**Lithium Protects against Anesthesia-induced Developmental Neuroapoptosis**

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Background: Ethanol and anesthetic drugs trigger neuroapoptosis in the developing mouse brain. Recently, it was found that ethanol-induced neuroapoptosis is preceded by suppressed phosphorylation of extracellular signal-regulated protein kinase (ERK), and lithium counteracts both the phosphorylated ERK suppressant action and ethanol-induced neuroapoptosis. The current study was undertaken to address the following questions. (1) Do ketamine and propofol mimic ethanol in suppressing ERK phosphorylation? (2) If they do, does lithium prevent this suppressant action and also prevent these anesthetic drugs from triggering neuroapoptosis?

Method: Postnatal day 5 mice were treated with propofol, ketamine, lithium, a combination of propofol or ketamine with lithium or saline, and their brains were prepared for Western blot analysis or histology. For Western blot, cytosolic lysates of caudate putamen were analyzed for expression of phosphorylated ERK and phosphorylated serine/threonine-specific protein kinase. For histology, brains were stained immunohistochemically with antibodies to activated caspase-3, and the density of activated caspase-3 positive cells was determined.

Results: Ketamine and propofol suppressed phosphorylated ERK, and lithium counteracted both the phosphorylated ERK suppressant action and neuroapoptotic action of these anesthetic drugs.

Conclusion: If further testing finds lithium to be safe for use in pediatric/obstetric medicine, administration of a single dose of lithium before anesthesia induction may be a suitable means of mitigating the risk of anesthesia-induced developmental neuroapoptosis.

TRANSIENT exposure of infant rodents to several classes of drugs, including N-methyl-D-aspartic acid antagonists and γ-aminobutyric acid-A agonists, triggers widespread neurodegeneration in the developing brain.1–6 The cell death process triggered by these drugs displays all of the classic ultrastructural characteristics of apoptosis,3,7,8 and it is mediated by the Bax-dependent mitochondrial intrinsic pathway involving cytochrome-c release and activation of caspases 9 and 3.9–11 The window of vulnerability to these agents coincides with the developmental period of rapid synaptogenesis,1,2 also known as the brain growth spur period, which occurs primarily during the first 2 weeks after birth in mice and rats but extends from about midgestation to several years after birth in humans.12

Ethanol, which has both N-methyl-D-aspartic acid antagonist and γ-aminobutyric acid-A mimetic properties, induces widespread neurodegeneration in the developing brain.1–5,6 Zhong et al. recently reported that a single dose of lithium (6 mEq/kg) coadministered with ethanol to infant mice protects against ethanol-induced neuroapoptosis.13 Further, it was hypothesized that the protective effect of lithium might be mediated by action of lithium on the glycogen synthase kinase 3 signaling system; however, no evidence for an interaction between either ethanol or lithium and the glycogen synthase kinase system was found.

Recent work in our laboratory has demonstrated that lithium suppresses the programmed cell death process that occurs naturally in the developing mouse brain and has confirmed the findings of Zhong et al. that lithium powerfully protects against ethanol-induced neuroapoptosis.14 To explore the mechanism of action of lithium, we focused on kinase signaling systems (extracellular signal-regulated kinase [ERK], serine/threonine-specific protein kinase [Akt], Jun N-terminal kinase [JNK]) that are believed to play a regulatory role in cell survival. We found that very rapidly (within 30 min) after ethanol administration, there is a marked suppression of ERK phosphorylation and that lithium stimulates ERK phosphorylation and prevents ethanol from suppressing this phosphorylation process.14 Ethanol also suppressed phosphorylated Akt, but lithium did not counteract this effect. We also found that ethanol activates the JNK system; but this does not explain the neurotoxic action of ethanol because JNK activation did not occur in the same neuronal populations that are killed by ethanol.

