Original Article

Activation of Tyrosine hydroxylase RNA Expression by Water Extract of *Vernonia cinerea* in Nicotine-pretreated PC12 Cells

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Background: *Vernonia cinerea* (VC) has been widely applied for smoking cessation. In tobacco users, the activation of dopaminergic neurons via nicotinic cholinergic receptors results in activated brain reward pathways by nicotine. However, molecular mechanism of this *Vernonia species* in tobacco-addiction is unclear.

Objective: The present study aimed to reveal a mechanism whether tyrosine hydroxylase (TH), a rate limiting enzyme in dopaminergic pathway, involved in the bioactivity of *Vernonia cinerea* on a cellular model of nicotine addict.

Materials and Methods: Rat pheochromocytoma 12 (PC12), dopaminergic cell line, was pre-treated and post-treated under medium containing nicotine and medium containing water extract of *Vernonia cinerea*, respectively. Treated cells were analyzed for the levels of transcripts of TH to determine the functional alteration of genes encoding for this enzyme.

Results: TH transcripts was up-regulated under treatment of various concentration of *Vernonia* extract. Upon cell cytotoxicity, its activation was not resulted from the promotion of cell death.

Conclusion: The present study was firstly described the TH activation of bioactive substance in the water extract of *Vernonia cinerea*.

Keywords: *Vernonia cinerea*, Tyrosine hydroxylase, Nicotine, Smoking cessation, PC12 cells

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Vernonia (*Asteraceae*), the largest genus in the tribe *Vernoniae*, is known for several species with food, medicinal and industrial uses. For the medical treatment, there was the knowledge on the ethnomedicinal, pharmacological evaluation and chemical diversity within the *Vernonia genus*. *Vernonia species* have traditional been used as the herbal drugs with various activities of antioxidants, anti-inflammation, anticancer, etc[1-3]. In this genus, *V colorata Drake, V guineensis Benth, V jugalis Oliv &Hiern, V lasiopus O Hoffm and V neocorymbosa Hilliardare* were reported to have neurological activities including anti-epileptic and anti-depressant activities[3]. Similar to the other *Vernonia species*, VC also plays a role in neurological control in many aspects. Reddy PJ et al[4] discovered the anti-cataleptic activity of VC for Parkinson's disease. Tupe et al[5] also demonstrated the anti-depressant activity in *Vernonia anthelmintica* and showed either methanolic or aqueous extracts had significant antidepressant activities in dose-dependent. Tea from dried VC leaves also has been documented and widely used as a traditional medicine for relieving cigarette craving in Thailand and other countries[6]. Supplement of *Vernonia cinerea* Less and exercise in cigarette smokers provided benefit in reduction of smoking rate[7].

The molecular mechanism in nicotine addiction involves in 6 signaling pathways via dopamine receptor, nicotinic receptor, GABAergic receptor, glutamate receptor, NMDA receptor, AMPA receptor. It is known well that the activation of brain reward pathways by nicotine in cigarette smokers is modulated by the activation of dopaminergic neurons via nicotinic cholinergic receptors[8]. TH is a key enzyme for catecholamine synthesis. TH expression is regulated under nicotine treatment. Chronic exposure of nicotine to an animal model for 2 weeks increased all levels of *TH* mRNA, TH protein and TH activity[9]. Phytochemical analysis of VC extracts under various types of solvents, methanol, ethanol, petroleum ether, benzene, acetone, ethyl aceteate chloroform or aqueous extracts, showed the chemical constitutes of flavonoids, tannins, saponins, steroids, triterpenoids, glycosides, and phenolic compounds[10]. Among these groups of substances, there has been no report for its effect on *TH* RNA expression. However, only ginsenosides and saponins from ginseng, showed their activities on

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promoting TH expression[11,12]. It remains unclear about the molecular mechanism of VC as an inhibitor for nicotine addiction. The recent study of Ketsuwan N showed the decreased levels of catecholamine by extracts of VC in rats after pre-treatment with nicotine[13], supporting down-regulation of dopamine synthesis. On the contrary, it is likely that activation of dopaminergic pathway may involve in interference of VC to smoking cessation, we, therefore, aimed to reveal whether *TH* RNA expression involves in the bioactivity of VC on cellular model of nicotine addict.

