[Ca]_i$ with an EC_{50} of 4.9 ± 1.7 μmol L^{-1} to a maximum of 365 ± 15%, while in cells expressing the 3xCys hTRPA1 mutant, the NDGA EC50 was 18 ± 3 μmol L^{-1} to a maximum of 85 ± 16% ($P < 0.01$ for both EC_{50} and maximum between wild-type and 3xCys-mutant hTRPA1).

the number of NDGA-induced localized hind limb responses displayed dose dependence, with an EC_{50} of 12.0 ± 2.1 nmol (Fig. 6B and C). In addition, intraplantar injection of terameprocol (30 nmol) produced an increase in the number of localized hind limb responses to acetone, similar to that observed for NDGA (Fig. 6C).

Finally, we examined the effect of systemic injection of the TRPA1 antagonist HC 030031 on the responses to near maximal doses of intraplantar CA (300 nmol, $n = 6$) and NDGA (30 nmol, $n = 6$). Systemic injection of HC 030031 (150 mg kg^{-1}) and vehicle did not produce a significant change in the localized hind limb responses to acetone (responses = 0.0 ± 0.3 and 1.1 ± 0.9 for HC 030031 and vehicle, respectively, $P = 0.9$, 0.2, $n = 12$, 9). The increase in localized hind limb responses to acetone produced by CA and NDGA were both significantly less in HC 030031 pretreated animals compared to vehicle pretreated animals (Fig. 6D, $P < 0.001$).

Discussion

The principle finding of this study is that the unspecific lipoxygenase inhibitor NDGA (Gregus et al. 2013) and its

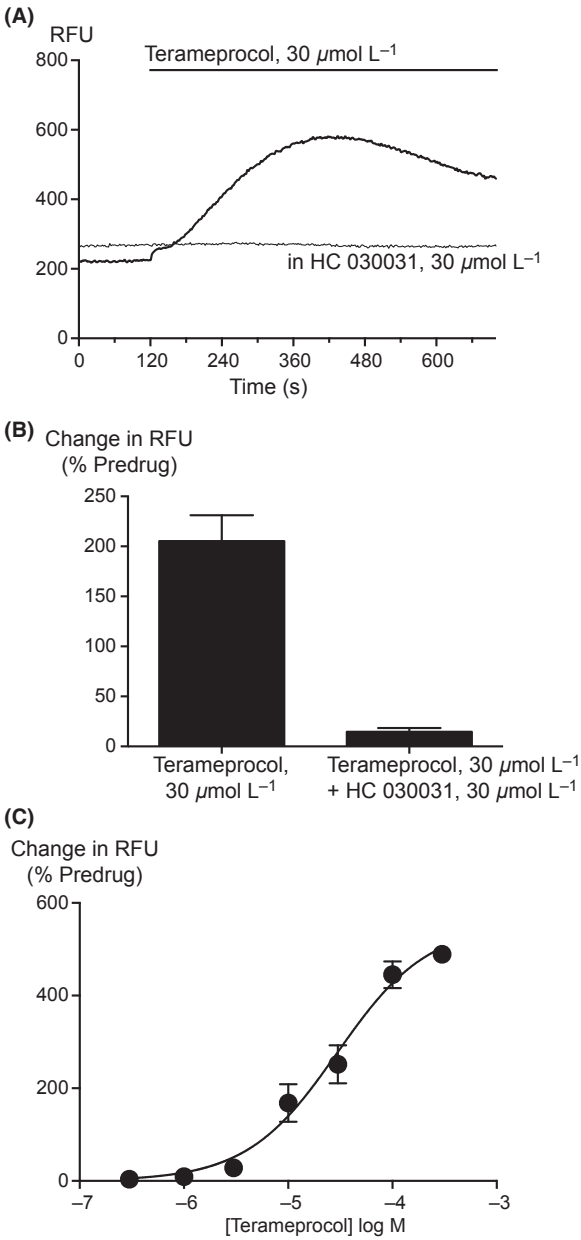

Figure 5. Terameprocol effectively activates hTRPA1. Changes in intracellular calcium ($[Ca]_i$) in HEK293 cells expressing hTRPA1 were measured as outlined as in the Materials and Methods section. (A) Example traces of terameprocol actions on HEK293-hTRPA1 cells with or without preincubation with the TRPA1 antagonist HC 030031. Traces represent the raw relative fluorescence units (RFU). Data from six similar experiments are summarized in (B), with each bar representing the mean ± SEM of the maximum change in calcium 5 fluorescence, HC 030031 significantly inhibited the effects of terameprocol ($P < 0.001$). (C) Concentration-effect curve for terameprocol were fit with a four-parameter logistic equation, each point represents the mean ± SEM of the change in fluorescence (RFU) from six experiments, each performed in duplicate or triplicate. Terameprocol elevated $[Ca]_i$ with an EC_{50} of 30 μmol L^{-1}.

