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Effect of tetrandrine on proto-oncogene c-*fos* expression in rat cerebrum

CHE Jian-Tu, ZHANG Jun-Tian, QU Zhi-Wei, WEI Guo
(*Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China*)

KEY WORDS tetrandrine; lindane; proto-oncogene proteins c-*fos*; intracellular fluid; calcium; Northern blotting; immunoblotting; thin layer chromatography

AIM: To detect the effect of tetrandrine (Tet) on c-*fos* gene expression in cerebrum induced by lindane, a neurotoxicant which activates Ca^{2+} channels. **METHODS**: Northern and dot blotting, dual wavelength thin layer chromatography scanner, were used in this study. **RESULTS**: Lindane 30 $mg \cdot kg^{-1}$ given by intragastric gavage (ig) increased the expression of c-*fos* gene to 146 mm^2 in rat cerebrum 1 h after treatment. Tet 1, 2, and 4 $mg \cdot kg^{-1}$ given by ig 30 min prior to lindane reduced c-*fos* gene expression in a concentration-dependent manner. Expressed genes reached only 86, 40, and 39 mm^2, respectively. **CONCLUSION**: Tet inhibited c-*fos* gene expression in rat cerebrum induced by Ca^{2+} agonist — lindane.

Intracellular calcium ($[Ca^{2+}]_i$) serves as the second signal to trigger a series of physiological and pathological events, one of which is alterations in gene expression[1]. Activation of Ca^{2+} channels leads to Ca^{2+} influx, inducing expression of immediate early genes (IEG) including the proto-oncogene c-*fos*[2]. IEG are rapidly and transiently transcribed via mediation of $[Ca^{2+}]_i$ in many cell types in response to a variety of extracellular signals[3]. c-*fos* gene, like other IEG, encode transcription factors and control secondary programs of gene expression[4].

Increases in free intracellular calcium ($[Ca^{2+}]_i$) play a key role in the brain to activate transcriptional events of c-*fos* which reflects neuronal injury[5]. In this situation, c-*fos* gene expression has been considered to be an early marker of neurotoxicity[5].

Tetrandrine (Tet), an alkaloid isolated from Radix *Stephaniae tetrandrae* inhibited the increases in $[Ca^{2+}]_i$ induced by certain Ca^{2+} agonists (such as glutamate and KCl) in fetal rat cerebral cells[6], and substantially attenuated the cortical neuronal injury[7]. These evidences are consistent with the hypothesis that Tet may inhibit c-*fos* gene expression induced by certain neurotoxicants in brain via restricting the increase in $[Ca^{2+}]_i$. To assure the hypothesis, we studied cerebral c-*fos* mRNA under Tet treatment of rats exposed to lindane (benzene hexachloride), a neurotoxicant which activates Ca^{2+} channels, leading to nerve cell injury.

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MATERIALS AND METHODS

Reagents Tet was gifted from Jinhua Pharmaceutical Factory (Jinhua, Zhejiang, China); lindane was from Dagu Chemical Plant (Tianjin, China); c-*fos* cDNA probe from Huamei Bioengineering Corp (Beijing, China); 4-morpholine ethanesulfonic acid (MOPS), diethyl pyrocarbonate (DEPC), formamide, ficoll.400, polyvinylpyrrolidone and bovine serum albumin (BSA) from Sigma (St Louis, USA); prime-alpha-Gene Labeling from Promega Corporation (Madison, USA); [α-^{32}P] dCTP from Yahui Biomedical Engineering Corporation (Beijing, China); nitrocellulose membrane from Hybond-N (Amerxham, UK).

Rats and treatments Wistar rats (♂, weighing 260 ± *s* 10 g) kept under 12-h dark/12-h light schedule, were given food and water *ad lib*.

Lindane was administered by intragastric gavage (ig) at a dose of 30 $mg \cdot kg^{-1}$ (subconvulsant dose) dissolved in olive oil 2 $mL \cdot kg^{-1}$. Tet-treated rats were divided into 3 groups according to the doses (1, 2, and 4 $mg \cdot kg^{-1}$ $n = 5$ for each group). Tet was dissolved in HCl 0.01 $mol \cdot L^{-1}$ and were given by ig 2 mL 30 min prior to lindane. Meanwhile, a group of age-matched, untreated rats served as the control ($n = 5$) and another treated with lindane alone as the model ($n = 5$).

