Increase of β₁-adrenergic receptor gene expression induced by nicotine in hippocampal slice of rat¹

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KEY WORDS nicotine; β-adrenergic receptors; hippocampus; reverse transcriptase polymerase chain reaction; Western blotting; radioligand assay

ABSTRACT

AIM: To investigate the effect of nicotine on β₁-adrenergic receptor (β₁-AR) in the hippocampal slice of rat.

METHODS: Hippocampal slices (400 µm thick) were incubated in artificial cerebrospinal fluid (ACSF) previously saturated with 95 % O₂ and 5 % CO₂ at 28 ºC for 120 min, and then incubated with nicotine 10 µmol/L for 30, 60, 90, and 120 min. mRNA of the β₁-adrenergic receptor was examined with semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), and the protein level was measured by Western blot and RIA. RESULTS: The mRNA gene expression and the protein level of β₁-adrenergic receptor in hippocampal slices were increased after nicotine treatment. The peak of protein occurred later but higher than that of mRNA level. CONCLUSION: Both expression of β₁-adrenergic receptor gene transcription and post-transcriptional protein level in rat hippocampus were altered by nicotine.

INTRODUCTION

The effect of nicotine on learning and memory function has been focused recently, some observation from animal and human being behavior experiments suggested that nicotine could improve animal and human being learning and memory[1-3]. Long-term potentiation (LTP) as the cellular model for learning and memory has been always used to explore the molecular and cellular mechanisms of the cognitive function. LTP involves a number of different neurotransmitter systems, and adrenergic system plays important role in learning and memory[4,5]. Norepinephrine (NE) is one of these major neuromodulators involved in the induction of LTP; activation of β-adrenergic receptor (AR) on synapse enhances LTP strongly[6], and β-AR blocker could block the LTP elicited by the titanic stimulation in the hippocampal slice of rat or induce distribution of learning and memory function in mice[7], which indicated that norepinephrine (NE) and β-adrenergic receptors in the CNS were involved in the learning and memory. It has been confirmed that nicotine can stimulate release of NE[8,9], facilitate or induce LTP in the hippocampal slice of rat[10,11]. LTP induced by nicotine with different parameters of electric stimulation could be blocked with proporanolol, a nonselective β-AR antagonist[7], so adrenergic receptor signaling is important in LTP induced by nicotine[12]. However, the molecular mechanisms responsible for the effects of that nicotine facilitate LTP induction via β₁ adrenergic receptors are unknown, the present ex-

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Received 2002-12-23 Accepted 2003-11-03
periment aimed to investigate whether nicotine influences the gene expression of β1 adrenergic receptors.

MATERIALS AND METHODS

Reagents and animals Nicotine was obtained from Sigma. Antibody for β1-AR was a product of Santa Cruz Biotechnology Inc. Tripure isolation reagent, PVDF membrane and Lumi-Light® Western blotting Substrate Kit were purchased from Rohe. [3H]DHA was from Amersham Pharmacia Biotech Ltd. Sprague-Dawley rats were from the Experimental Animal Center of Sun Yat-sen University (100-120 g, Grade II, Certificate No 26-001 conferred by Medical Animal Management Committee, Guangdong Province). Other chemicals from Sigma were of analytic grade.

Preparation of hippocampal slices Hippocampal slices (400 µm thick) used in the experiment were obtained from Sprague-Dawley rats. Animals were anesthetized with diethyl ether and then the brains were removed rapidly and placed in cold artificial cerebrospinal fluid (ACSF) consisting of (in mmol/L: NaCl 124, KCl 3.4, MgSO4·7H2O 1.7, NaHCO3 25, CaCl2 1.8, glucose 10; pH 7.4) and previously saturated with 95 % O2 and 5 % CO2. The hippocampal slices were transferred in ACSF and incubated at 28 ºC for 120 min, and then nicotine 10 µmol/L was added. After incubation for 30, 60, 90, and 120 min respectively, the samples were determined.

RNA preparation According to the protocol of Tripure™ isolation reagent, hippocampal slice with Tripure isolation reagent was homogenized, and then centrifuged at 12000×g for 10 min at 4 ºC. The supernatant was transferred and incubated for 5 min followed by incubation with chloroform for 2-15 min at room temperature. After isolation of the colorless upper aqueous phase isopropanol was added, and the sample was centrifuged again. The pellet was washed with 75 % ethanol.

