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Isoamyl alcohol odor promotes longevity and stress tolerance via DAF-16 in *Caenorhabditis elegans*

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ABSTRACT

The possibility that odor plays a role in lifespan regulation through effects on the nervous system is indicated by research on *Caenorhabditis elegans*. In fact, ablation of AWA and AWC, which are suggested as olfactory neurons, has been shown to extend lifespan via DAF-16, a homolog of FoxO. However, the effects of odor stimuli on the lifespan still remain unclear. Thus, we here aimed to clarify the effect of attractive and repulsive odors on longevity and stress tolerance in *C. elegans* and to analyze the pathways thereof. We used isoamyl alcohol as an attractive odor, and acetic acid as a repellent component, as identified by chemotaxis assay. We found that isoamyl alcohol stimulus promoted longevity in a DAF-16-dependent manner. On the other hand, acetic acid stimulus promoted thermotolerance through mechanisms independent of DAF-16. Above all, our results indicate that odor stimuli affect the lifespan and stress tolerance of *C. elegans*, with attractive and repulsive odors exerting their effects through different mechanisms, and that longevity is induced by both activation and inactivation of olfactory neurons.

1. Introduction

Recently, odor stimuli have attracted attention as potential aromatherapy for clinical use for central nervous system diseases. There are some clinical reports of, for example, Alzheimer patients smelling rosemary and lemon in the morning and lavender and orange at night showing higher score at cognitive function tests [1]. Moreover, in a rat experiment, the smell of grapefruit stimulated the sympathetic nervous system and decreased fat and food intake [1].

Meanwhile, the possibility that odor plays a role on lifespan regulation through the nervous system is indicated by research on *Caenorhabditis elegans*, a famous animal model used for lifespan experiments. *C. elegans* has, at least, 3 pairs of olfactory neurons, AWA, AWB, and AWC [2], and ablation of AWA and AWC, which are required for chemotaxis [3], has been shown to extend the lifespan via DAF-16, a homolog of FoxO [4]. Moreover, knockout of odr-2, odr-3, and odr-7, which are required for the function of AWA and AWC [5–8], has also been demonstrated to be involved in longevity via DAF-16 [8]. These researches suggest potential effects of aromatherapy on somatic health problems.

However, the effects of odor stimuli on lifespan remain unclear. In addition, the relation of AWB, which is important for avoidance [9], and longevity is also unclear. Hence, in this study, we aimed to clarify the effect of attractive and repulsive odors for longevity and stress tolerance in *C. elegans* and to analyze its pathway, as an aroma therapeutic research. Thus, we used isoamyl alcohol, which is receipted by AWC and induces chemotaxis [3], as an attractive odor, and acetic acid as a repellent component, as identified using chemotaxis assay. As a result, we found that isoamyl alcohol stimulus extended lifespan and that thermotolerance was promoted by both isoamyl alcohol and acetic acid, albeit through different mechanisms. These results support the notion that odor stimuli may have the potential to improve somatic health.

2. Materials and methods

2.1. Strains and culture

Wild-type (WT) *C. elegans* Bristol N_2 and *daf-16* (*mgDf50*) mutants were provided by the Caenorhabditis Genetics Center. Each strain was cultured on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* OP50 at 20 °C, as previously described [10].

Abbreviations: dDW, double-distilled water; NGM, nematode growth medium; PCR, polymerase chain reaction; WT, wild-type.

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2.2. Age synchronization

Adult worms were collected by S-basal (0.1 M NaCl; Kantokagaku, Tokyo, Japan, 50 mM Potassium Phosphate Buffer, pH 6.0), and subsequently treated with 20% NaClO Solution (10:1 NaClO:-NaOH; Hiter, KAO, Tokyo, Japan and Wako, Osaka, Japan, respectively); eggs were collected into S-basal. After 18 h, the hatched larvae were moved onto OP plates.

2.3. Odor

Isoamyl alcohol (3-methyl-1-butanol) (Wako) and acetic acid (Wako) were diluted with double-distilled water (dDW) to 0.01–1% and used as odor stimuli. A total of 2 μl of these odor liquids were dropped on the back of the lid of the NGM plate at 5 different places.

2.4. Chemotaxis assay

A circle with a diameter of 3 cm was drawn at the center of a 6-cm NGM plate, and 4 worms were placed at 4 different places on the periphery of the circle. Next, 10 μl of odor liquids were dropped at the center of the NGM plate, After 30 min incubation, worms that existed inside of the circle were assessed as “attractant,” while those that existed outside circle were assessed as “avoidance.” The tendency was calculated as follows: (number of worms inside the circle/total number of worms) × 100 (Fig. 1).

2.5. Longevity assay

Age-synchronized L1 larvae were transferred onto NGM plates seeded with OP50 and cultured at 20 °C for 3 days. After 3 days of culture, the worms were exposed to odor stimulus and cultured at 20 °C for an additional 24 h. Subsequently, the worms were transferred onto NGM plates dribbled with 100 μL OP50 (20 worms/plate) and cultured at 20 °C with odor. The day of transferring was decided as Day 0 and the survival rate of the worms was counted every 2 days. To prevent generation of progenies, the worms were treated with 0.5 mg/mL FUdR at Days −1, 0, 2, and 4.

