# Involvement of the Arachidonic Acid Cascade in the Hypersusceptibility to Pentylenetetrazole-Induced Seizure during Diazepam Withdrawal

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**The present study was designed to clarify whether the arachidonic acid cascade contributes to the decreased threshold for pentylenetetrazole-induced seizure under benzodiazepine withdrawal in mice. The seizure threshold for pentylenetetrazole was significantly decreased by the discontinuation of chronic treatment with diazepam. The decrease in the seizure threshold for pentylenetetrazole during diazepam withdrawal was significantly suppressed by intracerebroventricular (i.c.v.) pretreatment with the phospholipase $A_2$ inhibitor quinacrine (30, 100 nmol) and the lipoxygenase inhibitor nordihydroguaiaretic acid (10, 30 nmol). In contrast, the decreased seizure threshold in the diazepam-withdrawal group was intensified by pretreatment with the cyclooxygenase inhibitor diclofenac (56 nmol). These compounds did not alter the threshold for seizure in a control group. These findings suggest that enhancement of the arachidonic acid cascade may contribute to the hypersusceptibility to pentylenetetrazole-induced seizure during diazepam withdrawal.**

**Key words** diazepam withdrawal; arachidonic acid; glutamatergic pathway; quinacrine; nordihydroguaiaretic acid; diclofenac

Benzodiazepines have been used extensively as hypnotic, antiseizure and anxiolytic agents. However, the long-term use of benzodiazepines is known to induce several undesirable side effects such as tolerance and physical dependence accompanied by the expression of withdrawal signs in many patients.[1–3] In experimental animals, withdrawal signs include spontaneous seizure, increased muscle tone and a decreased seizure threshold for convulsants.[1,4,5]

Steppuhn and Turski reported that the expression of diazepam withdrawal signs is potently suppressed by treatment with ionotropic glutamate receptor antagonists.[6] Our previous study also showed that several *N*-methyl-D-aspartate (NMDA) receptor antagonists and group 1 metabotropic glutamate receptor (mGluR) antagonist suppress the expression of withdrawal signs after chronic treatment with diazepam.[4,5] Furthermore, we also demonstrated that NMDA receptor subunit (NR1 and NR2B) proteins as well as NMDA receptor in the cerebral cortex were increased in diazepam-withdrawn rats.[7,8] These findings suggest that a glutamatergic pathway may play a significant role in the expression of diazepam withdrawal signs.

It has been reported that arachidonic acid activates NMDA receptor,[9] inhibits glutamate uptake into neuronal and glial cell preparations[10–13] and induces the release of glutamate from presynaptic nerve terminals.[14–17] These findings led to the hypothesis that the arachidonic acid cascade accompanied by an increase in NMDA receptor tone is involved in the expression of diazepam withdrawal signs. Therefore, to clarify the role of the arachidonic acid cascade in the expression of benzodiazepine withdrawal signs, we examined the effects of the phospholipase $A_2$ ($PLA_2$) inhibitor quinacrine, the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) and the cyclooxygenase (COX) inhibitor diclofenac on the hypersusceptibility to pentylenetetrazole-induced seizure during diazepam withdrawal in mice.

## MATERIALS AND METHODS

**Animals** Male ddY mice (20–22 g) were obtained from Tokyo Animal Laboratories (Tokyo, Japan). The animals were housed at a temperature of 22±1°C with a 12 h light–dark cycle (light on 8:30 a.m. to 8:30 p.m.). Food and water were available *ad libitum*. The present study was conducted in accordance with the Declaration of Helsinki and with the Guiding Principles for the Care and Use of Laboratory Animals, adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**Chronic Diazepam Treatment** Mice were treated with diazepam (16 mg/kg, intraperitoneally (i.p.)) or vehicle (9% Tween 80/saline) once a day for 7 d. The seizure threshold for pentylenetetrazole was evaluated 48 h after the last injection of diazepam or vehicle.

