Synergistic protection of allopregnanolone and phenobarbital against maximal electroshock seizures in mice\textsuperscript{1}

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KEY WORDS steroids; allopregnanolone; phenobarbital; drug synergism; convulsions; GABA\textsubscript{A} receptors

ABSTRACT

AIM: To examine the interactions of allopregnanolone and phenobarbital for the protection against seizures.

METHODS: The protective activity of allopregnanolone and/or phenobarbital against seizures was studied in the C57 mice, using the maximal electroshock seizure (MES) test. The modulations by allopregnanolone and/or phenobarbital at the GABA\textsubscript{A} receptor were also characterized using the \textsuperscript{3}H-flunitrazepam binding in the membrane preparation of mouse cerebral cortex.

RESULTS: Pretreatment with phenobarbital produced a dose-dependent protective effect against seizures. The ED\textsubscript{50} value of phenobarbital was 2.61 (95\% confidence limits: 1.59 - 4.26) mg·kg\textsuperscript{-1}. Likewise, the ED\textsubscript{50} value of allopregnanolone was 0.11 (0.06 - 0.18) mg·kg\textsuperscript{-1}. The combination of allopregnanolone and phenobarbital (1:20) resulted in an ED\textsubscript{50} value of 0.73 (0.44 - 1.21) mg·kg\textsuperscript{-1} with the Q value smaller than 1. In measuring the enhancement of \textsuperscript{3}H-flunitrazepam binding, we found that the pattern for the concentration-effect curves of phenobarbital with or without allopregnanolone was consistent with that of the theoretical curves of functional synergism.

CONCLUSION: There was a synergism between allopregnanolone and phenobarbital for the protective activity against seizures. Also there was a functional synergism between these two agents for the enhancement of \textsuperscript{3}H-flunitrazepam binding to the GABA\textsubscript{A} receptor complex in the brain.

\textsuperscript{1} Project supported by the Science and Technology Foundation of Shanghai Education Committee, No 99JC05049.
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INTRODUCTION

Steroids are recognized for their genomic action as transcriptional regulators. Recently, they have been characterized in a nongenomic role of direct interaction with membrane-bound receptors\textsuperscript{1}. Several endogenous neurosteroids (steroids synthesized from cholesterol in the central nervous system) directly modulate GABA\textsubscript{A} receptor function\textsuperscript{2}. One of these neurosteroids allopregnanolone, is the most efficacious endogenous compound to enhance GABA\textsubscript{A} receptor function \textit{in vitro}\textsuperscript{3}.

The primary mechanism of action of barbiturates as an anticonvulsant agent is to enhance the inhibition through the GABA\textsubscript{A} receptor complex\textsuperscript{4}. Allopregnanolone also interacts with this GABA\textsubscript{A} receptor complex, but on a recognition site distinct from the barbiturate recognition site\textsuperscript{5}. So we examined previously if allopregnanolone shared with barbiturates the protective activity against seizures in maximal electroshock seizure (MES) test, and compared it to that of phenobarbital.

We noticed a possible synergistic interaction between these two agents\textsuperscript{6}. Therefore, in this study, we further investigated the interaction of allopregnanolone and phenobarbital in protection against seizures, and explored a similar interaction for modulating the GABA\textsubscript{A} receptor complex as a potential mechanism of this functional synergism.

MATERIALS AND METHODS

Animals Male C57 mice (Clean grade, Certificate No 02 - 229, 18 - 22 g) were obtained from Experimental Animal Resource, Medical Center of Fudan University. Animals were allowed to acclimatize with free access to food and water for a 48-h period before testing.

Drugs Allopregnanolone was purchased from Sigma Chemical Co (St Louis, MO, USA). Stock solution of allopregnanolone was prepared in Me\textsubscript{2}SO (SP Jinshan Chemical Plant) and further dilution was made using 0.9% saline. Phenobarbital was obtained from Shang-
hai Xinya Pharmaceutical Factory. All drug solutions were administered ip in a volume equal to 1 % of the animal’s body weight.

**MES test** Evaluations were carried out in a quiet, temperature controlled room (23 °C). Animals were fasted overnight and brought in the room 4 h prior to the experiment. Mice were injected ip with various doses of phenobarbital (0.5 – 50 mg · kg⁻¹), allopregnanolone (0.01 – 1.0 mg · kg⁻¹), and a combination (1:20) of allopregnanolone and phenobarbital (0.005 : 0.1 – 0.5 : 10) mg · kg⁻¹ 15 min before the MES test. Multifunctional Physiology Stimulator (Jialong Medical Instrument Co., Ltd) was applied for electrical stimulation (wave duration: 0.3 ms). Stimulating electrodes were placed on the mouse cornea. Normal saline was used to wet the electrode to guarantee the contact. Animals were observed right after the stimulation. Tonic extension was taken as the positive response. Stimulating voltage was adjusted to make all the mice have a positive response before treatment.

**[³H]Flunitrazepam binding** Instead of the traditional P2 membrane preparation, a mitochondrial and microsomal (P2 + P3) fraction was prepared as described, since the latter has a higher specific binding percentage. Briefly, mouse cerebral cortices were homogenized and centrifuged at 100 × g for 10 min. The supernatant was centrifuged at 140 000 × g for 30 min to obtain the mitochondrial and microsomal fraction. This fraction was washed three times by homogenization and centrifugation, and then was frozen. On the day of assay, the tissue was thawed, centrifuged as above, and washed two more times. Aliquots of membrane suspension were incubated with 1 nmol · L⁻¹ of [³H]flunitrazepam for 30 min. [³H]Flunitrazepam binding was measured by a filtration assay. Nonspecific binding was determined in the presence of 10 μmol · L⁻¹ diazepam.

