4-Oxo-(E)-2-hexenal produced by Heteroptera induces permanent locomotive impairment in crickets that correlates with free thiol depletion

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A R T I C L E   I N F O

Article history:
Received 10 February 2015
Revised 10 April 2015
Accepted 10 April 2015

Keywords:
2-Alkenals
Anti-predatory defense
Insect locomotion
Lipid-peroxidation product
4-oxo-(E)-2-hexenal
Paralysis

A B S T R A C T

Heteropterans produce 2-alkenals and 4-keto-2-alkenals that function as defense substances or pheromones. However, in spite of advances in heteropteran chemistry, it is still unclear how these compounds affect insect physiology. We found that exposure to 4-oxo-(E)-2-hexenal (OHE) induced permanent paralysis and death in crickets, an experimental model. The depletion of free thiols in leg tissues of OHE-treated crickets and the in vitro adduct formation of OHE with a thiol compound suggest that covalent binding of OHE to biologically active thiols is a potential cause affecting crickets’ locomotion.

http://dx.doi.org/10.1016/j.fob.2015.04.004

A B S T R A C T

1. Introduction

Heteropterans, or true bugs, are remarkable for their production of volatile chemicals such as (E)-2-hexenal, (E)-2-octenal, (E)-decen- al, and 4-oxo-(E)-2-hexenal (OHE) in scent glands located in the dorsal abdomen in nymphs and in metathoracic scent glands in adults (Fig. 1). In bugs, mixtures of these compounds are known to function as pheromones and as defense substances against predators in their natural habitats [1–5]. Of these compounds, (E)-2-hexenal has specifically been reported to be a predator repellent [6], inhibitor of microbial growth [7], and in the plant literature it is also reported for its role against herbivores [8–10]. In contrast, OHE is recognized for its deterrent effects and high toxicity but without repellent properties against insects or other predators [6,11,12]. Yet, in spite of the advances in heteropteran chemistry, it is still unclear how OHE and other (E)-2-alkenals affect the physiology of organisms.

Interestingly, OHE is also known as a peroxidation product of ω-3 polyunsaturated fatty acids (PUFAs) and similar to the cytotoxic compounds, 4-hydroxy-(E)-2-nonenal (HNE) and 4-oxo-(E)-2-nonenal (ONE), derived from peroxidation of ω-6 PUFAs [13]. Because they have an α,β-unsaturated carbonyl moiety, one of the primary targets of HNE and ONE is a cysteine thiol group of proteins such as v-ATPase, ionotropic receptors, and the synaptic-associated protein 25 (SNAP-25) that regulate enzyme activity and signal transduction in synaptic sites [14–17]. These two lipid peroxidation products are definitely associated with human aging and diseases, such as Alzheimer’s disease, Parkinson’s disease and atherosclerosis [18–23]. In fact, HNE adducts have been detected in sites of oxidative stress associated diseases [22,24,25]. Similarly, the accumulation of protein-bound 4-hydroxy-(E)-2-hexenal (HHE) has been observed in patients with neurodegenerative disorders [26]. The reaction properties are common, but the target proteins of HHE have been shown to be different from those of HNE [13]. Compared to HNE, ONE, and HHE, the effects of OHE on organisms are still largely unknown. What we know of OHE is that it has mutagenic properties and that it can form adducts with 2′-deoxyguanosine [27].
We investigated the effects of OHE inducing locomotive impairments in the house cricket (*Acheta domesticus*). Then, with this information, we explored the potential mode of action of OHE against insects by examining whether OHE affects the thiol content in crickets and whether OHE can react with thiol compounds using a model thiol compound.

2. Materials and methods

2.1. Insects and chemicals

Commercially available crickets (*A. domesticus*) in Tucson, AZ, USA, were reared on an artificial diet for tropical fishes in the laboratory. Nymphs between 120 and 150 mg in weight were used for all exposure assays. OHE was synthesized by a one-step reaction described in Moreria and Millar [28]. 1-Butanethiol (1-BuSH), (E)-2-hexenal, hexanal, and hexane were obtained from Sigma–Aldrich (St. Louis, MO, USA). (E)-2-Heptenal and (E)-2-nonenal were purchased from TCI America (Portland, OR, USA).