For analysis of mRNA expression, rats were decapitated 1 h after lindane treatment. Cerebrum was immediately frozen in dry ice.

Northern and dot blotting Total RNA was isolated from cerebral tissues using single-step method[8]. Extracted

RNA was stored at −70 ℃ for Northern blotting[9]. Samples containing 20 μg of total RNA were size fractionated by electrophoresis for Northern blotting through 1.2 % agarose 2 % formaldehyde gels stained with ethidium bromide 1 mg·L^{-1}, and transferred overnight to a nitrocellulose membrane by capillary blotting in 20 × SSC (NaCl 3 mol·L^{-1}, sodium citrate 0.3 mol·L^{-1}). Hybridizations with ^{32}P-labeled randomly primed c-*fos* c-DNA probe (1.6 MBq·L^{-1}) were carried out at 42 ℃ overnight in 50 % formamide, 5 × Denhardt's solution (1 × contains 0.2 % Ficoll, 0.2 % polyvinylpyrrollidone, 0.2 % bovine serum albumin) sodium phosphate 20 mmol·L^{-1}, 1 % SDS, and denatured salmon sperm DNA 50 mg·L^{-1}. After several washings, the filters were exposed to a X-ray film with an intensifying screen at −80 ℃.

Wet nitrocellulose membrane was placed in dot-blot apparatus. Total RNA sample (100 μL, 5 μg) was added to each well. Draw liquid through membrane by suction[10]. Consequent processes were the same as the method of Northern blotting after transfer of RNA to nitrocellulose membrane filter[10]. The signals from autoradiography in dot blotting were determined by dual wavelength TLC scanner.

RESULTS

Northern blotting Subconvulsant doses of lindane (30 mg·kg^{-1}) increased the expression of c-*fos* 1 h after treatment. While no expression was seen in control rats. Tet (1, 2, and 4 mg·kg^{-1}) 30 min prior to lindane decreased the proto-oncogene mRNA levels in a concentration-dependent manner (Fig 1A).

Dot blotting Lindane 30 mg·kg^{-1} increased the expression of c-*fos* gene with respect to control rats. Tet (1, 2, and 4 mg·kg^{-1}) 30 min prior to lindane reduced the c-*fos* gene expression in a concentration-dependent manner (Fig 1B, Tab 1).

Tab 1. Autoradiogram of c-*fos* mRNA dot blotting in rat cerebrum, n = 3 samples (each was pooled from 5 rats and assayed in triplicate). $\bar{x} \pm s$.
$^cP<0.01$ *vs* lindane 30 mg·kg^{-1}.

Lindane/mg·kg^{-1}	Tet/mg·kg^{-1}	Area/mm^2
0	0	44±16
30	0	146±14
30	1	86±33
30	2	40±20^c
30	4	39±17^c

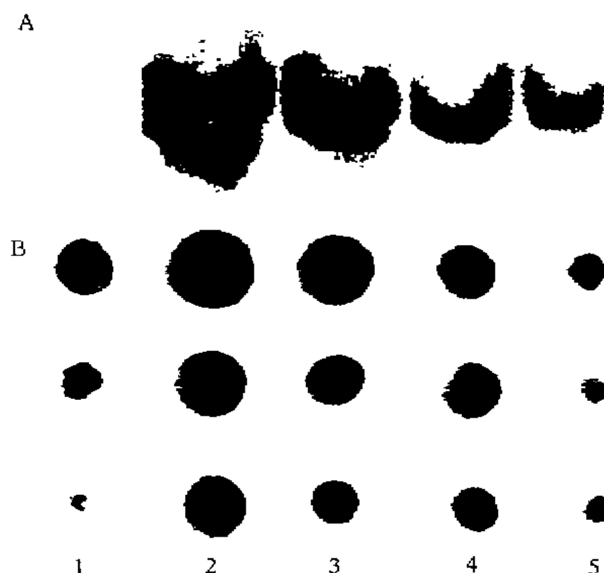

Fig 1. Northern blotting (A) and Dot Blotting (B) for induction of c-*fos* gene expression in rat cerebra.
Lane 1, control; lane 2, lindane 30 mg·kg^{-1};
lane 3, lindane 30 mg·kg^{-1} and Tet 1 mg·kg^{-1};
lane 4; lindane 30 mg·kg^{-1} and Tet 2 mg·kg^{-1};
lane 5; lindane 30 mg·kg^{-1} and Tet 4 mg·kg^{-1}.
The cerebra were collected 1 h after treatment with lindane.

