4-Oxo-2-nonenal (4-ONE): Evidence of Transient Receptor Potential Ankyrin 1-Dependent and -Independent Nociceptive and Vasoactive Responses In Vivo

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ABSTRACT

This study explores the in vivo effects of the proposed transient receptor potential ankyrin 1 (TRPA1) agonist 4-oxo-2-nonenal (4-ONE). Pharmacological inhibitors and genetically modified mice were used to investigate the ability of 4-ONE to act via TRPA1 receptors and possible mechanisms involving transient receptor potential vanilloid 1 (TRPV1). We hypothesized that 4-ONE activates sensory nerves, via TRPA1 or possibly TRPV1, and thus triggers mechanical hyperalgesia, edema formation, and vasodilatation in mice. An automated dynamic plantar aesthesiometer was used to determine hind paw withdrawal thresholds, and a laser Doppler flowmeter was used to measure skin blood flow. Edema formation was determined by measuring paw weights and thickness. 4-ONE (10 nmol) triggers unilateral mechanical hyperalgesia, edema formation, and vasodilatation in mice and is shown here to exhibit TRPA1-dependent and -independent effects. Neurogenic vasodilatation and mechanical hyperalgesia at 0.5 h postinjection were significantly greater in TRPA1 wild-type (WT) mice compared with TRPA1 knockout (KO) mice. Edema formation throughout the course time as well as mechanical hyperalgesia from 1 to 4 h postinjection were similar in WT and TRPA1 KO mice. Studies involving TRPV1 KO mice revealed no evidence of TRPV1 involvement or interactions between TRPA1 and TRPV1 in mediating the in vivo effects of 4-ONE. Previously, 4-ONE was shown to be a potent TRPA1 agonist in vitro. We demonstrate its ability to mediate vasodilatation and certain nociceptive effects in vivo. These data indicate the potential of TRPA1 as an oxidant sensor for vasodilator responses in vivo. However, 4-ONE also triggers TRPA1-independent effects that relate to edema formation and pain.

Introduction

Transient receptor potential ankyrin 1 (TRPA1) is the most recently identified mammalian member of the TRP superfamily that contains six transmembrane domains with a pore-forming region located between the fifth and sixth transmembrane domains (Clapham, 2003). Functional TRPA1, which is likely to assemble as a tetramer, conducts cations such as Na⁺ and Ca²⁺ upon activation (Barritt and Rychkov, 2005). TRPA1 is primarily considered to be expressed in sensory neurons, where it is coexpressed in approximately 50% of all transient receptor potential vanilloid 1 (TRPV1)-positive sensory neurons that also contain and release the neuropeptides substance P and calcitonin gene-related peptide (CGRP) (Gepetti et al., 2008; Nassenstein et al., 2008; Streng et al., 2008).

TRPA1 was initially identified as the receptor for a variety of pungent compounds that can be extracted from wasabi (6-(methylsulfinyl)hexyl isothiocyanate), mustard oil (allyl isothiocyanate), cinnamon (cinnamaldehyde), or garlic (alllicin and diallyl disulfide) (Bandell et al., 2004; Bautista et al., 2005, 2006; Macpherson et al., 2007b). It is now realized that TRPA1 can be activated by a range of compounds including reactive oxygen species such as hydrogen peroxide as well as...
products of lipid peroxidation such as 4-hydroxynonenal (4-HNE), and the prostaglandin metabolite 15-deoxy-Δ12,14-prostaglandin J2 (Trevisani et al., 2007; Andersson et al., 2008; Sawada et al., 2008; Taylor-Clark et al., 2008b). Activation is caused by the possession of an electrophilic carbon or sulfur (Macpherson et al., 2007a) that acts via covalent modifications of nucleophilic cysteine side chains in the intracellular N terminus of TRPA1 (Hinman et al., 2006; Macpherson et al., 2007a). Several TRPA1 agonists have been shown to trigger acute pain behaviors, hyperalgesia and neurogenic inflammation in animal and human studies (Eid et al., 2008). Our most recent evidence shows that mustard oil and cinnamaldehyde induce neurogenic vasodilation in mouse skin that is TRPA1-dependent (Pozsgai et al., 2010). Far less is known about the potential of lipid peroxidation products to act as endogenous mediators of TRPA1-dependent peripheral responses. Evidence indicates that 4-HNE acts in vivo to mediate nociception and neurogenic inflammation via TRPA1 (Trevisani et al., 2007), and 4-ONE activates mouse bronchopulmonary fibers via TRPA1 and TRPV1-dependent mechanisms in vitro (Taylor-Clark et al., 2008a). It is noteworthy that 4-ONE has not previously been used in vivo. However, it has been shown to be the most potent activator of TRPA1 in vitro through study of TRPA1 expressed in Chinese hamster ovary cells with an EC50 of 1.9 μM compared with an EC50 of 19.9 μM for 4-HNE (Andersson et al., 2008) and also in the mouse lung (Taylor-Clark et al., 2008b). These in vitro data are consistent with the higher chemical reactivity of 4-ONE (Lin et al., 2005). Here, we have examined the dependence of 4-ONE on TRPA1 in vivo to influence peripheral nociceptive and peripheral vascular responses, which is of potential importance in understanding the response of the peripheral tissues to oxidants. We have also investigated the possible involvement of TRPV1 in these responses.

