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Limonene, a natural cyclic terpene, is an agonistic ligand for adenosine A_{2A} receptors

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ABSTRACT

Limonene is a major aromatic compound in essential oils extracted from citrus rind. The application of limonene, especially in aromatherapy, has expanded significantly, but its potential effects on cellular metabolism have been elusive. We found that limonene directly binds to the adenosine A_{2A} receptor, which may induce sedative effects. Results from an *in vitro* radioligand binding assay showed that limonene exhibits selective affinity to A_{2A} receptors. In addition, limonene increased cytosolic cAMP concentration and induced activation of protein kinase A and phosphorylation of cAMP-response element-binding protein in Chinese hamster ovary cells transfected with the human adenosine A_{2A} receptor gene. Limonene also increased cytosolic calcium concentration, which can be achieved by the activation of adenosine A_{2A} receptors. These findings suggest that limonene can act as a ligand and an agonist for adenosine A_{2A} receptors.

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1. Introduction

Adenosine is an endogenous nucleoside that modulates numerous physiological processes, including oxygen and metabolic balance in tissues [1], immune response [2], and signaling in the nervous system [3]. Four mammalian subtypes of adenosine receptors (ARs) have been identified and their genes cloned as follows: A₁, A_{2A}, A_{2B}, and A₃. AR subtypes are distributed in a tissue-specific manner [4]. For example, A_{2A} receptors are highly expressed in the brain, immune cells, heart, lung, and blood vessels.

ARs differentially modulate the intracellular levels of adenosine 3',5'-cyclic monophosphate (cAMP). Thus, A₁ and A₃ receptor activation inhibits adenylyl cyclase to reduce cAMP concentration, whereas A_{2A} and A_{2B} receptor activation elevates cAMP levels [5]. Therefore, in the A_{2A} receptor subtype, ligand binding stimulates protein kinase A (PKA) and induces phosphorylation activation of cAMP response element-binding (CREB), which regulates the transcription of several target genes. In addition, ligand binding to the A_{2A} receptor induces the formation of inositol 1,4,5-triphosphate, which results in opening of endoplasmic reticulum calcium channels [6,7]. These changes are implicated in several clinical conditions, including cardiovascular disease [8], sleep disorders [9], and inflammation [10].

Limonene is a major aromatic compound in essential oils obtained from oranges, grapefruits, and lemons. These essential oils have long been popular in aromatherapeutic practice due to potent calming and sedative effects that alleviate nervous disorders, heart problems, colic, asthma, and depression. In addition, limonene has

anticancer activity [11], but its mechanism has not been elucidated. This study was designed to explore the effects of limonene on cellular metabolism through A_{2A} receptors. We first examined its affinity to the A_{2A} subtype. To further clarify limonene effects on A_{2A} receptor activation, we also investigated the resultant changes in each step of the A_{2A} receptor signaling pathway.

2. Materials and methods

2.1. Membrane preparation

Rat brain tissue membrane fractions were prepared as previously described [12]. Briefly, rat brain tissue was removed and the striata and cortices were dissected out at 4 °C in a 0.32 M sucrose solution containing 50 mM Tris–HCl and 2 mM EGTA (pH 7.6). The tissue was then homogenized in a Potter–Elvehjem homogenizer (Thermo Scientific, Rockford, IL, USA) at 4 °C and the resulting homogenates were centrifuged at 1000g for 10 min at 4 °C. The supernatants were re-centrifuged at 14,000g for 12 min at 4 °C; the pellets were resuspended in a buffer solution containing 50 mM Tris–HCl, 2 mM EGTA, and 1 mM EDTA (pH 7.4) and incubated for 30 min at 37 °C to remove endogenous adenosine. After centrifugation at 14,000 g, the pellets were resuspended in buffer including 50 mM Tris–HCl and 10 mM MgCl₂ (pH 7.4). Aliquots were then frozen at –80 °C until analysis.

2.2. Radioligand binding assays

The [³H]CGS21680 binding assay was performed in triplicate as previously described [13]. Briefly, aliquots of rat cerebral cortex membrane fraction (0.2 mg protein) were incubated in 200 μL of

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50 mM Tris-HCl buffer (pH 7.5) containing 10 mM MgCl₂ with [³H]CGS21680 (5 nM) in the presence or absence of unlabeled 5'-N-ethylcarboxamidoadenosine (NECA; 10 μM) for determination of nonspecific binding. After 90 min at 25 °C, the binding reaction was stopped by filtration through GF/C fiber glass filters (Whatman, Maidstone, Kent, UK), and then the membrane was washed five times with 5 mL ice-cold buffer. Radioactivity of the punched-out wet filters was counted after adding 5 mL of Ultima Gold scintillation cocktail (Perkin-Elmer, Waltham, MA, USA).