**Testing the Seizure Threshold for Pentylenetetrazole** The threshold for pentylenetetrazole-induced seizure was determined as described previously (Tsuda *et al.*, 1997). Mice were placed in a Perspex cylinder (10×10×10 cm; w×l×h) and infused with pentylenetetrazole *via* the tail vein. The threshold for seizure was determined as the time to the first clonic convulsion lasting more than 1 s. Infusions were not given for more than 240 s. The rate of infusion was 0.23 mL/min for pentylenetetrazole, and the pentylenetetrazole concentration was adjusted to 5 mg/mL. Mice were injected intracerebroventricularly (i.c.v.) with quinacrine (10–100 nmol) or NDGA (3–30 nmol) 30 min before, and diclofenac (30, 56 nmol) 60 min before pentylenetetrazole infusion. The i.c.v. injections were performed as described previously.[18] Briefly, one day before diazepam or vehicle treatment, the mice were anesthetized with ether and a 2-mm double-needle (tip: 27 gauge×2 mm and base: 22G×10 mm, Natsume Seisakusyo, Tokyo, Japan) attached to a 25-$\mu$L Hamilton microsyringe was inserted into the unilateral injection site; as a result, a simple hole for the injection was made in the skull. The drugs were injected through the hole with the mice unanesthetized, and the

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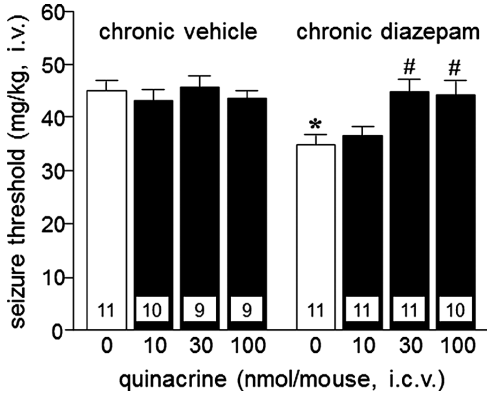


Fig. 1. Effect of Quinacrine on the Pentylenetetrazole-Induced Seizure Threshold in Chronically Vehicle- or Diazepam-Treated Mice

Ordinate: seizure threshold for pentylenetetrazole (mg/kg, i.v.). Each column represents the mean with S.E.M. The numbers inside each column represent the group sizes. Mice were injected with quinacrine (10–100 nmol, i.c.v.) 30 min before pentylenetetrazole i.v. infusion. One-way ANOVA: $F(7,81)=3.97$, $p<0.01$. $^{*}p<0.05$ *vs.* pretreatment with saline in the chronically vehicle-treated group (Tukey–Kramer test). $^{\#}p<0.05$ *vs.* pretreatment with saline in the chronically diazepam-treated group (Tukey–Kramer test).

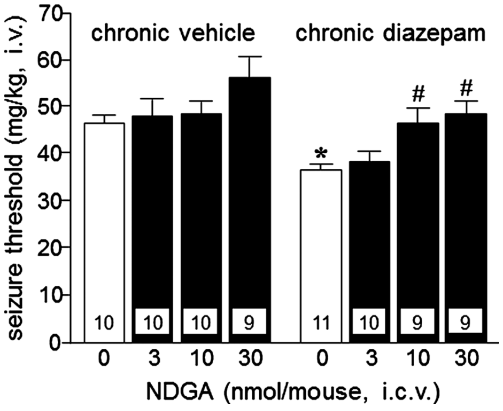


Fig. 2. Effect of NDGA on the Pentylenetetrazole-Induced Seizure Threshold in Chronically Vehicle- or Diazepam-Treated Mice

Ordinate: seizure threshold for pentylenetetrazole (mg/kg, i.v.). Each column represents the mean with S.E.M. The numbers inside each column represent the group sizes. Mice were injected with NDGA (3–30 nmol, i.c.v.) 30 min before pentylenetetrazole i.v. infusion. One-way ANOVA: $F(7,77)=4.45$, $p<0.01$. $^{*}p<0.05$ *vs.* pretreatment with vehicle in the chronically vehicle-treated group (Tukey–Kramer test). $\#p<0.05$ *vs.* pretreatment with vehicle in the chronically diazepam-treated group (Tukey–Kramer test).

volume for i.c.v. injection was 5 $\mu$L.

