Title: Protein Kinase C Involvement in Neuroprotective Effects of Thymol and Carvacrol Against Toxicity Induced by Amyloid-B in Rat Hippocampal Neurons

Running Title: Effects of thymol and carvacrol on PKC activity

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To appear in: Basic and Clinical Neuroscience

Received date: 2020/12/12
Revised date: 2021/02/14
Accepted date: 2021/05/25
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Please cite this article as:


DOI: http://dx.doi.org/10.32598/bcn.2021.666.2
Abstract

Introduction: We have reported that thymol and carvacrol can improve cognitive abilities in Alzheimer’s disease (AD) rat model. However, the mechanism of their action is not yet fully understood. Recently, our in vitro results suggested that PC12 cell death-induced by Aβ25-35 can be protected by thymol and carvacrol via PKC and ROS pathways. So, we hypothesize that the mechanisms of thymol and carvacrol in improving the learning impairment in AD rat model may be related to their effects on PKC. So, the activity of PKC and protein expression levels of PKCα was examined in the hippocampal cells of AD rat model.

Methods: To examine thymol and carvacrol effects, we performed behavioral test in AD rat model induced by Aβ25-35 neurotoxicity. To access the underlying mechanism of protective effects, western blotting was performed with antibodies against PKCα. We also measured PKC activity assay by Elisa. Histopathological studies were carried out in hippocampus by hematoxylin & eosin (H&E).

Results: It was shown that escape latency increased in Aβ-received rats compared to control group and thymol and carvacrol reversed this deficit. Furthermore, these compounds could enhance PKC activity, and increase the PKCα expression ratio. Moreover, H&E showed that Aβ caused shrinkage of the CA1 pyramidal neurons. However, thymol and carvacrol treatments could prevent this effect of Aβ peptides.

Conclusions: This study suggests that Aβ results in memory decline and histochemical disturbances in hippocampus. Moreover, these results revealed that thymol and carvacrol could have protective effects on cognition in AD-like models via PKC activation.

Keywords: Thymol, Carvacrol, PKC, Amyloid β, Alzheimer’s disease
Highlights

- Ability of rats to find invisible platform in the MWM was impaired by Amyloid Beta (Aβ) infusion in the hippocampus, while this effect was reversed by thymol or carvacrol administration.
- Aβ significantly downregulated PKC activity in the hippocampus of rats.
- Western blot analysis demonstrated that Aβ significantly reduced PKCα protein expression in AD rat model hippocampal cells.
- Expression ratio of PKCα was upregulated following the injection of thymol and carvacrol in rats.
- Injection of Aβ in hippocampus resulted in histochemical disturbances in CA1 pyramidal neurons.
- Carvacol and thymol can prevent several histological changes induced by Aβ.

Introduction

Alzheimer’s disease (AD) one of the most common causes of dementia, leads to progressive neurodegeneration affecting cognition (Duyckaerts, Delatour, & Potier, 2009). Neuropathologic characteristics of AD include amyloid β (Aβ) accumulation outside the neuronal cells and neurofibrillary tangle formation inside the cells in multiple brain regions, mainly hippocampus and cortical temporal lobe (Duyckaerts et al., 2009). AD causes progressive shrinkage of brain tissues and neuronal death. AD in early stages degenerates hippocampal cells leading to loss of short-term memory followed by diminished ability for performing routine tasks. AD progression causes nerve cell injury associating synapse loss, leading to gradual deteriorating behavioral changes (Reitz, Brayne, & Mayeux, 2011).

Recent reports suggest that a number of signaling pathways regulate patho-physiological processes involved in AD development and progress. One of these pathways includes protein kinase C (PKC), a central kinase in intracellular signal transduction (Crews & Masliah, 2010; Lucke-Wold et al., 2015). PKC is known as a “memory kinase” and several studies have shown its crucial role in memory function in normal and pathological experimental conditions including AD models (Lucke-Wold et al., 2015). Additionally, it has been reported that Aβ cytotoxicity could be protected by PKC activation (Han et al., 2004). 12 PKC isozymes have been identified in molecular studies that are classified into three subgroups: classical, novel, and atypical PKCs.
Different isoforms of PKC play an important role in a number of cognitive performances such as learning and memory (Khan, Nelson, Verma, Wender, & Alkon, 2009; Nelson & Alkon, 2009).