Materials and Methods

Animals. All experiments were carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act of 1986. Female CD1 mice (25–30 g) were purchased from Charles River (Margate, Kent, UK). We used age- and sex-matched male and female wild-type (WT) and TRPV1 knockout (KO) mice on a C57BL6/129SvJ strain (Clark et al., 2007) as well as WT and TRPA1 KO mice on a mixed genetic background as described previously (Kwan et al., 2006; Andersson et al., 2008; Stark et al., 2008) and also in the mouse lung (Taylor-Clark et al., 2008b). These in vitro data are consistent with the higher chemical reactivity of 4-ONE (Lin et al., 2005). Here, we have examined the dependence of 4-ONE on TRPA1 in vivo to influence peripheral nociceptive and peripheral vascular responses, which is of potential importance in understanding the response of the peripheral tissues to oxidants. We have also investigated the possible involvement of TRPV1 in these responses.

Chemicals, Trevillet, UK); ketamine (C13H16ClNOHCl) and medetom-idine (C24H44O6) (Sigma-Aldrich). 

Statistical Analysis. Results are presented as mean ± S.E.M. Prism software (GraphPad Software Inc., San Diego, CA) was used for two-sampled paired or unpaired Student’s t tests or one-way or two-way analysis of variance followed by Bonferroni’s multiple comparison test or a repeated-measures analysis of variance followed by Dunnet’s comparison test as required by the data. Values of p<0.05 were considered statistically significant.

Results

4-ONE Induces Dose- and Time-Dependent Mechanical Hyperalgesia and Edema Formation in CD1 Mice. The effects of intraplantar injections of 1 nmol to 30 nmol 4-ONE in 50 μl in the ipsilateral paw or 50 μl of vehicle, 1% ethanol in saline, in the contralateral paw were examined. Intraplantar injections of 10 and 30 nmol 4-ONE induced a significant reduction in paw withdrawal thresholds compared with respective baseline values, thus signifying the development of mechanical hyperalgesia (Fig. 1, a and b). Lower doses had no observed effect. Likewise, only the higher

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118 Graepel et al.
doses of 10 or 30 nmol 4-ONE were seen to induce a significant increase in paw mass compared with that of vehicle-treated paws (Fig. 1c). It has to be noted that both vehicle- and 4-ONE-treated paws at all concentrations showed a larger paw mass than untreated paws, which was probably caused by an injection volume artifact. Furthermore, 4-ONE could elicit a significant decrease in withdrawal threshold when injected into both hind paws of the same mouse (data not shown).

A dose of 4-ONE (10 nmol) was then chosen to study the time-dependent effects of 4-ONE. Paw withdrawal thresholds (g) were measured at baseline and throughout a 24-h time course. There was a significant reduction in paw withdrawal thresholds from 0.5 to 4 h postinjection of 4-ONE-treated paws compared with baseline values. This demonstrates that 10 nmol 4-ONE induced a unilateral mechanical hyperalgesia that had an immediate onset and lasted until 4 h postinjection (Fig. 1d). Paw thickness (mm) was followed throughout the time course. Both 4-ONE- and vehicle-treated paws showed significant initial increase in paw thickness until 1.5 h postinjection compared with baseline paw thickness, because of an injection volume artifact. It is noteworthy that 4-ONE induced a significant increase in paw thickness throughout the 24-h time course, whereas paw thickness of vehicle-treated mice returned to baseline values by 1.5 h postinjection (Fig. 1e).

