Original Article

Neurite Outgrowth of PC12 Mutant Cells Induced by Orange Oil and d-Limonene via the p38 MAPK Pathway

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We studied the effects of natural essential oil on neurite outgrowth in PC12m3 neuronal cells to elucidate the mechanism underlying the action of the oils used in aromatherapy. Neurite outgrowth can be induced by nerve growth factor (NGF), where ERK and p38 MAPK among MAPK pathways play important roles in activating intracellular signal transduction. In this study, we investigated whether d-limonene, the major component of essential oils from oranges, can promote neurite outgrowth in PC12m3 cells, in which neurite outgrowth can be induced by various physical stimulations. We also examined by which pathways, the ERK, p38 MAPK or JNK pathway, d-limonene acts on PC12m3 cells. Our results showed that neurite outgrowth can be induced when the cells are treated with d-limonene. After treatment with d-limonene, we observed that p38 MAPK is strongly activated in PC12m3 cells, while ERK is weakly activated. In contrast, JNK shows little activity. A study using an inhibitor of p38 MAPK revealed that neurite outgrowth in PC12m3 cells is induced via the activation of p38 MAPK by d-limonene. The results thus indicate that d-limonene may promote neural cell differentiation mainly via activation of the p38 MAPK pathway.

Key words: essential oil, d-limonene, p38 MAP kinase, PC12 mutant cells

Aromatherapy has gained significant attention in recent years. This therapy is one branch of phytotherapy, which aims to prevent or treat diseases using natural essential oil and is currently performed by health professionals as a complimentary or alternative treatment. The oils used in aromatherapy are highly concentrated and volatile fragrance substances consisting of 100% natural materials extracted from various parts of plants, including the flower, leaves, seeds, trunk, pericarp and resin [1, 2].

The aromatic substance stimulates the olfactory system, and the resulting responses can have favorable effects on the hypothalamus [3]. It is becoming clear that fragrant molecules in the essential oil have drug potency. The molecules can enter the bloodstream through the nasal membrane or alveoli. After reaching each organ, they may produce effects there, making people relaxed or restoring homeostasis. However, there is limited information on the mechanisms underlying the action of fragrant molecules.

In order for aromatherapy to be recognized as a medical treatment, the mechanism of action of the aromatic molecules should be elucidated in detail, and the effects of the treatment must be subjectively

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evaluated. One way to elucidate the mechanism is to investigate the effects of the aromatic molecules on intracellular signal transduction pathways.

Cells in living organisms undergo proliferation, differentiation or death in response to growth factors and other stimulations. The proliferation and differentiation of cells are controlled by the well-ordered expression of genes, which can be achieved because stimulations originating outside the cells can reach the genes inside the cells through the intracellular signal transduction pathways [4]. There are 3 primary mitogen-activated protein kinase (MAPK) pathways in mammalian cells. The pathways control the activities of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK [5, 6]. While ERK is activated by growth factors, JNK and p38 MAPK can be activated by environmental factors such as heat, osmotic pressure, chemicals, and radiation [7–10]. Working independently, these pathways can control cell proliferation and cell death to maintain homeostasis. Thus, it would be interesting to study the effects of aromatic molecules on the signal transduction pathway to elucidate the molecular basis of the effects of aromatherapy.

In this study, we investigated the mechanism by which an aromatic substance produces effects on the intracellular signal transduction pathways in specially prepared neuronal cells. We used orange oil and its major component, d-limonene. The cell line used in the studies, PC12m3, was previously developed in our laboratory [11]. The mutant cells do not show NGF-induced neurite outgrowth; instead neurite outgrowth is promoted in response to environmental stimuli.

The results clearly showed that the cell line is highly suitable for investigating the effects of aromatic molecules on the pathways and that the method used here is applicable to a wide variety of other essential oils used in aromatherapy. More interestingly, our results suggest that d-limonene can enhance the differentiation of cells via the p38 MAPK pathway.

