의학박사 학위논문

Effects of nicotine on the activity of glutamate transporter type 3 and the modulation mechanism

니코틴이 제 3 형 글루탐산염 운반자의 활성도에 미치는 영향 및 조절기전

2014 년 2 월

서울대학교 대학원

의학과 마취통증의학 전공

윤 희 조

니코틴이 제 3 형 글루탐산염 운반자의 활성도에 미치는 영향 및 조절기전

지도 교수 임 영 진

이 논문을 의학박사 학위논문으로 제출함
2013년 12월

서울대학교 대학원 의학과 마취통증의학 전공
윤 희 조

윤희조의 의학박사 학위논문을 인준함
2013년 12월

위 원 장 _____ (인)
부위원장 _____ (인)
위 원 _____ (인)
위 원 _____ (인)
위 원 _____ (인)

Effects of nicotine on the activity of glutamate transporter type 3 and the modulation mechanism

by
Hea Jo Yoon

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science (Anesthesiology and Pain Medicine) at Seoul National University College of Medicine

December 2013

Approved by Thesis Committee:

Professor _____ **Chairman**
Professor _____ **Vice chairman**
Professor _____
Professor _____
Professor _____

학위논문 원문제공 서비스에 대한 동의서

본인의 학위논문에 대하여 서울대학교가 아래와 같이 학위논문 제공하는 것에 동의합니다.

1. 동의사항

① 본인의 논문을 보존이나 인터넷 등을 통한 온라인 서비스 목적으로 복제할 경우 저작물의 내용을 변경하지 않는 범위 내에서의 복제를 허용합니다.

② 본인의 논문을 디지털화하여 인터넷 등 정보통신망을 통한 논문의 일부 또는 전부의 복제.배포 및 전송 시 무료로 제공하는 것에 동의합니다.

2. 개인(저작자)의 의무

본 논문의 저작권을 타인에게 양도하거나 또는 출판을 허락하는 등 동의 내용을 변경하고자 할 때는 소속대학(원)에 공개의 유보 또는 해지를 즉시 통보하겠습니다.

3. 서울대학교의 의무

① 서울대학교는 본 논문을 외부에 제공할 경우 저작권 보호장치(DRM)를 사용하여야 합니다.

② 서울대학교는 본 논문에 대한 공개의 유보나 해지 신청 시 즉시 처리해야 합니다.

논문 제목: Effects of nicotine on the activity of glutamate transporter type 3 and the modulation mechanism

학위구분: 박사
학 과: 의학과
학 번: 2005-30636
연 락 처:
저 작 자: 윤희조 (인)

제 출 일: 2013 년 12 월 22 일

서울대학교총장 귀하

ABSTRACT

Introduction: Nicotine, main ingredient of tobacco elicits seizure in animal model and cigarette smoking is regarded as behavioral risk factors associated with epilepsy or seizure. In hippocampus, origin of nicotine-induced seizures, most of the glutamate uptake could be performed primarily by excitatory amino acid transporter type 3 (EAAT3). It has been reported that dysfunction of EAAT3 is related to temporal lobe epilepsy. Reduction of EAAT3 activity may be mediated by PKC and PI3K, two intracellular signaling molecules. Therefore, I hypothesized that nicotine may attenuate EAAT3 activity by mediation of PKC and PI3K.

Methods: I investigated the effects of nicotine on the activity of EAAT3 by using *Xenopus* oocyte expression system and the involvement of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K). Rat EAAT3 was expressed in *Xenopus* oocytes by injecting EAAT3 mRNA. L-Glutamate (30 μM)-induced inward currents were recorded via the two-electrode voltage clamp method. Responses were quantified by integration of the current trace and reported in microCoulombs (μC).

Results: Nicotine decreased EAAT3 activity in a dose-dependent manner when oocyte was exposed to nicotine for 72 h. Since the inhibition reached maximal at nicotine concentrations at 0.03 μM, I used 0.03 μM for further experiments. Nicotine (0.03 μM for 72 h) significantly reduced V_{max}, but did not alter K_m value of EAAT3 for glutamate. When nicotine treated oocytes were incubated with phorbol-12-myrisate-13-acetate, a protein kinase C (PKC)

activator, PMA-induced increase in EAAT3 activity was abolished. PKC inhibitors (staurosporine, chelerythrine and calphostin C) significantly reduced basal EAAT3 activity. Whereas, there were no significant differences among the PKC inhibitors, nicotine, and PKC inhibitors + nicotine groups. In similar fashion, PI3K inhibitors (wortmannin and LY294002) significantly decreased EAAT3 activity, however no statistical differences were observed among PI3K inhibitors, nicotine, and PI3K inhibitors plus nicotine groups.

Conclusions: This study demonstrates that nicotine decreases EAAT3 activity and this effect seems to be mediated by PKC and PI3K. This study may provide an additional mechanism for nicotine induced seizure.

Keywords: Nicotine, Excitatory amino acid transporter type 3, Glutamate transporter, Phosphatidylinositol 3-kinase, Protein kinase C, *Xenopus* oocyte

Student number: 2005-30636

CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

EAAT3: excitatory amino acid transport type 3

PKC: protein kinase C

PI3K: phosphatidylinositol 3-kinase

PTZ: pentylenetetrazole

NMDA: N-methyl-D-aspartate

NO: nitric oxide

nAChR: nicotinic acetylcholine receptor

GSH: glutathione

GABA: *gamma*-aminobutyric acid

INTRODUCTION

Tobacco is a popular product world-wide despite the fact that nicotine causes toxicity and dependency. Pharmacologically, nicotine (methylpyridyl-pyrrolidine) is a water-soluble liquid alkaloid (1) which causes diverse central nervous system (CNS) effects, ranging from reduction of anxiety to seizure and coma (2). Smoking is regarded as a behavioral risk factor associated with epilepsy or seizure in epidemiological studies. People with epilepsy were found to smoke cigarettes more often than those without epilepsy (38.8% *vs.* 24.9%) (3). In addition, participants reporting current cigarette smoking have an increased risk of seizure in comparison with those who never smoke (relative risk 2.60, 95% confidence interval 1.53–4.42) (4). Moreover, accidental nicotine ingestion in children and adults has been shown to cause seizures (1, 5).

Glutamate transporters, also known as excitatory amino acid transporters (EAATs), are a family of high-affinity, sodium-dependent transporters that span the plasma membrane of glia and neurons and contribute to the clearance of glutamate from the extracellular space in order to maintain synaptic glutamate concentrations and prevent excitotoxicity (6). Substantial alterations in glutamate transport may be implicated in a wide spectrum of neurologic disorders, such as amyotrophic lateral sclerosis, epilepsy, Huntington’s disease, Alzheimer's disease, ischemic stroke injury, white matter injury, and schizophrenia (7-9).

