

## Limonene Enhances the cAMP Response Element (CRE)-Dependent Transcriptional Activity Activated via Adenosine A<sub>2A</sub> Receptor in a Neural-Crest Derived Cell Line, PC-12

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### Abstract

Prevention and treatment of Alzheimer's disease are urgent problems for elderly people in developed countries. To seek useful compounds that treat, delay the onset, or prevent the development of Alzheimer's disease, we screened natural resources using the cyclic AMP response element-dependent transcription in the PC12 cell line. We previously found that honey bee royal jelly stimulates the cyclic AMP response element-dependent transcription activity in PC12 cells. Here, we report that *R*-limonene, a monoterpene, enhanced the royal jelly-induced cyclic AMP response element-dependent transcription activity, whereas *R*-limonene showed no effect in absence of royal jelly. The enhancement was inhibited by ZM241385, an antagonist for adenosine A<sub>2A</sub> receptor. *R*-limonene also enhanced the cyclic AMP response element-dependent transcription activity activated by adenosine and AMP. Thus, *R*-limonene represents a novel type of modulator that enhances the CRE-dependent transcription via adenosine A<sub>2A</sub> receptor pathway.

### Key words

adenosine receptor · CRE-dependent transcription · limonene · memory · royal jelly

### Abbreviations

AD: Alzheimer's disease  
cAMP: cyclic AMP  
CRE: cAMP response element

LTP: long-term potentiation

RJ: royal jelly

Supporting information available online at <http://www.thieme-connect.de/products>

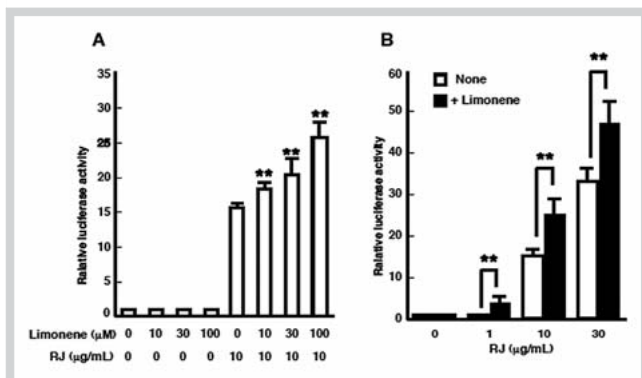
Neurodegenerative disorders, including AD, have a devastating impact on both patients and society [1]. Due to this expanding threat to our society, disease management and therapy are urgently needed. The biological basis of learning and the memory depends on the establishment of LTP in the hippocampus [2]. Biochemically, LTP is formed by activation of cAMP-dependent signaling and subsequent transcription of the target genes of the CRE-binding protein. Our screening of natural resources lead to the finding that nobiletin, a citrus flavonoid, activates the CRE-dependent transcription in PC12D cells, a rat pheochromocytoma cell line [3]. Our next studies proved that nobiletin improves the memory deterioration in beta-amyloid infused AD model rats [4], bulbectomy-induced cholinergic neurodegenerative mice [5], a transgenic mouse model of AD [6], and Parkinson disease model mice [7]. Using the same strategy, we found that honeybee RJ stimulates the CRE-dependent transcription in PC12D cells [8]. RJ contains AMP and AMP-N1-oxide that induces the neurite outgrowth via adenosine A<sub>2A</sub> receptor [9].