DISCUSSION

Lindane can cause an increased level of $[Ca^{2+}]_i$ in cerebrum in humans and other mammals[5]. Direct measurement of free intrasynaptosomal Ca^{2+} revealed that lindane increases $[Ca^{2+}]_i$ in a dose-dependent manner[11]. Lindane may thus exert its neurotoxic effects partly by affecting calcium homeostasis in neurons[12]. The studies indicated that the increases in $[Ca^{2+}]_i$ stimulated by lindane in the brain result in c-*fos* gene expression which is dependent on Ca^{2+} influx[13]. Intensity of c-*fos* expression reflects $[Ca^{2+}]_i$ level. Studies have also demonstrated that either of two types of Ca^{2+} channels — the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor and the voltage-gated L-type Ca^{2+} channel-on the neuronal membrane were the pathway of c-*fos* gene activation after lindane[14]. Obviously influx of Ca^{2+} through cell membrane channels will stimulate c-*fos* gene expression and been linked to glutamate neurotoxicity.

Our study showed that Tet could inhibit *c-fos* gene expression in rat cerebra induced by lindane. It prevented the induction considerably by lindane in a concentration-dependent manner. Previous studies of Tet showed that the increases in $[Ca^{2+}]_i$ stimulated by glutamate and K^+-depolarization were inhibited significantly by Tet in cerebral cells, but the rises $[Ca^{2+}]_i$ elicited by Bay-K-8644 (the agonist of L-type channel) was not sensitive to Tet[6], and there were protective effects of Tet on cortical cultures injury induced by glutamate and BOAA (excitatory amino acid of non-NMDA) except of NMDA[7]. These investigation are consistent with the hypothesis that there is another Ca^{2+} channel in neuronal membrane, which is different from NMDA and voltage-gated L-type Ca^{2+} channels, and is the pathway of activation of *c-fos* transcription induced by some neurotoxicants like lindane. This channel give the sensitive response to Tet, leading to the inhibition of *c-fos* gene expression. Although it is uncertain whether the inhibition of Ca^{2+} channels in nerve cells by Tet is the pathway by which *c-fos* gene expression was attenuated. The result is true that Tet definitely inhibited an early marker of neurotoxicity — *c-fos* gene expression in rat cerebrum, suggesting that this drug can protect cerebrum from the injury induced by some neurotoxicants like lindane.

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粉防己碱对大鼠大脑 *c-fos* 原癌基因表达的影响

车建途，张均田，屈志炜，卫 国

（中国医学科学院，中国协和医科大学药物研究所
药理二室，北京 100050，中国）

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关键词 粉防已碱；林丹；原癌基因蛋白 *c-fos*；细胞内液；钙；RNA 印迹；免疫印迹；薄层色谱

目的：本研究旨在探讨 Tet 对林丹——种通过细胞内游离钙（$[Ca^{2+}]_i$）介导大脑 *c-fos* 基因表达的神经毒剂—诱导的大鼠大脑 *c-fos* 基因表达的影响. **方法**：Northern 印迹杂交，斑点杂交技术及双波长薄层色谱扫描分析. **结果**：大鼠口服林丹 30 $mg \cdot kg^{-1}$ 1 小时后大脑 *c-fos* 基因表达明显增强至 146 mm^2，而在口服林丹前 30 min 分别口服 Tet 1，2 及 4 $mg \cdot kg^{-1}$，大鼠大脑 *c-fos* 基因表达被明显抑制（分别为 86，40 和 39 mm^2），其抑制程度呈剂量依赖关系. **结论**：Tet 抑制 Ca^{2+} 激动剂类神经毒剂—林丹诱导的大鼠大脑 *c-fos* 基因转录水平的表达.