ALPHA-PINENE ATTENUATES MICROGLIAL NF- κ B ACTIVATION AND INOS EXPRESSION IN GP120-INDUCED NEUROINFLAMMATION

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ABSTRACT

Background: Neuroinflammation plays a role in the pathogenesis of HIV-associated dementia (HIV). Activation of microglia is essential for triggering inflammatory-mediated neurotoxicity. HIV-1 120 kDa envelope glycoprotein (gp120) induces microglial NF- κ B signaling which in turn induces pro-inflammatory and iNOS gene transcription. Continuous or excessive activation of NF- κ B signaling leads to persistent production of TNF- α and nitric oxide by microglia and induces neuronal apoptosis. Alpha-pinene is a natural substance extracted from a pine tree and has efficacy in inhibiting NF- κ B signaling.

Objective: This study was designed as a true experimental study and aimed to investigate the effect of alpha-pinene administration toward inflammatory response represented by the percentage of microglia containing activated NF- κ B and iNOS expression.

Methods: Neuron-glia primary culture from brain tissue of rat fetus was divided into 5 groups as follows: negative control; positive control (gp120 1nM); treatment I, II, and III (gp120 1 nM + alpha-pinene 0.4 μ g/mL, 2 μ g/mL, and 10 μ g/mL, respectively). Microglial NF- κ B and iNOS expression were analyzed using the immunohistochemistry method. Neuronal apoptosis was measured by the TUNNEL method.

Results: Results showed that alpha-pinene administration on gp120-treated neuron-glia at all dosages decrease NF- κ B activation, iNOS expression, and apoptotic neuron significantly as compared to the gp120-only treated group ($p < 0.05$). Furthermore, alpha-pinene did not affect NF- κ B activation and neuronal apoptosis ($p > 0.05$), but significantly elevate iNOS expression ($p < 0.05$) mainly in dosage I and II.

Conclusion: We concluded that alpha-pinene has neuroprotective effect on gp120-treated neuron-glia cells through modulation of NF- κ B and iNOS expression thus inhibit neuronal apoptosis.

Keywords: apoptosis, gp120, iNOS, neuroinflammation, NF- κ B

Introduction

Human Immunodeficiency Virus -1 (HIV-1)-associated dementia (HAD) is subacute or chronic dementia characterized by impairment of neurocognitive, motoric, and behavior. Those symptoms result from neuronal death associated with HIV-1 infection in the central nervous system.^{1,2,3}

Human Immunodeficiency Virus -1 (HIV-1)-associated dementia (HAD) is accounted for approximately 20% of HIV-1-infected patients.⁴ It has been postulated that neuroinflammation plays a role in the pathogenesis of the neurodegenerative disease, including HAD.^{2,5} Even in a patient treated with highly active antiretroviral therapy (HAART), HAD still occurred, because HAART is only focused on reducing systemic viral load, but not targeting the neuroinflammation process.³ Therefore, it is required to administrate additional treatment to modulate the progressivity of HAD.

The population of brain cells consists mainly of the neuron and glial cells (microglia and astrocyte). Microglia play an essential role in the defense mechanism in the central nervous system and vulnerable to relatively slight physiological changes. Activated microglia have the same characteristics as a macrophage which is indicated by rapid proliferation, cytotoxic compound secretion, phagocytosis, and antigen presentation.⁵

Activation of microglia is initiated by binding of gp120 into its receptor, CD4, and its co-receptor, CXCR4 (or CCR5) on the microglia's cell membrane. This binding will induce mitogen-activated protein kinases (MAPK) signaling⁶ indicated by activation of c-Jun N-terminal kinase (JNK) and p38, and then sequentially activate transcription factor nuclear factor kappa B (NF- κ B). Induction of NF- κ B triggers upregulation and secretion of proinflammatory cytokines, TNF- α , and IL-1 β ^{7,8}, thus consequently lead to neurotoxic reactive oxygen species (ROS) production.⁹ Interestingly, TNF- α and ROS could activate NF- κ B^{10,11,12}, so there will be excessive TNF- α stimulation. Furthermore,

excessive TNF- α will induce apoptotic pathway through the activation of caspase.¹⁰