Materials and Methods

Reagents. Nerve growth factor (NGF) was purchased from Takara (Osaka, Japan). Essential oil (orange-bitter or orange-sweet) and d-limonene were purchased from NARD-JAPAN (Yamanashi, Japan, Fig. 1). The essential oil and d-limonene were diluted in ethanol at the concentration of 50% (v/v) prior to use. The final concentration of ethanol was 0.17 μl/ml in medium.

Cell cultures. PC12m3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.35% glucose, 10% horse serum, 5% fetal bovine serum (FBS), and 100 U/ml kanamycin. All cells were grown in culture flasks without coating by poly-D-lysine at 37°C in 5% CO2.

Determination of neurite outgrowth. A single-cell suspension of PC12m3 cells was obtained by trituration in DMEM. For culture experiments, cells were plated onto 25 cm² flasks at a density of 2.5 × 10⁵ cells per flask of serum-containing DMEM, then the cells were treated immediately with NGF and various essential oils (orange-bitter or orange-sweet) or d-limonene. After 7 days of incubation, the lengths and numbers of neurites were measured. Cells having neurites with lengths at least 1.5-fold greater than the diameter of the cell body were counted as previously described [12]. Each value is the mean ± SD for 200 cells sampled from 3 independent experiments.

Cytotoxicity of essential oil. The cytotoxicity of essential oil was determined using trypan blue (Sigma, St Louis, MO, USA) staining. PC12m3 cells were treated with orange-bitter oil or orange-sweet oil in the presence of NGF. After 7 days of incubation, cell counts were made and viability was assessed by 0.2% trypan blue dye exclusion and by using a hemocytometer [13]. Each value is the mean ± SD for cells sampled from 3 independent experiments.

Detection of activated MAPK. PC12m3 cells were treated with d-limonene in the absence of serum for 30 min, and then the presence or absence of the activated forms of ERK, JNK, and p38 MAPK was determined by using immunoblotting. MAP kinase activity was determined by the method modified as previously described [12]. Briefly, PC12m3 cells were plated at a density of 1 × 10⁶ cells/25 cm² in a flask of serum-containing medium and cultured for 5
days. The cultures were then replaced by 0.5% FBS-containing medium for 48h. Before d-limonene treatment, the cells were incubated for 2h in serum-free medium. The cells were stimulated for 30min by the addition of d-limonene (0.17 or 0.67µl/ml). MAP kinase activity was then assayed in the cell lysates. Cells were lysed in lysing buffer (20mM Tris, pH7.6, 1mM EDTA, 150mM NaCl, 1% TritonX-100, 1mM Na3VO4, 50mM NaF, 1mM phenylmethylsulfonyl fluoride, 10mg/ml pepstatin). Aliquots of the lysates (10-15µg) from each sample were fractionated on SDS-10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The blots were probed with antibodies specific for phospho-ERK1/2 (Thr202/Tyr204), phospho-p38MAPK (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), total p38 MAPK, total JNK and tubulin (New England BioLabs; Beverly, MA, USA) at a dilution of 1:1000 in blocking buffer (5% nonfat dry milk) for 12h at 4°C. The blots were probed with a secondary antibody, horseradish peroxidaseslinked anti-rabbit IgG, at a dilution of 1:2000 in blocking buffer for 60min at room temperature. The blots were stained for 1min using a nucleic acid chemiluminescence reagent (LumiGLO chemiluminescence reagent, Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) and exposed to X-ray film. The levels of activated kinase bands were quantified by densitometric scanning and NIH Image software analysis. The statistical significance of the differences between values (p < 0.05) was determined by analysis of variance (ANOVA).