2.2. Exposure assays

We prepared jars (8 cm i.d. × 9 cm ht, 500 ml) containing a small strip of filter paper with the test chemical compounds on a hand-made stainless stand (4.5 cm ht) to prevent crickets from getting in direct contact with compounds. After placing two randomly selected nymphs inside, jars were tightly closed with its cap and placed in an incubator set at 25 °C.

First, we examined the effects of OHE over time on ten randomly selected crickets with 0.4, 0.8, 1.0, 1.2, 1.6, 2.0, 3.2, 4.0 and 10 mg/l of OHE. Effects on nymph behavior in terms of paralysis and death were recorded every hour for 4 h. To determine the dose-dependent effect of OHE that induces paralysis in crickets in a 2 h exposure, we calculated its EC50 using JMP [29].

To compare the effects of OHE with the ones caused by related compounds, (E)-2-pentenal, (E)-2-hexenal, (E)-2-heptenal, (E)-2-octenal, (E)-2-nonenal, (E)-2-hexen-1-ol, (E)-2-hexene, hexanal, and hexane (control treatment) were used. For this, we exposed crickets in jars to 2.0 mg/l of each compound for 2 h in which all of the crickets exposed to OHE became paralyzed. After this time, the number of nymphs showing signs of paralysis and/or death were counted. Also, to test whether these compounds affected crickets permanently or temporarily, we subsequently moved these crickets into a new open jar and kept them under fresh air for 12–16 h. Again we recorded the number of crickets that had been affected only temporarily and showed signs of having recovered. Ten crickets per treatment and control were used.

2.3. Quantification of free thiols in crickets

To evaluate the biochemical causes of the paralysis caused by OHE, we quantified the amount of free thiols in crickets that showed paralysis after exposure to OHE. For this, we used newly prepared crickets that were subjected to a treatment of 4 h exposure to 2.0 mg/l of OHE. Crickets that died after being exposed were omitted from this analysis. As control, we used non-treated crickets. Crickets were kept frozen at –20 °C until thiol measurement. Then, hind legs of each OHE treated and non-treated cricket were collected separately and homogenized using a pestle in 250 µl of 100 mM phosphate buffer, pH 7.0 containing 1% Triton X-100 (buffer A) on ice. Homogenates were centrifuged at 11,000g for 15 min and the supernatant containing free thiols was recovered. The concentration of total protein in each sample was measured by the BCA assay [30] using a QuantiPro BCA Assay kit (Sigma–Aldrich) according to the manufacturer’s recommendations. Samples were diluted to 2 mg proteins/ml in buffer A. Amount of free thiols was determined according to the protocol described in Patsoukis and Georgiou [31] with a slight modification. Briefly, to prepare the sample containing non-protein free thiols, 90 µl of the sample (2 mg proteins/ml) were mixed with 10 µl of 50% (w/v) TCA solution, and then the mixture was kept on ice for 10 min. The mixture was centrifuged at 16,000g for 5 min, and then the supernatant was recovered as the sample containing non-protein free thiols. This sample (50 µl) was mixed with 100 µl of 400 mM Tris–HCl, pH 8.9 and 5 µl of 5 mM DTNB, and then kept for 30 min at room temperature. The absorbance of the resulting mixture at 412 nm was measured against a blank prepared using buffer A instead of sample solution in a DU 800 spectrophotometer (Beckman Coulter). The amount of total free thiols in the sample was measured using 10 µl of buffer A instead of TCA. The amount of free thiols derived from proteins was obtained by subtracting the amount of non-protein free thiols from total free thiols. Eight replicates were done for each assay. Differences in total amount of thiols, and amounts of protein and non-protein thiols, between treated and non-treated crickets were analyzed with ANOVA’s performed in JMP.

