**In Vivo** Study on Mechanism Underlying Increased Pharmacological Effects of Phenobarbital in Rats with Glycerol-Induced Acute Renal Failure

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The mechanism underlying the increased pharmacological effects of phenobarbital in rats with glycerol-induced acute renal failure (ARF) was examined. In the experiments, a surgical cannula was inserted in the lateral ventricle of the rats for phenobarbital infusion, and the ARF induction was performed by intramuscular administration of 50% glycerol. The onset time of anesthesia by phenobarbital was determined with the tail flick method. In addition, cerebral microsomes were prepared from excised cerebral cortices of sham and ARF rats, and the cerebral expression of the γ-aminobutyric acid (GABA)\(_\text{A}\) receptor and two cation-chloride transporters, KCC2 and NKCC1, was evaluated by Western blotting, as their functions are involved in the anesthetic effects of phenobarbital. When phenobarbital was infused in the ventricle, anesthesia was induced 2.2-times faster in ARF rats than in sham rats, and there was no detectable increase in the cerebral expression of the GABA\(_\text{A}\) receptor in ARF rats. It was additionally noted that the cerebral expression of KCC2 decreased, whereas that of NKCC1 was unaltered in ARF rats. These findings indicated that the anesthetic effects of phenobarbital are potentiated in ARF rats, probably due to imbalanced cerebral expression of KCC2 and NKCC1, suggesting that altered cation-chloride handling in nerve cells is associated.

**Key words** acute renal failure; anesthesia; KCC2; NKCC1; phenobarbital

**INTRODUCTION**

It was previously demonstrated that anesthetization by barbiturates is more readily induced in rats with decreased renal function.\(^1\)–\(^3\) A shorter onset time to loss of the righting reflex by phenobarbital in rats with acute renal failure (ARF) is one example.\(^5\) As the cerebral concentration of phenobarbital measured at the onset time of anesthesia was found to be lower in ARF rats than in sham rats,\(^1\) an altered susceptibility of the brain tissues was suggested to be involved in the ARF-related increase of the pharmacological effects of phenobarbital. Regarding the mechanism underlying this altered susceptibility, functional alteration of the γ-aminobutyric acid (GABA)\(_\text{A}\) receptor and/or cation-chloride handling in nerve cells may be considered because it is known that phenobarbital induces anesthesia by binding to the GABA\(_\text{A}\) receptor to influence the chloride concentration in the nerve cells.\(^5\) However, the precise mechanism remains to be clarified.

To conduct efficient pharmacotherapy with dosage optimization, the mechanism involved in the increased pharmacological effects and the factors responsible for the altered susceptibility are important. However, it is difficult to properly assess the ARF-related increase of the pharmacological effects because the pharmacokinetics of therapeutic compounds change when renal function is impaired due to altered hepatic drug handling and drug-metabolizing activities.\(^5\)–\(^7\) As a result, the blood concentration profile of the compound changes with the impairments, and its concentration in the brain tissues varies from that measured under normal conditions. This makes it difficult to assess whether the susceptibility of the brain tissues to phenobarbital increases in an intrinsic manner.

To overcome this interference by minimizing the ARF-related alteration of drug disposition in the brain tissues, we utilized a cannula insertion approach, in which the compound was immediately and continuously infused into the lateral ventricle via a surgically inserted cannula.\(^8\)\(^,\)\(^9\) With this approach, we examined phenobarbital-induced anesthetization in ARF rats, and assessed whether the cerebral susceptibility to phenobarbital differed by comparing the phenobarbital amounts required to induce anesthesia between sham and ARF rats. We also evaluated the effects of ARF on the cerebral expression of the GABA\(_\text{A}\) receptor and the cation-chloride transporters KCC2 and NKCC1 to elucidate the mechanism underlying the increased pharmacological effects following renal impairment.