Transcription factor nuclear factor- κ B (NF- κ B) is a critical mediator in neurodegenerative disorders include HAD and plays a role in the regulation of proinflammatory response. Several genes regulated by NF- κ B are iNOS, cytokines, chemokines, adhesion molecules, and immune receptors. In a physiologic state, NF- κ B activation is designed for the beneficial defense mechanism of neuronal cells in critical condition. However, excessive and continuous activation of this pathway will jeopardize the survival of neuronal cells and brain tissue.¹³

Activated microglia also produces and secretes nitric oxide (NO) that is formed through inducible nitric oxide synthase-mediated reaction, and NO formation could induce neuronal apoptosis.^{14,15} The amplification of NF- κ B activation could multiply proinflammatory, and NO signaling thus induces apoptosis of neurons. Developing neuroprotective drugs targeting NF- κ B signaling is potential because it could inhibit several pathways involved in neuronal apoptosis.

Essential oil and terpenoid which have anti-inflammatory properties could be used as phytotherapy.^{16,17} The main constituent of essential oil and terpenoid extracted from the pine tree is alpha-pinene. Alpha-pinene (C₁₀H₁₆) is a non-polar (hydrophobic) compound with molecular weight 136.24 g/mol, present in liquid form, and volatile.¹⁸ The volatile characteristic of alpha-pinene has a beneficial effect on its utilization for inhalation therapy in a particular case such as behavior disorders include dementia patients.¹⁹

Studies of neuroinflammation are commonly used lipopolysaccharide for the induction of inflammation. In this study, we used HIV-1 glycoprotein 120 (gp120) for inducing inflammation response in the model of HIV-1-related neurodegeneration *in vitro*.²⁰ This study was aimed to investigate the effect of alpha-pinene administration on the expression of microglia's activated NF- κ B, iNOS, and neuronal apoptosis.

Methods

Study Design

This study was designed as a true experimental study using primary cell culture of fetal rat neuron-glia which was divided into eight groups as follow: negative control; positive control (gp120 1 nM); alpha-pinene only dose I, II, and III (0.4 μ g/ml, 2 μ g/ml, and 10 μ g/ml, respectively); gp120 + alpha-pinene dose I, II, and III (0.4 μ g/ml, 2 μ g/ml, and 10 μ g/ml, respectively). This study was done in the Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya. In a gp120-treated group, gp120 1nM was administered 1 hour before treatment of alpha-pinene with three dosages.

Primary Culture of Rat Fetal Neuron-Glia

The procedures of fetal brain tissue isolation and primary culture of fetal rat neuron-glia were performed as described previously in the literature.²¹ Briefly, pregnant rats at gestation day 16-17 were euthanized by cervical dislocation. Rats were laid on the back, and the abdominal area was cleaned with 70% ethanol. By surgical procedure, the fetus was taken from the uterus. Fetuses were laid on a

petri dish containing sterile phosphate buffer saline (PBS) and then carried to the biological safety cabinet under laminar airflow. The head of a fetus was wiped with 70% ethanol and euthanized by decapitation. The fetal brain was rinsed with PBS Penstrep, incised and then fetal brain tissue was removed. Fetal brain tissue was separated from the meninges, and red blood cells were then washed again with PBS Penstrep. The brain tissue was then transferred into four microtubes which each microtube was added with 400 μ l Trypsin EDTA. Brain tissue then will be incubated in CO₂ 5% incubator with temperature 37°C for 15 minutes. After this process, each microtube was added with 600 μ l fetal bovine serum (FBS) 10% in PBS, then centrifuged at 1200 rpm, room temperature, for 2 minutes. The supernatant was removed, and the pellet was added with 800 μ l FBS 10% in PBS for each microtube, centrifuged again at 1200 rpm, room temperature, for 2 minutes. After this process, the supernatant was removed, then the pellet was added again with 800 μ l serum-free media for each microtube, centrifuged again at 1200 rpm, room temperature, for 2 minutes. The supernatant was removed, and the pellet was added with complete media until reach 1.5 mL volume, and then pipetted carefully. Cells were cultured on well-12, each well contains 450 μ l and then added with 500 μ l complete media, incubated in CO₂ 5% with temperature 37°C.²¹

Measurement of NF- κ B and iNOS Expression

Measurement of NF- κ B and iNOS expression were performed using an immunocytochemistry kit as instructed by the manufacturer.

Measurement of Apoptosis

Detection of apoptosis was done using a TUNEL assay kit as instructed by the manufacturer.