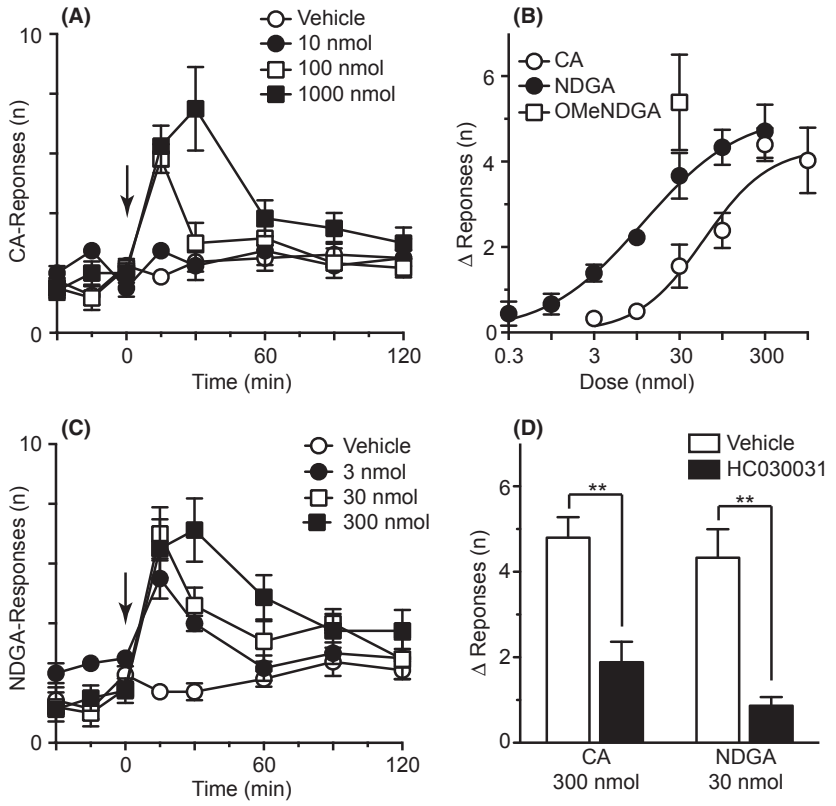

Figure 6. Nordihydroguaiaretic (NDGA) enhances responses to cool stimuli. Time plots of the number of hind paw lifts/shakes/licks in response to brief topical application of acetone (20 μL) before and after intraplantar injection of a range of doses of (A) cinnamaldehyde (CA), (B) NDGA, or matched vehicle. (C) Dose–effect curve of the average increases in acetone-induced hind paw lifts/shakes/licks produced by CA, NDGA and terameprocol (OMeNDGA). (D) Effect of the TRPA1 antagonist HC 030031on the increases in acetone-induced hind paw lifts/shakes/licks produced by CA, NDGA; animals were given an i.p. injection of HC 030031 (150 mg kg^{-1}), or vehicle prior to intraplantar CA/NDGA. In (A) and (B), CA and NDGA were injected at time 0. In (D) $^{**}P < 0.01$.

analog terameprocol are both efficacious agonists at hTRPA1. Consistent with this, both compounds produced enhanced responses to cold stimuli in vivo. These findings suggest that NDGA may not be a suitable probe for lipoxygenase-dependent process in studies of nociception or other responses where sensory nerves modulate tissue activity. They also raise the possibility of a TRPA1-related mechanism contributing to NDGA modulation of cancer cell growth, in addition to the well recognized pathways involving the inhibition of the Sp1 promoter (Hwu et al. 1998; Chang et al. 2004).

NDGA activation of TRPA1 was not likely to be associated with the inhibition of lipoxygenases because we demonstrated in a concomitant study that application of the lipoxygenase inhibitors caffeic acid and *N*-oleoyl dopamine to HEK 293-hTRPA1 cells did not activate TRPA1 (Redmond et al. 2014). The lack of any significant effect of the TRPA1 antagonist HC 030031 alone also indicates that basal activation of pathways producing fatty acid-derived modulators of hTRPA1 is low in HEK 293-hTRPA1 cells, making it unlikely that there is sufficient tone in these pathways for NDGA to shunt substrates to metabolic pathway that produces potent hTRPA1 agonists.

Many reactive molecules such as allyl isothiocyanate (mustard oil), acrolein, iodoacetamide, and CA, activate TRPA1 by binding to or modifying the reactivity of cysteine residues situated on the cytosolic N-terminal domain of the channel (Hinman et al. 2006; Macpherson et al. 2007). The apparent capacity of NDGA to activate hTRPA1 was strongly reduced in cells expressing mutant hTRPA1 with Cys621, Cys 641, and Cys665 mutated to serine. These findings are reminiscent of those for nonreactive activators of hTRPA1 such as arachidonic acid, 5-nitro-2-(3-phenylpropylamino) benzoic acid and lidocaine, which show significantly reduced activity at the 3xCys-mutant hTRPA1 (Leffler et al. 2011; Redmond et al. 2014). This is in contrast to agonists such as CA and allylisothiocyanate, whose activity is essentially abolished by mutation of the N-terminal Cys residues

(Hinman et al. 2006; Redmond et al. 2014). Our data further support the role of N-terminal domain cysteines as crucial for receptor function, in addition to their roles as targets for endogenous and environmental electrophiles.

