STEROIDS 71 (2006) 609–617

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Studies on neurosteroids XVIII LC–MS analysis of changes in rat brain and serum testosterone levels induced by immobilization stress and ethanol administration

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ARTICLE INFO

Article history:
Received 14 December 2005
Received in revised form 15 February 2006
Accepted 14 March 2006
Published on line 18 April 2006

Keywords:
Testosterone
5α-Androstane-3α,17β-diol
Liquid chromatography–mass spectrometry
Immobilization stress
Ethanol administration
Rat brain

ABSTRACT

The liquid chromatography–mass spectrometry (LC–MS) methods were developed and validated for the determination of testosterone (*T*) in the brain and serum of rats and of 5α-androstane-3α,17β-diol (ADIOL), a metabolite of *T*, in the brain of rats. After derivatization of *T* with 2-hydrazino-1-methylpyridine and of ADIOL with *p*-nitrobenzoyl chloride, the detection sensitivities of *T* and ADIOL using LC–MS were increased 70- and 400-times superior to those of intact *T* and intact ADIOL, respectively. Those LC–MS methods are specific and reliable for the analysis of trace amounts of *T* and ADIOL in small amounts of samples. The animal studies using the developed methods showed that the brain and serum levels of *T* and the brain levels of ADIOL were not changed by stress or ethanol administration but the concentration ratio of the brain *T* to serum *T* in the stressed rats was higher than that in untreated rats. The low levels of endogenous AIDOL in brain of stressed and unrestrained rats found in this study demonstrated that the contribution to anesthetic and anxiolytic effects of ADIOL via γ-aminobutyric acid type A receptors may be negligible.

1. Introduction

The term "neurosteroids (NSs)" was introduced to name steroids that are synthesized de novo in the central nervous system (CNS). On the contrary, neuroactive steroids (NASs) are commonly defined as steroids that have an effect on the CNS, regardless of their source. These steroids affect neurotransmission through action at membrane ion-gated and other neurotransmitter receptors [1]. For example, 3α-hydroxy-5α-pregn-20-one (allopregnanolone, AP) binds to γ-aminobutyric acid type A ($GABA_A$) receptors with high affinity and positively modulates the GABA action at these receptors [2]. Pharmacological dose of AP elicits anesthetic, sedative and anxiolytic effects. It has been also demonstrated that the endogenous AP in animal brain is rapidly elevated to the nM concentration following an increase of the brain level of its precursor, progesterone (PROG), by several acute stress paradigms (immobilization [3], forced swimming [4,5], electrical shock [6] and CO_2 inhalation [7]).

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doi:10.1016/j.steroids.2006.03.003

Recently, several reports described that the pharmacological doses of testosterone (*T*) and its metabolite, 5α-androstane-3α,17β-diol (ADIOL), elicit the anesthetic and anxiolytic effects in animal models [8–11]. ADIOL is structurally similar to AP (the both are 3α-hydroxy-5α-reduced steroids), so it also has allosteric activity at $GABA_A$ receptors [9–11]. Therefore, it is most probable that the anesthetic and anxiolytic effects of *T* may occur through its conversion to ADIOL, but the mechanisms are not well understood. Based on these findings, we constructed the hypothesis that the rat brain level of ADIOL should be increased by stressing the animal and the brain level of *T* should be either increased as a supply source of ADIOL. Therefore, the analysis of the changing levels of endogenous *T* and ADIOL in brains of the acute stressed animals should be helpful for understanding that *T* and ADIOL could contribute to the homeostasis of neuronal excitation.

The interaction of alcohol and *T* biosynthesis is another hot topic, mainly due to the effect of alcohol on aggression. Alomary et al. demonstrated that acute ethanol administration increases the *T* concentration in the brain [12] and *T* causes aggressive behavior in animals and humans [13]. However, a different study found that administration of ethanol decreases the plasma level of *T* in rats [14]. So, it is necessary to find out whether ethanol affects on the brain and circulating *T* levels.

In the present study, we first developed methods for precise determination of *T* and ADIOL in the brain and serum using liquid chromatography (LC)–mass spectrometry (MS) after derivatizations of *T* with 2-hydrazino-1-methylpyridine (HMP) and of ADIOL with *p*-nitrobenzoyl chloride (NBC). Next, these methods were applied to determination of the *T* and ADIOL levels in immobilization-stressed and ethanol-administered rats.

