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11 β -Hydroxylase Inhibitors Protect Against Seizures in Mice by Increasing Endogenous Neurosteroid Synthesis

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Abstract

Steroid 11 β -hydroxylase (CYP11B1; EC 1.14.15.4) is a mitochondrial enzyme located in the zona fasciculata of the adrenal cortex and also in the brain that mediates the conversion of 11-deoxycortisol to cortisol and 11-deoxycorticosterone (DOC) to corticosterone. Inhibitors of CYP11B1, such as metyrapone and etomidate, reduce glucocorticoid synthesis and raise levels of DOC providing greater availability for metabolic conversion to the GABA_A receptor modulating neurosteroid allotetrahydrodeoxycorticosterone (THDOC). Because THDOC is a potent anticonvulsant, it is plausible that CYP11B1 inhibitors could protect against seizures. Here we demonstrate that metyrapone affords dose-dependent protection against 6-Hz seizures 30 min after injection (ED₅₀, 191 mg/kg), but is markedly more potent at 6 h (ED₅₀, 30 mg/kg). Similarly, etomidate is also protective at 30 min and 6 h (ED₅₀ values, 4.5 and 1.7 mg/kg). Finasteride, an inhibitor of neurosteroid synthesis, attenuated the anticonvulsant effects of both CYP11B1 inhibitors at 6 h, but not 30 min following their injection. Plasma THDOC levels measured by liquid chromatography-mass spectrometry were markedly increased 6 h after injection of both CYP11B1 inhibitors and this increase was attenuated by finasteride pretreatment. We conclude that inhibition of CYP11B1 causes delayed seizure protection due to slow build-up of neurosteroids. Early seizure protection is independent of neurosteroids.

Keywords

Neurosteroid; Allotetrahydrodeoxycorticosterone; Steroid 11 β -hydroxylase (CYP11B1) inhibitor; Metyrapone; Etomidate; Finasteride

1. Introduction

Steroid 11 β -hydroxylase (CYP11B1; EC 1.14.15.4) is a mitochondrial enzyme located in the zona fasciculata of the adrenal cortex and also in the brain that converts 11-deoxycortisol to cortisol and 11-deoxycorticosterone (DOC) to corticosterone (Bureik et al., 2002). CYP11B1 inhibition leads to a reduction in cortisol levels, which activates the

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hypothalamic-pituitary-adrenal axis (HPA) via a positive feedback mechanism (Allolio et al., 1985; Sillence et al., 1987; Schulte et al., 1990). The resulting ACTH surge drives the adrenal glands leading to the buildup of upstream precursors, which are then shunted to alternative metabolic pathways. Specifically, levels of DOC are increased providing greater availability for metabolic conversion to the GABA_A receptor modulating neurosteroid allotetrahydrodeoxycorticosterone (THDOC) (Fig. 1). There may also be increases in the levels of the DOC precursor progesterone, which can be converted to the other prototypic GABA_A receptor modulating neurosteroid allopregnanolone. There may even be increases in androgens that are metabolized through testosterone to 5 α -androstenediol, androsterone and etiocholanolone, all of which positively modulate GABA_A receptors (Kaminski et al., 2005; Reddy and Jian, 2010). Because all of these neurosteroids have anticonvulsant properties (Kokate et al., 1994; Reddy and Rogawski, 2002; Kaminski et al., 2005), it is plausible that CYP11B1 inhibitors could protect against seizures. We investigated this possibility using two inhibitors of CYP11B1, metyrapone and etomidate (Carballeira et al., 1976; Dörr et al., 1984; De Coster et al., 1985; Lambert et al., 1986; Weber et al., 1993). Metyrapone is used clinically in the treatment of Cushing's syndrome (hypercorticism). Etomidate is a parenteral anesthetic agent that was discovered shortly after its introduction to induce hypoadrenalism. While this action is generally considered a liability, etomidate has been used to correct hypercortisolemia in seriously ill patients. The protective actions of metyrapone and etomidate were assessed with the 6-Hz seizure model in mice that has been previously shown to be sensitive to neurosteroids (Kaminski et al., 2004). To confirm that treatment with the CYP11B1 inhibitors is associated with increased neurosteroid synthesis we measured plasma levels of THDOC by liquid chromatography–mass spectrometry (LC–MS). We then used the neurosteroid synthesis inhibitor, finasteride (Kokate et al., 1999), to assess whether enhanced neurosteroidogenesis is causally related to the seizure protection conferred by the two CYP11B1 inhibitors.