It has been demonstrated that PKCα and PKCε signaling pathways have close correlation in neuropathological injury in AD, and Aβ production and related dementia in transgenic mouse AD model could be ameliorated by activation of these isoforms. Enhancing Aβ degradation and APP α-processing pathways have been suggested as mechanism of this action (Choi et al., 2006; Khan et al., 2009; Nelson & Alkon, 2009). Also, promotion of α-secretase-mediated APP processing and suppression of Aβ production by PKCα has been reported (Kinouchi et al., 1995). These studies, taking together show that activation of PKCα has a significant protective effect in many pathological states such as brain ischemia, neurodegeneration and aging.

Recent studies have examined potential of many medicinal plants and their active constituents to alleviate AD symptoms or affect disease process in various AD cellular and animal models and also in clinical trials with patients. These studies have indicated that a number of species show in-vitro and in-vivo activities including antioxidant, anti-inflammatory, anti-cholinesterase, neuroprotective, and even anti-amyloid and cognition improving effects (Bastianetto & Quirion, 2004; Kim MH, Kim SH, & Yang, 2014; Majlessi, Choopani, Kamalinejad, & Azizi et al., 2012).

Two main constituents in many aromatic plants essential oils such as Thymus, Zataria and Origanum species are thymol (2-isopropyl-5-methylphenol) and carvacrol (5-isopropyl-2-methylphenol) (Baser, 2008). These monoterpenic phenols possess some pharmacologic activities including antioxidant (Azizi, Salimi, Amanzadeh, Majlessi, & Naghdi, 2020), anti-inflammatory (Basch, Ulbricht, Hammerness, Bevins, & Sollars, 2004), and AChEI (Jukic, Politeo, Maksimovic, Milos, & Milos, 2007) effects that appear to be useful in managing AD cognitive deficit.

Our previous research showed that treatment with thymol and carvacrol could protect against many alterations resulting from administration of Aβ and scopolamine in two AD rat model (Azizi, Ebrahimi, Saadatfar, Kamalinejad, & Majlessi, 2012). Later, our in vitro results suggested that thymol and carvacrol can protect against PC12 cell death induced by Aβ25-35 via PKC and ROS mitochondrial pathways (Azizi et al., 2020). Considering the PKC hypothesis in the pathological process of AD (Lucke-Wold et al., 2015), we are interested in the development
of thymol and carvacrol as potential agents for AD treatment. In this study, we investigated the mechanism of protective effects of thymol and carvacrol in AD model using Aβ.

**Material and Methods**

**Chemicals and agents preparation**

Rabbit polyclonal anti-PKC α (sc-208) and goat anti-rabbit IgG horseradish peroxidase (HRP) (sc-2004) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Mouse anti-β-actin monoclonal antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). Amyloid β-Protein Fragment 25–35, thymol and carvacrol were all provided by Sigma-Aldrich Chemical Company (St. Louis, Missouri, USA). Tween 80 was provided from Merck, (Darmstadt, Germany). The protease inhibitor was obtained from Roche, (Mannheim, Germany). PKC-kinase activity kit and Bryostatin-1 were purchased from Enzo Life Sciences Company (Farmingdale, NY, USA) and Tocris Bioscience (Bristol, UK), respectively.

**Animal surgery and Morris water maze**

Male albino Wistar rats (200–250 g) were provided by Pasteur Institute of Iran. Animals were used according to the National Institute of Health Guide for the Care and Use of Laboratory Animals. Experiment procedures were performed in accordance with laboratory animals care and use guidelines of Pasteur Institute of Iran animal experiment administration committee. We made maximum efforts to reduce animal suffering and to minimize animal numbers.