All the binding assays were performed in triplicate.

**Data analysis** To construct the dose-effect curves, phenobarbital, allopregnanolone, and their combination were tested at 5 doses spanning the doses producing 50 % protection in the MES test. Twenty mice were tested at each dose. ED values and their 95 % confidence limits were determined by log-probit analysis using Bliss method. The computer program of Q-index test was used for the analysis of the combination study to determine the type of synergistic effect and the 95 % confidence limits of the experimental data. EC₂₀ values and E₅₀ values were the averages from three separate experiments in triplicate. Data were expressed as \( \bar{x} \pm s \).

**RESULTS**

Modulatory effects of allopregnanolone on the protective activity of phenobarbital in the MES test In the MES test, we found that pretreatment with either phenobarbital or allopregnanolone produced a dose-dependent protective effect against the seizures (Tab 1). The ED₃₀ value of phenobarbital in the MES test was 2.61 (95 % confidence limits: 1.59 – 4.26) mg · kg⁻¹. Likewise, allopregnanolone also produced a protective effect in a dose-dependent manner, with ED₃₀ of 0.11 (0.06 – 0.18) mg · kg⁻¹ (Tab 1). A ratio of 1:20 (approximately equal to the ED₃₀ ratio of the two agents) was applied for the combination study. The combination of allopregnanolone and phenobarbital resulted in an ED₃₀ value of 0.73 (0.44 – 1.21) mg · kg⁻¹. As the Q-index test revealed, the Q value was less than 1, indicating a significant synergism between the two agents (Fig 1).

**Effect of allopregnanolone and phenobarbital on the enhancement of [³H]flunitrazepam binding** Phenobarbital enhanced [³H]flunitrazepam binding in a concentration-dependent manner, with the EC₂₀ value

<table>
<thead>
<tr>
<th>Agent</th>
<th>ED₃₀/mg · kg⁻¹</th>
<th>ED₃₀/mg · kg⁻¹</th>
<th>ED₃₀/mg · kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital</td>
<td>0.45</td>
<td>2.61</td>
<td>15.25</td>
</tr>
<tr>
<td></td>
<td>0.19 – 1.02</td>
<td>1.59 – 4.26</td>
<td>7.25 – 32.08</td>
</tr>
<tr>
<td>Allopregnanolone</td>
<td>0.02</td>
<td>0.11</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>0.01 – 0.04</td>
<td>0.06 – 0.18</td>
<td>0.33 – 1.75</td>
</tr>
<tr>
<td>Allopregnanolone +</td>
<td>0.12</td>
<td>0.73</td>
<td>4.29</td>
</tr>
<tr>
<td>Phenobarbital (1:20)</td>
<td>0.05 – 0.30</td>
<td>0.44 – 1.21</td>
<td>2.11 – 8.72</td>
</tr>
</tbody>
</table>

ED values and their 95 % confidence limits (CL) were determined by log-probit analysis using Bliss method.

Tab 1. Comparison of the protective effects of phenobarbital and/or allopregnanolone against seizures in the MES test.
DISCUSSION

Several recent investigations have shown that the neurosteroid allopregnanolone allosterically modulate GABAergic transmission through a unique binding site on the GABA_A receptor Cl⁻ channel complex. This binding site has been found to be distinctly different from that of the benzodiazepine or the barbiturate site. The 3α-hydroxyl ring A-reduced metabolite of progesterone, allopregnanolone, has been observed to produce a potent enhancement of GABA_A receptor responses in vitro. Because phenobarbital is known to have protective activity against seizures by binding to the GABA_A receptor complex, and allopregnanolone is a potent modulator of the same receptor complex, we speculated a possible interaction between these two.

The protective effect of phenobarbital against the seizures is believed to be due to its modulation of the GABAergic transmission. Indeed, activation of the GABA_A receptor is the molecular event that underlies the treatment of this neurological disorder. Therefore, on the basis of the interaction of allopregnanolone and phenobarbital in vivo, we further explored the allopregnanolone potentiation in GABA_A receptor function as assessed by the measurement of [3H]flunitrazepam binding to mouse cerebral cortical membrane in vitro. We noticed, for the first time, that allopregnanolone had a synergistic relationship with phenobarbital in flunitrazepam binding as well as in MES test. Considering the relation of this binding with the functioning of the GABA_A receptor complex, the GABAergic transmission may be getting modulated in the same manner.

At this point, we think that this in vitro functional synergism, as revealed by the elevated binding at the GABA_A receptor complex, may contribute to the synergism between allopregnanolone and phenobarbital as anti-convulsant agents in vivo.

Although the exogenously applied allopregnanolone had a synergistic effect with phenobarbital in protection against the seizures, we are still not clear about the physiological significance of the endogenous allopregnanolone in the brain. There are many neurosteroids, acting as either positive or negative modulators of GABA_A receptor complex. For example, the negative modulator pregnnanolone sulfate can elicit seizures, which can be protected by the positive modulator allopregnanolone. Therefore, the potential changes in the neurosteroidal levels under pathophysiological conditions may be involved.
ACKNOWLEDGEMENT To Prof XU Duan-Zheng for his assistance in statistical analysis.

REFERENCES