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De novo biosynthesis of linoleic acid and its conversion to the hydrocarbon (*Z,Z*)-6,9-heptadecadiene in the astigmatid mite, *Carpoglyphus lactis*: Incorporation experiments with ^{13}C-labeled glucose

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ABSTRACT

De novo biosynthesis of linoleic acid (LA) and its conversion to (*Z,Z*)-6,9-heptadecadiene were examined in *Carpoglyphus lactis* (Acarina, Carpoglyphidae). Experiments involving ^{13}C-administration using [1-^{13}C]-D-glucose revealed that ^{13}C atoms were incorporated into LA of total lipid extracted from the mite, resulting in labeling of all even-numbered carbons. This result demonstrated that LA was produced from ^{13}C-labeled acetyl-CoA, which is indicative of direct *de novo* biosynthesis. In these feeding experiments involving [1-^{13}C]-D-glucose, ^{13}C atoms were also incorporated into (*Z,Z*)-6,9-heptadecadiene, which is one of the major secretory components in the mite. The labeling pattern of (*Z,Z*)-6,9-heptadecadiene at odd-numbered carbons agreed well with that of LA after loss of the carboxyl carbon. It was concluded that the mites could stably convert LA into (*Z,Z*)-6,9-heptadecadiene without the dietary requirement of this essential fatty acid.

1. Introduction

In general, animals are unable to biosynthesize linoleic acid (LA), rendering it essential in their diets. However, radiolabeling experiments with insects have indicated that some of them are capable of synthesizing LA, with 12 species among 40 examined known to be capable of producing it (Blomquist et al., 1982; Cripps et al., 1986; de Renobales et al., 1987). Apart from insects, the ability to synthesize LA has also been identified in protozoa (Sayanova et al., 2006), nematoda (Peyou-Ndi et al., 2000), and pulmonata (Weinert et al., 1993). Recently, it was concluded that *Tyrophagus* mites have the ability to convert hexadecanoic acid into LA through elongation and desaturation reactions (Aboshi et al., 2013). In many animals, LA is converted to arachidonic acid, a precursor of prostaglandins and other physiologically important eicosanoids by chain extension and desaturation in the endoplasmic reticulum. LA is also the presumed precursor of (*Z,Z*)-6,9-heptadecadiene, which is distributed sporadically among astigmatid mites. Based on the double-bond positions, it is reasonable to speculate that LA is converted to (*Z,Z*)-6,9-heptadecadiene by loss of the carboxyl group.

(*Z,Z*)-6,9-heptadecadiene, a simple but rather unusual hydrocarbon, was first identified as a major component of the secretion from the opisthonotal glands of *Carpoglyphus lactis* (Acarina, Carpoglyphidae) (Kuwahara et al., 1992). The mite uses neral as the alarm pheromone (Kuwahara et al., 1980), and neral has been demonstrated, using [1-^{13}C]-D-glucose, to be a biosynthetic product of the mevalonate pathway in *C. lactis* (Morita et al., 2004). Feeding experiments also indicate that (*Z,Z*)-6,9-heptadecadiene and other components in the extract contain ^{13}C atoms. This suggests that ^{13}C-labeled acetyl-CoA generated by glycolysis is utilized to produce LA, possibly by the mites themselves or by microorganisms in a symbiotic relationship with them. In unidentified *Tortonia* sp., (*Z,Z*)-6,9-heptadecadiene was identified as the alarm pheromone, whereas (*Z,Z*)-4,8-heptadecadiene, (*Z,Z,Z*)-4,8,11-heptadecatriene,

Abbreviations: FAs, fatty acids; FAMEs, fatty acid methyl esters; GC–MS, gas chromatography–mass spectrometry; LA, linoleic acid; LAME, linoleic acid methyl ester; NMR, nuclear magnetic resonance; HMQC, heteronuclear multiple quantum coherence.