2.3. Cell culture and transient expression of adenosine A_{2A} receptors in CHO cells

Cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a 10% CO₂ incubator at 95% humidity. The plasmid containing the human adenosine A_{2A} receptor (OriGene, Rockville, MD, USA) was transiently transfected into Chinese hamster ovary CHO cells with Hilymax transfection reagent (Dojindo, Seoul, Korea) according to the manufacturer's protocol. Transfections were performed in 100-mm tissue culture dishes and cells were treated with limonene for subsequent cAMP, PKA activity, and calcium assays.

2.4. Cyclic AMP measurement

Cells were washed with phosphate-buffered saline and treated with various concentrations of limonene. After incubation with limonene for 30 min, intracellular cAMP was quantified with a commercially available cAMP radioimmunoassay system (Enzo Life Sciences, Plymouth Meeting, PA, USA) according to the manufacturer's recommendations.

2.5. PKA activity assay

PKA activity was determined using a commercially available kit (Enzo Life Sciences) according to the manufacturer's instructions.

2.6. Measurement of limonene-induced cytosolic calcium release

Cells were seeded at a density of 30,000 cells/well into black 96-well plates with clear bottoms to allow cell visualization and fluorescence measurements from the bottom of each well. The dye-loading buffer consisted of a final concentration of 4 μM 4-(6-acetoxymethoxy-2,7-dichloro-3-oxo-9-xanthonyl)-4'-methyl-2,2'-(ethylenedioxy)dianiline-*N,N,N',N'*-tetraacetic acid tetrakis (acetoxymethyl) ester (fluo-3-acetomethyl ester; Sigma, St. Louis, MO, USA) in serum-free cell culture maintenance medium containing 20 mM HEPES and 2.5 mM probenecid. Assay conditions were as described previously [14]. Briefly, each 96-well plate containing dye-loaded cells was placed into a multilabel plate reader (VICTOR3; Perkin-Elmer) and laser intensity was adjusted to an optimum level to obtain basal values of approximately 10,000 fluorescence units. After determining the baseline fluorescence of the fluo-3-Ca²⁺ complex, limonene and NECA were added into the 96-well plate. Fluorescence readings were obtained every 10 s for 100 s. The maximum fluorescent signal was recorded and normalized to a positive control of 10 μM NECA. Each experiment was performed in quadruplicate on every plate. Each 96-well plate contained four wells dedicated to a positive control (10 μM NECA) and four wells to a negative control (assay buffer alone).

2.7. Immunoblotting analysis

Cells were lysed in RIPA buffer (10 mM Tris-HCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, pH 7.5)

containing protease inhibitor cocktail at 4 °C. Samples were separated using 7.5% SDS-PAGE and blotted onto a nitrocellulose membrane. Membranes were incubated with primary antibodies overnight at 4 °C. After washing several times with 0.5% TBS-E wash buffer, the membrane was incubated with the secondary antibody for 1 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence Western blotting detection reagents (Amersham-Pharmacia Korea, Seoul, Korea) and quantified with ChemiDoc™ XRS system (Bio-Rad, USA) using the Quantity One software.

3. Results and discussion

We demonstrated for the first time that a cyclic terpene, limonene, was a direct ligand for A_{2A} receptors and activated receptor-mediated signaling pathways, as confirmed by cytosolic cAMP and calcium concentrations, and induced phosphorylation of CREB transcription factor. Taken together, our data demonstrate that limonene is an A_{2A} receptor agonist.

First, we performed a radioligand binding assay to quantitate the binding affinity of limonene to A_{2A} receptors with a rat brain cerebral cortex membrane fraction. Specifically, we employed competitive binding of limonene with a known selective A_{2A} receptor agonist, [³H]CGS21680. We found that [³H]CGS21680 displayed a saturable binding profile in the absence of limonene, and the binding of [³H]CGS21680 to A_{2A} receptors decreased with increasing concentrations of limonene in a concentration-dependent manner (Fig. 1). At the highest limonene concentration, [³H]CGS21680 binding decreased by 91.2% as compared to the control group. This indicated that limonene competitively bound to the [³H]CGS 21680-binding site of the A_{2A} receptor. Therefore, we found that limonene has a high affinity toward A_{2A} receptors and can act as a ligand for them.

In the next step, we investigated whether limonene binding activated the A_{2A} receptor-mediated signaling pathway. A series of experiments was performed with CHO cells transfected with the human A_{2A} receptor gene. A_{2A} receptors are coupled to a G_s G protein [15] and activate adenylyl cyclase [16]. The activated G_s subunit can couple to downstream effectors to regulate the amount of second messengers, including cAMP and PKA. cAMP-induced gene transcription is generally accepted to be mediated through the activation of PKA, whose two regulatory subunits bind to cAMP and induce a conformational change, which produces subunit dissociation resulting in enzymatic activation [17] and subsequent phosphorylation of the transcription factor CREB.