**Drugs** Diazepam (Profarma Co., Italy) was suspended in vehicle consisting of 9% Tween 80 (Kishida Chemical Co., Osaka, Japan) in saline. Pentylenetetrazole (Sigma Chemical Co., St. Louis, U.S.A.), quinacrin dihydrochloride and diclofenac sodium (Sigma Chemical Co.) were dissolved in saline. NDGA (Cayman Chemical Co., U.S.A.) was dissolved 30% dimethyl sulfoxide (Kanto Chemical Co., Tokyo, Japan).

**Statistical Analysis** Data are presented as the mean with standard error of the mean (S.E.M.). All data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey–Kramer test. Differences were considered to be significant when $p$ values were less than 0.05.

## RESULTS

**Effects of Quinacrine on the Seizure Threshold for Pentylenetetrazole** Withdrawal from chronic treatment with diazepam elicited an increase in the susceptibility to seizure, with a significant decrease in the seizure threshold ($p<0.05$) (Fig. 1). In the chronically vehicle-treated group, the seizure threshold for pentylenetetrazole was not affected by pretreatment with quinacrine. In the chronically diazepam-treated group, the decreased seizure threshold for pentylenetetrazole in the diazepam-withdrawal group was significantly recovered by pretreatment with 30 ($p<0.05$) and 100 nmol ($p<0.05$) of quinacrine.

**Effects of NDGA on the Seizure Threshold for Pentylenetetrazole** The seizure threshold for pentylenetetrazole was significantly decreased by withdrawal from chronic treatment with diazepam ($p<0.05$) (Fig. 2). In the chronically vehicle-treated group, the seizure threshold for pentylenetetrazole was not affected by pretreatment with NDGA. In the chronically diazepam-treated group, the decreased seizure threshold for pentylenetetrazole in the diazepam-withdrawal group was significantly recovered by pretreatment with 10 ($p<0.05$) and 30 nmol ($p<0.05$) of NDGA.

**Effects of Diclofenac on the Seizure Threshold for Pentylenetetrazole** The seizure threshold for pentylenetetrazole

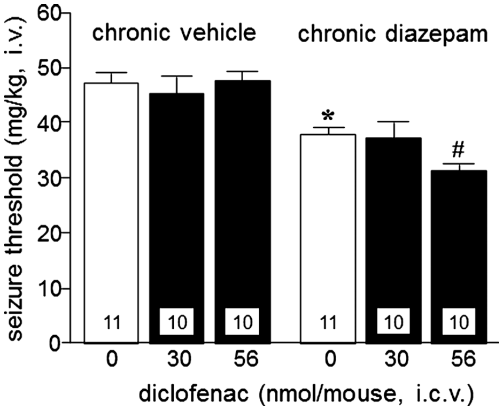


Fig. 3. Effect of Diclofenac on the Pentylenetetrazole-Induced Seizure Threshold in Chronically Vehicle- or Diazepam-Treated Mice

Ordinate: seizure threshold for pentylenetetrazole (mg/kg, i.v.). Each column represents the mean with S.E.M. The numbers inside each column represent the group sizes. Mice were injected with diclofenac (30, 56 nmol, i.c.v.) 60 min before pentylenetetrazole i.v. infusion. One-way ANOVA: $F(5,61)=9.60$, $p<0.01$. $^{*}p<0.05$ *vs.* pretreatment with saline in the chronically vehicle-treated group (Tukey–Kramer test). $\#p<0.05$ *vs.* pretreatment with saline in the chronically diazepam-treated group (Tukey–Kramer test).

was significantly decreased by withdrawal from chronic treatment with diazepam ($p<0.05$) (Fig. 3). In the chronically vehicle-treated group, the seizure threshold for pentylenetetrazole was not affected by pretreatment with diclofenac. In the chronically diazepam-treated group, the decreased seizure threshold for pentylenetetrazole in the diazepam-withdrawal group was further intensified by pretreatment with 56 nmol ($p<0.05$) of diclofenac.

