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An Odorant Derivative as an Antagonist for an Olfactory Receptor

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Abstract

Different odorants are recognized by different combinations of G protein-coupled olfactory receptors, and thereby, odor identity is determined by a combinatorial receptor code for each odorant. We recently demonstrated that odorants appeared to compete for receptor sites to act as an agonist or an antagonist. Therefore, in natural circumstances where we always perceive a mixture of various odorants, olfactory receptor antagonism between odorants may result in a receptor code for the mixture that cannot be predicted from the codes for its individual components. Here we show that stored isoeugenol has an antagonistic effect on a mouse olfactory receptor, mOR-EG. However, freshly purified isoeugenol did not have an inhibitory effect. Instead, an isoeugenol derivative produced during storage turned out to be a potent competitive antagonist of mOR-EG. Structural analysis revealed that this derivative is an oxidatively dimerized isoeugenol that naturally occurs by oxidative reaction. The current study indicates that as odorants age, they decompose or react with other odorants, which in turn affects responsiveness of an olfactory receptor(s).

Key words: antagonist, isoeugenol, odorant, olfactory receptor

Introduction

The detection of thousands of volatile odorous compounds is mediated by the G protein-coupled olfactory receptor (OR) superfamily, which are expressed by olfactory neurons in the olfactory epithelium (Buck, 1996; Firestein, 2001; Touhara, 2002; Mombaerts, 2004). Functional studies of ORs have demonstrated that individual odorants are recognized by multiple ORs, whereas an OR can recognize multiple odorants. Therefore, odorant identities are determined by a combinatorial receptor code wherein different odorants are recognized by specific sets of ORs (Malnic *et al.*, 1999; Touhara *et al.*, 1999; Kajiya *et al.*, 2001). Structure–activity relationship studies suggest that the ligand binding site of ORs can discriminate subtle differences in chemical structures of odorants, but also tolerate large changes in other molecular features (Zhao *et al.*, 1998; Touhara *et al.*, 1999; Wetzel *et al.*, 1999; Araneda *et al.*, 2000; Kajiya *et al.*, 2001; Gaillard *et al.*, 2002).

In natural circumstances, we always perceive a mixture of various odorants. According to the combinatorial receptor code hypothesis, a receptor code for an odorant mixture is expected to be the simple sum of codes for its components. However, psychophysical experiments have demonstrated that the perceived intensity of an odorant mixture tends to be smaller than the sum of the intensities of its components, a process called counteraction (i.e. reduction of odor intensity) or masking (i.e. modification of perceived odor) (Arctander, 1960; Cain and Drexler, 1974). The recent discovery of antagonism between odorants at the receptor level has revealed a molecular mechanism for odorant mixture counteraction (Oka *et al.*, 2004). Thus, odorants not only stimulate some ORs, but they also inhibit or antagonize other ORs (Araneda *et al.*, 2000; Spehr *et al.*, 2003; Oka *et al.*, 2004). In other words, individual odorants appear to have dual functions as OR agonists and antagonists, thus competing with each other at odorant binding sites in the olfactory epithelium.

The mOR-EG, a mouse OR encoded by the MOR174-9 gene (Zhang and Firestein, 2002) has been shown to recognize eugenol (EG), a main odorous compound in clove oil, and other structurally similar odorants (Kajiya *et al.*, 2001). We recently showed that methyl isoeugenol and isosafrol inhibited EG-induced Ca^{2+} responses of mOR-EG as competitive antagonists in HEK293 cells and in olfactory receptor neurons (Oka *et al.*, 2004). In addition, during the course of screening for antagonists, we found that isoeugenol inhibited EG responses in mOR-EG-expressing

HEK293 cells. Freshly purified isoeugenol, however, did not show an inhibitory effect, which let us to speculate that a compound(s) produced in isoeugenol during storage was an antagonist of mOR-EG. Herein we report that this naturally occurring compound is an oxidatively dimerized isoeugenol (OD-IEG) produced from isoeugenol and that it is a potent competitive antagonist for mOR-EG. The current study also points out an important caveat in olfactory research using odorous chemicals: a small amount of an impurity or derivative may have drastic effects on the odorant–OR interaction.

Materials and methods

Odorant compounds

Odorants utilized in this study were kindly provided by T. Hasegawa Co., Ltd or were purchased from Wako Co., Ltd (Tokyo, Japan) or Sigma-Aldrich (Tokyo, Japan). Odorants were kept at 4°C and their purities were checked by thin-layer chromatography (TLC) before experiments. Stored isoeugenol was utilized after storing pure isoeugenol for ~3 years at 4°C. Odorants were diluted to the indicated concentrations in Ringer's solution (140 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM sodium pyruvate, 9.4 mM glucose and 10 mM HEPES, pH 7.4).

TLC analysis

Silica-based TLC plates (0.25 mm thickness) used in this study were purchased from Merck Ltd, Tokyo, Japan. Odorant compounds were developed in ethyl acetate:hexane (1:1) and were detected by reaction with phosphomolibdic acid.

Preparation of OD-IEG and guaiacyl isoeugenol

To obtain large amounts of OD-IEG, pure isoeugenol was stirred in flask under an atmosphere of 100% O_2. Stirring for ~24 h resulted in ~10–20% conversion to OD-IEG. The sample was separated by preparative TLC as described for TLC analysis. The OD-IEG was purified by collecting the silica layer of the desired band and extracting it with ethyl acetate. To obtain guaiacyl isoeugenol, equal amounts of guaiacol and isoeugenol were mixed and stirred overnight, and the product was purified in the same way as for OD-IEG. The structure of the compounds was determined by nuclear magnetic resonance (NMR) and liquid chromatography–mass spectrometry (LC–MS).