4-ONE Has Both TRPA1-Dependent and -Independent Effects. To determine whether the in vivo effects of 4-ONE were mediated by TRPA1, we used both the established TRPA1 antagonist AP18 and WT and TRPA1 KO mice. 4-ONE induced a significant reduction in paw withdrawal thresholds (g) at 0.5 and 1 h postinjection when paws were pretreated with the vehicle for AP18. However, in paws that were pretreated with the TRPA1 antagonist AP18, 4-ONE...
induced mechanical hyperalgesia at 1 h postinjection but not at 0.5 h postinjection (Fig. 2a). In addition, there was no significant difference between paw thickness (mm) of vehicle- and 4-ONE-treated hind paws (Fig. 2b). To determine the selectivity of the dose of TRPA1 antagonist used, we tested it in WT and TRPA1 KO mice. Here, the response to 4-ONE in WT mice was significantly attenuated at 0.5 h, and a similar response was observed in TRPA1 KO mice, whether they received the antagonist or not (Fig. 3c). Again, 4-ONE-induced edema formation was not affected by deletion or antagonism of TRPA1 (Fig. 3d). Higher doses were not used, because preliminary evidence suggested that higher doses induced a nonspecific inflammation.

These results were followed up using WT and TRPA1 KO mice that were given intraplantar injections of 4-ONE (10 nmol) or vehicle. In WT mice, intraplantar 4-ONE induced a significant reduction in paw withdrawal thresholds from 0.5 h postinjection until 6 h postinjection (Fig. 2c). There was no change in paw withdrawal thresholds in vehicle-treated paws. The mechanical hyperalgesia seemed more sustained in TRPA1 WT mice than in CD1 mice (Fig. 1a). We were surprised to find that in TRPA1 KO mice intraplantar injections of 4-ONE triggered significant mechanical hyperalgesia at 1, 2, and 4 h postinjection but not at 0.5 and 6 h postinjection (Fig. 2c). 4-ONE induced a significant increase in paw thickness compared with baseline values in both WT and TRPA1 KO mice (Fig. 2d).

**TRPV1 Receptors Are Not Involved in Mediating 4-ONE-Induced Responses.** The above findings led to an investigation of the possible contribution of TRPV1 receptors to 4-ONE-induced in vivo responses in WT and TRPV1 KO mice. Previous evidence demonstrated that TRPA1 and TRPV1 receptors can be colocalized in sensory nerves and that 4-ONE activities may involve a TRPV1 component (Taylor-Clark et al., 2008a). In addition, to investigate a possible interaction of the TRPA1 and TRPV1 receptors these mice were pretreated with the TRPA1 receptor antagonist AP18. WT and TRPV1 KO mice pretreated with vehicle developed mechanical hyperalgesia at 0.5 and 1 h after the injection of 4-ONE. On the other hand, AP18 pretreatment in WT and TRPV1 KO mice blocked 4-ONE-induced mechanical hyperalgesia at 0.5 h postinjection but not at 1 h postinjection (Fig. 3a). Vehicle injections did not change paw withdrawal thresholds in any of the treatment conditions. Paw weights were taken at 1 h postinjection to determine 4-ONE-induced edema formation, and it was seen that 4-ONE-induced edema formation is independent of TRPV1 as well as TRPA1 receptors (Fig. 3b).

**4-ONE-Induced Vasodilatation Depends on TRPA1 and CGRP.** The effect of 10 nmol 4-ONE on hind paw blood flow was investigated. Previous studies have shown that topical application of mustard oil induces a potent TRPA1-dependent vasodilatation (Pozsgai et al., 2010), but 4-ONE is not absorbed into the skin via topical application. Here, intraplantar injections of 50 μl of 4-ONE or vehicle were given into either hind paw, and plantar blood flow was measured for 30 min. In genetically unaltered CD1 mice 10 nmol 4-ONE induced a significant and sustained increase in blood flow compared with vehicle-treated paws at corresponding time points postinjection (Fig. 3a). Vehicle injections did not change paw withdrawal thresholds in any of the treatment conditions. Paw weights were taken at 1 h postinjection to determine 4-ONE-induced edema formation, and it was seen that 4-ONE-induced edema formation is independent of TRPV1 as well as TRPA1 receptors (Fig. 3b).