Materials and Methods

Extraction of vernonia cinerea

Fresh plant harvested at Srinakharinwirot University-Ongkarak, was dried and kept cool at 4°C. Dried leaves of VC yielded one of 100th w/w powder of aqueous extract. Blended powder of dried leaves was macerated in distilled water at the controlled temperature for overnight. The mixture was sonicated at the controlled temperature, then filtrated. Water extract was freeze-dried then the dried extract powder was subjected to cell treatment. The effect of 4°C storage on cellular activity was further analyzed. To confirm whether the activity was stable or not, dried-leave powder of VC was stored at 4°C for 2 years. The stored powder was subjected into the preparation protocol as above.

Cell culture, treatment, and MTT viability test

PC12 cell lines were propagated under ATCC culture condition using 1: 1 DMEM-F12 with 10% fetal bovine serum and 5% horse serum (Hyclone). One million cells were plated in 6-well plates for overnight and continue-cultured under medium with 2 mM nicotine (Sigma-Aldrich)[14] for 2 days. Nicotine-pre-treated PC12 cells were replaced with new medium containing water-extract of VC to exclude the accumulation of toxic metabolites. Post-treatment after nicotine was done under medium containing various concentrations of water extracts of VC. After 2 day-incubation, cells were harvested for RNA preparation.

PC12 cells at 50,000 cells were cultured in 96-well plate was done and treated as above then assayed for cell viability by determination of NAD(P)H-dependent cellular oxidoreductase enzymes using MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, USB bioscience) at a final concentration of 0.4 mg/ml at 37°C for 2 hrs.

Reverse transcription, semi-quantitative PCR and real-time PCR

RNA of the complete treated cells were prepared in Trizol solution (according to protocol from Gibco-BRL). RT-PCR analysis was done using each set of primers for alpha-actin transcript as control house-keeping gene, and for *TH* transcript for determining gene activation in the dopaminergic pathway.

Primer sequences used in RT-PCR, designed by Primer3, for TH are sense primer [5'-ACCTATGCATTCAC CTGAGC-3'] and antisense [5'-AGTTCCTGAGCTTG TCCTTG-3']. Alpha-actin was used as a control house-keeping gene. Primers for alpha-actin transcript are sense primer [5'-TCTCTTCCAGCCTTCCTTC-3'] and antisense primer [5'-ATCTCCTTCTGCATCCTGTC-3']. Reverse transcription using enzyme from Roche Biosciences and other reagents from Fermentus was performed under 42°C for 1 hr. Real time-PCR was done in RotorGene 6,000. Conditions were optimized to primers for each amplicon.

$2^{-\Delta\Delta Ct}$ was calculated for determination of fold of expression of test genes to controls in triplicate runs. Statistic t-test was obtained from the analysis of duplicated isolated experiments.

Results

PC12 cells were cultured under medium with various concentrations of nicotine (0, 1, 2, 4, 8, 16 mM, respectively). By using MTT assay, the concentration of 2 mM nicotine was selected as a non-toxic condition. To evaluate the altered expression of *TH* transcript, the levels of RNA analyzed by real-time PCR were done from duplicated experiments of cell treatment and the levels of cDNAs were compared by calculation of $2^{-\Delta\Delta Ct}$ as expression of test genes to controls. The data of real-time PCR (Figure 1) showed expression analysis of *TH* in PC12 cells in comparing to a control alpha-actin gene. It was found about 4 to 8 fold of increased expression of *TH* RNA to actin RNA under the treatment with an increased concentration of water extract of VC, respectively. Statistical analysis showed significantly increased expression of *TH* RNA at all concentrations of VC water extracts (p<0.02).