2. Experimental

2.1. Material and reagents

T and ADIOL were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and Steraloids (Newport, RI, USA), respectively. Stock solutions of *T* and ADIOL were prepared as 100 μg ml^{-1} solutions in EtOH. Subsequent dilutions were carried out with EtOH to prepare 0.5, 1, 2, 5, 10 and 20 ng ml^{-1} solutions. [19,19,19-$^2H_3$]-*T* (D_3-*T*) [15] used as internal standard (IS) for the *T* assay was donated by Teikoku Hormone Medical Research Center (Kawasaki, Japan) and [16,16,17α-$^2H_3$]-ADIOL (D_3-ADIOL) [16] used as IS for the ADIOL assay was synthesized in our laboratories. These ISs were dissolved in and diluted with EtOH. HMP was synthesized in our laboratories as previously reported [17]. NBC was purchased from Tokyo Kasei Kogyo. Strata-X cartridges (60 mg adsorbent; Phenomenex, Torrance, CA, USA) were successively washed with AcOEt (2 ml), MeOH (2 ml) and H_2O (2 ml) prior to use. Bond Elut Si cartridges (500 mg adsorbent; Varian, Harbor, CA) were successively washed with $CHCl_3$–MeOH (30:1, v/v) (2 ml) and $CHCl_3$ (4 ml) prior to use. All other reagents and solvents were of analytical grade.

2.2. LC–MS

In the *T* assay, LC–MS was performed using an Applied Biosystems API 2000 triple stage quadrupole-mass spectrometer (Foster City, CA) connected to a Shimadzu LC-10AT chromatograph (Kyoto, Japan). A J'sphere ODS-H80 column (4 μm, 150 mm × 2.0 mm i.d.; YMC, Kyoto) was used at 40 °C. MeCN–MeOH–10 mM $HCOONH_4$ (10:3:7, v/v/v) was used as the mobile phase at a flow rate of 0.2 ml min^{-1}. *T* and D_3-*T* were analyzed as their HMP derivatives by electrospray ionization (ESI)–MS–MS [selected reaction monitoring (SRM)] in the positive-ion mode and the conditions were as previous study [18], that is, declustering potential: 80 V, focusing potential: 200 V, entrance potential: 10 V, ion spray voltage: 5 kV, curtain gas: 45 psi, ion source gas 1: 80 psi, ion source gas 2: 80 psi, turbo gas temperature: 500 °C and interface heater: on, collision energy: 30 eV, precursor ions: *m/z* 394.1 $[M]^+$ for *T* and *m/z* 397.1 $[M]^+$ for IS, monitoring ions: *m/z* 394.1 for *T* and *m/z* 397.1 for IS.

In the ADIOL assay, LC–MS was performed using a ThermoQuest LCQ ion trap-mass spectrometer (San Jose, CA) connected to a Jasco PU-980 chromatograph (Tokyo). A J'sphere ODS-H80 column (4 μm, 150 mm × 4.6 mm i.d.) was used at 40 °C. MeOH–H_2O (100:1, v/v) was used as the mobile phase at a flow rate of 1.0 ml min^{-1}. ADIOL and D_3-ADIOL were analyzed as their *p*-nitrobenzoates by electron capture atmospheric pressure chemical ionization (ECAPCI)-MS [19] with selected ion monitoring (SIM) mode and the conditions were as follows; vaporizer temperature: 425 °C, heated capillary temperature: 200 °C, sheath gas flow rate: 60 units, capillary voltage: −10 V, tube lens offset: −15 V, monitoring ion: *m/z* 590 $[M]^-$ for ADIOL and *m/z* 593 $[M]^-$ for D_3-ADIOL.

2.3. Derivatization of T with HMP

To a solution of the standard steroid, serum or brain samples in EtOH (30 μl), a freshly prepared solution of HMP (10 μg) in EtOH (50 μL) containing 25 μg of trifluoroacetic acid was added, and the mixture was kept at 60 °C for 1 h. After removal of solvents, the product was dissolved in MeOH–10 mM $HCOONH_4$ (1:1, v/v, 30 μl), 10 μl of which was subjected to LC–MS–MS.

2.4. Derivatization of ADIOL with NBC

The calibration curve samples or brain samples were dried and added with reagent, NBC (100 μg) in benzene (40 μl) and catalyst, quinuclidine (100 μg) in benzene (10 μl). The mixture was kept at 80 °C for 10 min, then, the additional reagent and catalyst (100 μg each) were added and entire mixture was further kept at 80 °C for 30 min. After addition of EtOH (30 μl) to decompose the excess reagent, the solvents were evaporated and the resulted residue was dissolved in EtOH (40 μl), 10 μl of which was subjected to LC–MS.

2.5. Animals

Wistar strain male rats (7-weeks old, 180–190 g) obtained from Japan S.L.C. (Hamamatsu, Japan) were assigned either to an untreated group ($n = 10$), a group subjected to immobilization stress ($n = 10$), a group receiving an intrapertioneal (i.p.)