Five different types of EAATs have been cloned. EAAT1 and EAAT2 are

localized mainly in glial cells, EAAT3 and EAAT4 in neurons, and EAAT5 in the retina. In the hippocampus, abundantly expressed EAAT3 (6) may play a major role as glutamate transporter because many synapses are not encompassed by astrocytes (10). Temporal lobe seizures are related to hippocampal sclerosis, a characteristic feature of hippocampal pathology (11, 12). In addition, dysfunction of EAAT3 has been reported to be related to temporal lobe epilepsy (13, 14), and in vivo electrophysiological studies have revealed that nicotine-induced seizures originate in the hippocampus (15-17). Reduction of EAAT3 activity by a variety of substances may be mediated by PKC and PI3K, two intracellular signaling molecules (18-20). Therefore, I hypothesized that nicotine could attenuate EAAT3 activity by mediation of PKC and PI3K.

In this study, I examined the effects of nicotine on EAAT3 activity expressed in *Xenopus* oocytes and investigated the roles of PKC and PI3K in the effect of nicotine on EAAT3.

MATERIALS AND METHODS

The study protocol was approved by the Institutional Animal Care and Use Committee at Seoul National University College of Medicine. Mature female *Xenopus laevis* frogs were purchased from *Xenopus* I (Dexter, MI). Molecular biology reagents were obtained from Ambion (Austin, TX). Nicotine and other chemicals were purchased from Sigma (St. Louis, MO, USA).

1. Oocytes preparation

Xenopus oocytes were harvested and microinjected as previously described (Do et al., 2002a). I anesthetized frogs in 500 ml of 0.2% 3-aminobenzoic acid ethyl ester (Sigma, St. Louis, MO, USA) in water. Frogs underwent an operation on ice after checking for unresponsiveness to toe pinching. Following a 5 mm incision in the lower lateral abdominal region, an ovarian lobule, containing about 150-200 oocytes, was removed. Oocytes were instantly immersed in calcium-free OR-2 solution (NaCl 82.5 mM, KCl 2 mM, $MgCl_2$ 1 mM, HEPES 5 mM, and 0.1% collagenase type Ia; pH =7.5) to remove the vitelline membrane. Oocytes were defolliculated by gentle shaking for nearly 2 h, and then incubated for one day in modified Barth's solution (NaCl 88 mM, KCl 1 mM, $NaHCO_2$ 2.4 mM, $CaCl_2$ 0.41 mM, $MgSO_4$ 0.82 mM, $Ca(NO_3)_2$ 0.3 mM, gentamicin 0.1 mM, and HEPES 15 mM; pH = 7.6) at 18^{o}C. Fully grown stage V or VI *Xenopus* oocytes were selected for the following experiments.

2. Expression of EAAT3

The complementary DNA (cDNA) of rat EAAT3 was provided by Dr. M.A. Hediger (Brigham and Women's Hospital, Harvard Institute of Medicine, Boston, MA, USA). The cDNA was subcloned into a commercial vector (Bluescript-SKm), and plasmid DNA was linearized using a restriction enzyme (Not I). I synthesized the messenger RNA (mRNA) in vitro using a transcription kit (Ambion, Austin, TX, USA). EAAT3 mRNA was quantified spectrophotometrically and diluted in sterile water. Thirty nanoliters of this mRNA (1 ng/nl) was injected into the cytoplasm of oocytes with an automated microinjector (Nanoject; Drummond Scientific Co., Broomall, PA, USA). The prepared oocytes were subsequently incubated at 18^{o}C for 3 to 4 days to express EAAT3 before electrophysiologic recording.

3. Electrophysiological recording

I measured electrophysiological changes at room temperature (21-23°C). Microelectrodes were prepared with a micropipette puller and 10 μl glass capillary tubes (Drummond Scientific Co.). The diameter of the microelectrode tips was adjusted to approximately 10 μm by breaking the tip and the resistance of the microelectrode was estimated at 1-5 MΩ when filled with 3 M KCl. Oocytes were perfused with Tyrode's solution (NaCl 150 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1 mM, dextrose 10 mM, and HEPES 10 mM; pH = 7.5) at a flow rate of 3 ml/min before measuring the currents. A single oocyte was voltage-clamped using a two-electrode oocyte voltage clamp amplifier (OC725-C: Warner Co., New Haven, CT, USA), with a

holding potential of -70 mV and the evoked currents were analyzed with the Ooclamp software program. Data from oocytes that did not show a stable holding current of less than 0.6 μA were discarded. L-glutamate was diluted in Tyrode's solution and perfused over an oocyte for 20 s at a rate of 3 ml/min. Inward currents yielded by superfusion of L-glutamate were recorded at 125 Hz for 1 min (baseline: 5 s, L-glutamate application: 20 s, washing with Tyrode's solution: 35 s). I measured the response induced by L-glutamate, which was calculated by integration of the inward currents and described as microCoulombs (μC). The response should reflect the sum of the total amount of transported glutamate. At least three different frogs were used for analysis in all of the experiments.

4. Experimental chemical treatment

To evaluate the dose-response effect of nicotine on EAAT3 activity, oocytes were incubated in nicotine at serial concentrations (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, and 1 μM) by dilution in modified Barth's solution for 72 h. In the control group, oocytes were incubated in modified Barth's solution alone. In a previous study, the median effective concentration of glutamate that induced EAAT3 activity was 27.2 μM (21), so I used 30 μM glutamate as an agonist in this study. To study the effects of nicotine on the V_{max} and K_m of EAAT3 with L-glutamate, I used serial concentrations of L-glutamate (3, 10, 30, 100, and 300 μM).

To investigate PKC involvement in the effects of nicotine on EAAT3 activity, PKC activator (100 nM PMA) or PKC inhibitor (100 μM chelerythrine, 2 μM

staurosporine, or 9 μM calphostin C) was applied to the oocytes for 10 min or 1 h, respectively, before recording the currents. Oocytes were exposed to the PI3K inhibitors (10 μM wortmannin and 50 μM LY294002) for 1 h to investigate the effect of PI3K inhibition on EAAT3 activity. To evaluate time course of the effects of nicotine exposure on the activity of EAAT3, oocytes were incubated in nicotine for 24, 48, and 72 h. The reversibility of the nicotine effect on EAAT3 activity was investigated by washing oocytes in modified Barth's solution for 12 or 24 h after incubation in nicotine for 72 h.