In addition, TH activation has been reported to be

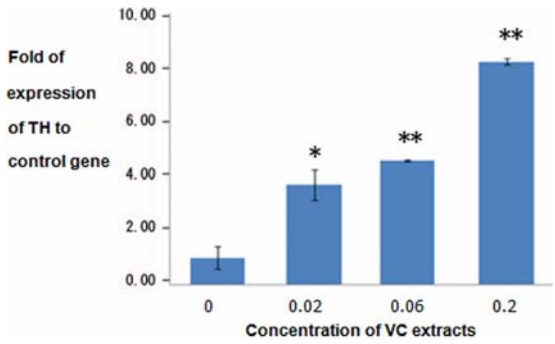

Figure 1. Effect of VC extract to *TH* RNA expression. The figure represents data of RT-PCR assay for transcripts of TH to control gene (alpha actin). Cells were pretreated with 2 mM nicotine and treated with concentrations of VC extract as indicated. Concentration at 0 mg/L is a negative control. The p-value from statistic t-test at each concentration of extracts to negative control are <0.05 (*) for 0.02 mg/L, <0.002 (**) for 0.06 mg/L and <0.002 (**) for 0.20 mg/L, respectively.

a stress-response to cell death of neurons, we had further analyzed the TH activation to cell viability. In Figure 2 the cell viability count (MTT test) showed that all tested cells remain viable. Moreover, calculation of *TH* RNA to cell death ration did not show any different pattern. *TH* expression was also activated by 6 to 10 folds, upon an increased concentration, respectively. This data indicated that the activation of *TH* RNA is not caused by activation of cell death.

Furthermore, we evaluated whether the substance in the water extract of VC was stable or not. Expression analysis was done using the newly prepared extracts obtained after stored at 4°C for 2 years. In comparison to old preparation of the water extracts of VC stored at 4°C for 2 years, new preparation of VC water extracts at a concentration of 0.02 mg/L and 0.2 mg/L showed the similar pattern of activation of *TH* RNA to the old preparation, as shown in Figure 3. This result confirmed that the biological activity of VC water extracts on the expression of *TH* RNA is stable.

Discussion

A folk way of Northern Thais has been used VC leaves for stopping smoke since long time ago. Literature review describes one mechanism of the activation of brain reward pathways by nicotine is resulted from the activation of dopaminergic neurons via nicotinic cholinergic receptors. However, the molecular mechanism of VC in smoking cessation by dopaminergic pathway has not yet clearly uncovered. In the present study, the authors have interested in the analysis of TH role in the dopaminergic pathway in nicotine addiction whether the activation of this pathway compensates stress release by nicotine. *TH* RNA expression level and TH activity are directly involved in regulating intracellular dopamine levels. A deficiency of TH leads to impaired synthesis of dopamine as well as epinephrine and norepinephrine. The activity of TH could be modulated by two mechanisms: medium- to long-term regulation of gene expression at molecular controlled levels, and short-term regulation of enzyme activity by protein modification, and feedback inhibition of catecholamine levels[15].

Previously, VC was shown its activity as antioxidants and activator for beta-endorphin level for reduction of smoking rate[7]. Part of mechanism of VC in nicotine addiction had been studied via determination of level of monoamine (MA), a degraded product of dopamine and cathecolamine which are catalyzed by monoamine oxidases (MAOs). Flavonoids in VC, such as apigenin, chrysoeriol, luteolin and aqercetin, were demonstrated to inhibit MAOs with variable degrees and were more prominent in inhibition toward MAO-A, probably resulting in an accumulated level of dopamine and catecholamine[16]. Moreover, VC also inhibits catalepsy, one of major symptom of Parkinson's disease, which is due to dopaminergic defect[7]. Therefore, it is likely that the activation of dopaminergic mechanism may involve in VC response. On the contrary, recent animal model on smoking cessation showed decreased levels of blood dopamine and catecholamine[13]. That may be due to uncontrollable

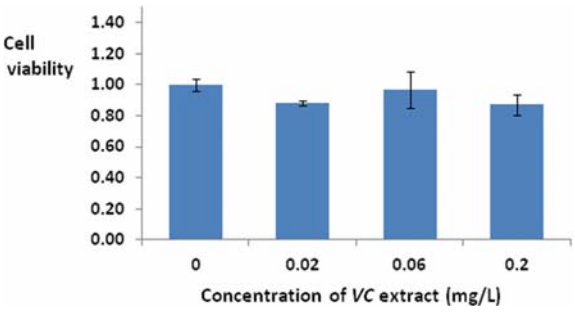

Figure 2. Effect of VC extract to cell viability of PC12 cell lines. The figure represents data of cell viability and expression of TH to control gene to cell viability. Bold bar indicates cell viability. Solid bar indicates % cell viability of PC12 treated with VC extracts at the indicated concentration. Concentration at 0 mg/L is a negative control. The *p*-value from statistic t-test at each concentration of extracts to negative control are >0.05.