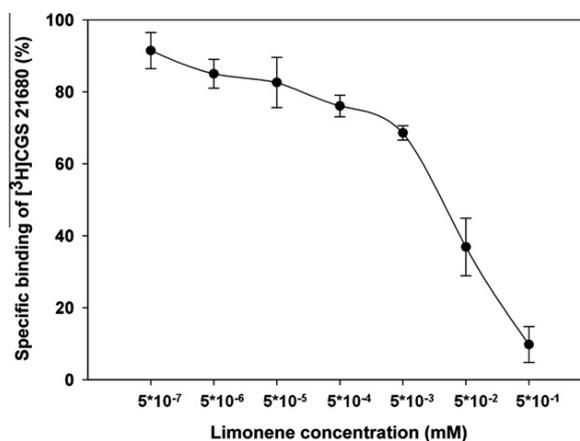


Fig. 1. Competitive binding assay of limonene with [³H]CGS21680 in a rat brain membrane fraction. Binding of [³H]CGS21680, an A_{2A} receptor ligand, was reduced by limonene in a concentration-dependent manner. Values are means ± SEM.

To quantitate cytosolic cAMP, cells expressing human A_{2A} receptors were incubated with limonene or the positive control, NECA (A_{2A} receptor agonist). The results showed that limonene increased intracellular cAMP levels from 2.2 to 7.3 pmol/mL in a concentration-dependent manner (Fig. 2A), whereas non-transfected cells did not show altered cAMP concentrations with limonene incubation (data not shown). Subsequently, PKA enzyme activity was quantified in cells expressing A_{2A} receptors, and the results showed that PKA activity was also induced in a concentration-dependent manner after limonene treatment; PKA activity increased by 28% following treatment with 25 μ M limonene (Fig. 2B). Next, we investigated the phosphorylation of CREB proteins with immunoblotting analysis. Total CREB protein levels remained unchanged with limonene treatment, although the level of phosphorylated CREB significantly increased after the treatment. Thus, the phospho-CREB to total CREB protein ratio significantly increased 2.5-fold with treatment by limonene (25 μ M) as compared to baseline (0 μ M; Fig. 3). This may have been due to increased PKA activity, as CREB is a PKA target protein. The non-transfected cells showed no significant changes in the same assay as compared to the transfected cells, suggesting that the limonene induction of PKA signaling was mediated by A_{2A} receptors.

Alternatively, we found that limonene treatment increased cytosolic calcium levels (Fig. 4). Limonene addition to CHO cells transfected with human A_{2A} receptors caused a rapid rise in cytosolic calcium concentrations. Limonene addition (25 μ M) increased intracellular calcium levels by 213% after 10 s and the level declined slightly afterward (Fig. 4). Intracellular cytosolic calcium concentration was unaltered in non-transfected CHO cells. G proteins have been reported to modify voltage-sensitive calcium channel (VSCC) activity by altering the activity of effector enzymes that produce intracellular signals or via a membrane-delimited pathway [18]. The best known effector enzyme involved in the regulation of VSCCs is adenylyl cyclase, whose activation leads to an increase in cAMP content and subsequent PKA activation. Several important studies involving cardiac and skeletal muscle [19–21] have reported an enhancement of calcium currents by cAMP levels, suggesting that the phosphorylation of VSCCs or of closely associated components can increase channel activity. Therefore, in this case, calcium increase can be explained by the increase in intracellular cAMP levels. Taken together, our results suggest that limonene activated A_{2A} receptors, resulting in the activation of the cAMP–PKA pathway and increase in intracellular calcium levels, both of which are typical effects of A_{2A} receptor agonists.

A_{2A} receptors are involved in various pathological processes. A_{2A} receptor activation causes vasodilation in the aorta and coronary artery [8]. In platelets, an A_{2A} receptor agonist facilitates platelet