**Results**

**Essential oil-induced neurite outgrowth and effects of p38 MAPK inhibitor.** Essential oil (orange-bitter or orange-sweet) treatment of PC12m3 cells induced their neuronal differentiation, as shown clearly by the appearance of neurite outgrowth (Fig. 2). NGF induced only minor neurite outgrowth in PC12m3 cells. However, PC12m3 cells showed 4.1- and 7.8-fold higher frequencies of neurite outgrowth induced by orange-bitter and by orange-sweet, respectively, than the frequency of neurite outgrowth of nontreated control cells (Fig. 3). We studied the effects of d-limonene, which is a dominant component of orange-sweet oil and orange-bitter oil (95-98%). When PC12m3 cells were treated with d-limonene, neurite outgrowth was greatly enhanced in the presence of NGF. On the other hand, d-limonene failed to induce additional neurite outgrowth in PC12 parental cells under the same conditions (Fig. 4). To examine the

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**Fig. 2** Stimulation of PC12m3 cell neurite outgrowth by essential oil (orange-bitter or orange-sweet) and d-limonene in the presence of NGF (30ng/ml). PC12m3 cells were grown as described in the experimental procedure. Phase-contrast photomicrographs of PC12m3 cells were taken 7 days after treatment with essential oil or d-limonene or after treatment with d-limonene in the presence of the p38 MAPK inhibitor SB203580 at the final concentration of 2µM (D-limonene + SB).
role of the MAPK signaling pathway in essential-oil-induced neurite outgrowth of PC12m3 cells, the cells were pretreated for 30 min with 2 µM of SB203580, a specific inhibitor of p38 MAPK, and then treated with d-limonene. As shown in Figs. 2 and 4, SB203580 inhibited the ability of PC12m3 cells to induce neurite outgrowth in response to d-limonene treatment.

**Cytotoxicity of essential oil.** The cytotoxicity

*Fig. 3* Essential oil-mediated enhancement of neurite outgrowth in PC12m3 cells in the presence of NGF (30 ng/ml). PC12m3 cells were treated with orange-bitter oil (0.17 µl/ml) or orange-sweet oil (0.17 µl/ml). The percentages of cells with neuritis were determined after 7 days. Each value is given as the mean ± S.D. for 200 cells sampled from 3 independent experiments.

*Fig. 4* Enhancement of neurite outgrowth in PC12m3 cells by d-limonene. PC12 parental and PC12m3 cells were treated with d-limonene in the absence or presence of NGF (30 ng/ml). PC12m3 cells stimulated with d-limonene (0.17 µl/ml) were pretreated with a specific p38 MAPK inhibitor SB203580 (2 µM) for 30 min prior to culturing (0.17 + SB). To examine the effects of ethanol on the inhibition of neurite outgrowth in PC12m3 cells in the presence of NGF, a concentration of ethanol, 0.17 µl/ml, was added to the medium. The percentage of cells with neuritis was determined after 7 days. Each value is given as the mean ± S.D. for cells samples from 3 independent experiments. *p < 0.05 denotes significant statistical difference compared with PC12m3 cells treated with NGF alone or treated with NGF and d-limonene without SB203580.

*Fig. 5* The effect of essential oil on the viability of PC12m3 cells. PC12m3 cells were treated with orange-bitter oil (0.17 µl/ml) or orange-sweet oil (0.17 µl/ml) in the presence of NGF (30 ng/ml). The cell number of viable or mortal cells was determined after 7 days using trypan blue staining as described in the experimental procedure. Each value is given as the mean ± S.D. for 200-500 cells sampled from 3 independent experiments.
of orange-bitter oil and orange-sweet oil was examined using trypan blue staining. The number of viable cells was 25.1% of the number of cells in the control for the orange-bitter oil treatment and 13.1% for the orange-sweet oil treatment, showing that the latter more strongly suppressed cell growth due to cell differentiation. The orange-bitter oil treatment resulted in a 1.8-fold greater number of dead cells in comparison to control cells, while the orange-sweet oil treatment resulted in a 0.7-fold greater count. These results show that orange-bitter oil had some cytotoxicity but that orange-sweet oil did not (Fig. 5).