**Fig. 2.** 4-ONE has both TRPA1 receptor-dependent and -independent in vivo effects. a, hind paws were pretreated with the TRPA1 receptor antagonist AP18 (25 nmol/25 μl) or vehicle (25 μl of 1% DMSO, 0.5% Tween 80 in PBS) and were then treated with 4-ONE (10 nmol/25 μl) or vehicle (25 μl of 1% ethanol in saline). Paw withdrawal thresholds (g) were measured at 0.5 and 1 h postinjection in CD1 mice. Results are shown as mean ± S.E.M. **+++, p < 0.001** versus baseline; **††, p < 0.01** versus 4-ONE-treated paws in vehicle-treated mice (n = 8–9). b, paw thickness (mm) at baseline and 0.5 and 1 h postinjection are shown as a measure of edema formation. Results are shown as mean ± S.E.M. **+++, p < 0.001** versus baseline values; **+++††, p < 0.001** versus vehicle-pretreated paws at corresponding time points postinjection (n = 8–9). c, paw withdrawal thresholds (g) were measured throughout a 24-h time course in WT and TRPA1 KO mice after the intraplantar injection of vehicle or 4-ONE (10 nmol). Results are shown as mean ± S.E.M. **+++, p < 0.01; ++++, p < 0.001** versus baseline (n = 8). d, paw thickness (mm) was measured in WT and TRPA1 KO mice after the intraplantar injection of vehicle or 4-ONE (10 nmol) throughout the 24-h time course. Results are shown as mean ± S.E.M. **++, p < 0.01 and ++++, p < 0.001** versus baseline for TRPA1 WT mice; **†, p < 0.05 and +++†††, p < 0.001** versus baseline for TRPA1 KO mice (n = 13).
A range of genetically modified mice were then used to investigate signaling mechanisms involved in 4-ONE-induced vasodilation. It is noteworthy that 4-ONE induced a vasodilatation measured over 0.5 h that depended on TRPA1 but was independent of TRPV1 (Fig. 4, b and c). In addition, 4-ONE...
induced a vasodilatation in CGRP WT mice that was not observed in CGRP KO mice, further pointing toward the activation of peripheral sensory nerves by 4-ONE (Fig. 4d). It is noteworthy that in both WT and CGRP KO mice 10 nmol 4-ONE triggered a significant reduction in paw withdrawal thresholds compared with baseline values from 0.5 to 2 h postinjection (Fig. 5a). In addition, 10 nmol 4-ONE triggered a significant increase in paw mass at 2 h postinjection in both WT and CGRP KO mice (Fig. 5a). This indicates that, although CGRP is pivotal for the development of 4-ONE-induced vasodilatation, it is not involved in mediating pain signals.

**Discussion**

This study has examined the in vivo effects of 4-ONE, a selective TRPA1 agonist in vitro (Andersson et al., 2008; Taylor-Clark et al., 2008a). We hypothesized that activation of TRPA1, which is expressed mainly on peptidergic C and Aδ-fibers, by 4-ONE would trigger the development of mechanical hyperalgesia and neurogenic edema and also lead to vasodilatation in vivo. Here, we demonstrate that 4-ONE (10 nmol) induced a dose- and time-dependent unilateral mechanical hyperalgesia and edema formation in vivo, with only a component of the mechanical hyperalgesia mediated by TRPA1 and no evidence for a role of TRPA1 in inflammatory swelling. We present novel evidence that 4-ONE has the ability to trigger TRPA1-dependent neurogenic vasodilatation in vivo.

4-ONE is an electrophilic ketoaldehyde that is derived from oxidized ω-6-polyunsaturated fatty acids such as arachidonic acid and can function as a mediator of oxidative stress (Lin et al., 2005). 4-ONE can form stable Michael adducts with cysteine and lysine residues in vitro and activate TRPA1 because of its electrophilic properties (Uchida, 2000; Lin et al., 2005; Andersson et al., 2008). These amino acids are commonly found in proteins and can thus be targeted by 4-ONE. 4-ONE is broken down enzymatically in vitro and in vivo, leading to the formation of reactive metabolites such as 4-HNE or 4-oxo-2-nonen-1-ol (Kuiper et al., 2008; Shimozu et al., 2009). In addition, it can form conjugates with other proteins such as glutathione (Kuiper et al., 2008). It is possible that 4-ONE metabolites or reactions with other proteins contribute to the TRPA1-independent effects seen in the present study. However, 4-ONE has been shown to selectively activate TRPA1 in dissociated dorsal root ganglion neurons in vitro from WT but not TRPA1 KO mice (Andersson et al., 2008). In addition, Taylor-Clark et al. (2008a) demonstrated that 4-ONE activates vagal bronchopulmonary C-fibers in vitro via TRPA1. In the present study, we have extended knowledge to demonstrate that 4-ONE induces unilateral mechanical hyperalgesia, edema formation, and vasodilatation in mice; 10 nmol 4-ONE was chosen for all detailed studies, and it was shown that 4-ONE-induced mechanical hyperalgesia is present from 0.5 h postinjection until 4 h postinjection. Similar results were previously seen with the related TRPA1 agonist 4-HNE, where the substantially higher dose of 150 nmol 4-HNE triggered unilateral mechanical hyperalgesia and edema in the rat hind paw (Trevisani et al., 2007). It is evident that 4-ONE is more potent at inducing mechanical hyperalgesia than 4-HNE in vivo, in keeping with in vitro data (Lin et al., 2005; Andersson et al., 2008). However, it seems that although 4-HNE displays a selectivity for TRPA1 in vitro and in vivo, 4-ONE-induced in vivo responses are only partially mediated by the TRPA1 receptor. It is noteworthy that Taylor-Clark et al. (2008) suggested that 4-ONE has the ability to activate TRPV1 receptors in vitro; however, we did not observe TRPV1 receptor-dependent responses using the present protocols in vivo.