Ca^{2+}-imaging of HEK293 cells

Ca^{2+}-imaging for mOR-EG-expressing HEK293 cells was performed essentially as described previously (Katada *et al.*, 2003). Briefly, 60–70% confluent HEK293 cells were transfected with 2.0 μg of pME18S-tagged mOR-EG and 1.5 μg of pME18S-Gα_{15} with 2.5 μl lipofectamine 2000/μg DNA (Invitrogen, Tokyo, Japan). Twenty-four to forty-eight hours after transfection, the transfected cells were loaded with 4 μM fura 2-AM for 30 min at 37°C. Odorant solutions were sequentially applied to the cells using a peristaltic pump at a flow rate of 1.5 ml/min for 15 s with a 2.5 min wash with Ringer's solution between stimulus applications. Intracellular Ca^{2+} levels were monitored using an AQUA COSMOS Ca^{2+}-imaging system (Hamamatsu Photonics, Shizuoka, Japan). Antagonist screening was performed essentially as described previously (Oka *et al.*, 2004). Briefly, when inhibitory odorants were screened (Figure 1), each odorant (1 mM) was co-applied with EG (100 μM). To perform quantitative pharmacological studies (Figure 3), an antagonist was applied 10 s prior to and during the application of EG (black bar in Figure 3a).

NMR analysis of OD-IEG

OD-IEG purified by a preparative TLC was dissolved in $CDCl_3$. NMR spectra were acquired at 25°C with a Varian Mercury 300 (^{1}H 300 MHz; Varian, Palo Alto, CA). The chemical shifts were shown on the i-scale in p.p.m. The ^{1}H-NMR spectrum of OD-IEG: δ(300 MHz, $CDCl_3$) 1.01 (3H, dd, J = 3.0, 3.3 Hz), 1.84 (3H, dd, J = 1.5, 5.1 Hz), 3.87 (3H, d, J = 2.1 Hz), 3.90 (3H, d, J = 2.1 Hz), 3.95 (1H, s), 4.06 (1H, dq, J = 6.3, 2.1 Hz), 4.42 (1H, d, J = 8.4 Hz), 5.70 (1H, s), 6.06 (1H, dq, J = 6.6, 9.0 Hz), 6.27 (1H, dd, J = 1.5, 14.1 Hz), 6.58 (1H, d, J = 8.4 Hz), 6.65 (1H, dd, J = 1.8, 6.3 Hz), and 6.80 to 6.91 (4H, m).

LC–MS analysis

The HPLC system consisted of model 1100 pumps, a tunable absorbance detector (Agilent Technologies, Palo Alto, CA) and an Xterra™ MS C_{18} column (C18, 3.0 × 100 mm; 3.5μm; Waters, Milford, MA). A 5–20 μl sample was injected and eluted with a gradient of H_2O (A) and methanol (B) at room temperature and at a flow rate of 0.5 ml/min using the following program: t = 0 min, 20% B; t = 8 min, 100% B; t = 8–11.4 min, 100% B. MS was performed in the ESI positive ion detection mode using a Thermo Electron LCQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA). The ionization source temperature was 270°C and the ionization voltage was 4.5 kV.

Results

Screening of an odorant that inhibits mOR-EG activity

Stimulation of mOR-EG-expressing HEK293 cells with its cognate ligand, EG, elicits an increase in intracellular Ca^{2+} level via Gα_{15} coupling to the mOR-EG, followed by activation of an inositol phosphate-mediated signaling cascade (Kajiya *et al.*, 2001; Katada *et al.*, 2003). Using this assay system, we previously identified methyl isoeugenol and isosafrol as antagonists of mOR-EG (Oka *et al.*, 2004). In addition to these compounds, further screening revealed that stored isoeugenol (see Materials and methods) significantly inhibited the response of mOR-EG in HEK293 cells to EG (Figure 1a, upper panel). The average response in the