Statistical Analysis

Data were analyzed using software SPSS version 17.0. Statistical analysis using in this study consist of independent T-test, One-way ANOVA, and correlation study. Data were analyzed using a confidence interval of 95% and assumed as significant of p-value < 0.05.

Results

Effect of Alpha Pinene on NF- κ B Activation

This study showed that alpha-pinene administration decrease the percentage of microglia containing activated NF- κ B in gp120-treated neuron-glia (mean 37.02 $\pm$ 18.55 %, 33.19 $\pm$ 2.82 %, and 32.69 $\pm$ 6.44 % at the dose I, II, and III, respectively vs. 57.57 $\pm$ 15.20 % at the gp120-only group) (One Way ANOVA, p<0.05). Furthermore, alpha-pinene administration at all dosages increases activation of NF- κ B in neuron-glia culture insignificantly as compared to the negative control group (Figure 1). All figures, tables, or informative illustrations are prepared as an HD image file.

Visualization of activated microglia is characterized by its processes. Microglia containing inactivated NF- κ B demonstrates a blue nucleus as shown in Figure 2(1), while microglia containing activated NF- κ B demonstrates dark cytoplasm and nucleus as shown in Figure 2.²

Figure 1. Percentage of microglia containing activated NF-κB in each group.

Note: (a) Negative control; (b) Positive control (gp120 1nM); (c) alpha-pinene I 0,4 ug/mL; (d) alpha-pinene II 2,0 ug/mL; (e) alpha-pinene III 10 ug/mL; (f) gp120 1nM + alpha-pinene I 0,4 ug/mL; (g) gp120 1nM + alpha-pinene II 2,0 ug/mL; (h) gp120 1nM + alpha-pinene III 10 ug/mL; Same notation indicates no significant differences and vice versa.

Figure 2. Microglia containing inactivated and activated NF-κB
Note: (1) negative control: rat brain neuron-glia culture without any treatment, (2) GP-120 treated neuron-glia culture, the figure demonstrates microglia containing activated NF-κB as indicated by dark nucleus as compared to control caused by NF-κB translocation into the nucleus, (3) neuron-glia culture treated with GP-120 + alpha-pinene 10 ug/mL. (Inverted microscope, Olympus, magnitude 400x)

Effect of Alpha Pinene on iNOS Expression

Figure 3. Percentage of microglia expressing iNOS.

Note: (a) Negative control; (b) Positive control (gp120 1nM); (c) alpha-pinene I 0,4 ug/mL; (d) alpha-pinene II 2,0 ug/mL; (e) alpha-pinene III 10 ug/mL; (f) gp120 1nM + alpha-pinene I 0,4 ug/mL; (g) gp120 1nM + alpha-pinene II 2,0 ug/mL; (h) gp120 1nM + alpha-pinene III 10 ug/mL; Same notation indicates no significant differences and vice versa.

This study showed that alpha-pinene administration decrease the percentage of microglia expressing iNOS in gp120-treated neuron-glia (mean 38.52 ± 28.3 %, 27.67 ± 1.98 %, and 56.78 ± 3.42 % at the dose I, II, and III, respectively vs. 91.76 ± 2.97 % in the gp120-only group) (One Way ANOVA, $p < 0.05$) (Figure 3). Furthermore,

alpha-pinene administration at all dosage increase expression of NF-κB in neuron-glia culture significantly as compared to the negative control group but significantly lower as compared to the gp-120 + alpha-pinene group (Figure 3). Figure 4 showed normal microglia¹, while microglia expressing iNOS indicated by red cytoplasm.

Figure 4. Microglia normal and highly expressing iNOS.

Note: (1) negative control: neuron-glia culture without any treatment, (2) gp120-treated neuron-glia (3) neuron-glia treated with gp120 + alpha-pinene 10ug/mL. Microglia expressing iNOS is indicated by dark cytoplasm (bold arrow). Microglia without iNOS expression is indicated by light cytoplasm (light arrow). (Inverted microscope, Olympus, magnitude 100x).

Effect of Alpha Pinene on Neuronal Apoptosis

This study showed that alpha pinene administration decrease the percentage of apoptotic neuron in gp120-treated neuron-glia (mean 21.77 ± 4.41 %, 8.07 ± 4.34 %, and 17.77 ± 13.59 % at the dose I, II, and III, respectively vs 63.49 ± 1.76 % in gp120-only group) (One Way ANOVA, $p < 0.05$) (Figure 5). Furthermore, alpha-pinene dose 2.0 decrease apoptotic neuron significantly as compared to the lowest dose in the gp120-treated group (8.07 ± 4.34 % vs 21.77 ± 4.41 %; $p < 0.05$).