The current study was undertaken to determine whether anesthetic drugs suppress ERK and/or Akt phosphorylation, whether lithium counteracts this suppressant action, and whether lithium protects against anesthesia-induced developmental neuroapoptosis. The anesthetic drugs focused on in this study were ketamine, an agent that interacts primarily with N-methyl-D-aspartic acid glutamate receptors, and propofol, an agent that interacts primarily with γ-aminobutyric acid-A receptors but also possibly interacts with N-methyl-D-aspartic acid glutamate receptors.15

Materials and Methods

In the first set of experiments, we sought to determine whether anesthetic drugs mimic ethanol in suppressing...
phosphorylation of ERK and Akt, and if they do, whether lithium counteracts this suppressant action. For this purpose, postnatal day 5 (P5) C57BL/6 mouse pups were treated with vehicle, ketamine (40 mg/kg, subcutaneous), propofol (50 mg/kg, intraperitoneal), lithium (6 mEq/kg, intraperitoneal), or a combination of ketamine (40 mg/kg) or propofol (50 mg/kg) and lithium (6 mEq/kg). These anesthetic dosing regimens were used because they have been shown to induce a significant neuroapoptosis response in C57BL6 infant mice.\(^{14,16}\) This dose of lithium was chosen because it was the dose Zhong et al.\(^{15}\) used in their original study showing that lithium protects against ethanol-induced neuroapoptosis. Pups were killed 120 min after administration of drug, brains were collected, and cytosolic extracts of caudate putamen were prepared for Western blot analysis of phosphorylated ERK 1/2 and phosphorylated Akt.

In a second set of experiments, we evaluated the ability of lithium (6 mEq/kg) to prevent apoptotic neurodegeneration induced by ketamine or propofol. For experiments with ketamine, P5 mouse pups were injected with saline vehicle, ketamine (40 mg/kg), lithium (6 mEq/kg), or a combination of ketamine (40 mg/kg) and lithium (6 mEq/kg). For experiments with propofol, P5 mouse pups were injected with intralipid vehicle, propofol (50 mg/kg), lithium (6 mEq/kg), or a combination of propofol (50 mg/kg) and lithium (6 mEq/kg). Pups were killed 5 h after drug administration, and brain tissue was prepared for immunohistochemical staining with antibodies against activated caspase-3.

We undertook a third set of experiments to determine whether a lower dose of lithium would be protective against the neuroapoptogenic actions of ketamine and propofol. Zhong et al.\(^{15}\) reported that blood lithium levels after administration of a 6 mEq/kg dose of lithium to infant mice remained in a therapeutic range 24 h later. We sought to confirm this and found that administration of 3 mEq/kg lithium results in blood concentrations at 12 h after drug administration that are at the upper end of the range considered therapeutic for adult humans, and they are at the lower end of the human therapeutic range at 24 h.\(^{14}\) but lithium at 6 mEq/kg produced blood levels approximately 2 times higher, which would potentially be in a toxic range. Therefore, in this third set of experiments, the ability of lithium at 3 mEq/kg to prevent ketamine or propofol from inducing neuroapoptosis was tested. For this purpose, P5 mouse pups were treated with saline or intralipid vehicle, ketamine (40 mg/kg), propofol (100 mg/kg), lithium (3 mEq/kg), or a combination of ketamine (40 mg/kg) or propofol (100 mg/kg) and lithium (3 mEq/kg). In this experiment, we not only lowered the dose of lithium, but we increased the dose of propofol (100 instead of 50 mg/kg) to achieve a more stringent test of lithium’s neuroprotective efficacy.

**Animals and Drugs**

All experiments and animal care procedures were performed in accordance with standards approved by the Washington University School of Medicine Animal Studies Committee (Saint Louis, Missouri). Postnatal day 5 infant C57BL6 mice (Harlan, Indianapolis, IN) were removed from the dam and maintained at an ambient temperature of 30°C for the duration of the experiment. Throughout all experiments, control and experimental pups were drawn randomly from the same litters. Drugs used in this experiment included ketamine, propofol, and lithium carbonate. Ketamine (Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) was obtained as an injectable solution (10 mg/ml) and administered subcutaneously as 40 mg/kg. Propofol (2,6-diisopropylphenol; AstraZeneca Pharmaceuticals LP, Wilmington, DE) was prepared as a 1% emulsion in intralipid and administered as an intraperitoneal dose of 50 mg/kg or 100 mg/kg. Vehicle controls for the ketamine experiments received 0.9% normal saline, and those for the propofol experiments received intralipid. Lithium carbonate was prepared in normal saline acidified with hydrochloric acid and neutralized with sodium hydroxide and administered as an intraperitoneal injection of either 3 or 6 mEq/kg.