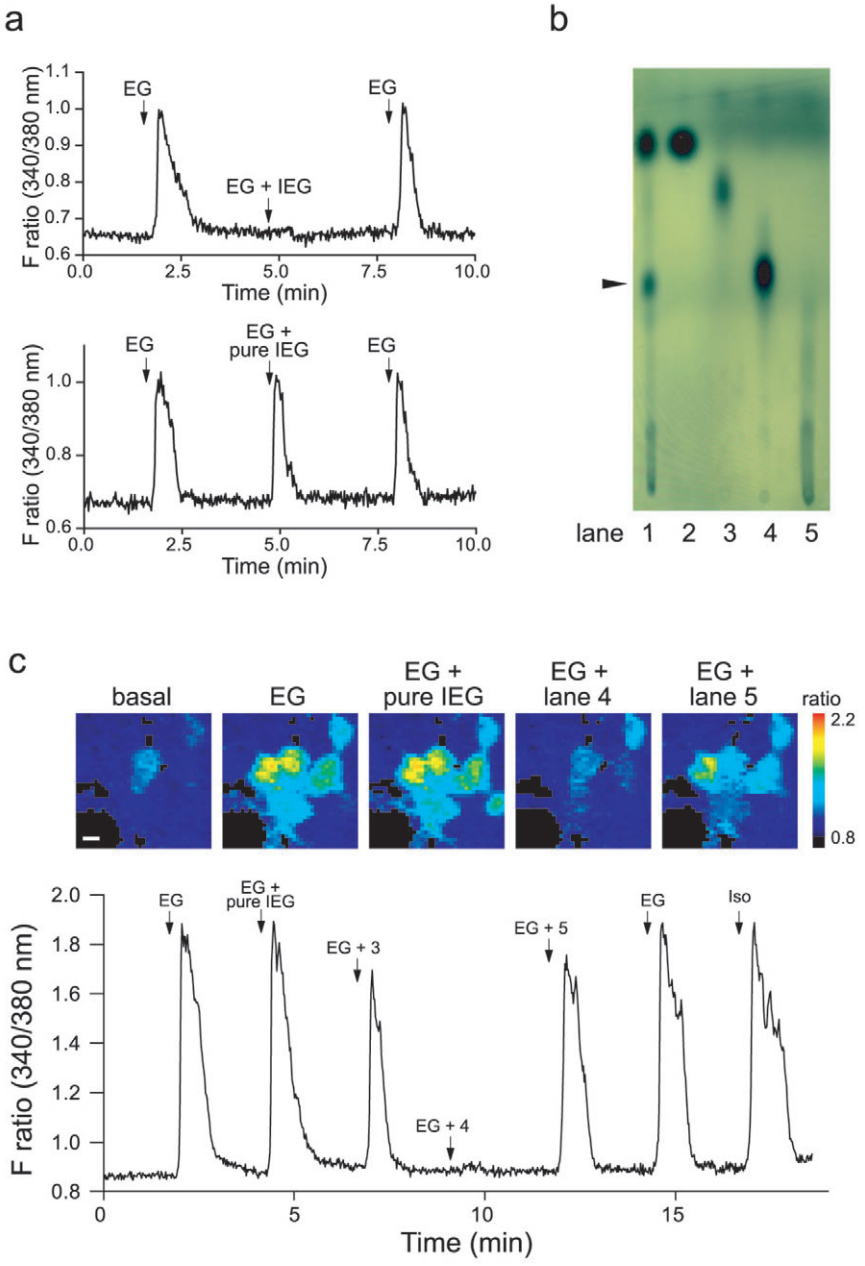

Figure 1 Stored isoeugenol contains a polar isoeugenol derivative that inhibits EG-induced Ca^{2+} responses in HEK293 cells expressing mOR-EG and $G\alpha_{15}$. **(a)** Ca^{2+}-response profiles showing inhibition of EG responses by stored isoeugenol but not by pure isoeugenol. Upper panel: EG (100 μM) was applied for 15 s at the times indicated by arrows with or without stored isoeugenol (1 mM). Bottom panel: pure isoeugenol (1 mM) did not inhibit Ca^{2+} responses. **(b)** Profiles of samples as separated by silica-based TLC in ethyl acetate:hexane (1:1) and stained with phosphomolybdic acid. Arrowhead indicates the major derivative in stored isoeugenol. Lane 1, stored isoeugenol; lane 2, pure isoeugenol; lane 3, compounds eluted before the major product; lane 4, the major derivative indicated by the arrowhead; and lane 5, compounds eluted after the major product. **(c)** Ca^{2+} imaging and a response profile showing inhibition of EG responses by a major derivative in stored isoeugenol. Upper panel: pseudocolored images of HEK293 cells at five representative time points following various stimulations. The change in the fluorescent ratio intensities before and after application of each stimulus is shown in the pseudocolored images, in which red equals the largest change in fluorescent ratio. Scale bar = 10 μm. Bottom panel: a representative Ca^{2+} response profile. The major derivative (arrowhead in b) had inhibitory activity on mOR-EG expressed in HEK293 cells. Other compounds had little effect on the response. Isopreterenol (iso), a β-adrenergic receptor agonist, was applied as a control.

presence of both EG and stored isoeugenol (1:10 ratio) was 16.0 ± 3.3% (mean ± SE; n = 8) of that elicited by EG alone. However, when the same amount of column-purified isoeugenol (pure isoeugenol) was utilized in the experiments, no inhibition was observed (Figure 1a, lower panel). Thus, the average response in the presence of pure isoeugenol was 97.7 ± 3.3% (n = 8) of that elicited by EG alone. Furthermore, although pure isoeugenol is a colorless oil, it gradually becomes yellow and viscous during storage. Thus, we speculated that an isoeugenol derivative(s) produced during

storage was responsible for the inhibition activity on mOR-EG.

To examine this possibility, we used TLC to analyze the purity of stored isoeugenol. In addition to isoeugenol (Figure 1b, lane 2), there was one major and some other minor compounds in the stored sample (Figure 1b, lane 1). We purified the major product (arrowhead in Figure 1b) by preparative TLC (Figure 1b, lane 4) and we found that it significantly inhibited EG responses, whereas the other compounds in the stored isoeugenol sample (Figure 1b, lanes 3 and 5) had little effect (Figure 1c). These results suggested that a derivative in the isoeugenol sample that was produced during storage acted as a mOR-EG antagonist.