**MATERIALS AND METHODS**

**Materials** Phenobarbital and ethyl carbamate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Butemotanide was from MP Biomedicals (Santa Ana, CA, U.S.A.). The purified monoclonal antibody against mouse β\(_2\)/β\(_3\) subunits of the GABA\(_\text{A}\) receptor (catalogue No. MAB241) was purchased from Millipore (Billerica, MA, U.S.A.). According to the product information, the monoclonal antibody exhibited cross-reactivity with the rat GABA\(_\text{A}\) receptor. Two other purified rabbit polyclonal antibodies, an antibody against rat NKCC1/SCL12A5 (potassium, chloride cotransporter) (catalogue No. 07-431) were also purchased from Millipore. Anti-rat α\(_1\)-acid glycoprotein (AGP) was obtained from Life Diagnostics Inc. (West Chester, PA, U.S.A.) as an affinity isolated polyclonal antibody (catalogue No. 18130). All other reagents used were

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Creatinine concentration and plasma AGP level. The creatinine concentration and plasma AGP level were examined in the study with bumetanide. The creatinine concentration and plasma AGP level were measured using a previously reported method. In brief, the rats were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneal injection (i.p.)). Then, a hole of 1 mm in diameter was made by drilling the skull, paying close attention to avoid damaging the brain tissue. Next, a guide cannula (AG-8, Eicom, Kyoto, Japan) was carefully inserted into the right lateral ventricle through the skull, paying close attention to avoid damaging the brain tissue. The guide cannula was then firmly glued to the skull, and was temporarily plugged with a dummy cannula (AD-8, Eicom) and cap nut (AC-8, Eicom).

After a 24-h recovery period from the insertion surgery, rats were randomly allocated to either the sham or ARF group. The cerebral protein concentration and plasma AGP level were examined in the study with phenobarbital. In brief, the rats were anesthetized with sodium pentobarbital (80 mg/kg, i.p.). Then, a hole of 1 mm in diameter was made by drilling the skull, paying close attention to avoid damaging the brain tissue. Next, a guide cannula (AG-8, Eicom, Kyoto, Japan) was carefully inserted into the right lateral ventricle through the skull, and its tip was placed in the ventricle at the following brain atlas coordinates originating from the bregma: anterior, 0.0 mm; lateral, 1.5 mm; ventral, 3.2 mm. The guide cannula was then firmly glued to the skull, and was temporarily plugged with a dummy cannula (AD-8, Eicom) and cap nut (AC-8, Eicom).

After a 24-h recovery period from the insertion surgery, rats were randomly allocated to either the sham or ARF group. Rats in the ARF group were deprived of water for 24 h, and then injected with 50% glycerol (10 mL/kg) in the left and right thighs to induce ARF. They were then normally fed with free access to water for a 24-h incubation period. ARF induction was assessed based on elevation of the serum creatinine concentration and plasma AGP level. The creatinine concentration was conventionally assessed with the Jaffe reaction. The AGP level was examined by Western blotting, as described later. Sham rats were treated with 0.9% sodium chloride solution in the same manner as those in the ARF group. No water deprivation was applied to sham rats.

In the protein expression study, rats were used without the cannula insertion surgery, but were still randomly allocated to either the sham or ARF group to be treated in the same manner as described above. They were then supplied for the study as described later.