Infusion of Aβ25–35 into the rats’ hippocampus was performed as previous study (Majlessi et al., 2012). Seven days after surgery, spatial reference memory was evaluated using the Morris water maze test (MWM). The water maze and experimental procedure were explained previously (Majlessi, Choopani, Bozorgmehr & Azizi, 2008). In brief, rats received four trials during five days. During first 4 days, the platform was hidden and placed in the southwest quadrant center to examine spatial learning. The position of platform was stable during 4 days and acquisition of this task was investigated. On the fifth day, the platform was situated in southeast quadrant center and was elevated above water. Each rat swimming path in each trial was recorded.
automatically using a computerized system and several parameters such as escape latency and swimming speed were computed and analyzed.

Groups (n=10) received 0.2 nM bryostatin-1, 0.5, 1, or 2 mg/kg thymol, or carvacrol, or 2ml/kg 0.1% v/v Tween 80 (vehicle). All drugs were injected intraperitoneally 30 min before MWM test each day. One Aβ-received group was tested in MWM with no injection. (Azizi et al., 2012).

**Kinase activity assay**

After removing the brains and immediate dissection of hippocampi on a cold plate, they were weighed, and homogenized quickly in ice-cold normal saline. Then, the homogenate was centrifuged at 3,000 r/min for 10 min at 4°C, the supernatants were separated and stored at 4°C for PKC activity assay, according to the manufacturer’s instruction for enzyme linked immunosorbent assay kit (ENZO, ADI-EKS-420A). The kit was based on a solid-phase ELISA that uses a polyclonal antibody recognizing the phosphorylated form of the substrate. This assay was designed for PKC activity analyses in the solution phase. Optical density was measured at 450 nm with a Bio-Rad microtiter plate reader. Results are indicated as percentage of control values.

**Western blotting analysis**

Individual rat hippocampus were homogenized and suspended in 500 µl lysis buffer containing 2.5 ml 1M Tris-HCl (pH 7.4), 1500 µl 5M NaCl, 250 mg sodium deoxycholate, 1% SDS 50 mL plus freshly added protease inhibitor. The suspension was centrifuged at 12,000 RPM for 10 minutes at 4°C. Tissue lysates were briefly sonicated and the supernatant protein content was determined using the Bradford assay. Then, by adding the homogenization buffer, tissue lysates were adjusted to 1 mg protein/ ml, divided into aliquots, frozen and stored at -70°C for subsequent western blotting. For western blot, equal amounts of protein were heated to 95 °C, separated in 8% SDS-poly acrylamide gels and transferred to PVDF membranes. Then, the membrane was blocked for 2 h in TBST (50 mmol/L Tris-Cl, pH 7.6, 150 mmol/L NaCl and 0.1 % Tween 20) containing 1 % (w/v) casein, and then incubated with primary antibodies (rabbit polyclonal anti-PKCε (1 : 1000), rabbit polyclonal anti-PKCα (1 : 500), and b-actin (1 : 1000) overnight. Then, they were incubated with horseradish peroxidase (HRP)-conjugated goat anti-
rabbit IgG for 2 hours. Then, the blots were developed by ECL advance western blotting detection kit, as described. Chemiluminescence detection was linear at 30–60 sec of exposure for all antibodies. The exposed films were scanned and densitometric analysis was done with Image J software. All tests were performed 3-5 times with similar results and the data were analyzed using ANOVA. The control expression level was designated as value “1”, and the treatment expression ratios were expressed in compare to the control.

**Tissue preparation and histological staining**

After the behavioral experiment, animals were anesthetized with 100 mg/kg sodium pentobarbital, and perfused transcardially with 0.9 % saline and then 4% paraformaldehyde (PF) in buffered saline. The rats were decapitated and the brain tissues were immediately fixed in 4% paraformaldehyde for 2-3 days. The brain tissue were washed under slow running tap water overnight, and then dehydration process was done in ascending grades of ethanol (70% , 80% , 90% , absolute alcohol) for 45 minutes in each change. Then, brain tissues were cleared for 4 hours in two changes of xylene. After that, paraffin was impregnated in an oven, and the paraffin blocks were prepared. The blocks were cut using a rotary microtome (Leica RM 2245™) and 4 μm paraffin section were obtained. The sections were mounted on grease free albumenized slides, air dried, and fixed using a slide drier for better fixation of the section. Finally, the tissue sections were stained by hematoxylin and eosin according to the standard method (Suvarna, Layton & Bancroft, 2019). Photography was performed at ×100 and ×400 magnifications under the microscope (Leica DM 2500, Germany). 5 microscopic fields were randomly chosen to observe them in each rat.