**Western Blot Analysis**

Two hours after drug administration, pups were killed by decapitation, and brains were removed and placed on ice while caudate/putamen was dissected. Tissue was maintained at −80°C until the time of cell lysate preparation. Tissue was homogenized in 10× cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing phosphatase and protease inhibitors (Pierce, Rockford, IL), sonicated and maintained at 4°C for 60 min before centrifugation. Equal amounts (40 μg) of the resulting cell lysate was resolved by sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene fluoride membrane. Blots were incubated in 3% milk in 0.125% Tween-20 for 60 min and then incubated for 16 h at 4°C with antibodies raised to phospho-p44/42 Map Kinase (Cell Signaling Technology) or phospho-Akt (Ser473) (1:1000; Cell Signaling Technology) and ifElF4E (internal standard, 1:12500, Cell Signaling Technology). After incubation with primary antibody, blots were thoroughly washed and then incubated with anti-rabbit IgG horseradish peroxidase-linked secondary antibody (1:1666; Cell Signaling Technology) for 60 min. The horseradish peroxidase signal was detected with Pierce SuperSignal West Dura Extended Duration Substrate (Pierce), and the resulting image was captured with a FujiFilm LAS 3000 Imaging Station. Blots were analyzed with Science Lab 2005 Multi Gauge Ver3.0 (FujiFilm, Tokyo, Japan). To quantify the blots, signal strength was measured in arbitrary units and

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expressed as a ratio of signal of antibody of interest to signal of the eIF4E internal standard.

**Histology and Quantitative Cell Counts**

Five hours after drug administration, pups were deeply anesthetized with sodium pentobarbital and perfused with 4% paraformaldehyde in Tris buffer through the left cardiac ventricle and ascending aorta. Brains were removed from the skull and immersed in fixative for 3 days before being cut into serial 70-μm-thick sections in the sagittal plane. Every fourth section was selected for immunohistochemical staining with antibodies raised to activated caspase-3 (AC3, Cell Signaling Technology) by methods previously described. The AC3 stain has been validated as a marker of cell death in earlier studies through demonstration that the pattern of neuronal degeneration revealed by AC3 immunohistochemistry after neuroapoptogenic drug treatment is exactly reproduced by either terminal deoxynucleotidyl transferase dUTP nick-end labeling or DeOlmos cupric silver staining. Further, electron microscopy has been used to confirm that the dying cells have ultrastructural features of neurons and are undergoing pathomorphological changes characteristic of apoptosis.

After staining with activated caspase-3 antibody, quantitative counts were made in the cerebral cortex and caudate/putamen regions of the brain using a stereology system consisting of Stereo Investigator (MicroBrightField, Inc, Colchester, VT) on a Pentium III personal computer, connected to a Prior Optiscan motorized stage (ES103 XYZ system; Prior Scientific Inc, Rockland, MA) mounted on a Nikon Labophot-2 microscope (Nikon, Tokyo, Japan). The boundaries of the area of interest were traced into the computer, and Stereo Investigator used these tracings to calculate the area of each section from which counts were obtained. Activated caspase-3-positive neurons larger than 6 μm were counted. The population estimator function of Stereo Investigator was used to mark each profile after it was counted to ensure that no profile would be missed or counted twice. The counts from three sections per region of interest from a given brain were summed and divided by the tissue volume (total area times thickness of the sections) from which the counts were obtained to yield an estimate of the density of AC3-positive cell profiles (count per mm³). A single investigator who remained blind to treatment condition performed quantification of AC3-positive cell profiles for each brain region.

