



Linalool Exerted a Neuroprotective Activity against Corticosterone-induced Apoptosis of PC12 cells

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ABSTRACT

Introduction: Linalool is an active compound found in many essential oils having sedative, anxiolytic and analgesic properties. Therefore, these essential oils have been usually used in aromatherapy to reduce stress. However, the mechanism underlying anti-stress of linalool has not been investigated.

Objective: To investigate the mechanism of neuroprotective action of linalool on corticosterone-induced neurotoxicity in PC12 cells.

Methods: PC12 cells were treated with 650 μ M of corticosterone in the absence or presence of linalool or fluoxetine for 24 hours. PC12 was treated with corticosterone 650 μ M and difference concentration of linalool (100, 200, 300, 400, 500, 600 and 700 μ M) for 24 hours. Methyl thiazolyl tetrazolium (MTT) assay and lactate dehydrogenase (LDH) detection were investigated to confirm the neuroprotective effect of linalool against cell damage caused by corticosterone. Then, the intracellular Ca^{2+} content was measured by fluorescent labeling.

Results: Linalool 100 and 200 μ M significantly increased cell viability compared with the group treated with corticosterone alone ($p<0.05$). Linalool 100, 200, 300 and 400 μ M significantly ($p<0.05$) reduced LDH release. Linalool (100, 200, 300, 400, 500 and 600 μ M) tend to reverse the increased intracellular Ca^{2+} produced by corticosterone.

Conclusion: These findings indicated that linalool exerted a neuroprotective activity against corticosterone-induced neuronal apoptosis in PC12 cells. The mechanism underlying this action may be partly due to the attenuation of intracellular Ca^{2+} overloading under stress conditions. However, further studies need to investigate the precise cellular and molecular mechanisms underlying this neuroprotective activity of linalool.

Keywords: linalool; corticosterone; PC12; apoptosis; intracellular calcium

Introduction

Depression is one of the most common mental disorders, leading to a decline in social ability, disability and many diseases [1,2]. The hypothalamic-pituitary-adrenal (HPA) axis activation is one of the acceptable mechanisms underlying the pathophysiology of depression. Dysregulation of the HPA axis results in elevated cortisol, the stress hormone as well as corticotropin-releasing hormone as well as hippocampal volume reduction [3,4]. Antidepressant drugs such as

fluoxetine can inhibit the activation of the HPA axis [5]. However, long-term treatment of antidepressant can cause many side effects, including nausea, insomnia, agitation, weight gain, somnolence, sexual dysfunction, and cardiovascular adverse events. Therefore, complementary and alternative medicines (CAM) have been increasingly used for treating depression [6]. Aromatherapy, one of the CAM options, has been frequently chosen by patients with depressive symptoms since it is an invasive and inexpensive treatment [7].

Lavender [8,9] and bergamot [10], essential oils commonly used in aromatherapy to reduce depressive symptoms, contain the active components including linalool [11]. The previous study found that linalool demonstrated antidepressant-like effects by decreasing immobility time in the tail suspension test in mice [12]. There are many studies investigating the effect of linalool in various stress conditions. For example, linalool attenuated the decreased cell viability against glucose/serum deprivation (GSD)-induced cytotoxicity, indicating its neuroprotective effect [13]. Recently, Migheli et al (2021) also reported that linalool protected PC12 cells from H₂O₂-induced oxidative stress by increasing cell viability, reducing LDH release and ROS production [14]. However, the neuroprotective action of linalool on stress induced by corticosterone, another stress hormone, has not been investigated. Therefore, the aim of the present study was to investigate the mechanism of neuroprotective action of linalool on corticosterone-induced neurotoxicity in PC12 cells.

Methodology

Cell culture

PC 12 cells were ordered from the American type culture collection (ATCC) (Manassas, USA). PC12 cells were cultured in collagen-coated plate and maintained with Dulbecco's modified Eagle's medium (DMEM) cell culture, consisting of 0.35% glucose, 10% horse serum, 5% fetal bovine serum (FBS) and 100 U/ml kanamycin and subsequently incubated at 37°C for 48 hours until 70% cell growth. Then, the cells were initially incubated with linalool solution plus 650 µM corticosterone for 24 hours and then studied 1) survival of PC12 cells against corticosterone, 2) LDH secretion from PC12 cells, and 3) intracellular calcium level in PC12.

Chemicals

Linalool, fluoxetine and corticosterone were purchased from Sigma–Aldrich (St Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from Millipore (Billerica, MA, USA) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from MP Biomedical (Illkirch, France). Fura-2/AM dye was purchased from Invitrogen (Carlsbad, CA, USA).