5. Statistical analysis

The results are expressed as means ± S.E.M. The batch-to-batch responses of oocytes were variable owing to different expression levels of EAAT3. Therefore, data were normalized to the results of the same day controls for each oocyte batch. Statistical analysis was conducted using the one way analysis of variance (ANOVA) or Student's t-test appropriately. Post hoc comparison was performed with Student-Newman-Keuls correction. Data were analyzed using SPSS, version 18.0 (Chicago, IL) and Prism version 5.0 (Graphpad, San Diego, Ca). Statistical differences were considered significant for P values < 0.05.

RESULTS

Inward currents were produced by oocytes that were provided with L-glutamtate only when they had been injected with EAAT3 mRNA, compared to no response to L-glutamtate from oocytes that had not been injected with EAAT3 mRNA independent of incubation in 0.03 and 0.3 μM nicotine for 72 h (data not shown). When oocytes were exposed to sequentially higher concentrations of nicotine (0.001-1 μM) for 72 h, the responses decreased in a concentration-dependent manner, with a significant decrease at 0.03 μM (P = 0.004), 0.1 μM (P = 0.024), 0.3 μM (P = 0.033), and 1 μM (P = 0.02) nicotine compared to the control group (Fig. 1). Nicotine resulted in a concentration-dependent reduction in EAAT3 activity at low concentrations, although the IC_{50} value of nicotine could not be calculated from the dose-response data because they did not fit the Hill equation. Since the inhibition reached a maximum at concentrations of 0.03 μM or higher, I used 0.03 μM for further experiments. The EAAT3 response to L-glutamate in the presence of 0.03 μM nicotine was reduced by approximately 25% (Fig.1).

When oocytes were exposed to L-glutamate at 3-300 μM with or without 0.03 μM nicotine for 72 h, the responses decreased significantly at concentrations of 10, 30, 100, and 300 μM L-glutamate (Fig. 2). Further analysis of the data by Prism version 5.0 (Graphpad, San Diego, CA) showed that nicotine significantly decreased the V_{max} of EAAT3 for glutamate (control: 2.7 ± 0.1 μC and nicotine: 2.1 ± 0.1 μC, $P < 0.001$) but caused no significant change in

the K_m value (control: 18.6 ± 2.9 μM and nicotine: 20.4 ± 2.9 μM, P = 0.662.; Fig. 2).

EAAT3 activity was significantly increased in oocytes pretreated with 100 nM PMA (control: 1.00 ± 0.07 and PMA: 1.28 ± 0.11, P = 0.01; Fig. 3). When nicotine-treated oocytes (0.03 μM for 72 h) were exposed to PMA (100 nM for 10 min), the nicotine-induced inhibition of EAAT3 activity was abolished (nicotine: 0.73 ± 0.05 and PMA+nicotine: 0.99 ± 0.06, P = 0.02; Fig. 3).

Preincubation of oocytes with 2 μM staurosporine significantly reduced EAAT3 activity (control: 1.00 ± 0.05 and staurosporine: 0.76 ± 0.06, P = 0.004). Oocytes exposed to staurosporine, nicotine, or staurosporine plus nicotine showed a significant decrease in EAAT3 activity as compared with untreated controls. However, the EAAT3 activity was not significantly different among oocytes treated with staurosporine, nicotine, or staurosporine plus nicotine (Fig. 4), suggesting the absence of an additive or synergistic interaction between the effects of staurosporine and nicotine on EAAT3 activity. Similar patterns of responses were recorded for other PKC inhibitors (chelerythrine [100 μM for 1 h] and calphostin C [9 μM for 1 h]) (Fig. 4).

EAAT3 activity was significantly decreased by pretreatment of oocytes with PI3K inhibitor wortmannin (10 μM for 1 h) (control: 1.0 ± 0.04 and wortmannin: 0.74 ± 0.06, P = 0.013; Fig. 5), however, there were no differences in response among the wortmannin, nicotine, and wortmannin plus nicotine groups (Fig. 5). Likewise, another PI3K inhibitor, LY294002 (50 μM for 1 h) showed a similar pattern of responses (Fig. 5).

A time course of the effect of nicotine exposure on EAAT3 activity for 24, 48, or 72 h showed a time-dependent reduction in EAAT3 activity with a significant difference only between the control and 72 h treated groups (P = 0.031; Fig. 6).

Oocytes exposed to nicotine (0.03 μM) were removed after exposure for 72 h and then incubated in modified Barth's solution for 12 or 24 h. In this washout study, EAAT3 activity still showed a significant inhibition compared to that of the control at 12 h (P = 0.006). However, EAAT3 activity recovered toward the control level at 24 h, which indicated that the effects of nicotine exposure on EAAT3 activity were reversible (P = 0.231; Fig. 7).

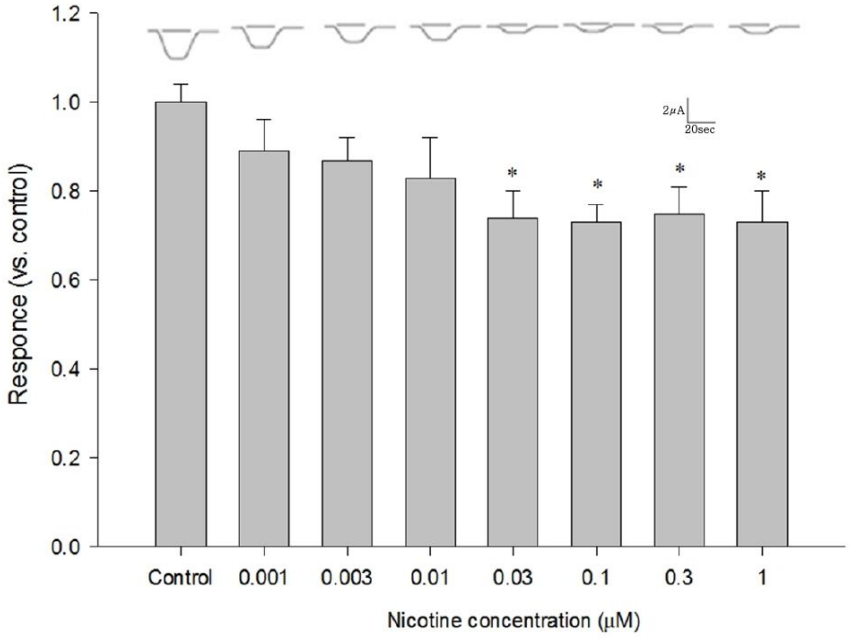

Figure 1. Effects of nicotine exposure on the activity of EAAT3. Oocytes were superfused with various concentration of nicotine (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, and 1 μM) for 72 h before the responses to 30 μM of L-glutamate were measured. Each set of data has been normalized by using the mean value of the control group from the same batch. Data are described as the mean ± S.E.M. (n = 15 - 27) *$P < 0.05$ compared to control.