Structural determination of the antagonist in stored isoeugenol

We carried out structural analysis of the compound that exhibited an inhibitory activity on mOR-EG by NMR and LC–MS. The NMR spectrum of the compound suggested that it possessed two isoeugenol moieties (Figure 2a). LC

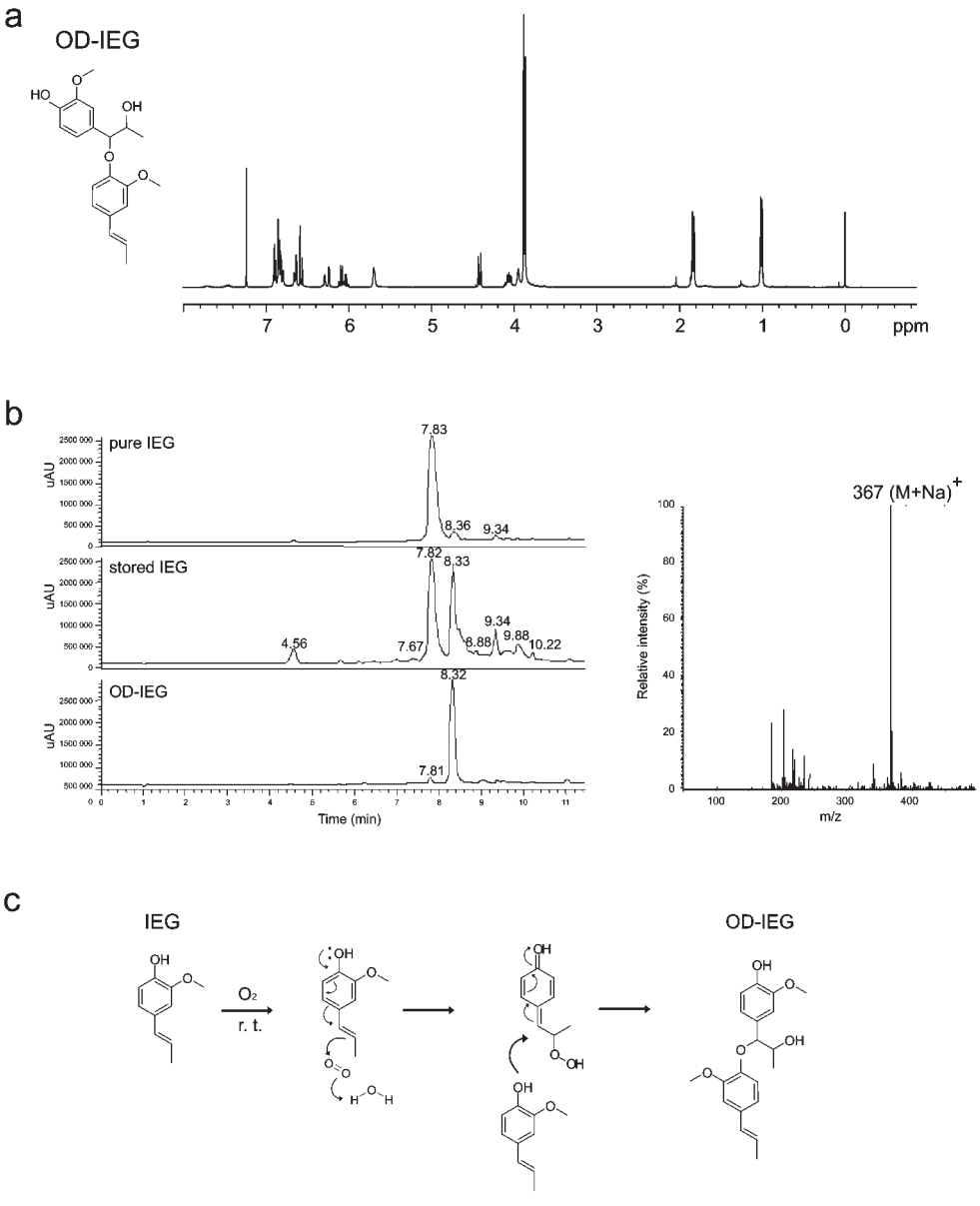

Figure 2 Structural analysis of the isoeugenol derivative that inhibited mOR-EG. **(a)** NMR spectrum of the major derivative in stored isoeugenol (see Figure 1) and its deduced structure. **(b)** LC–MS analysis. Left panel: LC spectra of pure isoeugenol (top), stored isoeugenol (middle) and a purified major derivative (bottom). Pure isoeugenol was eluted at ~7.8 min in chromatograms as detected by absorption at 220 nm. In contrast, the major derivative, which represented ~10% of stored isoeugenol, eluted at ~8.3 min. Right panel: the product ion mass spectrum of the major derivative peak eluted at 8.32 min. The base peak at *m/z* 367 corresponding to $(M + Na)^+$ was detected in the positive mode. **(c)** A proposed mechanism for the oxidative coupling of two isoeugenol molecules. Oxygen assists in the generation of a transition peroxide state that, in turn, is attacked by another isoeugenol molecule. This results in the production of OD-IEG.

analysis showed that isoeugenol eluted at retention time of 7.83 min (Figure 2b, left upper), whereas the major product (arrowhead in Figure 1b, lane 4) was found at 8.32 min (Figure 2b, left bottom). The stored isoeugenol contained a significant amount of this compound (~10%; Figure 2b, left middle). The product ion mass spectrum of the sample eluting at 8.32 min had a base peak corresponding to $(M+Na)^+$ at *m/z* 367 (Figure 2b, right), which was consistent with the mol. wt calculated for the structure deduced by NMR analysis. Because this compound (4-(2-hydroxy-1-[2-methoxy-4-(prop-1-en-1-yl)phenoxy] propyl)-2-methoxyphenol) has not previously been reported, we have named it oxidatively dimerized isoeugenol (OD-IEG) for convenience.