Cell viability assay

Cell viability was determined by using (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) MTT assay. In brief, PC12 cells were cultured in 96 well plates at a density of 5×10³ cells/well in serum-free DMEM and allowed to adhere at 37 °C, 5%

CO₂ incubator for 24 hours. The cells were co-treated with various concentrations of linalool (100, 200, 300, 400, 500, 600, 700 µM) or 10 µM fluoxetine and 650 µM corticosterone and incubated for 24 hours. Then, a 20 µl MTT solution was added and the plate was further incubated in a CO₂ incubator at 37 °C for 4 hours. The medium was aspirated off and 100 µL DMSO was added to dissolve formazan crystals. The absorbance of the purple solution was measured at 545 nm by spectrophotometer (Epoch™ microplate spectrophotometer; Biotek, Winooski, VT, United States). Cell viability was shown as a percentage of non-treated control.

Lactate Dehydrogenase (LDH) Assay

This assay is based on the measurement of LDH release from the cells due to damage of plasma membrane. Thus, the presence of stable LDH in cell culture medium can be detected and used as an indicator of cellular toxicity. LDH release was measured by following the instruction manual of LDH activity assay kit and using spectrophotometer (Epoch™ microplate spectrophotometer; Biotek, Winooski, VT, United States). All experiments were performed in triplicate.

Measurement of Intracellular Ca²⁺ Concentration

Quantitative intracellular calcium was determined using Fura-2/AM, fluorescence indicator of free calcium. Initially, PC12 cells were co-treated with various concentrations of linalool (100, 200, 300, 400, 500, 600, 700 µM) or 10 µM fluoxetine and 650 µM corticosterone and incubated for 24 hours. Then, the cells were collected and incubated with Fura-2/AM and pluronic F-127 for 45 min at 37 °C. After washing with ice-cold PBS solution, the fluorescence was measured by microplate reader (Epoch™ microplate spectrophotometer; Biotek, Winooski, VT, United States) at 400 nm excitation and 505 nm emission. Cell viability was shown as a percentage of non-treated control.

Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). The statistical analysis was performed with one-way ANOVA followed by Newman-Keuls multiple comparison test using GraphPad Prism 9 software. The differences between mean values were accepted as statistically significant at $p \leq 0.05$. Each experimental group consisted of 6 cell samples.

Results

Effect of linalool on viability of PC12 cells after inactivation by corticosterone

As shown in figure 1, 650 μ M corticosterone significantly decreased cell viability of PC12 ($p<0.01$). The 100 and 200 μ M linalool significantly attenuated the decreased cell viability produced by corticosterone. ($p<0.05$), while fluoxetine 10 μ M resulted in less cell survival than incubating with corticosterone alone ($p<0.01$).

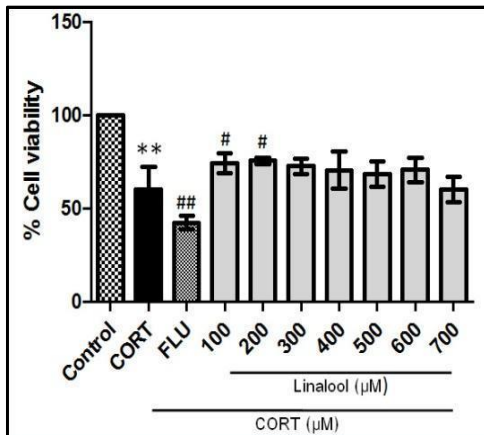


Figure 1 Effects of linalool on corticosterone (CORT)-induced cytotoxicity of PC12 cells measured by MTT assay (mean $\pm$ SD, n= 4-6). ** $p<0.001$ as compared with control (CONT) group; # $p<0.05$ and ## $p<0.01$ as compared with the CORT group.

Effect of linalool on corticosterone-induced LDH release from PC12 cells

As indicated in figure 2, the percentage of LDH release was significantly increased for corticosterone-treated PC12 cells ($p<0.001$). Linalool (100, 200, 300 and 400 μ M) but not 10 μ M fluoxetine significantly attenuated the increased LDH release produced by corticosterone ($p<0.01$).

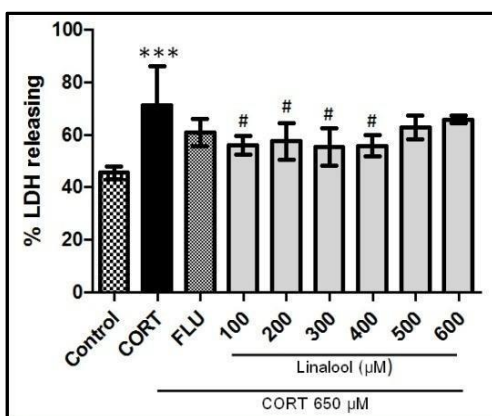


Figure 2 Effects of linalool on LDH release in corticosterone (CORT)-induced PC12 cells (mean $\pm$ SD, n= 4-6). *** $p<0.001$ as compared with control (CONT) group; # $p<0.05$ as compared with the CORT group.