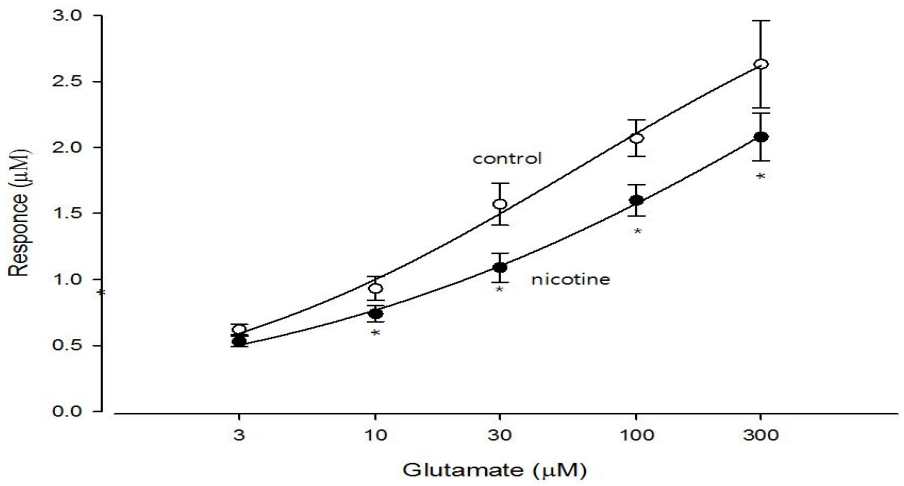

Figure 2. Dose-response curves of EAAT3 to L-glutamate in the presence or absence of nicotine. Oocytes were exposed to nicotine (0.03 μM for 72 h in the nicotine group. Nicotine significantly decreased the responses induced by 10, 30, 100, and 300 μM L-glutamate. Data are described as the mean ± SEM. (n = 16 - 20). *$P < 0.05$ compared to the corresponding controls.

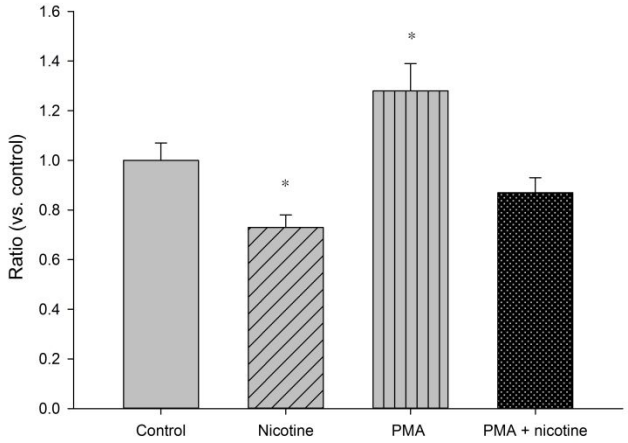

Figure 3. Effects of protein kinase C (PKC) activation on EAAT3 activity in the presence or absence of 30 μM nicotine for 72 h. When nicotine-treated ooctyes (0.03 μM for 72 h) were exposed to PMA (100 nM for 10 min), the nicotine-induced reduction in EAAT3 activity was attenuated. PMA, phorbol-12-myristate-13-acetate. Data are described as mea ± S.E.M. (n = 20 - 23). *P < 0.05 compared to control.

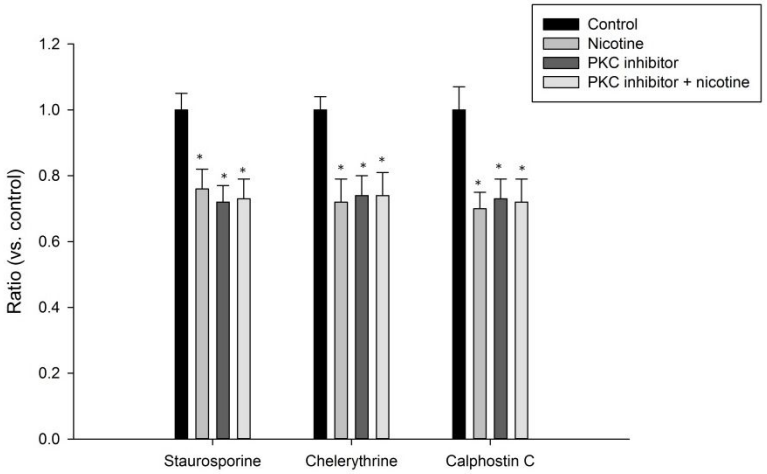

Figure 4. Effects of protein kinase C (PKC) inhibition on EAAT3 activity in the presence or absence of 0.03 μM nicotine for 72 h. Whereas oocytes exposed to PKC inhibitor, nicotine, or PKC inhibitor plus nicotine showed a significant decrease in EAAT3 activity compared to control; the EAAT3 activity in oocytes treated by PKC inhibitor, nicotine, or both was not different significantly. Data are described as mean ± S.E.M. (n = 17 - 23). *P < 0.05 compared to control.

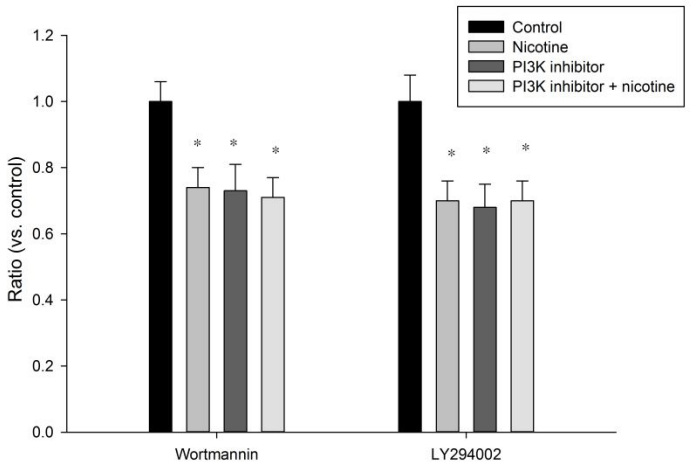

Figure 5. Effects of PI3K inhibition on EAAT3 activity in the presence or absence of 0.03 μM nicotine for 72 h. Whereas preincubation of oocytes with a PI3K inhibitor, PI3K inhibitor significantly reduced basal EAAT3 activity, the activity was not different in oocytes exposed to PI3K inhibitor, nicotine, or PI3K inhibitor plus nicotine. PI3K, phostphatidylinositol 3-kinase. Data are described as mean ± S.E.M. (n = 17 - 22). *$P < 0.05$ compared to control.