OD-IEG appears to be produced via oxygen-assisted reactions during storage. Stirring of pure isoeugenol for 24 h in the presence of 100% oxygen resulted in ~10–20% conversion to OD-IEG, suggesting that this compound was easily generated by exposure to air. Therefore, this compound was likely produced via the reactions shown in Figure 2c. This intermolecular reaction of isoeugenol appears to be accelerated in the presence of oxygen.

Antagonistic activity of OD-IEG on mOR-EG

To determine whether the reduction of Ca^{2+} responses was derived from competitive inhibition, we examined the effects of various concentrations of OD-IEG on EG-induced responses of HEK293 cells expressing mOR-EG (Figure 3a). OD-IEG was prepared from isoeugenol by stirring overnight in the presence of oxygen and was purified by preparative TLC. The purified OD-IEG was then utilized immediately for experiments. In HEK293 cells expressing mOR-EG and $G\alpha_{15}$, the Ca^{2+}-responses elicited by EG were inhibited by OD-IEG in a dose-dependent manner with an IC_{50} of 10 μM (Figure 3b). It should be of note that the IC_{50} value was much lower than for methyl isoeugenol (66 μM; see Oka *et al.*, 2004), which had previously been the most potent antagonist of mOR-EG. A Ca^{2+}-response was not observed when OD-IEG was applied alone, even at 1 mM, suggesting that OD-IEG was not an agonist or a partial agonist of mOR-EG (data not shown). In addition, the Ca^{2+}-responses elicited by isoproterenol via endogenous β-adrenergic receptors were not affected by OD-IEG, excluding the possibility that the inhibitory activity was due to non-specific effects on signaling downstream of $G\alpha_{15}$ (data not shown). These results demonstrated that OD-IEG is a potent antagonist of the mOR-EG and that it inhibits the response to EG by competing at the receptor ligand binding site.

Identification of OD-IEG in natural fragrant oils

Although OD-IEG has not previously been reported as a natural compound, our results indicated that it might exist in natural fragrant oils containing isoeugenol. Clove oil is known to contain ~1% isoeugenol, although the content depends on its origin. We separated clove oil by preparative TLC and extracted the compounds that migrated around the same Rf value as OD-IEG. LC analysis showed a peak that eluted at 8.28 min and that contained a compound with an *m/z* = 367 on MS, demonstrating that OD-IEG is present in clove oil (Figure 4a). We also found that mace oil, which is made up of ~0.6% IEG, contained OD-IEG. MS/MS analysis confirmed that the *m/z* 367 compounds in clove and mace oil were identical to OD-IEG (Figure 4b). These results indicate that OD-IEG is naturally produced in fragrant oils that contain a modest amount of isoeugenol.

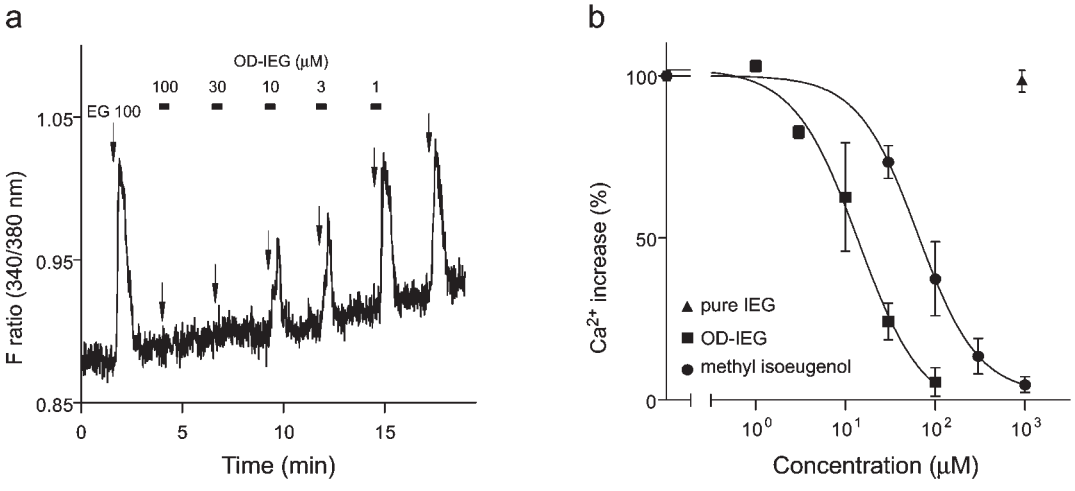

Figure 3 Antagonism of mOR-EG expressed in HEK293 cells by OD-IEG. **(a)** Dose-dependent inhibition of EG-induced Ca^{2+}-increases by OD-IEG. A dose of 100 μM EG (EG 100) was applied to mOR-EG-expressing HEK293 cells for 15 s at the times indicated by arrows. Black bars indicate the periods during which 100, 30, 10, 3, or 1 μM OD-IEG was included in the buffer. The antagonist was applied 10 s prior to and during the application of EG. **(b)** Dose-dependent inhibition of mOR-EG by OD-IEG, methyl isoeugenol, or pure isoeugenol. The data are shown as a percentage of the response to 100 μM EG without OD-IEG, methyl isoeugenol, or pure isoeugenol. Each point represents the mean ± SE from three to six responding cells. Squares, OD-IEG; triangles, pure isoeugenol; circles, methyl isoeugenol. The IC_{50} for OD-IEG was 10 μM.