The present results indicate that NDGA enhances behavioral responses to cool stimuli and that this is TRPA1 mediated. First, intraplantar injection of NDGA and CA alone had no behavioral effects at room temperature, but increased the behavioral nocifensive responses to evaporative cooling during the local topical application of acetone. This is similar to that previously reported for CA and other TRPA1 agonists (del Camino et al. 2010; Gentry et al. 2010) and is indicative of a specific enhancement of responses to cold stimulation. Some studies have observed that TRPA1 agonists produce behavioral responses at room temperature, however, these observations were made with other agents, or at higher doses of CA than used here (Trevisani et al. 2007; Andrade et al. 2008; Eid et al. 2008; Tsagareli et al. 2010). NDGA- and CA-induced behavioral effects displayed dose dependence with similar potency and efficacy, consistent with results in vitro. Finally, the behavioral effect of both NDGA and CA were reduced by the TRPA1 antagonist HC 030031, which has previously been shown to inhibit TRPA1-mediated behaviors in vivo (Eid et al. 2008). The agonist actions of NDGA on a key detector of a subset of noxious sensory information suggests that its usefulness in animal studies of sensory function is limited, as it seems to inhibit the activity of enzymes producing pronociceptive compounds (Gregus et al. 2012, 2013), but at the same time be directly activating their potential target. Hitherto unexplained effects of NDGA have been reported in airway smooth muscle (Henry 1994), a tissue where TRPA1 may be expressed on both neuronal and non-neuronal cells (Caceres et al. 2009; Nassini et al. 2012).

NDGA and its derivative terameprocol are potential chemotherapeutic agents. Their anti-cancer activity has been demonstrated to arise through to their binding to the Sp1 transcription site on genes involved in regulation of cell cycle progression. These effects of NDGA and terameprocol on cell survival and Sp1-mediated transcription occur at concentrations between 10 and 100 μmol L^{-1} (Chen et al. 1998; Hwu et al. 1998; Castro-Gamero et al. 2013), concentrations that produce a significant activation of TRPA1. By contrast to the inhibitory effects of Sp1 inhibition on cell growth, recent findings indicate that activation of TRPA1 may promote the survival of small cell lung cancer cells (SCLC, Schaefer et al. 2013), and contribute to a more invasive phenotype in vitro (Du et al. 2014). TRPA1 has also been strongly implicated as a mediator of chemotherapy-induced peripheral neuropathy (CIPN, Nassini et al. 2011), a major dose-limiting effect of oxaliplatin and cisplatin. Interestingly, available evidence suggests that unlike NDGA or terameprocol, oxaliplatin and cisplatin activate TRPA1 indirectly via ROS (Nassini et al. 2011). Given that continuous activation of TRPA1 can lead to profound channel desensitization (Ibarra and Blair 2013; Redmond et al. 2014), it is an open question whether chronic administration of a TRPA1 agonist like NDGA or terameprocol will lead to exacerbation or inhibition of CIPN or SCLC invasiveness. There is very little information about the systemic administration of terameprocol to humans, however, neuropathic pain was not noted in the most comprehensive report to date (Grossman et al. 2012). The inhibition of SP1 activity by NDGA and derivatives has also been explored in the context of inhibition of viral replication (Chen et al. 1998; Hwu et al. 1998), although this has not progressed as far as clinical trials.

NDGA also affects a variety of voltage-gated Ca (I_{Ca}, Korn and Horn 1990; Hatton and Peers 1997) and K channels (Hatton and Peers 1997), as well as the Mg-inhibitable transient receptor potential melastatin-like 7 channel (TRPM7, Chen et al. 2010), independently of effects on lipoxygenase. The effects on I_{Ca} and TRPM7 are inhibitory and occur over concentration ranges of (5–50 μmol L^{-1}). NDGA effects on K currents (I_K) are more complex, with inhibition of voltage-gated I_K contrasting with an efficacious stimulation of large-conductance Ca-activated I_K. NDGA activity on these channels may contribute to the chemotherapeutic or other effects of the compound, however, the effects of NDGA on TRPA1 in vitro and cold stimulation in vivo were blocked by an antagonist of TRPA1, indicating a central role for the interaction of NDGA with this channel in the effects we observed.

In summary, NDGA and its derivative terameprocol are ligands with a similar efficacy and affinity for the human TRPA1 receptor in vitro. As would be expected from a TRPA1 ligand, local administration of NDGA caused enhanced behavioral responses to noxious cold stimuli. The identification of TRPA1 as another potential site of action for NDGA and its derivatives raises interesting possibilities about new therapeutic mechanisms of action as well as potential adverse effects of this class of compounds.

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Author Contributions

M. C. Sr. and W. J. R. conceived the study. W. J. R. performed the calcium measurements, M. C. Jr. did the elec-

trophysiology, C. W. V and V. M. did the behavioral work. W. J. R. and M. C. Sr. wrote the paper.

Disclosures

None declared.

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