Determination of the level of β1-AR mRNA by RT-PCR The level of β1-AR mRNA was detected by RT-PCR based on the method of Miyahara et al[13]. The cDNA was synthesized with MluV reverse transcriptase. Reaction mixture 20 µL containing 0.2-3.2 µg sample RNA, RNasin ribonuclease, oligo d (T)16 primer, dNTP 0.2 mmol/L, and 1 U Tag DNA polymerase. Amplified reaction was performed with a thermocycler for a single 5-min heating step at 95 ºC followed 28 cycles under the conditions: 94 ºC (60 s), 55 ºC (60 s), 72 ºC (60 s); and final extension at 72 ºC for 10 min. The amplification resulted in an expected product of 188 bp. Initially, the number of cycles was titrated for sufficient but still exponential amplification. Aliquot of 8 µL was removed from PCR mixture after different cycles of amplification and were electrophoresed on a 1.5 % agarose gel containing ethidium bromide in TAE 40 mmol/L. Amplified DNA band was scanned and the relative density was quantified. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal standard was simultaneously amplified using 5'-TCA CCA TCT TCC AGG AGC GAG A-3' as a sense primer and 5'-ATG AGG AGC GAG A-3' as an antisense primer. The amplified fragment of GAPDH was 303 bp.

Determination of β1-AR protein level by Western blot analysis Western blot analysis was performed according to the method of Podlowski et al[14] with minor modification. The slices were transferred to a microcentrifuge tube and subsequently homogenized by sonication in 120 µL of a cold PBS buffer consisting of phenanthroline 1 mmol/L, iodoacetamide 1 mmol/L, PMSF 0.4 mmol/L, pepstatin A 1 µmol/L. Protein concentrations were determined by nucleic acid and protein analyzer (BECKMAN DU 640) based on the Bradford method. Samples containing amounts of total protein (20 µg) were boiled for 5 min, separated on 12 % SDS-PAGE gel, transferred on PVDF membranes and blocked for 3 h at room temperature. Then the blots were incubated overnight with primary antibody. A rabbit polyclonal antibody (1:1000 Santa Cruz) was used as a primary antibody. An horseradish peroxidase-conjugated (1:1000) anti-rabbit Ig was used as a secondary antibody. Protein signals were visualized with enhanced chemiluminescence (Donpoint) and quantified with Gel Doc 1000 system (BIO-RAD).

Radioligand binding assay Rat hippocampal crude membrane was prepared according to the method based on Lai et al with modification[15]. Hippocampal slice was homogenized with 20 fold volume buffer con-
taining Tris-HCl 50 mmol/L, MgCl₂ 10 mmol/L, and then centrifuged. The concentration of protein was measured by nucleic acid and protein analyzer with Bradford method. β₁ Adrenergic receptor binding assay was carried out according to Stutz et al. Sample 50 µL was incubated with [³H]DHA at different concentration in a total volume of 250 µL for 30 min at 25 ºC. The incubation buffer consisted of Tris-HCl 50 mmol/L and MgCl₂ 10 mmol/L, pH 7.4. Non-labelled competitor propranolol was added to measure non-specific binding. After incubation, samples were filtered under vacuum over Whatman GF/G filter, and washed with ice-cold buffer. The amount of [³H]DHA was determined by LKB-1214-Rackbeta Counter at 60 % efficiency. Bₘₐₓ and Kᵅ values were calculated by nonlinear regression analysis and normalized to the control.

Statistical assay The data were presented as mean±SD. Semiquantitative data of RT-PCR and Western blot were expressed as the percentage relative to controls in the same experiment. Statistical assay was performed using 2-Way ANOVA of SPSS software. P<0.05 was considered statistical significant.

RESULTS

RT-PCR RNA extracted from sample was scanned on the 1.5 % agarose gel with ethidium bromide staining, no degradation of RNA and contamination of DNA were found. The reverse transcription and amplification of total RNA without digestion isolated from hippocampal slice resulted in a single band of the expected size of 188 bp using β₁-AR specific primer. No amplified products could be revealed in RNA preparation digested with RNase A, suggesting no contamination of DNA in the RNA samples. The amplified products were increased proportionally to the increase in the amount of template RNA and the number of cycles, indicating that the RT-PCR used in this study is sufficiently sensitive and accurate to detect changes in β₁-AR mRNA level. On the basis of similar titration, 28 cycles were adopted for measurements of β₁-AR mRNA and GAPDH mRNA (Fig 1).

Determination of β₁-AR mRNA in the hippocampal slice of rat No change in β₁-AR mRNA was found in the hippocampal slice without nicotine treatment (data not shown), but significant increase in β₁-AR mRNA level was demonstrated after nicotine 10 µmol/L treatment. The mRNA level of β₁-AR was increased significantly to 153 %±20 % of control after nicotine treatment for 60 min, but after 90-min and 120-min treatment, the mRNA level of β₁-AR was 136.8 %±15.6 % and 128.2 %±17.7 % of control, respectively. Prolonged application of nicotine did not have further effects on β₁-AR mRNA expression (Fig 2).