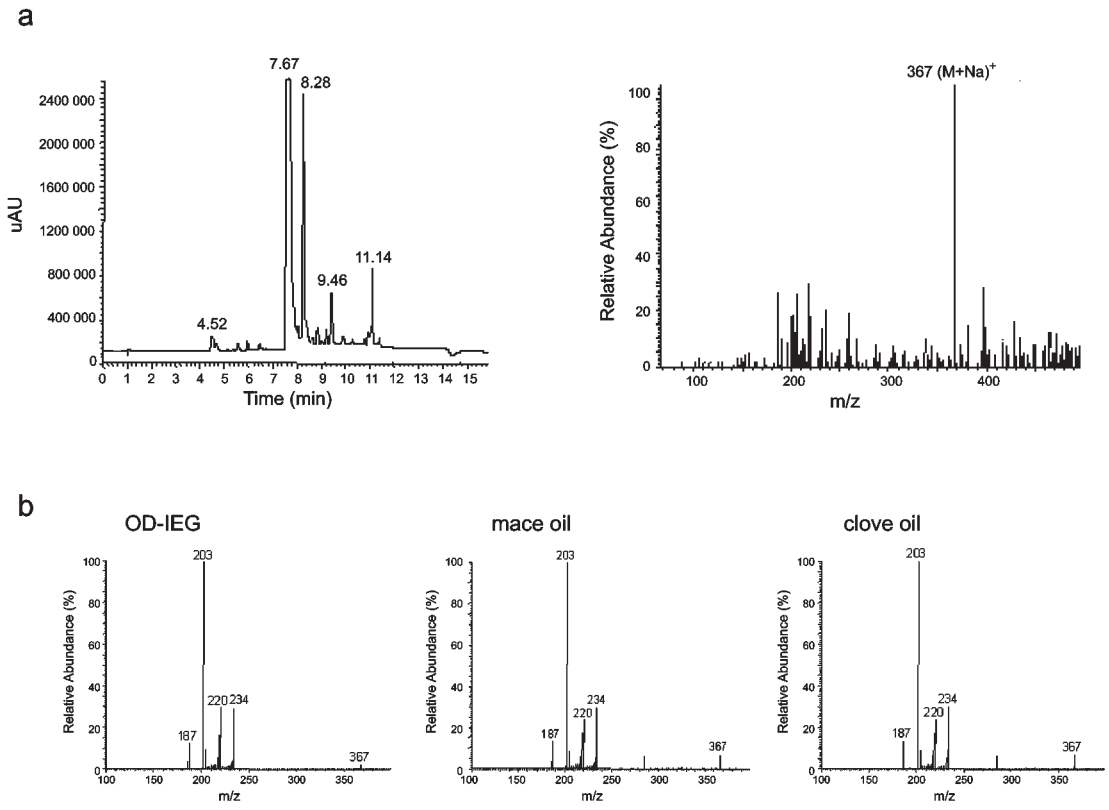

Figure 4 Detection of OD-IEG in various natural fragrant oils by LC–MS. **(a)** Left: LC profile of clove oil components that were extracted from silica TLC plates at the same Rf value as OD-IEG. Right: the product ion mass spectrum of the peak eluted at 8.28 min. The peak corresponding to OD-IEG at *m/z* 367 was detected. **(b)** MS/MS analysis of the peak at *m/z* 367 from clove and mace oils. The spectrum for both oils was identical to that for OD-IEG.

Discussion

In the current studies, we identified a novel, naturally occurring compound that is oxidatively produced by the intermolecular reaction of two isoeugenol molecules. This compound, OD-IEG, is a potent antagonist for the mouse olfactory receptor mOR-EG. This is the first evidence of a natural derivative of an odorant that affects the responsiveness of a particular OR. Our results also reveal a caveat in experiments using odorous chemicals: a small amount of an impurity or a derivative may cause drastic effects on the odorant-OR interaction.

OD-IEG appears to be a novel natural compound that had not previously been identified. OD-IEG was probably overlooked in earlier studies because it is easily pyrolysed in a GC column at high temperature, producing isoeugenol, vanillin and 4-hydroxy-3-methoxy phenylacetate (data not shown). Exposure to oxygen accelerates the coupling reaction of isoeugenol, suggesting that the generation of OD-IEG in the stored isoeugenol sample proceeds via an oxygen-assisted reaction. Isoeugenol appears to be highly reactive with alcohols at low temperature in the presence of oxygen due to the formation of a peroxide transition state. Indeed, when isoeugenol and guaiacol were mixed under 100% oxygen, the isoeugenol peroxide reacted with an alcohol group of guaiacol, resulting in the coupling product of isoeugenol and guaiacol, which we named guaiacyl isoeugenol (structure shown in Figure 5:2). It appears that OD-IEG was detected in natural fragrant oils, such as clove and mace oils, because isoeugenol is reactive in natural conditions.

Because the mol. wt of OD-IEG (344) is near the upper limit of odorous compounds, OD-IEG may not be volatile. Therefore, in physiological conditions, it may not enter the nasal cavity to antagonize mOR-EG-expressing neurons. Thus, our findings may not be physiologically relevant; however, they clearly illustrate the following: (i) ORs can be a target of non-volatile molecules that are present in the mucus, thereby affecting olfactory sensitivity or perception and (ii) it is conceivable that, after odorants activate an OR repertoire specific to each odorant, they are easily degraded or converted in the mucus into forms that act as agonists or antagonists of other ORs.