Hyperalgesia develops as a result of activation of sensory C-fibers that express a range of receptors and ion channels, including TRPA1 and TRPV1, to be able to integrate various noxious stimuli. We hypothesized that 4-ONE induces mechanical hyperalgesia as well as edema formation by activating TRPA1 and possibly TRPV1 that are coexpressed on these sensory C-fibers. Petrus et al. (2007) have discovered a small-molecule TRPA1 receptor antagonist, AP18. This TRPA1 antagonist has been shown to block human and mouse TRPA1 in vitro and coinjection with 1 mM AP18 reduces mustard oil-induced nocifensive behavior in vivo (Petrus et al., 2007; C. Gentry, personal communication). We demonstrated that local pretreatment with 25 μl of a 1 mM AP18 solution inhibits 4-ONE-induced mechanical hyperalgesia at 0.5 h postinjection but not at 1 h postinjection (Fig.
2a). It is noteworthy that Petrus et al. only showed early time points when they examined the inhibition of TRPA1 activation. In their studies, coinjection with 1 mM AP18 inhibited the acute nocifensive behavior induced by cinnamaldehyde during the first 5 min after injection (Petrus et al., 2007), and AP18 partially blocked the early phase of bradykinin-evoked mechanical hyperalgesia, which is thought to be mediated by activation of TRPA1 (Petrus et al., 2007; Wang et al., 2008). This indicates that AP18 has a short half-life in vivo; however, intraplantar injections of 1 mM AP18 that were given 24 h after complete Freund’s adjuvant treatment were able to reverse complete Freund’s adjuvant-induced mechanical hyperalgesia at 1 and 2 h postinjection (Petrus et al., 2007; Fernandes et al., 2010). In the present study, TRPA1 KO mice did not develop 4-ONE-induced mechanical hyperalgesia at 0.5 h postinjection although mechanical hyperalgesia was observed from 1 to 4 h postinjection in TRPA1 KO mice. This indicates that TRPA1 is not a necessary prerequisite for 4-ONE-induced mechanical hyperalgesia, although a TRPA1 component exists. There was no difference in 4-ONE-induced edema formation between WT and TRPA1 KO mice, indicating that 4-ONE-induced edema formation is independent of TRPA1. In addition, pretreatment with the TRPA1 antagonist AP18 at a dose shown here to be selective blocked 4-ONE-induced mechanical hyperalgesia at 0.5 h postinjection, consistent with the results obtained in TRPA1-deficient mice. Thus these results indicate that 4-ONE is able to act via TRPA1-dependent and -independent pathways to mediate mechanical hyperalgesia.

We next investigated whether TRPV1 underlies the TRPA1-independent effects of 4-ONE. It is well documented that TRPV1 acts as a molecular integrator of several noxious stimuli and thus plays a key role in sensing tissue injury and inflammation (Caterina et al., 1997). Indeed, Taylor-Clark et al. (2008a) have demonstrated that activation of TRPV1 by capsaicin inhibited 4-ONE-induced responses. This study highlights the importance of in vivo research, because previous in vitro experiments suggested that 4-ONE could be a selective TRPA1 agonist for in vivo studies. The present study supports the concept that TRPA1 receptors can act as oxidant sensors in vivo and provides evidence that this leads to increased blood flow, in addition to nociceptive responses.

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Authorship Contributions

Participated in research design: Graepel, Bevan, and Brain.
Conducted experiments: Graepel, Fernandes, and Aubdool.
Contributed new reagents or analytic tools: Andersson and Bevan.
Performed data analysis: Graepel, Fernandes, and Aubdool.
Wrote or contributed to the writing of the manuscript: Graepel, Aubdool, Andersson, Bevan, and Brain.
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