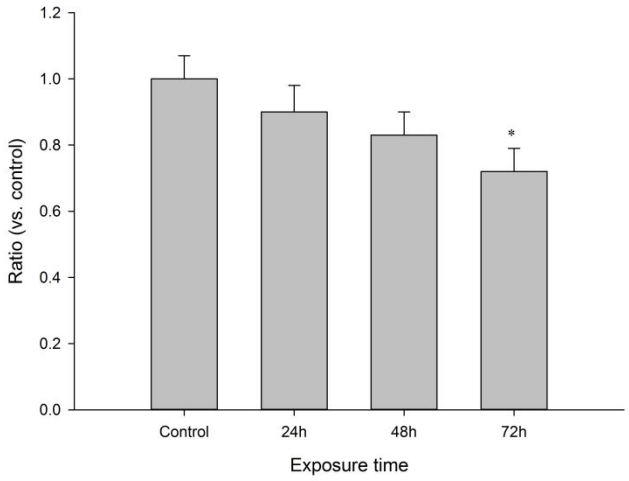

Figure 6. Time course of the effects of nicotine exposure on the activity of EAAT3.

Oocytes were exposed to 0.03 μM nicotine for 24, 48 and 72 h. Incubation in 0.03 μM nicotine for 72 h showed a significantly lower EAAT3 activity than controls. Data are described as mean ± S.E.M. (n = 18 - 23). *$P < 0.05$ compared to control

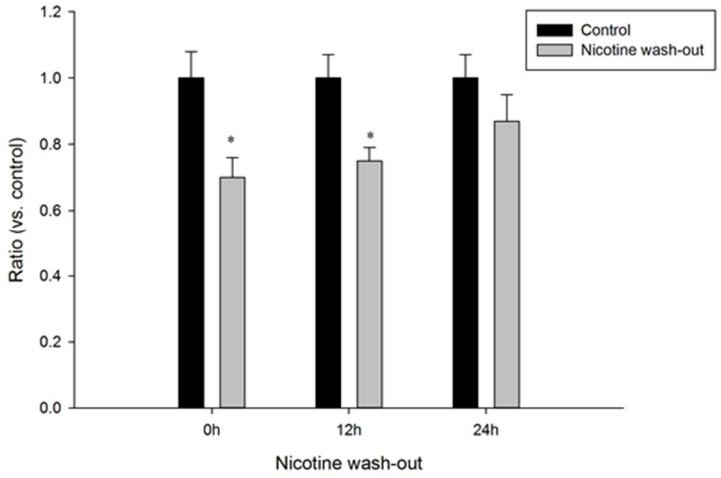

Figure 7. Effects of nicotine wash-out on the activity of EAAT3.

Oocytes were exposed to 0.03 μM nicotine for 72 h and washed out in modified Barth's solution for 12, 24 h. EAAT3 activity returned to the control level at 24 h. Data are described as mean ± S.E.M. (n = 14 - 16). *$P < 0.05$ compared to control

DISCUSSION

Nicotine readily crosses the blood-brain barrier and binds to brain tissue with high affinity (22). The brain/blood ratio for nicotine concentration was 3-4 when nicotine concentrations plateaued in both blood and brain, indicating preferential distribution of nicotine toward brain tissue (23-25). Considering that plasma nicotine concentrations in smokers range from 0.625 nM to 0.31 μM (median value: 0.07μM) (26, 27), the expected concentration in the brain would be 1.875 nM - 1.24 μM (approximate median value: 0.245 μM). To approximate the putative brain concentration of nicotine while smoking, I thus studied nicotine concentrations from 1 nM to 1 μM for evaluating the dose-response influence of nicotine on EAAT3 activity. In addition, I exposed oocytes to nicotine for 72 h to study the chronic effects of nicotine.

In experimental mouse models of seizure, nicotine administration leads to seizure in a dose-dependent manner (28, 29). In this study, 0.03 μM nicotine decreased EAAT3 activity by 25% and the inhibition reached a plateau at concentrations above 0.03 μM. In the previous mouse models of seizures, nicotine administration led to seizures in a dose-dependent manner without saturation, but nicotine was administered intravenously or intraperitoneally, which resulted in higher nicotine levels compared to absorption by smoking (28-30).

A 0.03 μM concentration of maximal effect found in this study is much lower than the median value of the nicotine brain concentration (0.245 μM). A prospective study of smoking as a risk factor for seizure or epilepsy found no

correlation between the risk of seizure and the number of cigarettes smoked daily, but seizure development increased incrementally with pack-years of smoking (relative risk 1.03, 95% confidence interval 1.02–1.05, per pack-year) (4). Thus, the duration of exposure to nicotine could be more important for seizure development than the amount of smoking or blood concentration of nicotine. In my time course study, nicotine attenuated the activity of EAAT3 in a time-dependent manner.

Several underlying mechanisms of nicotine-induced seizure have been proposed, all of which involve initial activation of central nicotinic acetylcholine receptor (nAChR). First, glutamate release and increased glutamatergic synaptic transmission could elicit seizures in animal experimental models. Damaj et al. (31) suggested the involvement of mainly α7 nAchR, L-type calcium channels, N-methyl-D-aspartate (NMDA) receptors, and nitric oxide (NO) formation in nicotine-induced seizures. In addition, nicotine is known to enhance the production of NO through glutamate release and activation of NMDA receptors in the rat hippocampus (32). Second, reduced GABAergic input to CA1 pyramidal cells through the nAchR in the hippocampus may mediate nicotine-induced excitability or seizures (33, 34). Third, oxidative stress resulting from the depletion of glutathione (GSH) may trigger nicotine-induced seizure (35, 36). Oxidative free radicals may play a pivotal role in epileptogenesis (37). Oxidative stress has been shown to be associated with decreased bioavailable NO, and increased production of reactive oxygen species and reactive nitrogen species (38). Interestingly, NO excess or depletion suggested in the first and third

mechanisms could induce seizures because NO acts as a neuromodulator with dual proconvulsive and anticonvulsive effects (39, 40).

EAAT3 was reported to have functions which are related to the suggested mechanisms of nicotine-induced seizure: [1] EAAT3 could block excessive NMDA activity (41); [2] EAAT3 plays a role in direct transport of glutamate which is the precursor for GABA synthesis into GABAergic neurons of the hippocampus (42) and reduced EAAT3 activity may cause seizures as a result of diminished presynaptic GABA release (8); and [3] since the affinity of EAAT3 for cysteine is equivalent to that for glutamate, EAAT3 may mediate uptake of cysteine into neurons (41, 43), which is related to the synthesis of glutathione, a major antioxidant agent (44). Neuronal glutathione deficiency was reported in EAAT3-deficient mice (45).

My results showed that nicotine attenuated the activity of EAAT3. Therefore, dysfunction of EAAT3 may be involved in the mechanism and pathway of nicotine-induced seizure.