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and other monoene hydrocarbons present in the species were inactive in a pheromonal bioassay (Kuwahara et al., 1995a). Furthermore, the presence of (*Z*,*Z*)-5,9-octadecadienoic acid and (*Z*,*Z*,*Z*)-5,9,12-octadecatrienoic acid have been demonstrated to be possible biosynthetic precursors of (*Z*,*Z*)-4,8-heptadecadiene and (*Z*,*Z*,*Z*)-4,8,11-heptadecatriene, respectively (Kuwahara et al., 1995b). Unusual fatty acids, (*Z*,*Z*,*Z*)-9,12,17-octadecatrienoic acid and (*Z*,*Z*,*Z*)-2,7,10-octadecatrienoic acid, have also been predicted to be precursors of the new hydrocarbon (*Z*,*Z*)-1,6,9-heptadecatriene found in *Caloglyphus polyphyllae* (Shimizu et al., 1999).

Based on the preliminary findings and methods of Morita et al. (2004), the present study aimed to elucidate *de novo* biosynthesis of LA and (*Z*,*Z*)-6,9-heptadecadiene in *C. lactis*. We examined the biosynthesis from acetyl-CoA with a ^{13}C atom at the C2 position derived from [1-^{13}C]-D-glucose by examining ^{13}C-labeling patterns using ^{13}C-nuclear magnetic resonance (^{13}C NMR) spectroscopic analysis after purification and isolation. The results demonstrated production of LA labeled at the even-numbered carbons, which is indicative of *de novo* biosynthesis from acetyl-CoA. The presence of (*Z*,*Z*)-6,9-heptadecadiene labeled at the odd-numbered carbons suggests that biosynthesized LA is converted to (*Z*,*Z*)-6,9-heptadecadiene after loss of the carboxyl carbon.

2. Materials and methods

2.1. Chemical analysis

Column chromatography was performed on a Wakosil silica gel C-200 with the specified solvents. ^{1}H and ^{13}C NMR, and HMQC spectra were recorded on a Bruker Biospin AC400M spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C), using tetramethylsilane as the internal standard. Gas chromatography–mass spectrometry (GC–MS) analysis was conducted using a Network GC System (6890N; Agilent Technologies Inc.) coupled with a mass selective detector (5975 Inert XL; Agilent Technologies Inc.) operated at 70 eV using an HP-5MS capillary column (0.25 mm i.d. × 29 m, 0.25 μm film thickness; Agilent Technologies Inc.). Helium was used as the carrier gas at a flow rate of 1.00 ml/min, with a split-less mode at a temperature programmed to change from 60 °C (2 min) to 290 °C at a rate of 10 °C/min. The temperature was maintained at 290 °C for 5 min.

2.2. Rearing of mites

C. lactis Linnaeus (Acari: Carpoglyphidae) was originally a strain maintained at Tokyo Women's Medical College and was received from the Laboratory of Chemical Ecology, Kyoto University, Japan, in 2009. The strain was maintained at 20 °C and 75% relative humidity in a glass container (10 cm in diameter, 6 cm in height) and fed on a mixture of dry yeast and sugar (1:1, w/w) at our laboratory.

2.3. Feeding experiment with a [1-^{13}C]-D-glucose-enriched diet

For the feeding experiment, mites were fed on a mixture of dry yeast and [1-^{13}C]-D-glucose (>99% ^{13}C enrichment, Sigma–Aldrich Co., Ltd., Tokyo, Japan) (1:1, w/w) for 3 weeks. A set amount of feed (1.2–1.8 g) was provided once every 4 days. No adverse effect was observed on changing the sugar component from sucrose to glucose. The rate at which ^{13}C atoms were incorporated into (*Z*,*Z*)-6,9-heptadecadiene was monitored by GC–MS during a given time interval over several days. At each timepoint, 10 mites were transferred to a conical glass insert (5 mm in diameter × 30 mm in height, Agilent Technologies Inc.) using a needle and soaked for 5 min in hexane (3 μl). The extract was directly subjected to GC–MS analysis. To monitor incorporation of ^{13}C atoms into LA, 30–50 mites were collected in a conical glass insert and were extracted with chloroform:methanol (2:1, v/v, 10–20 μl) for 1 h. After the solvent was evaporated under N_2 flow, extracts that contained free fatty acids (FAs) were subjected to the following methyl esterification without hydrolysis of total lipids. To the residue, benzene:methanol (4:1, v/v, 30–50 μl) and trimethylsilyldiazomethane (ca. 0.6 M, 15–25 μl, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were added to obtain FAMEs. After 30 min, the reaction mixture was directly subjected to GC–MS analysis.