**Neurite outgrowth-inducing action of d-limonene was mediated by the p38 MAPK pathway in PC12m3 cells.** The activation of ERK has been shown to play an important role in neuronal differentiation in PC12 cells. However, PC12m3 cells have been shown to have poor neurite

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**Fig. 6** D-limonene-induced activation of p38 MAPK, ERK and JNK. PC12m3 cells were treated with d-limonene for 30 min, and the extracts were subjected to immunoblotting with anti-phospho-ERK1/2, anti-phospho-p38 MAPK and anti-phospho-JNK or anti-p38 and anti-JNK antibodies. Equal protein loading was confirmed with anti-tubulin antibody. The levels of activated ERK1/2, p38 MAPK and JNK were quantitated by densitometric scanning. Each value represents the percentage of that in response to non-treated control cells and represents the mean with S.D. of 3 independent experiments.
outgrowth despite normal sustained activation of ERK by NGF treatment [11]. On the other hand, the activation of p38 MAPK in addition to ERK activity has been observed in NGF-treated PC12 cells [14]. We therefore examined d-limonene-induced activation of three MAPK pathway including ERK, p38 MAPK and JNK in PC12m3 cells. The results showed that d-limonene enhanced ERK and p38 MAPK activity but that JNK was not activated by d-limonene (Fig. 6). Interestingly, p38 MAPK was strongly activated by d-limonene in PC12m3 cells. On the other hand, neurite outgrowth induced by d-limonene was inhibited by a specific p38 MAPK inhibitor, SB203580 (Fig. 4). These results indicate that activation of p38 MAPK is required for d-limonene-induced neurite outgrowth in PC12m3 cells.

Discussion

In this study, we investigated the effects of orange oil and its major component, d-limonene. Orange oil has been shown to have anti-stress effects, and 95 to 98% of the oil consists of d-limonene [1].

Our results show that treatment of cells with orange-bitter oil and orange-sweet oil resulted in 3.7- and 6.4-fold higher frequencies of neurite outgrowth compared with non-treated control cells. The different effects might be explained by the trace amount of geraniol that is contained in orange-sweet oil but not in orange-bitter oil. The low-level cytotoxicity of orange-bitter oil might be related to furocoumarin contained in the oil, which has a phototoxic property.

The presence of d-limonene greatly changes intracellular environments by activating p38 MAPK, resulting in the promotion of the synthesis of a series of proteins. p38 MAPK contributes to the survival of cells [15]. In other words, the kinase plays an important role in stress-dependent differentiation. Furthermore, ERK and p38 MAPK have a greater effect on cell differentiation and protection than does JNK since d-limonene more strongly activates ERK and p38 MAPK than JNK. The results of our study revealed that d-limonene functions to prevent cell death and circumventing stress [16–20].

Orange oil is often used in air-refreshing units, air refreshers, and perfume lotion due to its familiar citrus fragrance. The major component (95–98%) of the oil is a monoterpen hydrocarbon called d-limonene. D-limonene is known to activate the digestive system [1]. Since the oil contains a trace amount of furocoumarins, attention should be paid to its phototoxic effects. When added to massage oil, its relaxing effects are expected to increase because of synergistic effects. Orange oil may also have synergistic effects of psychological and physical sedation and fatigue relaxation when used together with esters and terpene aldehydes, which themselves have sedative and relaxing effects.

There are many reported effects of d-limonene: blood-pressure-lowering effects, promotional effects on peristaltic movement in the digestive system, antimutagenic effects, anticancer effects (gastric cancer, lung cancer, etc.), sedative effects due to suppression of central nervous system activity, and immunomodulatory effects [21–23]. D-limonene is also thought to have strong anti-carcinogenic effects and is useful in chemophylaxis and chemotherapy of tumors [24].