2.4. in vitro Reaction of OHE with 1-butanethiol

To determine whether OHE can react with free thiol groups, OHE (1 mmol) was mixed with 1-butanethiol (1-BuSH, 0.2 mmol) in 1 ml of 20 mM phosphate buffer/acetoniitrile (2/1, v/v, pH 6.5) at 25–27 °C for 14 h following the method described in Sasai et al. [32]. The mixture was extracted with ether and the organic layer was washed with brine and dried over anhydrous Na2SO4. The crude extract was analyzed for structure elucidation by GC–MS (an Agilent 6890N gas chromatograph linked to an Agilent 5975MS mass spectrometer, operated at 70 eV, with a HP-5MS capillary column, 30 m × 0.25 mm i.d., 0.25 µm in film thickness). To quantify the product compositions, a GC with a flame ionization detector and a DB-5MS capillary column, 25 m × 0.32 mm i.d., 0.52 µm in film thickness was used. The oven temperature was programmed from 50 °C (3 min holding) to 300 °C at a rate of 10 °C/min and then held for 5 min. The injector temperature was maintained at 200 °C. Chemical ionization (CI) mass spectra were obtained in the positive mode at an ion source temperature of 250 °C with the GC–MS system using methane as regent gas. Four replicates of the reaction were done for chemical analyses.

To determine the structures of OHE adducts, four reaction mixtures were combined (422 mg) and applied onto a silica gel column (30 g), and successively eluted in a sequence with 300 ml each of hexane, 1%, 3%, 4%, 5%, 10% and 50% ethyl acetate in hexane. The compositions of each elute were monitored by GC/MS. The 4% ethyl acetate fraction that contained two OHE adducts (SHx and SHy) was further fractionated on another silica gel column (6 g) eluted with 60 ml of 4% ethyl acetate in hexane into 10 fractions (6 ml each) to isolate these two compounds. SHa was isolated from the hexane fraction. 1H and 13C NMR spectra of the isolated OHE adducts were acquired on a Bruker DRX-500 (1H at 500 MHz and 13C at 125 MHz) and a Varian Inova-600 spectrometer (1H at 300 MHz and 13C at 150 MHz).
600 MHz and $^{13}$C at 150 MHz) in a CDCl$_3$ solution with tetramethylsilane as an internal standard.

3. Results

3.1. Permanent locomotive impairment in crickets induced by the exposure of OHE

Each cricket showed signs of distress and paralysis after being exposed to OHE. They moved around in the jar and frequently groomed their antennae, mouthparts, and fore and hind legs within 15 min after the exposure. Then they became gradually less active, could not support their body, and lost their capability of movement. OHE seemed to affect more markedly the crickets’ hind legs, which became rigidly stretched and flipped up dorsal ward (Fig. 2). Paralysis and death induced by OHE in crickets occurred more frequently when exposed to higher doses and for a longer time (Fig. 3). The theoretical EC$_{50}$ of 2 h exposure of OHE was 1.24 mg/l (Fig. 4). Free thiols derived from both protein and non-protein sources were also significantly decreased to 79% (OHE treated = 27.9 ± 5.4 pmol/mg protein in legs, not treated = 35.2 ± 2.0 pmol/mg protein in legs; P = 0.003; DF = 15) and 55% (OHE treated = 2.9 ± 0.7 pmol/mg protein in legs, not treated = 5.3 ± 1.9 pmol/mg protein in legs; P = 0.004; DF = 15), respectively (Fig. 5A and C).

3.2. Depletion of the amount of free thiols in crickets by the exposure of OHE

OHE reacted with 1-BuSH to form three OHE adducts, tentatively named SHa, SHx and SHy (Fig. 6). Of the adducts, SHx was the major product (mean ± SD = 63.6 ± 1.2%) followed by SHy (29.7 ± 1.0%) and SHa (6.6 ± 0.2%) (N = 4). These OHE adducts were identified as 2-buthylthio-5-ethylfuran (SHa, peak 2), 3-buthylthio-4-oxo-hexanal (SHx, peak 3) and 2-buthylthio-4-oxo-hexanal (SHy, peak 4) by GC/MS and NMR analyses (Fig. 7). SHx and SHy were 1,4-Michael type adducts, while SHa was a product of 1,2-addition followed by cyclization. The mass and NMR spectra of the identified OHE adducts are summarized as follows:

- **Compound SHa (peak 3):** colorless oil; EIMS m/z (%): 184 (M$^+$, 71.6), 169 (8.5), 128 (72.4), 113 (100), 57 (7.5); CIMS (methane): m/z 185 [M+H]$^+$, 213 [M+C$_2$H$_5$]$^+$, 225 [M+C$_3$H$_5$]$^+$; $^1$HN M R (600 MHz, CDCl$_3$): $^6$ 6.39 (1H, d, J = 3.0 Hz), 5.96 (1H, d, J = 3.0 Hz), 2.72 (2H, t, J = 7.4 Hz), 2.64 (2H, q, J = 7.5 Hz), 1.56
4. Discussion