Because the IC_{50} value of OD-IEG for mOR-EG was ~10 μM, a concentration of 30–100 μM was high enough to completely inhibit the response of the mOR-EG to EG. Given that the old isoeugenol contained ~10% of OD-IEG, it was a reasonable that it showed antagonistic activity at 1 mM in the initial antagonist screening. This implies that contaminants or decomposition products often present in commercially available compounds may greatly affect the results of odorant–OR experiments. Indeed, it is widely known that most odorants undergo decomposition,

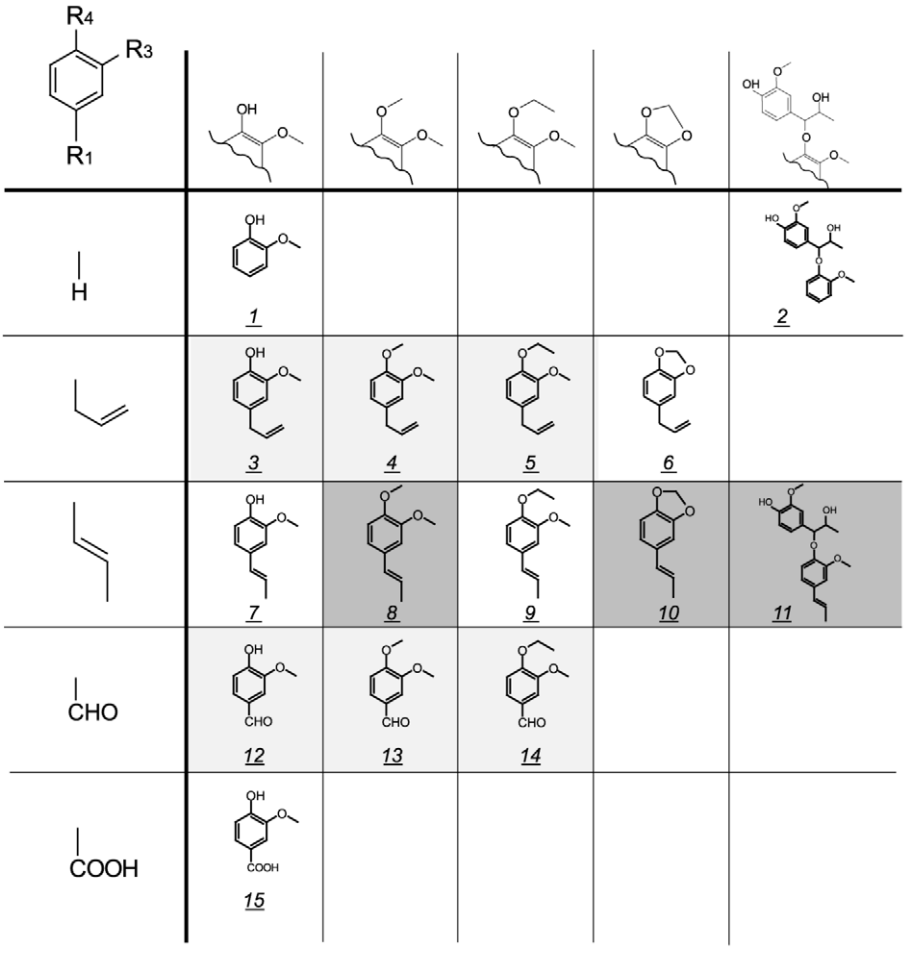

Figure 5 Structure–activity relationship of mOR-EG agonists and antagonists. The compounds in the same column have the same functional groups at positions R_3 and R_4. The compounds in the same line have the same functional group at position R_1. Many of the compounds with an allyl or aldehyde group at the R_1 position are mOR-EG agonists (3~5, 12~14: gray), whereas many of the compounds with a propenyl group at this position are mOR-EG antagonists (8, 10, 11: dark gray). 1, guaiacol; 2, guaiacyl isoeugenol; 3, EG; 4, methyl eugenol; 5, ethyl eugenol; 6, safrole; 7, isoeugenol; 8, methyl isoeugenol; 9, ethyl isoeugenol; 10, isosafrole; 11, OD-IEG; 12, vanillin; 13, methyl vanillin; 14, ethyl vanillin; and 15, vanillic acid.

resulting in the generation of many derivatives (Arctander, 1960). For example, limonene and linalool, the most frequently incorporated fragrance chemicals in scented products, are easily oxidized in air during handling or storage (Skold *et al.*, 2002; Matura *et al.*, 2003). Most aldehyde compounds are also degradable unless they are handled properly. Therefore, it is important that odorants are stored at low temperature under argon without oxygen and that the purity is checked prior to use.

Several studies on odorant-OR interaction have revealed that an individual OR recognizes multiple structurally-related compounds with distinct ligand specificity (Touhara *et al.*, 1999; Araneda *et al.*, 2000; Kajiya *et al.*, 2001; Spehr *et al.*, 2003). Identification of multiple antagonists for mOR-EG enabled us to further investigate the structure–activity relationship of an OR. Figure 5 shows a structure–activity matrix for odorants based on the EG structure that have different functional groups at three positions on the benzene ring. Agonists for mOR-EG (*3~5*, *12~14*: gray) possess an allyl or aldehyde group at the R_1 position, whereas a propenyl group was always found at this position in antagonists (*8*, *10*, *11*: dark gray). This suggests that the R_1 position is a major factor in determining whether an odorant is an agonist or antagonist for mOR-EG. Consistent with this rule, guaiacyl isoeugenol (Figure 5: *2*), which has the same structure as OD-IEG except that it lacks the propenyl group, was not an antagonist (data not shown). In contrast, a subtle difference at the R_3 or R_4 positions leads to a change in agonist and antagonist potency, indicating that these positions are determinants of ligand specificity for mOR-EG. For example, ethyl isoeugenol (*9*) was neither an antagonist nor an agonist, suggesting that ORs can discriminate small differences in an odorant molecule. This information will be of great help in the analysis of the mode of agonist and antagonist binding to an OR and the molecular dynamics involved in transition of an OR from an inactive to an active state.