**Statistical Analyses**

Data are presented as mean ± SD. Statistical analyses were conducted and graphs were prepared with the aid of GraphPad Prism software, version 4.0a (GraphPad Software, Inc., La Jolla, CA). For comparison of only two data sets (for example, lithium-treated vs. vehicle control) an unpaired two-tailed t test with Welch correction was used. For Western blot or histologic analyses involving multiple comparisons (e.g., vehicle vs. anesthetic drug vs. lithium + anesthetic drug) a one-way ANOVA and Bonferroni multiple comparison test was used. In all experiments, pups from each litter were assigned in approximately equal numbers to experimental and control groups. Sample sizes in all experiments were at least 6 per group. For the exact number of animals in each treatment group, see figure legends.

**Results**

**Effect of Lithium and/or Anesthetic Drugs on Erk1/2 and Akt Expression**

Western blot analysis of the caudate/putamen of P5 mouse pups revealed that lithium alone significantly increased phosphorylation of ERK1 (P = 0.006) and ERK2 (P = 0.002) compared to vehicle controls (fig. 1, A and B), whereas both ketamine and propofol reduced pERK-1 and pERK-2 to about 50% of vehicle control levels (P < 0.001 for all comparisons) (fig. 1, A and C). When lithium was combined with ketamine or propofol, the suppressant effect of these anesthetics on phosphorylated ERK-1 and -2 was eliminated, leaving the resultant values for phosphorylated ERK-1 and -2 not significantly different from and almost at the same level as vehicle controls. Phosphorylation of Akt was suppressed significantly by ketamine (P < 0.01) and by propofol (P < 0.001) (fig. 1, A and C). Lithium alone had no effect on phosphorylated Akt expression (fig. 1B) and did not counteract the suppressant effect of propofol on phosphorylated Akt. Lithium did counteract the effect of ketamine on phosphorylated Akt, but only to a marginally nonsignificant degree (P = 0.0503). In the 2-h period during which the phosphorylated forms of ERK1/2 and Akt showed changes in response to anesthetic drugs and/or lithium, there was no change in total ERK1/2 or Akt (data not shown).

**Effect of Lithium and/or Anesthetic Drugs on Activated Caspase-3 Immunohistochemistry**

In experiments aimed at determining whether lithium influences the natural rate of neuro apoptosis in the normal developing brain it was found that 6 mEq/kg lithium reduced the density of AC3-positive profiles to 51% of that in a group of littermates treated with saline (P = 0.004) (fig. 2A). In a separate series of experiments, the neuro apoptosis response after administration of ketamine (40 mg/kg) or propofol (50 mg/kg) with or without lithium (6 mEq/kg) was evaluated. One-way ANOVA of AC3-positive profile counts in the cerebral cortex and caudate/putamen after treatment with vehicle, ketamine, propofol, or a combination of ketamine or propofol and lithium (fig. 2B) revealed a highly
There was a significant increase in AC3-positive profiles compared to litter-matched vehicle controls after treatment with ketamine (P < 0.001) or propofol (P < 0.001). This increase was completely abolished by coadministration of lithium together with either anesthetic drug, resulting in an apoptosis rate significantly lower than the anesthetic drug alone (P < 0.001) but not significantly different from vehicle controls. The histologic appearance of AC3 staining in the cortex and caudate/putamen after treatment with lithium, ketamine, or propofol alone or with ketamine or propofol in combination with lithium is illustrated in figure 3. Although other brain regions were not quantified, qualitative evaluation of serial sections encompassing all brain regions support the conclusion that lithium suppressed spontaneous apoptosis or apoptosis induced by ketamine or propofol to a similar degree throughout the brain.