Our results indicate that while exposure to ω,β-unsaturated aldehydes with six to eight carbon chain lengths caused only moderate and temporary paralysis in crickets, exposure to OHE impaired the cricket’s locomotive ability resulting in permanent paralysis and death. The crickets’ hind leg stretching reaction after being treated with OHE is similar to the symptoms exhibited by locusts when treated with the insecticide, pymetrozine [33]. However, while locusts treated with pymetrozine do not show any signs of paralysis, and their fore and mid legs are not affected by the insecticide, in OHE treated crickets all legs were affected. This suggests that the mode of action of OHE is probably different to the one of pymetrozine.

Table 1
The effect of OHE and related compounds on cricket.

| Compound                  | Movement disorder (%) |          |
|---------------------------|-----------------------|----------|
|                           | Temporary*            | Permanent* |
| OHE                       | 100                   | 100      |
| (E)-2-Hexenal             | 50                    | 0        |
| (E)-2-Hexen-1-ol          | 40                    | 0        |
| (E)-2-Hexene              | 0                     | 0        |
| Hexanal                   | 0                     | 0        |
| (E)-2-Pentenal            | 0                     | 0        |
| (E)-2-Heptenal            | 20                    | 0        |
| (E)-2-Octenal             | 20                    | 0        |
| (E)-2-Nonenal             | 0                     | 0        |
| Hexane                    | 0                     | 0        |

* Percentage of paralyzed crickets after 2 h exposure to 2.0 mg/l of each compound.

** Percentage of paralyzed crickets first exposed to 2.0 mg/l of each compound for 2 h and then kept under fresh air for 12–16 h.

Fig. 5. Amount of free thiols in hind legs of crickets. (A) Whole free thiols, (B) protein-derived free thiols, (C) non-protein-derived free thiols. Asterisks indicate that the differences among treatments were significantly different (P < 0.01).

Fig. 6. Gas chromatogram of (A) reaction products of 1-butanethiol with OHE and (B) OHE. 1. Dibutyl disulfide (artifact from 1-BuSH); 2. 2-buthylthio-5-ethylfuran (SHa); 3. 3-buthylthio-4-oxo-hexanal (SHx); 4. 2-buthylthio-4-oxo-hexanal (SHy); imp. impurity.
The temporal inhibition of movement occurred by only (E)-2-alkenals with six to eight carbon chain lengths suggests that there could be size-dependent certain mode of actions of these (E)-2-alkenals. One possibility is the penetration rate of the tested compounds into crickets' body. It has been previously shown that longer carbon chain aldehydes have a lower penetration rate through the cuticle than shorter chain aldehydes \[11,34\]. (E)-2-Hexen-1-ol also induced moderate temporary paralysis in crickets, suggesting that \( \alpha,\beta \)-unsaturated C-6 compounds might inhibit the function of molecules associated with insect locomotion, for example, neurotransmission, by competitive antagonistic effect or loosely blocking the target molecules. (E)-2-Hexenal, (E)-2-heptenal, (E)-2-octenal and (E)-2-hexen-1-ol might reversibly or slowly affect the function of these biological molecules, while OHE could irreversibly and quickly inactivate these molecules. The toxic potencies of aldehydes are recognized to be linked to their electrophilicities, size and solubility\[17\], thus, the differences in our results can be also explained by the electrophilic reactivity of the tested aldehydes. In fact, a previous report showed that OHE is a stronger electrophile than (E)-2-hexenal and (E)-2-octenal by comparing their lowest unoccupied molecular orbital (LUMO) energy level\[7\].

The in vitro reaction of OHE with 1-BuSH resulted in two major thioether-type compounds (SHx and SHy) produced by a 1,4-Michael addition reaction similar to the reported reactions of HNE and OHE\[16,18,22\]. The remaining aldehyde and ketone moiety of SHx and SHy could be involved in a secondary reaction with other nucleophiles (e.g. lysine) to form cross-linked dimer or oligomer as previously described\[23,35\].