## DISCUSSION

Consistent with previous results, the seizure threshold for pentylenetetrazole was significantly decreased when chronic treatment with diazepam was discontinued, which reflects hyperexcitability in response to physical dependence on diazepam. In the present study, we first demonstrated that the

$PLA_2$ inhibitor quinacrine and lipoxygenase inhibitor NDGA, but not COX inhibitor diclofenac, suppressed the increase in seizure susceptibility to pentylenetetrazole in diazepam-withdrawn mice. These suppressions by quinacrine and NDGA were observed at doses that did not affect the seizure threshold in control mice. Arachidonic acid is a cellular signaling mediator and is catalyzed by two major groups of enzymes, including lipoxygenases and COX. Therefore, it is likely that activation of $PLA_2$ and lipoxygenase, but not COX, contributed to the hypersusceptibility to pentylenetetrazole-induced seizure during diazepam withdrawal.

Many reports have indicated that arachidonic acid enhances a glutamatergic pathway. Arachidonic acid increases the release of glutamate in synaptosomes,[14–17] inhibits glutamate uptake into neuronal and glial cell preparations,[10–13,19] and potentiates NMDA receptor activity.[9] Moreover, 12-lipoxygenase metabolites of arachidonic acid also increase the release of glutamate from synaptosomes.[15] Thus, it is hypothesized that the increase in arachidonic acid and its 12-lipoxygenase metabolites during diazepam withdrawal in the brain may lead to an increase in the release of glutamate in the synaptic cleft and to the potentiation of NMDA receptor activity, which may induce hypersusceptibility to pentylenetetrazole-induced seizure. Since NDGA is a nonselective lipoxygenase inhibitor, the metabolites from other types of lipoxygenase, such as 5-lipoxygenase metabolites, leukotrienes, may also be involved in the hypersusceptibility to pentylenetetrazole-induced seizure during diazepam withdrawal.

The COX inhibitor diclofenac intensified the increase in the susceptibility to seizure with pentylenetetrazole in diazepam-withdrawn mice. While this result surprised us a bit, previous research has shown that pharmacological inhibition[20] or genetic deletion[21,22] of COX-2, but not COX-1, increases the susceptibility to glutamate-related excitotoxicity or seizure. The inhibition of COX by diclofenac may increase arachidonic acid and/or lipoxygenase metabolites, which may contribute to the hypersusceptibility to pentylenetetrazole-induced seizure. On the other hand, previous reports have shown that i.c.v. administration of COX-derived metabolites such as prostaglandin (PG) $D_2$,[23–25] $E_2$ and $F_{2\alpha}$[23] blocks seizure induced by pentylenetetrazole. Therefore, PGs may serve as an endogenous protective element against seizure. These findings may explain why diclofenac exacerbates the hypersusceptibility to pentylenetetrazole-induced seizure during diazepam withdrawal, which is mediated by the inhibition of COX-2.

In conclusion, the present study demonstrates that quinacrine and NDGA recovered the decreased seizure threshold for pentylenetetrazole induced by diazepam withdrawal. Although further investigation will be required to elucidate the precise biochemical alterations associated with chronic treatment with diazepam, our present results indicate that the increase in arachidonic acid and/or its metabolites followed by the activation of lipoxygenase may contribute to the hypersusceptibility to pentylenetetrazole-induced seizure during diazepam withdrawal. Thus, $PLA_2$ or lipoxygenase inhibitors may have therapeutic potential as palliative agents for treating signs of benzodiazepine withdrawal.

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