One of the effects of essential oil on intracellular signal transduction pathways is the induction of anticancer detoxicating enzymes that inhibit cancer initiators. D-limonene suppresses cancer-causing factors such as c-jun and c-myc, which are overexpressed in response to stress [22, 23, 25]. D-limonene also suppresses cholesterol synthesis through the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [26]. In addition, it inhibits the binding of mevalonate-derived isoprene complex and Ras or Ras-related proteins. The inhibition is stronger in cancer cells because cancer cells are sensitive to the inhibition. The inhibition depresses actin activities in cells through lowering the levels of Rho proteins, cell chemotaxis, and the secretion of urokinase (which is required for tumor metastasis). Perilllic acid, an isomer of d-limonene in living organisms, has the same effects and strictly blocks the MAPK pathways [27]. Perilllic acid interrupts signaling via the Ras/MAP kinase pathway by depleting farnesylated Ras levels, an effect that contributes to the inhibition of IL-2 production and T cell activation.

Furthermore, d-limonene lowers the levels of BCL-XL and Bax, which cause immortalization of cells, and promotes apoptosis leading to the destruction of cancer cells [28]. It arrests the cell cycle at G0/G1, which also helps prevent cancer. D-limonene increases the expression level of p53, a tumor suppression gene. Cancer cells need to decrease their
adhesiveness for metastasis. D-limonene increases gap-junctional communication complexes, which means that d-limonene can help prevent cancer metastasis.

The present study suggests that d-limonene may not have strong effects on cytotoxin because it only causes a low level of JNK activation. On the other hand, d-limonene causes higher levels of activation of p38 MAPK. This means that p38 MAPK promotes cell differentiation and protection more strongly than does JNK. Thus, the results clearly show that d-limonene can prevent cell death and help circumvent stress.

Morooka and Nishida reported that p38 MAPK appears to be involved in a variety of functions, including differentiation and nervous functions [14]. Furthermore, p38 MAPK has been demonstrated to be required for growth factor or cAMP-induced neuronal differentiation in rat PC12 cells. We previously showed that the p38 MAPK pathway inhibitor SB203580 but not the ERK pathway blocker U0126 inhibited the activity of PC12m3 cells to induce neurite outgrowth in response to osmotic shock [9, 11]. In the present study, we showed that d-limonene promotes neurite outgrowth of PC12m3 cells, but not that of PC12 parental cells, via activation of the p38 MAPK pathway (Fig. 2). Several reports indicate that p38 MAPK is activated in the brain. The formation of short-term and long-term memory for inhibitory avoidance requires p38 MAPK activation in the rat hippocampus [29]. Guo found that p38 MAPK was activated by sleep deprivation in the hippocampus [30]. The transportability of d-limonene through the blood brain barrier (BBB) has been reported [31]. The BBB, constituted by the cerebral capillary endothelium, serves as a regulatory interface between circulating blood and brain tissue. The essential oils used in aromatherapy are volatile constituents. Inoue et al. studied the relative volatilities of some aroma compounds [32]. The most volatile of the aroma compounds studied in their work, including octanal, β-ionone and limonene, was limonene based on its hydrophobicity and its relatively high vapor pressure. When a mouse was exposed to an aroma bath for 20 min at 37°C, the concentrations of d-limonene in the blood and brain were 0.63 μg/ml and 2.07 μg/g, respectively, due to percutaneous absorption [32]. These findings indicate that d-limonene absorbed through the BBB may affect the nervous functions via the p38 MAPK pathway in the brain.

As one of the effects of essential oils on living organisms, the aromatic substance in the oil stimulates the olfactory system and then works directly on the limbic system leading to stimulation of the hippocampus, amygdalae and cingulated gyrus, which control memory, feelings of pleasure and pain and motivation, respectively. When the brain recognizes the stimulation of a fragrance, there is a feeling of pleasure, and pleasure hormones are secreted in the body [33–36]. A comfortable smell can make people feel relaxed and can increase the natural capability to heal that is present in everyone. Thus, aromatherapy, which can heal the mind and body using aromatic materials from flowers, herbs and plants, has attracted much attention as a medical treatment. Our results clarify that d-limonene in the essential oils used in aromatherapy contributes to the protection and differentiation of cells. It is also clear from our results that d-limonene helps prevent cell death and circumvent stress [19]. Thus, we believe that aromatherapy has the potential to cure and prevent diseases, to prevent infection, to promote the natural healing capability, and to stabilize the mind and body.

References

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