Figure 5. Percentage of microglia contained apoptotic neuron
Note: (a) Negative control; (b) Positive control (gp120 1nM); (c) alpha-pinene I 0,4 ug/mL; (d) alpha-pinene II 2,0 ug/mL; (e) alpha-pinene III 10 ug/mL; (f) gp120 1nM + alpha-pinene I 0,4 ug/mL; (g) gp120 1nM + alpha-pinene II 2,0 ug/mL; (h) gp120 1nM + alpha-pinene III 10 ug/mL; Same notation indicates no significant differences and vice versa.

Note: (1) Negative control; (2) Positive control (gp120 1nM); (3) alpha-pinene II 2,0 ug/mL. Black arrow showed apoptotic cell with brown nucleus. (Inverted microscope, Olympus, 400x)

Discussion

In this study, alpha-pinene did not affect the number of microglia containing NF-κB in untreated neuron-glia, but alpha pinene could reduce the number of microglia containing NF-κB in gp120-treated neuron-glia. This

finding suggested that alpha pinene could selectively alter the neuron-glia cells in an inflammatory state and did not affect healthy cells.

Exposure of gp120 will increase its binding to the CD4 receptor and chemokines receptor (CXCR4 and CXCR-5). This binding activates the signaling pathway for the activation of protein kinase MAPK²², which in turn leads to I κ B degradation and release of NF- κ B. Activated NF- κ B translocates to the nucleus and binds to specific DNA thus induces the transcription process of several proinflammatory genes as well as Inos.²³

This study demonstrated that alpha pinene administration to gp120-treated neuron-glia decreases the number of microglia expressing iNOS. iNOS has been known as a catalyst for nitric oxide (NO) formation.²⁴ As alpha pinene decreases NO production, it could be used as anti-inflammatory agents particularly in gp120-induced neuroinflammation.

Alpha-pinene is contained in essential oil chamomile and has been used for a century for its anti-inflammatory properties in the treatment of eczema, dermatitis, and irritation.²⁵ Chemical analysis on essential oil from *J. Oxycedrus* has been reported, and it showed that one fraction from essential oil which contains 93% alpha pinene could induce I κ B degradation and activates NF- κ B. A previous study using chondrocyte cell line C-28/I2 demonstrated that alpha pinene reduces I κ B phosphorylation thereby reduce the activation of NF- κ B and its DNA binding activity.²⁶

These studies confirm the role of alpha-pinene as anti-inflammatory agents mainly through its modulation of NF- κ B signaling. However, further study is required for investigating the effect of alpha-pinene especially for the treatment of neuroinflammation mediated neurodegenerative disease, including HAD.

Our study also showed that alpha pinene decreases the expression of iNOS in gp120-treated neuron-glia significantly in all dosages of alpha-pinene as compared to the gp120-treated group. This result was in accordance with the previous study reported that alpha pinene administration dosage 2,0 ug/mL and 0,4 μ g/mL inhibit iNOS expression in LPS-treated neuron-glia.²⁷ Together with this result, we could hypothesize that alpha pinene administration could be beneficial in several situations highlighted by neuroinflammation such as HAD and other neurodegenerative disorders such as Parkinson disease and Alzheimer disease.

Further experiment showed that alpha pinene dosage 10 ug/mL for 24-hour decrease the number of apoptotic neuron in untreated neuron-glia culture. On the other hand, the previous study showed that alpha pinene administration (dosage 10 ug/mL and 100ug/mL for 2 hours) induces neuronal apoptosis in neuron-glia culture.²⁸ From these studies, we could say that the dosage of alpha-pinene is correlated with pharmacological activity. Low dose alpha-pinene (10 ug/mL) has a dual effect, neuroprotective and neurotoxic, while high dose alpha-pinene (100 ug/mL) possesses a neurotoxic effect. Considering the duration of treatment, acute treatment (2 hours) with low dose alpha pinene has a neurotoxic effect, while low dose administration in 24 hours has a neuroprotective effect.

Conclusion

We concluded that alpha pinene has a potential role in inhibiting neuroinflammation mediated by gp120 as indicated by NF- κ B expression, iNOS expression, and also neuronal apoptosis.

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