2.6. Viability after heat treatment

Age-synchronized L1 larvae were transferred onto NGM plates seeded with OP50 and cultured at 20 °C for 3 days. After 3 days of culture, the worms were exposed to odor stimulus and cultured at 20 °C for 24 h. Subsequently, the worms were transferred onto NGM plates dribbled 100 μL OP50 and cultured at 37 °C for 3.5 h (20 worms/plate). After heat stress treatment, the odor stimulus was given again and the plates were cultured at 20 °C. The number of living worms was counted every 2 days.

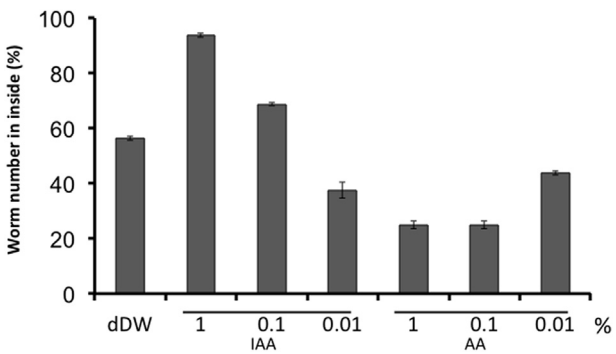

Fig. 1. Chemotaxis on isoamyl alcohol (IAA) and acetic acid (AA). A circle with a diameter of 3 cm was drawn at the center of a 6-cm nematode growth medium (NGM) plate, and 4 adult worms were placed in 4 spots on the periphery of the circle. Ten μl of odor liquid was placed at the center of the NGM plate. After 30 min incubation, worms that existed inside of the circle were measured as “attracted.” The tendency was calculated as follows: (number of worms inside the circle/total number of worms) × 100. The data are presented as the mean ± SE.

2.7. Gene expression

Age-synchronized L1 larvae were transferred onto NGM plates seeded with OP50 and cultured at 20 °C for 3 days. After 3 days of culture, adult worms were exposed to odor stimulus and cultured at 20 °C for 24 h. RNA was purified from whole-cell extracts of worms by using RNAiso PLUS (Takara, Shiga, Japan). cDNA was synthesized using the PrimeScript® RT reagent kit with gDNA Eraser (Perfect Real Time; Takara). cDNA was amplified using Thermal Cycler Dice® Real Time System Lite (Takara) and Thunderbird SYBR qPCR Mix (TOYOBO, Osaka, Japan) according to the manufacturers' instructions. We used *actin* as the internal control. The primers and polymerase chain reaction (PCR) condition have been previously described [11,12].

2.8. Statistical analysis

Statistical analyses were performed using the analysis software SPSS (IBM, NY, USA). Statistical significance for the survival curves was analyzed using the log-rank test, and that for the values plotted on the graph was analyzed using Dunnett's test, with statistically significant differences defined at $^{*}p < 0.05$ and $^{**}p < 0.005$.

3. Results

3.1. C. elegans escaped from acetic acid

First, we performed a chemotaxis assay to decide the optimum concentration of odors. The rate of worms that showed attractive or avoidance movement was measured at different concentrations of isoamyl alcohol or acetic acid. As a result, the number of worms inside the circle was approximately 50% in dDW, while the number of worms staying inside the circle upon isoamyl alcohol stimulus was increased in a dose-dependent manner (Fig. 1). Conversely, the number of worms staying inside the circle was decreased to as low as 20% by 0.1% and 1% acetic acid stimuli. Therefore, these results suggested that *C. elegans* prefers isoamyl alcohol and dislike acetic acid and we determined that 1% isoamyl alcohol and 0.1% acetic acid were appropriate odor doses for the subsequent experiments.

3.2. Odor stimulus of isoamyl alcohol prolongs the lifespan via DAF-16

Next, we analyzed the effects of stimulation with 1% isoamyl alcohol and 0.1% acetic acid on lifespan. The group given isoamyl alcohol showed changes in the survival rate at day 14 compared to the other groups; especially, it showed a 30% higher survival rate compared to the dDW group (Fig. 2A). Meanwhile, the acetic acid group showed a similar tendency as the dDW group, suggesting that while odor stimulus of isoamyl alcohol could prolong the lifespan, acetic acid could not.

Previous studies have indicated that olfactory neurons and longevity are related to *daf-16* [4,8], suggesting that the extended life span by odor stimuli may involve DAF-16 signaling. To clarify the relevance of DAF-16 on the extended life span by odor stimulus, we next observed the lifespan using a *daf-16* mutant (*mgDf50*). Interestingly, while isoamyl alcohol extended the lifespan of WT worms, isoamyl alcohol did not prolong the lifespan of the *daf-16*

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