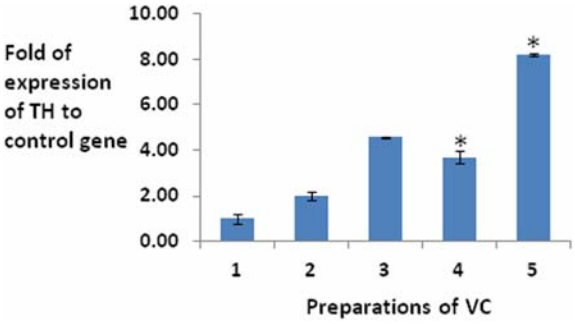

Figure 3. Effect of 2-year storage of VC leaf dried powder to its bioactivity on TH RNA expression. The figure represents data of RT-PCR assay for *TH* transcripts to control gene. Cells were pretreated with 2 mM nicotine and treated with concentrations of *VC* extract as indicated. No. 1 is data of negative control (2 mM nicotine without the addition of VC extract). No. 2 and 3 are obtained from 0.02 and 0.2 mg/L of *VC* extract which was prepared and stored for 2 years. No. 4 and 5 are obtained from 0.02 and 0.2 mg/L of VC extract which was stored for 2 years and freshly prepared after storage. The *p*-values from statistic t-test of 2 year stored VC extracts to fresh preparation of VC extracts at same concentrations, 2 to 4 and 3 to 5, are <0.05 (*).

stress in rats. In the present study, a role of VC in a cellular model of nicotine addiction was performed. We had tested for RNA analysis of *TH*, a key rate-limiting step enzyme, in a pathway of synthesis of dopamine-catecholamine, the dopaminergic pathway is activated through increase synthesis of *TH* transcript. This activation is not related to any process of cell death since the number of PC12 cells is nearly the same in samples treated with different concentrations of VC extract. Activation of TH by phosphorylation depending 1 on 4-3-3 proteins binding, which are likely to be associated with neurodegenerative diseases, makes an indirect link between TH and these diseases[15]. Regulation of *TH* RNA expression is dependent on transcriptional responsive elements (TRE), such as CRE, AP-1, AFT-2 and Nurr[14]. Oxidative stress increases *TH* RNA expression via AP-1[17] but reduces the level of reduced tetrahydropterin (BH4), a cofactor for TH protein, resulting in reduced enzyme activity[15]. Some bioactive compounds in VC such as anti-oxidative flavonoids may modulate *TH* expression via direct control of TREs or regulation of active transcription factors.

Conclusion

The recent researches reviewed no report of bioactive substance from *Vernonia species* in regulating *TH* RNA expression, therefore, the present study showed preliminary evidence of VC in the activation of dopaminergic pathway, via the increase of *TH* transcript levels. However, the levels of active TH protein or intermediate substances in dopaminergic pathway could be further evaluated to confirm the role of TH in neuronal responsiveness for nicotine treatment.

What is already known on this topic?

Role of flavonoids isolated from VC in nicotine addiction has been shown the inhibitive activities of monoamine oxidase A and B (MAO-A, MAO-B). These enzymes degrade dopamine and catecholamine, therefore, the upstream substances in this pathway could be increased.

In an animal model of Parkinson's disease which dopamine level is defective, VC also inhibits catalepsy.

What this study adds?

Vernonia cinerea (VC) could activate the dopaminergic pathway in PC12 cell lines via an increase of *TH* transcript levels.

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Potential conflicts of interest

The authors declare no conflicts of interest.

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