Evaluation of the Pharmacological Effects of the Compounds Following the 24-h incubation period, the rats were used for the pharmacological study. In addition to phenobarbital, we employed two other compounds, ethyl carbamate and bumetanide, to examine the mechanism underlying the increased pharmacological effects of phenobarbital by utilizing their central nervous system (CNS)-affecting functions. Phenobarbital induces anesthesia by binding to the GABA_A receptor, whereas ethyl carbamate induces anesthesia in a GABA_A receptor-independent manner. Bumetanide is an NKCC1 inhibitor. As NKCC1 regulates action potentials by controlling the cellular chloride concentration in the nerve cells, the study with bumetanide was conducted to explore factors responsible for the increased pharmacological effects of phenobarbital. Regarding the preparation of the compound solutions, phenobarbital and ethyl carbamate were dissolved in distilled water at concentrations of 70 mg/mL and 0.67 g/mL, respectively. Bumetanide was first dissolved in 1 M sodium hydroxide solution at a concentration of 10 mg/mL, and was then diluted with isotonic sodium chloride solution at 1.5 mg/mL. For the animal experiments, each fully alert rat was transferred to an animal cage, and the dummy cannula used for plugging the guide cannula was removed. A 40-cm of polyethylene tube (0.5 mm i.d. × 0.8 mm o.d., SP-31, Natsume Seisakusho, Tokyo, Japan) was prepared, in which the air in its lumen was purged by the compound solution, and one of its ends was firmly attached to the guide cannula. The other end of the polyethylene tube was connected to the 24-gauge needle of a tuberculin syringe filled with the compound solution. The cannula swivel (TSC-21, Sugiyamagen, Tokyo, Japan) was utilized to ensure that the rat was able to freely move in the cage without the tube becoming twisted. The syringe was set on an infusion pump (SPE-1, As One, Osaka, Japan).

The pharmacological effects of the compound were evaluated based on the onset time, and the amount of the compound infused into the ventricle by the onset time was calculated. We evaluated the pharmacological effects based on the onset time, considering the simplicity and the apparatus availability. In the study with phenobarbital, the solution was infused at a rate of 2.6 µL/min according to the bulk flow rate of the cerebrospinal fluid. The onset time of anesthesia was determined with the tail flick method, in which the rat was pinched every minute by a binder clip at a pre-defined site near the tail tip. The noxious stimulus was pinching, which was started 10 min before a tentative onset time that was determined in a preliminary examination. If a rat did not react to the noxious stimulus, second, third, and fourth pinches were performed every 30 s. If a rat did not react to any of these additional stimulations, the time point when the first stimulation was performed was considered as the onset time of anesthesia. After the onset time had been determined or the pre-determined experimental period had elapsed, the rat was deeply anesthetized with phenobarbital to be sacrificed by blood draw from the abdominal aorta following midline incision. A blood specimen was obtained to measure the serum creatinine concentration and plasma AGP level. The rat brain was then excised, and a small volume of dye was gently infused into the ventricle via the cannula. It was then dissected to confirm whether the cannula had been properly inserted in the ventricle. In the study with ethyl carbamate, the onset time of anesthesia was determined in the same manner as that in the study with phenobarbital.

In the study with the ventricular infusion of bumetanide, its effects on the CNS function were evaluated based on the onset time of seizure induction. Bumetanide infusion was performed at a rate of 3.2 µL/min. The experiment ended after the confirmation of seizure induction or was terminated at 180 min if no seizure was inducted. The data from the study with bumetanide were handled as censored data.

Western Blotting Analysis The cerebral protein expression of the GABA_A receptor and the cation-chloride transporters NKCC1 and KCC2 was evaluated by Western blotting analysis. First, pooled microsomes of the rat cerebral cortex were prepared from sham and glycerol-induced ARF rats using a reported method of ultracentrifugation with slight modification. Five rats were used from each group, and under deep anesthetization with sodium pentobarbital (80 mg/kg, i.p.), they were sacrificed by blood draw from the abdominal
aorta. Immediately after the blood draw, the brain was perfused with ice-cold isotonic sodium chloride solution to wash out the blood remaining in the brain vessels. Perfusion was performed using common carotid arteries and the superior vena cava. The whole brain was then excised. The excised brain was gently washed in ice-cold isotonic sodium chloride solution, and was then cut lengthwise into three pieces. The cortical region of each piece was manually sectioned for collection. Next, all cortexes collected were homogenized together with 3 volumes of ice-cold isotonic phosphate buffer (pH 7.4). The homogenate was centrifuged at 9000 × g for 20 min, and the supernatant was then centrifuged again at 105000 × g for 60 min. The pellet obtained was suspended in 50 mM Tris buffer (pH 7.4) containing 20% glycerol and 1 mM ethylenediaminetetraacetic acid (EDTA) to form a microsome preparation. The preparation was stored at −80°C until use. The protein content of the preparation was measured using a protein assay kit (Bio-Rad, Hercules, CA, U.S.A.).