An additional set of experiments sought to determine whether a lower dose of lithium (3 mEq/kg) would suppress natural apoptosis or prevent apoptosis induced by ketamine (40 mg/kg) or propofol (100 mg/kg). Administration of lithium (3 mEq/kg) alone resulted in a significant treatment effect [F(4,45) = 45.76, P < 0.0001]. There was a significant increase in AC3-positive profiles compared to litter-matched vehicle controls after treatment with ketamine (P < 0.001) or propofol (P < 0.001). This increase was completely abolished by coadministration of lithium together with either anesthetic drug, resulting in an apoptosis rate significantly lower than the anesthetic drug alone (P < 0.001) but not significantly different from vehicle controls. The histologic appearance of AC3 staining in the cortex and caudate/putamen after treatment with lithium, ketamine, or propofol alone or with ketamine or propofol in combination with lithium is illustrated in figure 3. Although other brain regions were not quantified, qualitative evaluation of serial sections encompassing all brain regions support the conclusion that lithium suppressed spontaneous apoptosis or apoptosis induced by ketamine or propofol to a similar degree throughout the brain.

An additional set of experiments sought to determine whether a lower dose of lithium (3 mEq/kg) would suppress natural apoptosis or prevent apoptosis induced by ketamine (40 mg/kg) or propofol (100 mg/kg). Administration of lithium (3 mEq/kg) alone resulted in a reduction in the neuroapoptosis rate to a level 52% that of littermate saline controls (fig. 4A; P = 0.0011, lithium vs. saline). One-way ANOVA of AC3-positive profile density in the cortex and caudate/putamen of pups treated with vehicle, ketamine, propofol, or a combination of ketamine or propofol and lithium revealed a highly significant treatment effect [fig. 4B; F(4,41) = 45.36, P < 0.0001]. Compared to vehicle controls, the density of AC3-positive profiles was significantly increased in pups treated with ketamine (P < 0.001) or propofol (P < 0.001). Administration of lithium in combination with ketamine or propofol prevented the suppressant effects of ketamine and propofol on pERK-1 and pERK-2, leaving these values unchanged from vehicle control. Lithium counteracted the suppressant effect of ketamine on pAkt, but only to a marginally nonsignificant degree (P = 0.0593).

Discussion

Our findings document that lithium exerts a strong neuroprotective effect in the in vivo mouse brain against the apoptogenic action of two anesthetic drugs, ketamine and propofol. Lithium conferred complete or nearly complete protection in 2 brain regions evaluated...
Lithium also reduced the density of AC3-positive profiles when administered in combination with either ketamine or propofol (50 mg/kg, intraperitoneal injection). Ketamine and propofol increased the density of AC3-positive cell profiles in both the cortex and caudate/putamen compared to saline, whereas lithium alone reduced the density of AC3-positive cell profiles compared to saline.

Whether this dose of lithium can potently protect against fully anesthetizing doses of these drugs will have to be tested in a larger animal species, because their mean values were not significantly different. Overall treatment effects were highly significant (ANOVA P < 0.0001), and post hoc analysis revealed that the density of AC3-positive profiles was significantly increased by ketamine (P < 0.001) and propofol (P < 0.001). Lithium abolished the neuroapoptosis response to ketamine (P < 0.001) and propofol (P < 0.001), leaving the AC3-positive profile count at or below the vehicle control level.

The finding that lithium confers protection against the apoptogenic action of these two anesthetic drugs and also suppresses spontaneous neuroapoptosis, suggests that the drug-induced and spontaneous phenomena are the same process with respect to the final pathway through which the apoptosis trigger is tripped. Although the initiating mechanisms are not identical, the data suggest that there is a convergence at some point in the intracellular signaling pathways into a single pathway through which lithium can exert a counteractive influence that arrests further propagation of the apoptosis signal. Our findings identify phosphorylation of ERK-1 and -2 as a step in the signaling process where the actions of lithium and those of several other agents (ketamine, propofol, ethanol) impinge with opposing actions and the action of lithium is strong enough to result in a neuroprotective effect.
In separate studies, we have demonstrated that lithium protects against neuroapoptosis induced by isoflurane and chloral hydrate in the infant mouse brain. The efficacy of lithium in protecting against neuroapoptosis induced by ethanol, ketamine, propofol, isoflurane, and chloral hydrate not only recommends lithium as a potential antiapoptotic neuroprotective agent, but also suggests the important possibility that other agents, acting by a similar mechanism, can be developed that can stabilize apoptosis pathways in the developing brain and totally prevent unwanted apoptotic processes.