Fig 1. (A) Representative picture of ethidium bromide staining of agarose gel for assessment of DNA-free RNA preparation from the hippocampal slice of rats. Lane 1: RNA samples were digested with RNase A; Lane 2: without reverse transcription before PCR; Lane 3-5: Normal RT-PCR with β₁-AR specific primer, GAPDH specific primer, and bis β₁-AR and GAPDH primers, respectively. Lane M: marker. (B) RT-PCR was performed using 3.0 µg total RNA with 18, 21, 24, 27, 30, and 35 cycles, respectively (Lane 1-6). (C) Lane1-5 with 28 cycles using 0.2, 0.4, 0.8, 1.6, and 3.2 µg total RNA respectively with 28 cycles.
Effect of nicotine on β1-AR protein level in hippocampal slice

No signal was found in lane 1 because of no β1-AR protein in loading sample. However, one band weight of 63 kDa appeared at the other lanes loaded with sample extracted from hippocampus, consistent to the other investigation[9]. This finding indicated that the antibody was specific for β1-AR. β1-AR protein level in hippocampal slice began to increase at 30 min. It was elevated by 54.3 % at 60 min (P<0.05) and reached peak level (by 81.5 %, P<0.01) at 90 min. It dropped at 120 min (67.4 % vs 81.5 %) (Fig 3).

Effect of nicotine on maximum binding capacity of β1-AR

The maximal binding capacity (Bmax) for [3H]DHA binding was greatly increased to 171.4 %±17.3 % of control after treatment with nicotine 10 µmol/L for 60 min and reached to the peak (193.1 %±20.2 % of control) after nicotine treatment for 90 min. But when the period was prolonged to 120 min, Bmax was 155.4 %±11.3 % of control (P<0.01 vs 90 min). The results reinforced the data that β1-AR protein expression was up-regulated in the hippocampal slice after nicotine 10 µmol/L treatment. In contrast, no effect of nicotine on the affinity of β1-AR was investigated (data not shown).
DISCUSSION

Adrenergic system regulates forms of synaptic plasticity involved in memory formation. LTP induced by nicotine is blocked by propranolol, a nonselective antagonist\(^7\), which indicated that β-AR activation was involved in cognitive function improved by nicotine and other nicotinic receptor agonists. In present study, we directly observed that nicotine up-regulated β₁-AR mRNA and protein level.

Both β₁ and β₂ receptors are expressed in hippocampus, but β₁-AR is predominant for cognitive function because it was expressed on neurons, while β₂-AR was expressed in blood vessels, thus we selected β₁-AR in present study. β₁-AR mRNA level and protein level were increased in hippocampal slice after nicotine treatment. The fact that the peak of protein level appeared later than that of mRNA indicated the protein increase was referred to gene transcription. However, mRNA level was unchanged after treatment with nicotine for 30 min while the protein expression was markedly increased, which suggested there was at least a pathway independent to gene transcription. So the change of protein level of β₁-AR induced by nicotine was due to not only the altered β₁-AR gene transcription but also regulation of post-transcription, such as post-transcriptional modification, translation, even degradation of protein. The effect of nicotine on β₁-AR gene post-transcription may be more significant. In this study, it should be noticed that the magnitude of changes in the β₁-AR density measured by radioligand assay is not corresponded with that determined by Western blot analysis, which may be due to the difference in the sensitivity of the two methods. Western blot is specific for β₁-AR but radioligand binding assay is not.

Induction of LTP is mediated by G protein signaling pathway. Activation of β₁-AR is one of the important components to G protein signaling pathway. β₁-AR agonist elicited increase in cAMP level and ionic concentration, which caused phosphorylation of ERK\(^17\)-19. So the increase in β₁-AR mRNA and protein expression will improve cognitive function through G protein signaling pathway.

Interestingly, prolonged treatment with nicotine did not further up-regulated both mRNA and protein expression of β₁-AR. One of the possibility is that acute release of neurotransmitter induced by nicotine will up-regulate β₁-AR mRNA expression and protein synthesis, but lasting neurotransmitter release will inhibit up-regulation of β₁-AR mRNA transcription and protein synthesis\(^20\). Another possibility is that nicotine receptor will desensitize after long-lasting stimulation by nicotine. Fu et al reported that nicotine receptor desensitized at 40 min after consecutive stimulation by high concentration of nicotine\(^21\).

In conclusion, nicotine induced LTP by up-regulating mRNA and protein expression of β₁-AR in hippocampal slice.

REFERENCES