**Statistical analyses**

Data were shown as means ± standard error of mean (S.E.M) and analyzed with one-way ANOVA followed by Tukeys honestly significant difference. $p < 0.05$ was considered as criteria for statistical significance compared with Aβ25-35 treatment or control group.
Results

Thymol and carvacrol improved Aβ25-35 induced cognitive impairment

Escape latency was significantly different between Aβ and the control groups \((p< 0.01)\), showing cognitive impairment caused by Aβ administration into the rat hippocampus. However, in animals received thymol and carvacrol, this deficit was significantly attenuated. As shown in Figure 1, escape latencies during the four days with hidden platform were significantly different among the groups tested. Post-hoc analysis indicated that Aβ25-35 increased escape latency significantly \((p< 0.01)\) compared to control. Thymol and carvacrol at doses of 0.5, 1 or 2 mg/kg reduced Aβ25-35 effects (Figure 1).

![Figure 1](image_url)

**Figure 1.** Mean escape latencies in seconds in each group to locate the platform in the Morris water maze. The latency time in Aβ received rats was significantly more than control group \((##p< 0.01)\); while it was significantly shorter in the thymol and carvacrol groups than Aβ group \((*p< 0.05, **p< 0.01)\). Ten groups were tested (n =10): control, Aβ, Aβ + Tween, Aβ + 0.5 mg/kg Thy, Aβ + 1 mg/kg Thy, Aβ + 2 mg/kg Thy, Aβ + 0.5 mg/kg Car, Aβ + 1 mg/kg Car, Aβ + 2 mg/kg Car. Error bars indicate ± SEM. Car, carvacrol; Thy, thymol.


The effect of thymol and carvacrol on PKC activity in hippocampal neurons

Enzyme activity was determined using an assay kit obtained from Enzo Life Science (USA). Protein samples extracted from hippocampus of Aβ group considerably inhibited PKC activity in comparison with control. Treatment with thymol and carvacrol at concentrations of 0.5, 1 and 2 mg/kg could also significantly induce the PKC activity (Figure 2).

**Figure 2.** Thymol and carvacrol effect on PKC activity inhibited by Aβ.

The PKC activity was significantly lower in Aβ samples compare to control (#p< 0.05). While compare to Aβ, the PKC activity was significantly higher in the carvacrol (a), thymol (b) (1 mg/kg and 2 mg/kg) and Bryostatin (0.2 nM) groups. The data were shown as mean ± SEM. #p< 0.05 vs. control, *p< 0.05, **p< 0.01, ***p< 0.001 vs. Aβ. Experiments were performed three times in triplicate. Car, carvacrol; Thy, thymol.
Thymol and carvacrol increased the expression of PKCα

To determine the mechanism of thymol and carvacrol effect, the protein expression of PKCα was accessed with western blotting in hippocampus. In Aβ+ thymol, carvacrol or Bryostatin groups, PKCα expression was significantly higher than Aβ group ($p < 0.01$, Figures 3(a) and 3(b)). Therefore, thymol and carvacrol upregulated PKCα expression in the brain and they can exert their neuroprotective effects by activating PKC.
Figure 3. The effect of carvacrol (a) and thymol (b) on PKCα expression in rat hippocampus. Representative Western blots of levels of PKCα expression detected with Rabbit polyclonal anti-PKCα (sc-208). This figure shows the western blot of PKCα and the quantification with densitometry. There was significant reduction of PKCα expression in Aβ group compared to control (#p< 0.05). While compared to Aβ group, PKC expression was significantly higher in carvacrol (a), thymol (b) (1 mg/kg and 2 mg/kg) and Bryostatin (0.2 nM) groups (*p< 0.05). Densitometric analysis is expressed after normalizing to the levels of β-actin. Data are presented as the mean ± SEM. Experiments were carried out 3 times in triplicate. #p< 0.05 vs. control, *p< 0.05, **p< 0.01 vs. Aβ.
Figure 4. Neuroprotective activity of thymol and carvacrol on morphological changes and neuronal injury of rat hippocampus caused by Aβ neurotoxicity.
H&E staining of mice hippocampal CA1 slices presents:

a) Control group neurons showed normal shape, size, and regularly arrangement with nucleus colored blue marked with arrow. Neuronal count was less in b) Aβ-treated and c) Aβ+ Tween 80 in comparison with control. d) Car and e) Thy groups showed size increase in nerve cell bodies when compared to Aβ treated group. Mixed pattern of normal and shrinkage neurons was observed. f) In Bryostatin group, neurons have almost normal morphology and their arrangement was regular and few condensed neuronal cells. Representative of at least three experiments.

Normal cells marked with ▲
Shrinkage cells marked with ▲

Protective effect of thymol and carvacrol on hippocampal neurons injury

The cell nuclei become blue-black by hematoxylin that shows intranuclear details clearly, while cell cytoplasm and connective tissue fibers show pink, orange and red shades by eosin (Suvarna, Layton & Bancroft, 2019). The neuropathological damage was evaluated by irregular neuronal shape, arrangement and death. Irregular neuronal shape, arrangement and also hippocampal neuronal death were observed following Aβ administration. This injury was dramatically reduced by thymol, carvacrol and Bryostatin, and the number of neurons survived was significantly higher in comparison with Aβ group. This suggests that thymol and carvacrol can prevent Aβ induced neuronal damage.

Discussion

Our previous research suggested thymol and carvacrol potential to protect PC12 cells against toxicity induced by Aβ25-35. Our results indicated that these compounds might insert the neuroprotective action via increased PKC activity (Azizi et al., 2020). We also demonstrated the neuroprotective effects of thymol and carvacrol in AD animal models (Azizi et al., 2012). Our present results indicated that, thymol and carvacrol significantly enhanced PKC activity. Further, these compounds increased the PKCα expression ratio. Furthermore, the results of H&E staining showed the protective effects of thymol and carvacrol on hippocampal cells. As a result,
dysfunction and death of hippocampal neurons induced by Aβ could be protected by thymol and carvacrol, and consequent learning and memory deficits could be prevented. We confirmed that thymol and carvacrol treatment improved Aβ25-35 induced cognitive impairment in rat hippocampus through increasing PKC activity.

Recently, it has been indicated that the neuropharmacological properties of phytochemicals derived from medicinal plants may be clinically beneficial in AD (Dey, Bhattacharya, Mukherjee, & Pandey, 2017). So, an increasing number of herbal extracts and active constituent derived from plants have been investigated for their protective effects in AD in recent years (Kalász et al., 2018). Thymol and carvacrol, natural monoterpenoid phenols, are obtained from many plants belonging to Lamiaceae family and have shown in-vivo or in-vitro activities including antioxidant, neuroprotective, and memory enhancing (Sharifi-Rad, 2018; Salehi, 2018). Neuroprotective effects of these compounds have been reported in a variety of animal models, including AD (Azizi, et al., 2012; Asadbegi, Yaghmaei, Salehi, Komaki, & Ebrahim-Habibi, 2017; Guan et al., 2019) and Parkinson disease (Manouchehrabadi, Farhadi, Azizi, & Torkaman-Boutorabi, 2020). One recent study has demonstrated significant attenuation of negative effects of Aβ25-35 injection on escape latency and traveled distance and improving Aβ induced by thymol and suggested the neuroprotective properties of this compound (Asadbegi et al., 2017). Another recent in vivo study has shown that carvacrol could ameliorate brain AD induced by AlCl3, and attributed this effect to anti-inflammatory and antioxidant activities of carvacrol (Medhat et al., 2019). These results indicated that carvacrol attenuates memory impairments through inhibiting hippocampal neuronal injury inhibition. Another study showed improvements in bradykinesia, motor coordination and the locomotor activity, and reduction in apomorphine-induced rotation by carvacrol in 6-OHDA-stimulated rats. These findings suggested that carvacrol exerts neuroprotective effects in Parkinson's disease model (Manouchehrabadi, 2020). In the present study, we evaluated thymol and carvacrol effects on Aβ induced cognitive deficits in an AD rodent model. Aβ infusion in the rat hippocampus CA1 region was shown to significantly impaired rats performance to find the invisible platform in MWM. Moreover, this deficiency was reversed by thymol or carvacrol administration. So our findings provided evidence for thymol and carvacrol efficacy for enhancing cognitive performance.
More recently, researchers have demonstrated that PKC activation might affect pathophysiology of AD and attenuate related cognitive deficit (Schrott et al., 2015; Amiri et al., 2020). It has been shown that Bryostatin-1 reduces amyloid accumulation in AD transgenic mice brains (Hongpaisan, Miao-Kun Sun, & Alkon, 2011). Our previous results also indicated that thymol and carvacrol can cause PKC activation like Bryostatin-1 (Azizi et al., 2020). Consistent with these data, our findings obtained from PKC activity assay revealed that thymol and carvacrol reversed Aβ-induced neurotoxicity and consequently improved behavioral outcomes in AD model rats. In accordance with our results, Chen et al. have displayed that the antioxidant property of carvacrol might lead to its cardioprotective activity through MAPK/ERK signaling pathway activation, as PKC common downstream effectors. Therefore, considering the possible neuroprotective role of PKC in AD, it is reasonable to suggest administration of thymol and carvacrol as potential disease-modifying agents.