Adenosine A<sub>2A</sub> receptor is a G-protein coupled receptor that distributes in the central nervous system [10]. The role of adenosine A<sub>2A</sub> receptor in the formation of LTP is puzzling [11]. Although an agonist of the receptor increased LTP in the rat hippocampus [12], mice deficient in the receptor exhibited the improved spatial recognition [13]. Thus, modulators of the receptor are potential pharmacological tools to treat AD, although there is a controversy over the function of adenosine A<sub>2A</sub> receptor in the brain activity. To find a substance that potentiates the antidementia activity of RJ, we screened extracts derived from herbal plants. Preliminary screening identified limonene as a candidate for the activator of the RJ-induced stimulation of CRE-dependent transcription in PC12 cells. To confirm this finding, we tested commercially available limonenes for the CRE-dependent transcription induced by RJ in PC12 cells. As shown in **Fig. 1A**, *R*-limonene dose-dependently enhanced the CRE-dependent transcription activity induced by 10 µg/mL RJ. Interestingly, *R*-limonene had no effect on the CRE-dependent transcription activity in the absence of RJ. *R*-limonene was effective on both low and high concentrations of RJ (**Fig. 1B**).

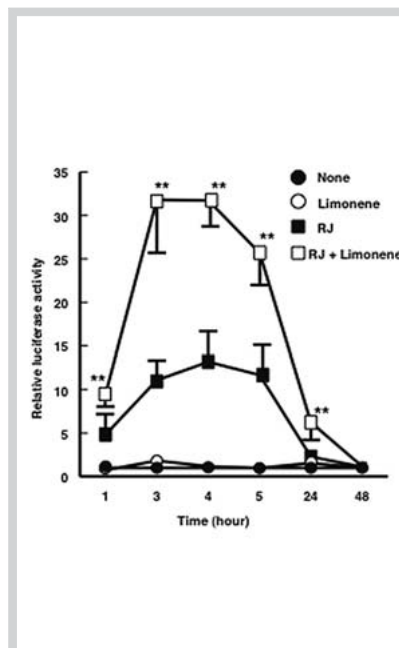
We next examined the effect of *R*-limonene on RJ along the time-course of its stimulation of CRE-dependent transcription (**Fig. 2**). RJ at 10 µg/mL significantly increased the CRE-dependent transcription activity 1–5 h after administration. The activating effect of RJ diminished at 24 and 48 h after administration. The addition of 100 µM *R*-limonene to RJ significantly enhanced the CRE-dependent transcription activity induced by RJ from 1 to 24 h. Again, *R*-limonene alone had no effect on the CRE-dependent transcription activity at all time points examined.

We next tested the effect of several analogues of *R*-limonene on the CRE-dependent transcription induced by RJ (**Fig. 3**). (*S*)-(-)-limonene and (4*R*)-limonene oxide enhanced the RJ-induced CRE-dependent transcription comparable to the level of *R*-limonene. (4*S*)-limonene oxide significantly increased the CRE-dependent transcription induced by RJ, but the potency of the limonene oxide was weaker than that of *R*-limonene. Interestingly, all these limonene analogues had no effect on the CRE-dependent

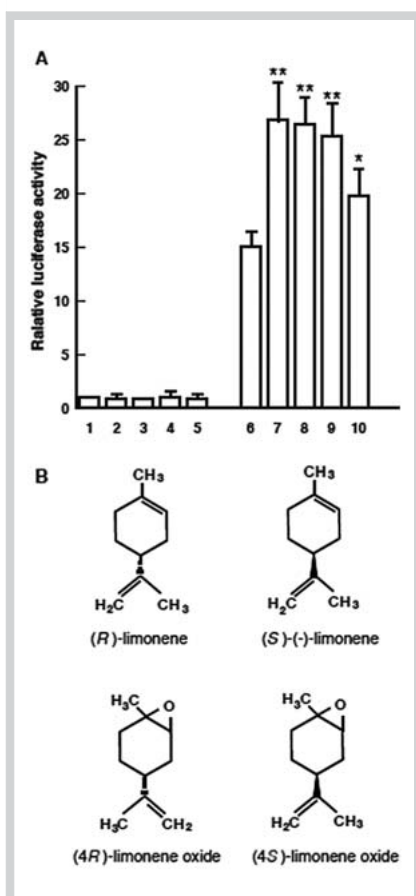
\* These authors contributed equally to this work.