Odor perception appears to be regulated by complex interactions between odorants and ORs that are based on a combinatorial coding strategy and antagonism between

odorants. We identified a novel, naturally occurring compound that was oxidatively produced from isoeugenol and that was a potent antagonist for an OR. Thus, degradation or conversion of an odorant can affect odorant recognition by ORs. Furthermore, we identified a previously unrecognized chemical reaction between odorants that occurs under natural conditions that must be considered when examining molecular recognition in olfaction. These studies demonstrate that odorant recognition by ORs and subsequent sequestration of odorant compounds in the olfactory mucus, where the components are largely unknown, might involve chemistry much more complex than previously imagined.

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References

Araneda, R.C., Kini, A.D. and **Firestein, S**. (2000) *The molecular receptive range of an odorant receptor*. Nat. Neurosci., 3, 1248–1255.

Arctander, **S.** (ed.) (1960) Perfume and Flavor Materials of Natural Origin. Arctander, Elizabeth, NJ.

Buck, **L.B.** (1996) *Information coding in the vertebrate olfactory system*. Annu. Rev. Neurosci., 19, 517–544.

Cain, **W.S.** and **Drexler, W.S.C.M.** (1974) *Scope and evaluation of odor counteraction and masking*. Ann. N. Y. Acad. Sci., 237, 427–439.

Firestein, **S.** (2001) *How the olfactory system makes sense of scents*. Nature, 413, 211–218.

Gaillard, I., Rouquier, S., Pin, J.P., Mollard, P., Richard, S., Barnabe, C., Demaille, J. and **Giorgi, D.** (2002) *A single olfactory receptor specifically binds a set of odorant molecules*. Eur. J. Neurosci., 15, 409–418.

Kajiya, K., Inaki, K., Tanaka, M., Haga, T., Kataoka, H. and **Touhara, K.** (2001) *Molecular bases of odor discrimination: reconstitution of olfactory receptors that recognize overlapping sets of odorants*. J. Neurosci., 21, 6018–6025.

Katada, S., Nakagawa, T., Kataoka, H. and **Touhara, K.** (2003) *Odorant response assays for a heterologously expressed olfactory receptor*. Biochem. Biophys. Res. Commun., 305, 964–969.

Malnic, B., Hirono, J., Sato, T. and **Buck, L.B.** (1999) *Combinatorial receptor codes for odors*. Cell, 96, 713–723.

Matura, M., Goossens, A., Bordalo, O., Garcia-Bravo, B., Magnusson, K., Wrangsjo, K., and **Karlberg, A.T.** (2003) *Patch testing with oxidized R-(+)-limonene and its hydroperoxide fraction*. Contact Dermatitis, 49, 15–21.

Mombaerts, **P.** (2004) *Odorant receptor gene choice in olfactory sensory neurons: the one receptor–one neuron hypothesis revisited*. Curr. Opin. Neurobiol., 14, 31–36.

Oka, Y., Omura, M., Kataoka, H. and **Touhara, K.** (2004) *Olfactory receptor antagonism between odorants*. EMBO J., 23, 120–126.

Skold, M., Borje, A., Matura, M., and **Karlberg, A.T.** (2002) *Studies on the autoxidation and sensitizing capacity of the fragrance chemical linalool, identifying a linalool hydroperoxide*. Contact Dermatitis, 46, 267–272.

Spehr, M., Gisselmann, G., Poplawski, A., Riffell, J.A., Wetzel, C.H., Zimmer, R.K. and **Hatt, H.** (2003) *Identification of a testicular odorant receptor mediating human sperm chemotaxis*. Science, 299, 2054–2058.

Touhara, **K.** (2002) *Odor discrimination by G protein-coupled olfactory receptors*. Microsc. Res. Techn., 58, 135–141.

Touhara, K., Sengoku, S., Inaki, K., Tsuboi, A., Hirono, J., Sato, T., Sakano, H. and **Haga, T.** (1999) *Functional identification and reconstitution of an odorant receptor in single olfactory neurons*. Proc. Natl Acad. Sci. USA, 96, 4040–4045.

Wetzel, C.H., Oles, M., Wellerdieck, C., Kuczkowiak, M., Gisselmann, G. and **Hatt, H.** (1999) *Specificity and sensitivity of a human olfactory receptor functionally expressed in human embryonic kidney 293 cells and* Xenopus laevis *oocytes*. J. Neurosci., 19, 7426–7433.

Zhang, **X.** and **Firestein, S.** (2002) *The olfactory receptor gene superfamily of the mouse*. Nat. Neurosci., 5, 124–133.

Zhao, H., Ivic, L., Otaki, J.M., Hashimoto, M., Mikoshiba, K. and **Firestein, S.** (1998) *Functional expression of a mammalian odorant receptor*. Science, 279, 237–242.

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