PKCα belongs to serine/threonine kinases family and its signal transduction is mediated by calcium and diacylglycerol as second messengers (Newton, 2010). It has been demonstrated that activation of PKCα could elevate α-secretase activity that inhibits Aβ production (Murakami et al., 2020). In the present study we also observed that PKCα expression level is significantly increased in the thymol and carvacrol groups compared to Aβ. In accordance with our results, Yabuki et al. (2019) have displayed that ST101, as a PKCα activator, improved schizophrenic symptoms and memory deficits in rats through CaMKII/PKCα signaling stimulation in hippocampal cells (Yabuki, Wu, & Fukunaga, 2019). Hippocampus in the temporal lobe is considered as a main target of AD pathophysiology. (Bondolfi et al., 2002; Li et al., 2013; Zhang, Dong, Zhao, & Ma, 2014). So, histochemical alterations were assessed in the hippocampal neurons of the rat model of AD developed by injection of Aβ25-35. H&E showed shrinkage in the CA1 pyramidal neurons and neuronal loss in the rat model of AD. Furthermore, daily treatment with thymol and carvacrol were effective to prevent several histological disturbances induced by Aβ injection into CA1. In the present study, decreased hippocampal volume due to neuronal shrinkage and loss through an accelerated apoptosis in AD is detectable using imaging and pathological studies. Shrinkage and neuronal loss have been reported in the rat models of AD (Bondolfi et al., 2002; Li et al., 2013; Zhang, Dong, Zhao, & Ma, 2014) and clinical studies (Simić, Kostović, Winblad, & Bogdanović, 1997; Zarow et al., 2005). In vitro
studies have indicated that hippocampal neuronal cells can be protected against toxicity induced by Aβ through PKC pharmacologic activation.

Our study suggests that thymol and carvacrol improved learning and memory deficit by stimulating hippocampal PKC signaling in AD model rat brains. Since, stimulation of PKC activity might have a potential ability to enhance cognitive function and perhaps modulate pathophysiology of AD, thus our findings offer an encouraging target for drug design and development.

In conclusion, our study demonstrated that thymol and carvacrol stimulated the PKC pathway in AD model rats. Considering PKC activating capacity of thymol and carvacrol, these terpenoid compounds could be suggested as therapeutic agents for AD management. It warrants further study, and holds promise for AD patients.

Acknowledgments
The financial support of the study has been provided by the graduate Department of the Pasteur Institute of Iran for the first author’s Ph.D.

Conflict of interest
There is no conflict of interest for declaration.
References