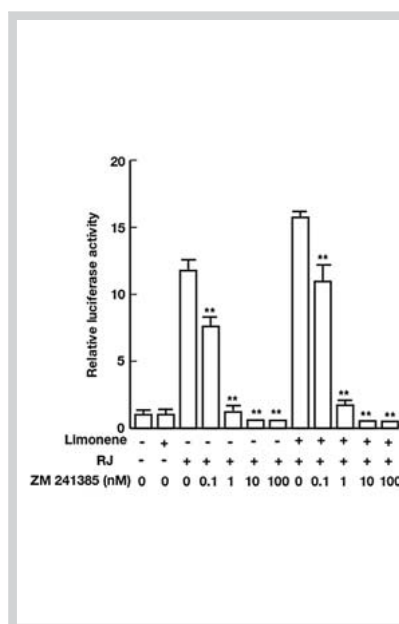
**Fig. 1** Limonene enhances the CRE-dependent transcription induced by RJ in PC12 cells. **A** Limonene dose-dependently enhanced the CRE-dependent transcription induced by RJ. Note that limonene alone had no effect on the CRE-dependent transcription. **B** Limonene was effective on various concentrations of RJ in enhancing the CRE-dependent transcription. PC12 cells were transfected with a CRE reporter plasmid and incubated with RJ in the presence or absence of 100 μM limonene for 5 h. The luciferase activity was relative to the control value. The assay was performed in quadruplicate. \*\**P* < 0.05.



**Fig. 2** Time-course of the effect of limonene on the CRE-dependent transcription induced by RJ. PC12 cells were transfected with a CRE reporter plasmid and stimulated with RJ and/or limonene for the indicated times. The luciferase activity was relative to the control value (1 h without any additives). ●, none; ○, 100 μM limonene; ■, 10 μg/mL RJ; □, 10 μg/mL RJ + 100 μM limonene. The assay was performed in quadruplicate. \*\*Significantly different from the value with RJ at the same time point, *p* < 0.05.



**Fig. 3** Effect of limonene analogues on the CRE-dependent transcription induced by RJ in PC12 cells. PC12 cells were transfected with a CRE reporter plasmid and incubated with RJ and/or various types of limonene analogues for 5 h. The luciferase activity was relative to the control value. 1, control; 2, *R*-limonene; 3, (*S*)-(-)-limonene; 4, (*4R*)-limonene oxide; 5, (*4S*)-limonene oxide; 6, 10 μg/mL RJ; 7, 10 μg/mL RJ + *R*-limonene; 8, 10 μg/mL RJ + (*S*)-(-)-limonene; 9, 10 μg/mL RJ + (*4R*)-limonene oxide; 10, 10 μg/mL RJ + (*4S*)-limonene oxide. Limonene analogues were tested at 100 μM. The assay was performed in quadruplicate. \*\*Significantly different from the value with RJ alone, *p* < 0.05.



**Fig. 4** Effect of ZM241385, an antagonist of adenosine  $A_{2A}$  receptor, on the CRE-dependent transcription induced by RJ and limonene. PC12 cells were transfected with a CRE reporter plasmid and incubated with 10 μg/mL RJ and/or 100 μM limonene in the presence of various concentrations of ZM241385 for 5 h. The luciferase activity was relative to the control value. The assay was performed in quadruplicate. \*\*Significantly different from the value without ZM241385, *p* < 0.05.

transcription in absence of RJ. These results suggest the propenyl moiety that attaches to the cyclohexene or cyclohexane might be important for the activating effect of limonenes.