2. Material and methods

2.1. Animals

Male NIH Swiss mice (25–30 g) were housed three per cage in a vivarium under controlled laboratory conditions (temperature, 22–26°C; humidity, 40–50%) with an artificial 12-h light/dark cycle and free access to food and water. Animals were allowed to acclimate to the vivarium conditions for at least 5 days. The experiments were performed during the light phase of the light/dark cycle after at least a 0.5 h period of acclimation to the experimental room. Animals were maintained in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and studies were performed under protocols approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke (NINDS) in strict compliance with the Guide for the Care and Use of Laboratory Animals of the National Research Council (National Academy Press, Washington, DC; <http://www.nap.edu/readingroom/books/labrats/>).

2.2. Drug administration and neurosteroid measurement

Solutions of etomidate (Sigma-Aldrich, St. Louis, MO) and finasteride (Steraloids, Newport, RI) were made fresh daily in 40% hydroxypropyl- β -cyclodextrin (Trappsol; Cyclodextrin Technologies Development, High Springs, FL) in sterile saline. Further dilutions were made using sterile saline. Metyrapone (Sigma-Aldrich) was dissolved in sterile saline. All drug solutions were administered intraperitoneally in a volume equaling 10 ml/kg at different times before seizure testing or blood collection. The mice were decapitated under brief carbon dioxide narcosis. Trunk blood was collected into tubes containing anticoagulant (EDTA sodium) and centrifuged at 5000 rpm for 15 min at 4 °C. Subsequently blood plasma

was transferred to Eppendorf tubes and stored at -70°C until THDOC measurements were made by LC-MS as previously described (Reddy and Rogawski, 2002).

2.3. 6-Hz seizure test

Anticonvulsant activity at different time points after drug treatment was assessed using a low-frequency electrical stimulation seizure test (Barton et al., 2001) applied according to the protocol of Kaminski et al. (2004). In brief, 3-s corneal stimulation (200-s duration, 32-mA monopolar rectangular pulses at 6 Hz) was delivered by a constant-current device (ECT Unit 5780; Ugo Basile, Comerio, Italy). Ocular anesthetic (0.5% tetracaine) was applied to the corneas 15 min before stimulation. Immediately before stimulation, the corneal electrodes were wetted with saline to provide optimal electrical contact. After the stimulation, the animals exhibited a “stunned” posture associated with rearing and automatic movements that lasted from 60 to 120 s in untreated animals. An animal was considered to be protected from seizures if it resumed its normal exploratory behavior within 10 s of stimulation.

2.4. Data analysis

To construct dose—response curves, metyrapone and etomidate were tested at several doses spanning the dose estimated to produce 50% protection (ED_{50}), which was determined together with its 95% confidence interval by non-linear curve fitting with GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Statistical comparisons between ED_{50} values were made with the same software. Statistical comparisons in the time-course experiments assessing protection against 6-Hz seizures following treatment with the CYP11B1 inhibitors were made with the Fisher’s exact test. Mean plasma levels of THDOC were compared with the Student’s *t*-test. At least 6–8 mice were included in each experimental group.

3. Results

Administration of metyrapone (100 mg/kg) resulted in the slow development of seizure protection in the 6-Hz test, reaching maximum effect at 6 h post-treatment (Fig. 2A). Seizure protection declined during the subsequent 18 h of monitoring. In dose—response experiments, treatment with metyrapone at doses of 10 to 300 mg/kg resulted in dose-dependent seizure protection (Figs. 2B and 3). The ED_{50} values at 0.5 and 6 h after treatment were 191 and 30 mg/kg, respectively. Finasteride (50 mg/kg) administered 0.5 h prior to metyrapone did not affect the anticonvulsant potency assessed at 0.5 h post-treatment but it did significantly decrease the potency of metyrapone at 6 h post-treatment (Fig 2B). Mean baseline plasma THDOC levels in vehicle treated mice were 0.30 ± 0.07 ng/ml (Fig. 4). Six h after treatment with metyrapone mean THDOC levels increased to 2.0 ± 0.2 ng/ml. Finasteride pretreatment reduced the metyrapone-induced increase in THDOC levels at 6 h by one-half to 1.0 ± 0.3 ng/ml.