There was a minor but unique adduct with a furan ring (SHA) produced by a 1,2-addition from the reaction between OHE and 1-BuSH. According to the hard and soft acids and bases theory\[36\], 1,4-Michael addsucts occur more predominantly than 1,2-adducts, and our results are consistent with this theory. The 1,2-addition between a thiol group and \( \alpha,\beta \)-unsaturated aldehydes such as 4-hydroxy-2-alkenals has not been reported before. Our results showed that (E)-2-hexenal only affected crickets temporarily, thus, the furan ring formation specific to OHE could be involved in the irreversible effect seen in crickets. Previously, Sasaki et al.\[32\] showed that \( \alpha \)-acaridial, a conjugated dial found in astigmatid mites, can react with 1-BuSH to form 2,3-substituted furan compound, and thus furan ring formation is supposed to be specific to conjugated dicarbonyl compounds.

The adduction formation of OHE with 1-BuSH and the depletion of thiol compounds after OHE exposure suggest that this compound could covalently react with a cysteine residue in proteins, enzyme active sites, glutathione, and other thiol compounds such as coenzyme A in vivo, and impair these biologically active molecules. Oral administration of a thiol inactivator, N-ethylmaleimide, to crickets resulted in very similar symptoms to the exposure of OHE (personal observation of KN), suggesting that the irreversible reaction of OHE with certain thiol compounds can inhibit the cricket's locomotion. The effects of OHE and HNE show interesting similarities. HNE is reported to reduce the contractility of smooth muscle. With HNE, it appears to occur as an irreversible alkylation of thiol groups on the receptor system, L-type calcium channels and/or contractile apparatus of muscle\[37,38\]. Furthermore, actin is known as another target of HNE\[39\]. Thus, OHE might affect insect neurotransmission and/or muscular components in similar fashion to HNE. Further study to identify the target molecules of OHE in insects could not only contribute toward a deeper understanding of the ecological significance of OHE as a defense substance in heteropterans, but it could also contribute to the understanding of human neurodegenerative diseases caused by lipid peroxidation products. Heteropteran bugs protect themselves by discharging unpleasant volatile components that can repel their predators immediately. Having toxic compound(s), such as OHE, as another chemical barrier together with repellent in a blend, could fortify the bug's chemical defense system.

Acknowledgements

We thank Neil Jacobsen and Guangxin Lin (University of Arizona) for acquiring the NMR spectra. We are also grateful to Xianchun Li (University of Arizona) and all of the members in his lab for their considerable help. We thank Naoki Mori (Kyoto University) for providing the information about the reaction of 1-BuSH. This work was supported by a Grant from the BIO5 Institute of the University of Arizona to JXB. KN designed and performed the experiments, KN and JXB analyzed the data and wrote the paper.

References

[1] Aldrich, J.R. (1988) Chemical ecology of the Heteroptera. Annu Rev Entomol 33, 211–238.
[2] Blum, M.S. (1961) The presence of 2-hexenal in the scent gland of the pentatomid Brochymena quadrupunctata. Ann Entomol Soc Am 54, 1907–1920.
[3] Eisner, T., Eisner, M. and Siegler, M. (2005) Secret weapons: defenses of insects, spiders, scorpions, and other many-legged creatures, Belknap Press of Harvard University Press, Cambridge.
[4] Millar, J.G. (2005) Pheromones of true bugs. Top Curr Chem 240, 37–84.
[5] Moresa, M.C.B., Pareja, M., Laumann, R.A. and Borges, M. (2008) The chemical volatiles (semiochemicals) produced by Neotropical stink bugs (Hemiptera: Pentatomidae). Neotrop Entomol 37, 489–505.
[6] Noge, K., Prudic, K.L. and Becerra, J.X. (2012) Defensive roles of (E)-2-alkenals and related compounds in Heteroptera. J Chem Ecol 38, 1050–1056.
[7] Noge, K., Kimura, H., Abe, M., Becerra, J.X. and Tamogami, S. (2012) Antibacterial activity of 4-oxo-(E)-2-hexenal from adults and nymphs of the heteropteran, Dolycoris baccarum (Hemiptera: Pentatomidae). Biosci Biotechnol Biochem 76, 1975–1978.
[8] Andrushko, S.A., Brown, G.C., Dahlman, D.L. and Hildebrand, D.F. (1997) Methyl jasmonate exposure induced insect resistance in cabbage and tobacco. Environ Entomol 26, 642–654.
[9] Comi, K., Satoh, M., Ozawa, R., Shimosaga, Y., Sanada, S., Sasaki, K., Matsumura, M., Ohashi, Y., Kanno, H., Akimitsu, K. and Takabayashi, J. (2010) Role of hydroperoxide lyase in white-backed planthopper (Sogatella furcifera Horváth)-induced resistance to bacterial blight in rice, Oryza sativa L. Plant J. 61, 46–57.
[25] Lauderback, C.M., Ceballo, R.A., Saedi, V. and Becerra, J.X. (2012) Synergy versus potency in the defensive secretions from nymphs of two pentatomomorphan families (Hemiptera: Coreidae and Pentatomidae). J Chem Ecol 38, 1358–1365.