Next, we examined the effect of the adenosine receptor  $A_{2A}$  antagonist ZM241385 on the RJ-induced stimulation of CRE-dependent transcription. The antagonist dose-dependently suppressed

the CRE-dependent transcription induced by RJ (● Fig. 4). The limonene-induced enhancement of the CRE-dependent transcription was also inhibited by ZM241385 in a similar manner. These results suggest that the action of RJ on the CRE-dependent transcription is probably mediated via the adenosine receptor  $A_{2A}$ . We examined the effect of limonene on the CRE-dependent transcription induced by adenosines. Both adenosine and AMP activated the CRE-dependent transcription in PC12 cells in a dose-dependent manner (Fig. 1S, Supporting Information). Adenosine was more potent than AMP in activating the CRE-dependent transcription. The addition of limonene dose-dependently enhanced the CRE-dependent transcription induced by adenosine (Fig. 2A S, Supporting Information). (*S*)-(-)-limonene and (*4R*)-limonene oxide significantly enhanced the adenosine-induced CRE-dependent transcription like *R*-limonene (Fig. 2B S, Support-

ing Information). (4*S*)-Limonene oxide slightly increased the CRE-dependent transcription induced by adenosine, but the increase was insignificant. These results confirmed the effect of limonenes on RJ and that limonene acts on the signaling pathway that includes adenosine receptor A<sub>2A</sub>.

We have shown here that limonene enhanced the CRE-dependent transcription induced by RJ and adenosine via adenosine receptor A<sub>2A</sub> in PC12 cells. RJ has been known to induce the neurite outgrowth mediated by the adenosine receptor in PC12 cells [9]. Both the neurite outgrowth and the CRE-dependent transcription induced by RJ appear to share the similar signal transduction pathways. In this regard, it is interesting that limonene competitively decreased the binding of [<sup>3</sup>H]CGS 21 680, an A<sub>2A</sub> receptor agonist, to a rat brain cerebral cortex membrane, suggesting that limonene is a direct ligand for A<sub>2A</sub> receptor [14]. But limonene alone failed to stimulate the CRE-dependent transcription in PC12 cells (● **Figs. 1A, 2 and 3**), whereas AMP and adenosine activated the CRE-dependent transcription (**Fig. 1S**, Supporting Information). Thus, the interaction of limonene with the adenosine receptor may be different from that of the authentic ligands. Limonene may act on the adenosine A<sub>2A</sub> receptor in an allosteric manner.

That limonene itself has no activity to induce the CRE-dependent transcription, while it augments the activity of ligands of adenosine A<sub>2A</sub> receptor, makes limonene a unique compound. A comparison of the activities of limonene analogues revealed that the propenyl moiety that attaches to the cyclohexene or cyclohexane might be important in modulating the activity of ligands of the adenosine A<sub>2A</sub> receptor (● **Fig. 3**). The structure will become a lead compound to develop a novel type of agonists with higher activity than limonene.

## Materials and Methods

### Materials

Native RJ was obtained by the Yamaguchi's organic bee culture method [15]. The RJ used in this study was prepared from frozen powder of native RJ as described [8]. *R*-limonene was purchased from Nacalai Tesque. (*S*)-(-)-Limonene, (4*R*)-Limonene oxide (mixture of *cis* and *trans*), (4*S*)-Limonene oxide (mixture of *cis* and *trans*), adenosine 5'-monophosphate, adenosine, and ZM 241 385 (purity > 98%) were obtained from Sigma-Aldrich. PC12 cells were obtained from the Riken BioResource Center.

### Measurements of CRE-dependent transcriptional activity in PC12 cells

To measure the CRE-dependent transcriptional activity in PC12 cells, we adopted the reporter gene assay as described previously [8]. Details are provided in the Supporting Information. The concentration of limonene and its analogues used in the present study (100 μM) had little effect on the morphology of PC 12 cells.

### Statistical analysis

Data are expressed as the means ± SD. Comparison between the groups were performed using paired Student's t-test using Microsoft Excel.

## Supporting information

Details on the measurement of the CRE-dependent transcriptional activity in PC12 cells and on the effects of adenosine and AMP on the CRE-dependent transcription in PC12 cells, and of limonene on the CRE-dependent transcription induced by adenosine are available as Supporting Information.

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## Conflict of Interest

▼ The authors declare no conflict of interest.

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