Xenopus oocytes used in my study may have cholinergic receptors but these receptors are muscarinic (46). Furthermore, oocytes that were not injected with EAAT3 mRNA and exposed to nicotine were unresponsive to L-glutamate in my study. Most of the CNS effects of nicotine are mediated by nAChR. However, nicotine could have effects on brain independent of nAChR (47).

The activity of EAAT3 can be regulated by cell surface expression (redistribution between plasma membrane and cytosol, trafficking) and/or affinity for glutamate (48, 49). My kinetic study showed decreased V_{max} but no

significant change in K_m of EAAT3 for glutamate. This suggested that nicotine redistributed EAAT3 from the plasma membrane to intracellular pools without changing the affinity for glutamate (49). EAAT3 expressed on cell membrane can be evaluated directly by immunofluorescence analysis or biotinylation method (50, 51).

I investigated the involvement of PKC and PI3K in nicotine effects on EAAT3 activity. *Xenopus* oocyte has its basal activities of PKC and PI3K (52-55). PKC activation was shown to increase the expression of the transporter at the plasma membrane (56). Activation of PKC was demonstrated to stimulate glutamate uptake in brain homogenates (57) and was shown to increase EAAT3 activity, whereas it was reported to reduce EAAT2 function (58). Among various isoforms of PKC, PKCα increased EAAT3 activity by association of EAAT3 with PKCα at the plasma membrane from an intracellular store, thereby enhancing the transporter rate (49, 59, 60). In contrast to PKCα, PKCε induced an increase in EAAT3 activity without changing its cell surface expression in C6 glioma cells (59). However, *Xenopus laevis* oocytes express PKC isoforms α, β1, β2, γ, δ and ζ, but not PKCε (61).

PI3K has also been demonstrated to modulate EAAT3 activity independently by cell trafficking, like PKC (49, 62). However, PKC could function as a downstream signal of PI3K (63-65). This study using wortmannin and LY294002, the PI3K inhibitors, also supported the involvement of PI3K in mediating the effect of nicotine on EAAT3 activity.

In experiments using PKC inhibitors (staurosporine, chelerythrine, and calphostin C) and PI3K inhibitors (wortmannin and LY294002), I could not find additive or synergistic interactions between nicotine and PKC inhibitors or PI3K inhibitors. These results suggest the attenuation of EAAT3 activity through PKC and PI3K. My study demonstrated that downregulation of EAAT3 activity by nicotine is time-dependent and reversible.

In conclusion, I found that nicotine exposure decreased EAAT3 activity presumably by mediation of PKC and PI3K. This study may suggest a novel mechanism for nicotine-induced seizure.

REFERENCES

1. Lavoie FW, Harris TM. Fatal nicotine ingestion. The Journal of emergency medicine. 1991;9(3):133-6.
2. Picciotto MR, Brunzell DH, Caldarone BJ. Effect of nicotine and nicotinic receptors on anxiety and depression. Neuroreport. 2002;13(9):1097-106.
3. Kobau R, DiIorio CA, Price PH, Thurman DJ, Martin LM, Ridings DL, et al. Prevalence of epilepsy and health status of adults with epilepsy in Georgia and Tennessee: Behavioral Risk Factor Surveillance System, 2002. Epilepsy & behavior : E&B. 2004;5(3):358-66.
4. Dworetzky BA, Bromfield EB, Townsend MK, Kang JH. A prospective study of smoking, caffeine, and alcohol as risk factors for seizures or epilepsy in young adult women: data from the Nurses' Health Study II. Epilepsia. 2010;51(2):198-205.
5. Smolinske SC, Spoerke DG, Spiller SK, Wruk KM, Kulig K, Rumack BH. Cigarette and nicotine chewing gum toxicity in children. Human toxicology. 1988;7(1):27-31.
6. Danbolt NC. Glutamate uptake. Progress in neurobiology. 2001;65(1):1-105.
7. Beart PM, O'Shea RD. Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. British journal of pharmacology. 2007;150(1):5-17.
8. Maragakis NJ, Rothstein JD. Glutamate transporters in neurologic disease. Archives of neurology. 2001;58(3):365-70.
9. Sheldon AL, Robinson MB. The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. Neurochemistry international. 2007;51(6-7):333-55.
10. Bergles DE, Diamond JS, Jahr CE. Clearance of glutamate inside the synapse and beyond. Current opinion in neurobiology. 1999;9(3):293-8.
11. Wieser HG. ILAE Commission Report. Mesial temporal lobe epilepsy with hippocampal sclerosis. Epilepsia. 2004;45(6):695-714.
12. Bouilleret V, Ridoux V, Depaulis A, Marescaux C, Nehlig A, Le Gal LSG. Recurrent seizures and hippocampal sclerosis following intrahippocampal kainate injection in adult mice: electroencephalography, histopathology and synaptic reorganization similar to mesial temporal lobe epilepsy. Neuroscience. 1999;89(3):717.
13. Crino PB, Jin H, Shumate MD, Robinson MB, Coulter DA, Brooks-Kayal AR. Increased expression of the neuronal glutamate transporter (EAAT3/EAAC1) in hippocampal and neocortical epilepsy. Epilepsia. 2002;43(3):211-8.
14. Mathern GW, Mendoza D, Lozada A, Pretorius JK, Dehnes Y, Danbolt NC, et al. Hippocampal GABA and glutamate transporter immunoreactivity in patients with temporal lobe epilepsy. Neurology. 1999;52(3):453-72.
15. Floris V, Morocutti C, Ayala GF. Effects of Nicotine on the Cortical, Thalamic and Hippocampal Electrical Activity in Rabbits. Journal of