2.4. Isolation of ^{13}C-labeled LAME

C. lactis of all developmental stages and both sexes were separated from the culture medium by suspending them in saturated saline. Medium-free mites (6.62 g) were then extracted with chloroform:methanol (2:1, v/v, 30 ml) for 1 day. After filtration, the filtrate was concentrated *in vacuo* and the total lipid was hydrolyzed with sodium methoxide (100 mg) in methanol (5 ml) under reflux for 2 h. After acidifying the reaction mixture with 1 N HCl at room temperature, ethyl acetate was added. The organic layer was successively washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. To the residue, methanol (5 ml) and a catalytic amount of H_2SO_4 were added and the reaction mixture was refluxed for 1 h. The mixture was then added to saturated $NaHCO_3$ and extracted with ethyl acetate. The organic layer was separated and successively washed with water and brine. After drying over anhydrous Na_2SO_4, the solvent was removed *in vacuo*, leaving FAMEs (120 mg) in the form of oil. These esters were applied to an SiO_2 column (6 g, Wako-gel C-200) and successively eluted with 50 ml of hexane, mixtures of ether in hexane (1%, 3%, 5%, 10%, and 30%), and ether. For isolation and purification of LAMEs, a mixture of methyl esters eluted with 5% ether in hexane was applied to a 10% $AgNO_3$–SiO_2 column (2 g) and successively eluted with 20 ml of hexane, mixtures of ether in hexane (1%, 3%, 5%, 10%, 20%, 30%, and 50%), and ether. Each eluate was monitored by GC–MS. LAME (10 mg) was eluted with 10% and 20% ether in hexane and analyzed by ^{13}C NMR spectroscopy. A sample of authentic unlabeled LAME was obtained from a commercial source for use as a standard (Sigma–Aldrich Co., Ltd., Tokyo, Japan).

2.5. Isolation of ^{13}C-labeled and unlabeled (*Z*,*Z*)-6,9-heptadecadiene

Mites (4.56 g) separated from culture medium were extracted with hexane (25 ml) for 10 min to obtain the secretion of the laterodorsal opisthonotal glands. After evaporation of the solvent, the extract (20 mg) was applied to an SiO_2 column (1 g, Wako-gel C-200) and successively eluted with 10 ml of hexane, mixtures of ether in hexane (1%, 3%, 5%, 10%, and 30%), and ether. For isolation and purification of (*Z*,*Z*)-6,9-heptadecadiene, a mixture of hydrocarbons eluted with hexane was applied to a 10% $AgNO_3$–SiO_2 column (500 mg) and eluted with 5 ml of hexane, mixtures of ether in hexane (1%, 3%, 5%, 10%, 20%, 30%, and 50%), and ether successively. Each eluate was monitored by GC–MS. (*Z*,*Z*)-6,9-heptadecadiene (1 mg) was eluted with 3% ether in hexane and analyzed by ^{13}C NMR spectroscopy. ^{13}C-unlabeled (*Z*,*Z*)-6,9-heptadecadiene (1 mg) was prepared in a similar manner from mites (5–10 g) reared on [1-^{13}C]-D-glucose-free media for use as the standard.

2.6. Determination of ^{13}C-labeling patterns

^{13}C NMR spectra of the compound labeled at the ^{13}C atoms and of the naturally occurring compound were recorded under the same experimental conditions. Chemical shifts were assigned for