Lithium has long been used as a mood stabilizer in the treatment of manic-depressive (bipolar) disorder; more recently, it has been reported to have neuroprotective properties relevant to adult neurologic disorders such as Alzheimer disease, Huntington disease, and ischemic stroke. Mechanisms underlying lithium’s mood stabilizing and neuroprotective properties in the adult brain remain to be elucidated, but putative candidates include inhibition of glycogen synthase kinase-3β, stimulation of heat shock protein-70, inhibition of Ca influx through the N-methyl-D-aspartic acid glutamate receptor, and activation of the ERK signaling pathway. However, these postulated mechanisms are based on evidence generated in the adult nervous system or in the petri dish. To the best of our knowledge, with the exception of a recent article from our laboratory, there are no previously published studies demonstrating in the in vivo developing brain that lithium stimulates ERK phosphorylation or that increased phosphorylation of ERK is protective against drug-induced developmental neuroapoptosis.

The neuroapoptosis literature is massive, but models for inducing apoptotic neurodegeneration in the in vivo developing nervous system are scarce, the two best characterized examples being the phenomenon under investigation herein, and degeneration of sympathetic neurons induced by trophic factor deprivation, as originally described by Levi-Montalcini and others. As Dikranian et al. recently pointed out, these two phenomena have identical ultrastructural characteristics, and both meet all of the morphological criteria originally stipulated by Wyllie et al. for recognizing apoptosis. Neuroapoptosis induced by trophic factor deprivation has been studied extensively and reportedly involves activation of the JNK intracellular signaling pathway. We have demonstrated that ethanol activates the JNK pathway, but we deemed this to be irrelevant to the neuroapoptogenic action of ethanol because it did not occur in the neuronal populations that underwent apoptosis after ethanol treatment.

Although our findings point to the ERK pathway as a major intersection where lithium and anesthetic drugs have opposing actions, this is very likely not a full explanation for either lithium’s protective action or the apoptogenic action of anesthetic drugs. There is strong evidence that apoptosis can be triggered through kinase signaling systems, but it may require a combined impingement on two or more kinase systems to efficiently induce apoptosis. For example, Marushige and Marushige found that activation of JNK can trigger apoptosis if the Akt pathway is simultaneously suppressed, but JNK activation alone was ineffective. Similarly, Akt suppression alone was ineffective, but it became markedly effective if the ERK pathway was simultaneously suppressed. This conceptual framework provides a potential explanation for the apoptotic actions of ketamine and propofol, in that these drugs suppressed both the ERK and Akt pathways. Whether the Akt pathway contributes to the protective action of lithium is unclear in that lithium did not significantly alter the actions of either ketamine (marginally nonsignificant) or propofol (not approaching significance) on the Akt pathway.

Lithium is an FDA-approved drug for use in the treatment of bipolar affective disorder and has been used extensively off-label for several other neuropsychiatric disorders. However, it has not been approved nor proposed for uses targeting the developing brain. The risk of lithium causing harmful side effects depends on the dose, duration of treatment, and age of the patient at the time of treatment. Lithium is known to have weak teratogenic effects in humans, but this has been described only after chronic exposure of the fetus during the first trimester of pregnancy. There are no known toxic effects associated with exposure of either immature animals or humans to a single, clinically relevant dose of lithium in late gestation or early childhood. However, before lithium could be recommended as a protective therapy against drug-induced developmental neuroapoptosis, a more complete evaluation is needed to further establish both its efficacy and safety. Among the issues that must be carefully investigated is whether lithium’s ability to suppress spontaneous developmental neuroapoptosis has any lasting consequences.