neuropsychiatry. 1964;5:247-51.
16. Stumpf C GG. Actions of nicotine in the limbic system. Ann NY Acad Sci. 1967;15(142):143-58.
17. Cohen SL, Morley BJ, Snead OC. An EEG analysis of convulsive activity produced by cholinergic agents. Progress in neuro-psychopharmacology. 1981;5(4):383-8.
18. Na HS, Park HP, Kim CS, Do SH, Zuo Z. 17beta-Estradiol attenuates the activity of the glutamate transporter type 3 expressed in Xenopus oocytes. European journal of pharmacology. 2012;676(1-3):20-5.
19. Shin H-J, Ryu J-H, Kim S-T, Zuo Z, Do S-H. Caffeine-induced inhibition of the activity of glutamate transporter type 3 expressed in< i> Xenopus</i> oocytes. Toxicology letters. 2012.
20. Casado M, Bendahan A, Zafra F, Danbolt N, Aragon C, Gimenez C, et al. Phosphorylation and modulation of brain glutamate transporters by protein kinase C. Journal of Biological Chemistry. 1993;268(36):27313-7.
21. Do SH, Kamatchi GL, Washington JM, Zuo Z. Effects of volatile anesthetics on glutamate transporter, excitatory amino acid transporter type 3: the role of protein kinase C. Anesthesiology. 2002;96(6):1492-7.
22. Matta SG, Balfour DJ, Benowitz NL, Boyd RT, Buccafusco JJ, Caggiula AR, et al. Guidelines on nicotine dose selection for in vivo research. Psychopharmacology. 2007;190(3):269-319.
23. Ghosheh OA, Dwoskin LP, Miller DK, Crooks PA. Accumulation of nicotine and its metabolites in rat brain after intermittent or continuous peripheral administration of [2'-(14)C]nicotine. Drug metabolism and disposition: the biological fate of chemicals. 2001;29(5):645-51.
24. Rowell PP, Li M. Dose-response relationship for nicotine-induced up-regulation of rat brain nicotinic receptors. Journal of neurochemistry. 1997;68(5):1982-9.
25. Benowitz NL. Pharmacokinetic considerations in understanding nicotine dependence. Ciba Foundation symposium. 1990;152:186-200; discussion -9.
26. Schneider NG, Olmstead RE, Franzon MA, Lunell E. The nicotine inhaler: clinical pharmacokinetics and comparison with other nicotine treatments. Clinical pharmacokinetics. 2001;40(9):661-84.
27. Taylor RG, Woodman G, Clarke SW. Plasma nicotine concentration and the white blood cell count in smokers. Thorax. 1986;41(5):407-8.
28. Sood N, Sahai AK, Medhi B, Chakrabarti A. Dose-finding study with nicotine as a proconvulsant agent in PTZ-induced seizure model in mice. Journal of biomedical science. 2008;15(6):755-65.
29. Miner LL, Collins AC. Strain comparison of nicotine-induced seizure sensitivity and nicotinic receptors. Pharmacology, biochemistry, and behavior. 1989;33(2):469-75.
30. Shoaib M, Stolerman IP. Plasma nicotine and cotinine levels following intravenous nicotine self-administration in rats. Psychopharmacology (Berl). 1999;143(3):318-21.
31. Damaj MI, Glassco W, Dukat M, Martin BR. Pharmacological characterization of nicotine-induced seizures in mice. The Journal of pharmacology and experimental therapeutics. 1999;291(3):1284-91.

32. Fedele E, Varnier G, Ansaldo MA, Raiteri M. Nicotine administration stimulates the in vivo N-methyl-D-aspartate receptor/nitric oxide/cyclic GMP pathway in rat hippocampus through glutamate release. British journal of pharmacology. 1998;125(5):1042-8.
33. Dobelis P, Hutton S, Lu Y, Collins AC. GABAergic systems modulate nicotinic receptor-mediated seizures in mice. The Journal of pharmacology and experimental therapeutics. 2003;306(3):1159-66.
34. Chiodini FC, Tassonyi E, Hulo S, Bertrand D, Muller D. Modulation of synaptic transmission by nicotine and nicotinic antagonists in hippocampus. Brain research bulletin. 1999;48(6):623-8.
35. Yildiz D, Ercal N, Armstrong DW. Nicotine enantiomers and oxidative stress. Toxicology. 1998;130(2-3):155-65.
36. Yildiz D, Liu YS, Ercal N, Armstrong DW. Comparison of pure nicotine- and smokeless tobacco extract-induced toxicities and oxidative stress. Archives of environmental contamination and toxicology. 1999;37(4):434-9.
37. Aguiar CC, Almeida AB, Araujo PV, de Abreu RN, Chaves EM, do Vale OC, et al. Oxidative stress and epilepsy: literature review. Oxid Med Cell Longev. 2012;2012:795259.
38. Tabima DM, Frizzell S, Gladwin MT. Reactive oxygen and nitrogen species in pulmonary hypertension. Free Radic Biol Med. 2012;52(9):1970-86.
39. Rundfeldt C, Koch R, Richter A, Mevissen M, Gerecke U, Loscher W. Dose-dependent anticonvulsant and proconvulsant effects of nitric oxide synthase inhibitors on seizure threshold in a cortical stimulation model in rats. European journal of pharmacology. 1995;274(1-3):73-81.
40. Banach M, Piskorska B, Czuczwar SJ, Borowicz KK. Nitric oxide, epileptic seizures, and action of antiepileptic drugs. CNS & neurological disorders drug targets. 2011;10(7):808-19.
41. Nieoullon A, Canolle B, Masmejean F, Guillet B, Pisano P, Lortet S. The neuronal excitatory amino acid transporter EAAC1/EAAT3: does it represent a major actor at the brain excitatory synapse? Journal of neurochemistry. 2006;98(4):1007-18.
42. Sepkuty JP, Cohen AS, Eccles C, Rafiq A, Behar K, Ganel R, et al. A neuronal glutamate transporter contributes to neurotransmitter GABA synthesis and epilepsy. J Neurosci. 2002;22(15):6372-9.
43. Shanker G, Allen JW, Mutkus LA, Aschner M. The uptake of cysteine in cultured primary astrocytes and neurons. Brain research. 2001;902(2):156-63.
44. Dringen R. Metabolism and functions of glutathione in brain. Progress in neurobiology. 2000;62(6):649-71.
45. Aoyama K, Suh SW, Hamby AM, Liu J, Chan WY, Chen Y, et al. Neuronal glutathione deficiency and age-dependent neurodegeneration in the EAAC1 deficient mouse. Nature neuroscience. 2006;9(1):119-26.
46. Kusano K, Miledi R, Stinnakre J. Cholinergic and catecholaminergic receptors in the Xenopus oocyte membrane. The Journal of physiology. 1982;328:143-70.
47. Ferrea S, Winterer G. Neuroprotective and neurotoxic effects of nicotine.