Effect of linalool on corticosterone-induced intracellular Ca^{2+} concentration in PC12 cells

As shown in figure 3, after treatment of PC12 cells with 650 μ M corticosterone for 24 h, the concentration of intracellular Ca^{2+} markedly increased as compared with the control group ($p<0.01$). By contrast, linalool (100, 200, 300, 400, 500 and 600 μ M) tend to reverse the increased intracellular Ca^{2+} produced by corticosterone. Surprisingly, 10 μ M fluoxetine did not attenuate the increased Ca^{2+} release produced by corticosterone. ($p<0.01$).

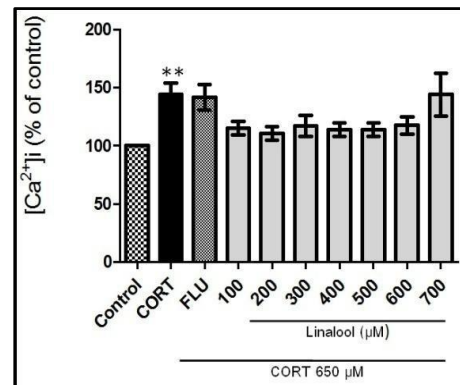


Figure 3 Effects of linalool on corticosterone-induced intracellular Ca^{2+} concentration in PC12 cells (mean $\pm$ SD, n= 4-6). ** $p<0.01$ as compared with the control (CONT) group.

Discussion

Linalool is the principal component of many essential oils produced from aromatic plants including members of the Lamiaceae (mints), Lauraceae (laurels, cinnamon, rosewood) and Rutaceae (citrus) [15-17]. Linalool, a lipophilic monoterpene, may pass the blood brain barrier to exert effects on brain function. Many studies have demonstrated that linalool exerts neuroprotective, anti-inflammatory, and antioxidant effects in the brain [18-21]. Linalool demonstrated antidepressant-like effects in animal models and neuroprotective action against glucose/serum deprivation and H_2O_2 induced oxidative stress. However, the neuroprotective action of linalool on stress induced by corticosterone, another stress hormone, has not been investigated. These findings indicated for the first time that linalool exerted a neuroprotective activity against corticosterone-induced neuronal apoptosis in PC12 cells. The previous studies have shown that high corticosterone level caused severe PC12 damage including apoptosis and decreased nerve growth factor (NGF) mRNA levels in cells [22], but antidepressants can alleviate this damage [23]. This is consistent with the present study that showed that linalool attenuated the decreased cell viability of PC12 induced by corticosterone. Unexpectedly, fluoxetine, the standard

antidepressant, potentiated the decreased cell viability produced by corticosterone. Similarly, after fluoxetine, the cell viability showed a significant increase at day1 but a slight decrease at day 2 [24]. Recently, it was also shown that neuronal death was markedly increased with treatment of 10-30 μ M fluoxetine [25]. Therefore, our results suggest that linalool may protect PC12 cells from the toxic effect of the hormone corticosterone superior to fluoxetine, used with high dose or longer duration. LDH is an enzyme present in the cytoplasm of all mammalian cells. Normally, the plasma membrane does not allow the secretion of LDH, but LDH is released into the extracellular fluid following cell damage. Thus, the LDH leakage has been commonly used as the marker of cell damage or injury. The previous study showed that antidepressant medications can attenuate PC12 cell damage from corticosterone by reducing LDH release [26]. Consistent with this study, it was found that high corticosterone level increased LDH secretion, likely due to cell damage as shown in the MTT experiment with reduced cell viability. In contrast, linalool attenuated the increased LDH secretion and decreased cell viability induced by corticosterone treatment. Thus, this study suggests that linalool has a protective effect on PC12 cell damage from corticosterone exposure, while fluoxetine does not have a protective effect against cell damage from this stress hormone. Intracellular calcium is a key signaling ion involved in neuronal cell function including synaptic activity, cell communication and adhesion [27]. After corticosterone exposure, intracellular calcium was elevated, resulting in increased LDH release and ROS production which subsequently cell damage [28]. This study also found that the concentration of intracellular Ca^{2+} was upregulated in corticosterone-treated PC12 cells but slightly downregulated in the cells co-treated with linalool.

Conclusion

These results indicate that the neuroprotective effect of linalool against corticosterone-induced apoptosis may be partly mediated by the inhibition of intracellular Ca^{2+} concentration intracellular calcium overload. However, further studies need to investigate the precise cellular and molecular mechanisms underlying this neuroprotective activity of linalool.

Competing Interests

No conflicts of interest, financial or otherwise, are declared by the authors.

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Author contributions

All authors contributed to the final version of the manuscript.

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