[26] Shibata, T., Iio, K., Kawai, Y., Shibata, N., Kawaguchi, M., Toi, S., Kobayashi, M., Yamamoto, T. and Kobayashi, M. (2004) Accumulation of protein-bound 4-hydroxy-2-hexenal in spinal cords from patients with sporadic amyotrophic lateral sclerosis. Brain Res 1019, 170–177.

[27] Maekawa, M., Kawai, K., Takahashi, Y., Nakamura, H., Motani, T., Sawada, R., Hachiya, T. and Kasi, H. (2006) Identification of 4-oxo-2-hexenal and other direct mutagens formed in model lipid peroxidation reactions as dGuo adducts. Chem Res Toxicol 19, 130–138.

[28] Moreira, J.A. and Millar, J.G. (2005) Short and simple synthesis of 4-oxo-(E)-2-hexenal and homologs: pheromone components and defensive compounds of Hemiptera. J Chem Ecol 31, 965–968.

[29] SAS Institute Inc. JMP version 10. Cary, NC, 2005.

[30] Smith, P.K., Krohn, R.L., Hermansson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150, 76–85.

[31] Patsoukis, N. and Georgiou, C.D. (2004) Determination of the thiol redox state of organisms: new oxidative stress indicators. Anal Bioanal Chem 378, 1783–1792.

[32] Sasai, T., Hirano, Y., Maeda, S., Matsunaga, I., Otsuka, A., Morita, D., Nishida, R., Nakayama, H., Kuwahara, Y., Sugita, M. and Mori, N. (2008) Induction of allergic contact dermatitis by astigmatid mite-derived monoterpenes, α- and β-pinene. Biochem Biophys Res Commun 375, 336–340.

[33] Kaufmann, L., Schurmann, F., Viale, V., Haenni, J., Ader, M. and Kayser, H. (2004) The serotonergic system is involved in feeding inhibition by pymetrozine. Comparative studies on a locust (Locusta migratoria) and an aphid (Myzus persicae). Comp Biochem Physiol C 138, 469–483.

[34] Remold, H. (1963) Scent-glands of land-bugs, their physiology and biological function. Nature 198, 764–766.

[35] De, T., Aron, J.S., Lee, S.H. and Blair, I.A. (2003) A novel lipid hydroperoxide-derived cyclic covalent modification to histone H4. J Biol Chem 278, 42098–42105.

[36] Pearson, R.G. (1990) Hard, soft acids and bases – the evolution of a chemical concept, Coord Chem Rev 100, 403–425.

[37] de Jongh, R., Haene, G.R.M.M., van Koeveringe, G.A., Dambros, M. and van der Vlist, A. (2008) Lipid peroxidation product 4-hydroxyxenon to rats. Food and Chemical Toxicology 46, 2368–2377.

[38] de Jongh, R., Haene, G.R.M.M., van Koeveringe, G.A., Dambros, M. and van der Vlist, A. (2008) Lipid peroxidation product 4-hydroxyxenon to rats. Food and Chemical Toxicology 46, 2368–2377.

[39] de Jongh, R., Haene, G.R.M.M., van Koeveringe, G.A., Dambros, M. and van der Vlist, A. (2008) Lipid peroxidation product 4-hydroxyxenon to rats. Food and Chemical Toxicology 46, 2368–2377.