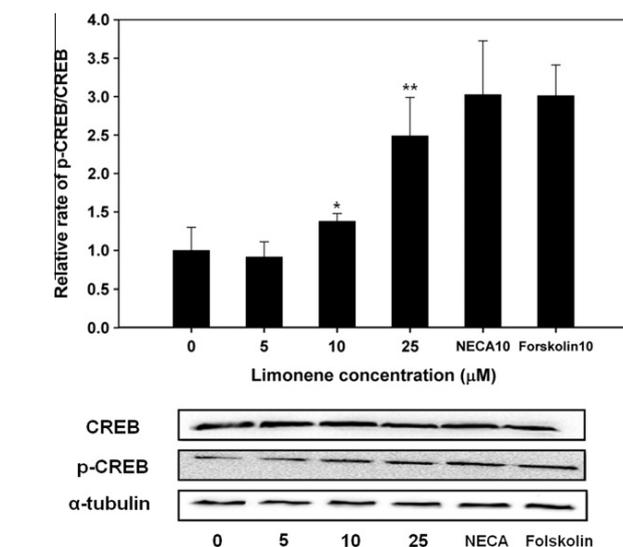
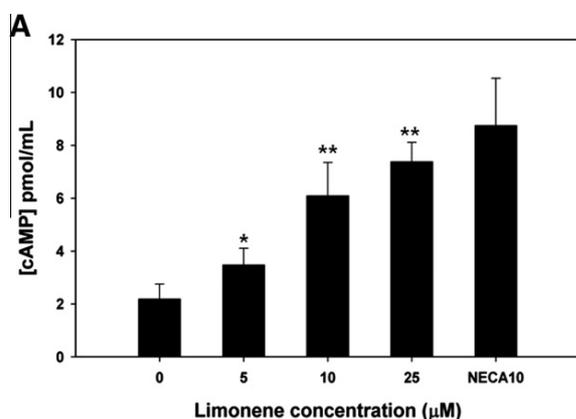


Fig. 3. Western blot analysis of CREB and phosphorylated CREB. Values are means \pm SEM. * $P < 0.05$, ** $P < 0.005$ vs. control.

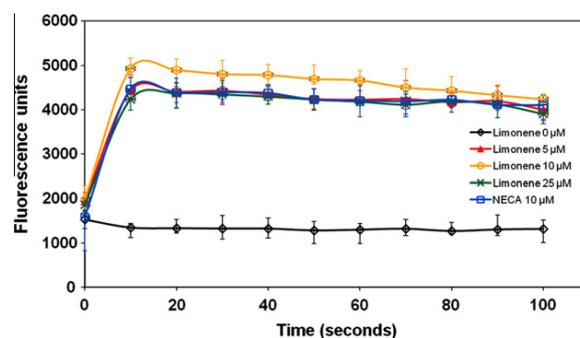


Fig. 4. Quantification of cytosolic calcium concentrations. Cytosolic calcium was quantified by incubation with fluo-3-AM in CHO cells transfected with human A_{2A} receptors and measured with a multilabel plate reader. Calcium signal time-course following limonene and NECA addition on CHO cells expressing A_{2A} receptors was measured using a multilabel plate reader. Values are means \pm SEM. $N = 4$ for each time point.

aggregation through activation of the PKA signaling pathway and thus could act as a potential antithrombotic agent [22]. A_{2A} receptor ligands can protect against tissue injury by reducing inflamma-

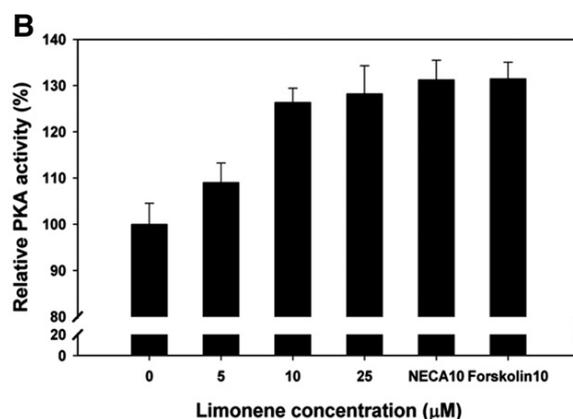


Fig. 2. Cytosolic cAMP concentration and PKA activity. CHO cells expressing human A_{2A} receptors were treated with limonene for 30 min. (A) Cytosolic cAMP concentrations and (B) PKA activity. Values are means \pm SEM. * $P < 0.05$, ** $P < 0.005$ vs. control.

tion during reperfusion following ischemia, potentially by reducing pro-inflammatory cytokine release (e.g., TNF α , IL-6, IL-8, and IL-12) and enhancing anti-inflammatory cytokine release (e.g., IL-10) by monocytes [23].

A_{2A} receptor ligands also have therapeutic potential as sleep inducers. A_{2A} receptors play an important role in sleep process regulation, as demonstrated by the intracerebroventricular infusion of an A_{2A} receptor agonist, CGS21680, which promotes sleep in rodents [24,25]. One potential mechanism may be histaminergic system inhibition through increased GABA release in the tuberomammillary nucleus by A_{2A} receptor agonists [26]. The detailed mechanism of A_{2A} receptor agonists in sleep will be investigated in the future. Recent efforts have focused on further improvement in A_{2A} receptor agonist subtype selectivity with specific and novel therapeutic applications. To the best of our knowledge, this is the first work demonstrating that naturally occurring limonene acts as an A_{2A} receptor agonist, thus opening the door to its potential therapeutic applications.

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