Western blotting was performed as follows: As a specimen for electrophoresis, the microsome preparation was mixed with isotonic phosphate buffer (pH 7.4), and the protein concentration was adjusted to 1.0 mg/mL. The specimen was then boiled for 5 min with 2-mercaptoethanol. After boiling, the specimen was applied to an sodium dodecyl sulfate (SDS)-polyacrylamide (10%) gel at a protein concentration of 10 µg/lane for GABA_A receptor expression, or at a protein concentration of 20 µg/lane for NKCC1 and KCC2 expression. The gel was subjected to electrophoresis for approximately 150 min at 4°C, followed by transfer to a nitrocellulose membrane. After overnight blocking, the membrane was incubated for 60 min with the anti-GABA_A anti-NKCC1 or anti-KCC2 antibody. The antibodies were diluted to 1:1000 for use. The migration pattern was visualized with the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, U.S.A.) and DAB substrate kit (Vector Laboratories). The migration patterns were analyzed in a semi-quantitative manner based on densitometric readings.

The plasma AGP level was also evaluated by Western blotting. In this case, the plasma specimen was applied to the SDS-polyacrylamide (15%) gel at a protein amount of 1 µg/lane, followed by electrophoresis, membrane transfer, and blocking in the same manner as described above. The membrane was then incubated for 1 h with the anti-rat AGP antibody diluted to 1:5000. The migration pattern was visualized as described above, followed by semi-quantitative analysis based on densitometric readings.

**Data Analysis** Data were expressed as the mean ± standard error (S.E.) if normally distributed. If not normally distributed, they were summarized with a box-and-whisker plot, in which the first and third quartiles were indicated at the bottom and top of the box, respectively. The median was expressed as a bar in the box. The whisker beneath the box indicated the range from the first quartile to the lowest data point or to the point at a 1.5-interquartile, whichever was shorter. The whisker above the box indicated the range from the third quartile to the highest data point or to the point at the interquartile. The differences between the groups were examined by the Mann–Whitney U-test, and p < 0.05 was considered significant.

The censored data in the bumetanide study were expressed as a Kaplan–Meier plot. The intergroup difference was evaluated by the log-rank test, with p < 0.05 being significant.

**RESULTS**

**Evaluation of Anesthetic Effects of Phenobarbital and Ethyl Carbamate in ARF Rats** Prior to the experiments, the development of glycerol-induced ARF was confirmed by elevation of the serum creatinine concentration. The creatinine concentrations in sham and ARF rats were 5.5 ± 0.7 and 23.4 ± 1.5 µg/mL, respectively, being significantly higher in ARF rats than in sham rats (p < 0.05). The plasma AGP level in ARF rats also increased to 4.8 ± 0.9 times higher than that in sham rats (p < 0.05).

The anesthetic effects of phenobarbital and ethyl carbamate were then evaluated in sham and glycero-induced ARF rats. Phenobarbital induced anesthesia faster in the ARF rats than in sham rats. When the doses required to achieve anesthesia were compared, the value for the ARF rats was markedly lower, being approximately 46% of that for sham rats (Fig. 1A). On the other hand, when the anesthetic effects were examined with ethyl carbamate whose anesthetic effects are induced in a GABA_A receptor-independent manner, there was no difference regarding the onset time between sham and ARF rats (Fig. 1B).