Pharmacopsychiatry. 2009;42(6):255-65.
48. Lee G, Huang Y, Washington JM, Briggs NW, Zuo Z. Carbamazepine enhances the activity of glutamate transporter type 3 via phosphatidylinositol 3-kinase. Epilepsy research. 2005;66(1-3):145-53.
49. Davis KE, Straff DJ, Weinstein EA, Bannerman PG, Correale DM, Rothstein JD, et al. Multiple signaling pathways regulate cell surface expression and activity of the excitatory amino acid carrier 1 subtype of Glu transporter in C6 glioma. The Journal of neuroscience : the official journal of the Society for Neuroscience. 1998;18(7):2475-85.
50. Trotti D, Peng J-B, Dunlop J, Hediger MA. Inhibition of the glutamate transporter EAAC1 expressed in Xenopus oocytes by phorbol esters. Brain research. 2001;914(1):196-203.
51. Zhu Y, Fei J, Schwarz W. Expression and transport function of the glutamate transporter EAAC1 in Xenopus oocytes is regulated by syntaxin 1A. Journal of neuroscience research. 2005;79(4):503-8.
52. Chen X, Zhang X, Jia C, Xu J, Gao H, Zhang G, et al. Membrane depolarization increases membrane PtdIns (4, 5) P2 levels through mechanisms involving PKC βII and PI4 kinase. Journal of Biological Chemistry. 2011;286(46):39760-7.
53. Johnson J, Capco DG. Progesterone acts through protein kinase C to remodel the cytoplasm as the amphibian oocyte becomes the fertilization-competent egg. Mechanisms of development. 1997;67(2):215-26.
54. HEHL S, STOYANOV B, OEHRL W, Schonherr R, WETZKER R, HEINEMANN S. Phosphoinositide 3-kinase-γ induces Xenopus oocyte maturation via lipid kinase activity. Biochem J. 2001;360:691-8.
55. López-Hernández E, Santos E. Oncogenic Ras-induced germinal vesicle breakdown is independent of phosphatidylinositol 3-kinase in Xenopus oocytes. FEBS letters. 1999;451(3):284-8.
56. Guillet BA, Velly LJ, Canolle B, Masmejean FM, Nieoullon AL, Pisano P. Differential regulation by protein kinases of activity and cell surface expression of glutamate transporters in neuron-enriched cultures. Neurochemistry international. 2005;46(4):337-46.
57. Casado M, Bendahan A, Zafra F, Danbolt NC, Aragon C, Gimenez C, et al. Phosphorylation and modulation of brain glutamate transporters by protein kinase C. The Journal of biological chemistry. 1993;268(36):27313-7.
58. Gonzalez MI, Robinson MB. Protein kinase C-dependent remodeling of glutamate transporter function. Molecular interventions. 2004;4(1):48-58.
59. Gonzalez MI, Kazanietz MG, Robinson MB. Regulation of the neuronal glutamate transporter excitatory amino acid carrier-1 (EAAC1) by different protein kinase C subtypes. Mol Pharmacol. 2002;62(4):901-10.
60. Gonzalez MI, Bannerman PG, Robinson MB. Phorbol myristate acetate-dependent interaction of protein kinase Calpha and the neuronal glutamate transporter EAAC1. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2003;23(13):5589-93.
61. Johnson J, Capco DG. Progesterone acts through protein kinase C to remodel the cytoplasm as the amphibian oocyte becomes the fertilization-competent egg. Mech Dev. 1997;67(2):215-26.

62. Sims KD, Straff DJ, Robinson MB. Platelet-derived growth factor rapidly increases activity and cell surface expression of the EAAC1 subtype of glutamate transporter through activation of phosphatidylinositol 3-kinase. The Journal of biological chemistry. 2000;275(7):5228-37.
63. Toker A, Cantley LC. Signalling through the lipid products of phosphoinositide-3-OH kinase. Nature. 1997;387(6634):673-6.
64. Ettinger SL, Lauener RW, Duronio V. Protein kinase C delta specifically associates with phosphatidylinositol 3-kinase following cytokine stimulation. The Journal of biological chemistry. 1996;271(24):14514-8.
65. Frey RS, Gao X, Javaid K, Siddiqui SS, Rahman A, Malik AB. Phosphatidylinositol 3-kinase gamma signaling through protein kinase Czeta induces NADPH oxidase-mediated oxidant generation and NF-kappaB activation in endothelial cells. The Journal of biological chemistry. 2006;281(23):16128-38.

국문 초록

서론: 담배의 주요 성분인 니코틴은 동물실험에서 발작을 유발하고, 흡연은 간질, 발작의 행동학적 위험 요인으로 생각된다. 니코틴 유발 발작의 기시부인 해마는 대부분의 글루탐산이 제 3 형 글루탐산염 운반자에 의해서 주로 이루어진다. 측두엽 간질은 제 3형 글루탐산염 운반자 기능이상과 관련이 있다. 또한 protein kinase C (PKC)와 phosphatidylinositol 3-kinase (PI3K)는 제 3 형 글루탐산염 운반자의 기능 저하를 중재하는 것으로 알려졌다. 그러므로 저자는 니코틴이 PKC와 PI3K의 매개에 의해 제 3형 글루탐산염 운반자의 기능이상을 유발할 수 있다고 가정하였다.

방법: 저자는 Xenopus oocyte 를 이용하여 제 3 형 글루탐산염 운반자의 활동에 니코틴이 미치는 영향과 PKC 와 PI3K 의 관여를 조사하였다. 제 3 형 글루탐산염 운반자 mRNA 를 주입하여 Xenopus oocyt 에 제 3 형 글루탐산염 운반자를 발현한 후, two-electrode voltage clamp 방법을 이용하여 글루탐산염 (30 μM)에 의해 유발된 내향성 전류를 측정하였다. 반응은 전류를 정량화하여, microCoulombs (μC)단위로 기록되었다.

결과: 니코틴을 72 시간동안 oocyte 에 노출한 후 니코틴에 의한 제 3 형 글루탐산염 운반자의 활동저하가 용량 의존적으로 관찰되었다. 활동저하는 니코틴 농도가 0.03 μM 에서 최대치를 보였으므로, 이 농도를 이후의 실험에서 사용하였다. 니코틴을 0.03 μM 을 72 시

간동안 노출시켰을 때 글루탐산염에 대한 제 3 형 글루탐산염 운반자의 V_{max} 는 유의하게 감소하였지만 K_m 은 변화가 없었다. 니코틴 노출된 oocyte 에 phorbol-12-myristate-13-acetate (PMA, a PKC activator)를 처치하면, PMA 에 의해 유발된 제 3 형 글루탐산염 운반자 활동증가가 관찰되지 않았다. PKC inhibitor (staurosporine 과 chelerythrine)는 니코틴 노출이 없는 oocyte 의 제 3 형 글루탐산염 운반자 활동을 감소시켰으나 PKC inhibitor, 니코틴, PKC inhibitors + 니코틴 군들간에는 유의한 차이를 보이지 않았다. 비슷하게 PI3K inhibitor (wortmannin 과 LY294002)도 제 3 형 글루탐산염 운반자의 활동을 감소시켰으나, PI3K inhibitor, 니코틴, PI3K inhibitor + 니코틴 군들간에 유의한 차이가 나타나지 않았다.

결론: 결론적으로 니코틴은 제 3 형 글루탐산염 운반자의 활동을 감소시키고, PKC 와 PI3K 에 의해서 중재되는 것으로 보여진다. 이 실험은 니코틴 유발 발작의 하나의 기전을 제공할 수 있다.

주요어: 니코틴, 제 3 형 글루탐산염 운반자, Phosphatidylinositol 3-kinase, Protein kinase C, *Xenopus* oocyte
학 번: 2005-30636