Etomidate (10 mg/kg) displayed a different time-course for seizure protection than metyrapone (Fig. 2C). Significant seizure protection was observed at 0.5 and 6 h after injection. In dose—response experiments, etomidate had potent activity at both time points (Fig. 2D and 3). However, as in the case of metyrapone, finasteride pretreatment reduced the potency of etomidate at the 6 h post-treatment time point but not at the 0.5 h time point (Fig. 2D). Mean plasma levels of THDOC were also markedly elevated 6 h after etomidate injection (1.8 ± 0.1 ng/ml) and finasteride pretreatment once again attenuated this increase (0.8 ± 0.1 ng/ml) (Fig. 4).

4. Discussion

Our results demonstrate that metyrapone and etomidate exhibit a substantial protective action in the 6-Hz seizure test. Both drugs displayed a peak effect 6 h following administration, which was associated with a marked increase in plasma concentrations of the neurosteroid THDOC. Furthermore, finasteride pretreatment was associated with a reduction in the increase in plasma THDOC at 6 h and an elevation in the drugs' ED₅₀ values for seizure protection. It is noteworthy, however, that finasteride did not have any effect on the anticonvulsant potency of metyrapone and etomidate at 0.5 h following injection. At that time metyrapone's anticonvulsant potency was more than 6-fold lower than its peak potency at 6 h whereas etomidate's potency was less than 3-fold lower.

Etomidate is well recognized to directly enhance the activity of GABA_A receptors (Ashton and Wauquier, 1985; Proctor et al., 1986; Belelli et al., 1997; Hill-Venning et al., 1997; Rüscher et al., 2004.). Agents that positively modulate GABA_A receptors have been found to confer robust protection in the 6-Hz seizure test (Kaminski et al., 2004). Therefore, while etomidate has not previously been shown to have activity in the 6-Hz test, its early, finasteride-insensitive anticonvulsant activity is likely mediated by direct actions on GABA_A receptors. There is no evidence that metyrapone acts directly on GABA_A receptors. However, metyrapone does enter the brain (Stith et al., 1976). Its weak anticonvulsant activity at 0.5 h could be due to low potency effects on any of a number of anticonvulsant targets; an action on GABA_A receptors cannot be excluded.

Metyrapone and etomidate are known to inhibit CYP11B1, resulting in a suppression of glucocorticoid synthesis (Jenkins et al., 1958; Carballeira et al., 1976). Reduced negative feedback of the HPA axis leads to elevated ACTH, which stimulates steroidogenesis. In the face of CYP11B1 inhibition, DOC increases markedly (Allolio et al., 1985), which can be shunted to the production of the anticonvulsant neurosteroid THDOC (Fig. 1). Indeed, our measurements indicate dramatic increases in plasma THDOC levels with both agents. Although it is plausible that CYP11B1 inhibition would also lead to an elevation in progesterone, which could be shunted to the production of allopregnanolone (also a powerful anticonvulsant), at most, modest increases in progesterone have been observed with metyrapone (Schöneshöfer et al., 1980) and etomidate (Dörr et al., 1984; Allolio et al., 1985), suggesting that allopregnanolone does not play a substantial role in the anticonvulsant action of the CYP11B1 inhibitors. The marked reduction induced by pretreatment with finasteride in the anticonvulsant potencies of metyrapone and etomidate at 6 h supports the concept that delayed production of neurosteroids is a major factor in the anticonvulsant activity at this time point. The fact that finasteride does not affect the anticonvulsant potencies at 0.5 h demonstrates that it does not induce a proconvulsant action which could lead to an erroneous conclusion regarding the involvement of neurosteroids. The failure of finasteride to alter baseline seizure susceptibility in the absence of stimulated neurosteroid production has been noted previously (Kokate et al., 1999; Lawrence et al., 2010).