**Evaluation of Cerebral Expression of the GABA_A Receptor, and the Cation-Chloride Transporters NKCC1 and KCC2 in Sham and ARF Rats** The cerebral protein expression of the GABA_A receptors in sham and glycerol-induced ARF rats was then evaluated, as phenobarbital exerts its anesthetic effects by associating with the GABA_A receptor to open the chloride channel in the receptor. As shown in Fig. 2A, GABA_A receptor expression was lower in the ARF rats than in sham rats, suggesting that alteration of the GABA_A receptor expression was minimally involved in the increased pharmacological effects of phenobarbital. Next, the cerebral expression of the cation-chloride transporters NKCC1 and KCC2 was examined. Cooperating with the GABA_A receptor, these transporters regulate action potentials in the nerve cells. As a result, the expression of NKCC1 was unaltered, whereas...
that of KCC2 was lower in the ARF rats than in sham rats (Figs. 2B, 2C).

**Evaluation of the Pharmacological Effects of Bumetanide in ARF Rats** As NKCC1 and KCC2 regulate action potentials in a collaborative manner, inhibition of the NKCC1 function was expected to enhance the influence of the decreased expression of KCC2 on CNS function. With this consideration, the pharmacological effects of bumetanide, an NKCC1 inhibitor, on CNS function were then examined in sham and glycerol-induced ARF rats. When bumetanide was directly infused into the ventricle, tonic seizures were induced in sham rats. As shown in Fig. 3, the amount of bumetanide required for seizure induction was 0.34–0.46 mg in sham rats. On the other hand, the frequency of seizure induction was significantly lower in the ARF rats (Fig. 3).

**DISCUSSION**

Although anesthesia was reported to be readily induced by barbiturates under renal impairment, the mechanisms underlying the increased pharmacological effects remain to be clarified. We examined the pharmacological effects of phenobarbital in this study with the compound being directly infused into the lateral ventricle. As a result, phenobarbital-induced anesthesia at a lower dose in glycerol-induced ARF rats than in sham rats (Fig. 1A), consisting with previous reports and supporting that the cerebral susceptibility to barbiturates increases in the ARF rats in an intrinsic manner. On the other hand, as shown in Fig. 1B, the anesthetic effects of ethyl carbamate occurred in the same manner in sham and ARF rats. Phenobarbital exerts its anesthetic effects by associating with the GABA \( \alpha \) receptor, whereas ethyl carbamate induces anesthesia in a GABA \( \alpha \) receptor-independent manner by affecting neurotransmitter-gated ion channels. Therefore, the increase in pharmacological effects may be a phenomenon specific to compounds that associate with the GABA \( \alpha \) receptor.

Based on these findings, functional alteration of the GABA \( \alpha \) receptor was thought to be involved in the increased pharmacological effects of phenobarbital. The GABA \( \alpha \) receptor functions as a chloride ion channel to let ambient chloride ions flow into the nerve cell, leading to an increase in the intracellular chloride concentration to regulate action potentials. Although the cerebral expression of the GABA \( \alpha \) receptor was expected to increase in the ARF rats, its expression decreased (Fig. 2A), suggesting that the GABA \( \alpha \) receptor is only minimally involved in the increased pharmacological effects of phenobarbital. Expression of the GABA \( \alpha \) receptor likely increases in a specific region of the brain. However, such an increase may not largely influence the pharmacological effects because phenobarbital exerts its anesthetic effects evenly throughout the brain. Regarding the involvement of the synaptic GABA concentration, it is unlikely a factor involved in the increased pharmacological effects of phenobarbital in the ARF rats because phenobarbital opens the chloride channel in a direct manner by associating with the GABA \( \alpha \) receptor regardless of the ambient GABA concentration.

In addition to the GABA \( \alpha \) receptor, two cation-chloride transporters, NKCC1 and KCC2, are also known to control...
the cellular chloride concentration to regulate action potentials in nerve cells.\textsuperscript{22–24} We therefore hypothesized that alteration of their functions impacts the cerebral susceptibility to phenobarbital, leading to an increase in the pharmacological effects of phenobarbital. As shown in Figs. 2B and C, when their expression was examined with the cerebral microsomes prepared from the cortical region of the excised brains in sham and ARF rats, the expression of NKCC1 was unchanged, whereas that of KCC2 decreased in the ARF rats. As NKCC1 and KCC2 participate in inward and outward chloride transport in nerve cells, respectively,\textsuperscript{22–24} the current finding that their expression is unevenly changed in the ARF rats suggests that the intracellular chloride concentration is equilibrated in a different manner between sham and ARF rats.