In conclusion, our results demonstrate that CYP11B1 inhibition produces delayed seizure protection at least in part due to slow build-up of THDOC. Whether other neurosteroids such as the other major pregnane neurosteroid allopregnanolone or the androstane neurosteroids 5 α -androstenediol, androsterone and etiocholanolone play a role remains to be determined. THDOC that is produced in the periphery readily enters the brain to induce anticonvulsant actions. However, CYP11B1 is widely expressed in brain (Mellon and Deschepper, 1993) and there is evidence for in situ production of neurosteroids, including THDOC, that is independent of the adrenal gland (Purdy et al., 1991). Therefore, it is conceivable that

inhibition of brain CYP11B1 and enhancement of local neurosteroidogenesis could contribute to the anticonvulsant action of metyrapone and etomidate.

Etomidate is a general anesthetic that has previously been reported to have anticonvulsant properties in animals (Ashton, 1983; Lowson et al., 1991; Borowicz and Czuczwar, 2003) and may have clinical utility in the treatment of status epilepticus (Yeoman et al., 1989). In some instances, however, etomidate paradoxically induces epileptiform discharges and seizures (Gancher et al., 1984; Ebrahim et al., 1986; Sen et al., 2010). Although metyrapone is not ordinarily considered to be an anticonvulsant agent, doses similar to those used in the present study may produce delayed seizure protection in some circumstances (Krugers et al., 1998). We have now shown that neurosteroids contribute to the anticonvulsant activity of both agents. Moreover, our results indicate that targeted inhibition of CYP11B1, by boosting endogenous anticonvulsant mechanisms, may provide a new strategy for epilepsy therapy, although approaches to address the adverse effects of CYP11B1 inhibition such as co-medication with corticosteroids may be required. Our results also suggest that the high efficacy of etomidate in the treatment of status epilepticus (Shorvon, 1994) and possibly its anesthetic actions as well (Vanlersberghe and Camu, 2008) may be due to the combination of a direct action on GABA_A receptors and the indirect effects mediated by enhanced neurosteroidogenesis.

Abbreviation

THDOC allotetrahydrodeoxycorticosterone

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Fig. 1.

Adrenal steroid biosynthetic pathways illustrating shunting toward the production of the neurosteroids allopregnanolone and THDOC when 11 β -hydroxylase is inhibited by metyrapone and etomidate. Finasteride inhibits 5 α -reductase isoenzymes, reducing neurosteroid production. 3 α -HSD, 3 α -hydroxysteroid oxidoreductase isoenzymes. Open arrowheads denote enzyme inhibition.

Fig. 2.

Metyrapone and etomidate confer protection in the 6-Hz seizure test that is sensitive to finasteride at 6 h but not 0.5 h. A, C: Time courses for the protective effects of metyrapone (A) and etomidate (C) in the 6-Hz seizure test. The data values shown represent the percent of mice protected in groups of 6–8 animals; all vehicle-treated mice experienced seizures. B, D: ED₅₀ values with 95% confidence intervals for protection by metyrapone (B) and etomidate (D) in the 6-Hz seizure test with and without finasteride pretreatment (50 mg/kg, i.p., 0.5 h prior to metyrapone or etomidate). The values are derived from dose—response experiments (shown in Fig. 3) with doses of metyrapone (10–300 mg/kg) and etomidate (0.3–20 mg/kg). ζ , $p < 0.05$ vs. vehicle-treated mice; Ψ , $p < 0.05$ vs. metyrapone or etomidate alone at 6 h post-injection.

Fig. 3.

Dose—response data for seizure protection conferred by metyrapone and etomidate at 0.5 and 6 h in the 6-Hz seizure test with and without pretreatment with finasteride. Curves indicate best logistic fits to the data by global nonlinear curve fitting with Hill slopes constrained to 2.3 for metyrapone and 1.3 for etomidate. The global R^2 values are 0.92 and 0.78 for metyrapone and etomidate, respectively. ED_{50} values with 95% confidence intervals are presented in Fig. 2B and D.

Fig. 4.

Plasma levels of allotetrahydrodeoxycorticosterone (THDOC) 6 h following injection of metyrapone (100 mg/kg) and etomidate (10 mg/kg) with and without finasteride pretreatment (50 mg/kg, i.p., 0.5 h prior to drug treatment). ζ , $p < 0.05$ vs. vehicle-treated mice; Ψ , $p < 0.05$ vs. metyrapone or etomidate alone.