Based on the current finding that the NKCC1 and KCC2 expressions unevenly change in ARF rats, we carried out an additional study to examine the influence of the unevenly charged expression on the brain function, in which we performed the ventricular infusion of an NKCC1 inhibitor, bumetanide, with an expectation that inhibition of the NKCC1 function accentuates any difference in equilibration between sham and ARF rats. As shown in Fig. 3, the pharmacological response to bumetanide in the glycerol-induced ARF rats was different from that in sham rats, in which sham rats exhibited tonic seizures in all cases, but they were rare in the ARF rats. It was speculated that inhibition of NKCC1 function by bumetanide impaired the regulation of cellular membrane depolarization, triggering seizures in sham rats.\textsuperscript{15,22,29} The observed difference in the response to bumetanide in sham and ARF rats may be related to the cerebral expression of NKCC1 and KCC2 being uneven in the ARF rats (Figs. 2B, C). Neural action potentials are known to be largely influenced by the cellular chloride concentration,\textsuperscript{22–24} which NKCC1 and KCC2 coordinately regulate. As KCC2 handles chloride efflux in nerve cells, the decreased expression of KCC2 increases the intracellular chloride concentration.\textsuperscript{22,24} The observation that bumetanide hardly induces seizures in the ARF rats is thought to reflect the efficient suppression of stimulus action potentials in nerve cells. Due to the decreased expression of KCC2, the chloride concentration in nerve cells in the ARF rats may have been equilibrated at a different level than compared with that in sham rats, resulting in the neural action potentials being efficiently suppressed. With these considerations, altered chloride handling and the resultant change in the cellular chloride concentration in nerve cells increasing the susceptibility to phenobarbital may be an underlying mechanism of the increased pharmacological effects in the ARF rats.

As we investigated the pharmacological effects in vivo, several factors that may be involved in the increased susceptibility were not examined in this study. Alteration of the cellular entry of sodium and/or calcium ion results in potentiated phenobarbital anesthetization in the ARF rats because electrolyte entry plays an important role in the depolarization of nerve cells.\textsuperscript{26,27} Histamine is also likely involved in this altered susceptibility by influencing neural depolarization.\textsuperscript{28} In addition, the mechanism accounting for how renal failure influences the cerebral KCC2 expression remains unexamined, although brain-derived neurotrophic factor (BDNF) and its receptor TrkB are known factors that regulate KCC2 expression.\textsuperscript{29} It should be noted that although the increased pharmacological effects were similarly observed in the rats suffering from renal impairments which were induced with an intravenous injection of uranyl nitrate or bilateral ligation of ureter,\textsuperscript{1–3} we cannot exclude the possibility that the current findings are influenced with a specific phenomenon in the glycerol-induced ARF rats, and that the insertion of guide cannula in the ventricle may affect the neural protein expressions. These factors and concerns need to be investigated in future studies in order to fully understand the mechanism underlying the increased susceptibility in the ARF rats.

Collectively, we demonstrated in this study that phenobarbital more readily induced anesthesia in glycerol-induced ARF rats when it was directly introduced into the lateral ventricle. The increased pharmacological effects of phenobarbital reflect an intrinsic increase in the cerebral susceptibility to phenobarbital, which is likely related to the uneven cerebral expression of the two cation-chloride transporters NKCC1 and KCC2 in the ARF rats. The uneven alteration in their expressions may cause the intracellular chloride concentration in nerve cells to be differentially equilibrated in the ARF rats, allowing the action potentials in the nerve cells to be more readily suppressed.

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Conflict of Interest The authors declare no conflict of interest.

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