BRIEF COMMUNICATION

Decreased allopregnanolone levels in cerebrospinal fluid obtained during status epilepticus

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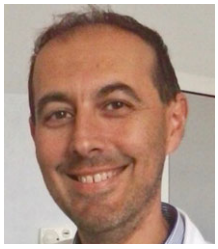

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Summary

Neuroactive steroids are increasingly considered as relevant modulators of neuronal activity. Especially allopregnanolone (AP) and pregnenolone sulfate (PS) have been shown to possess, respectively, anticonvulsant or proconvulsant properties. In view of the potential role of these steroids, we aimed at evaluating AP and PS levels in cerebrospinal fluid (CSF) and blood samples obtained from patients with status epilepticus (SE). To this purpose, we enrolled 41 patients affected by SE and 41 subjects investigated for nonepileptic neurologic disorders. Liquid chromatographic procedures coupled with electrospray tandem mass spectrometry and routine laboratory investigations were performed. Significantly lower AP levels were found in the CSF of patients affected by SE (−30%; $p < 0.05$, Mann-Whitney test). Notably, AP was not detectable in 28 of 41 patients affected by SE ($p < 0.01$ vs. controls, Fisher's exact test). In serum, AP levels did not differ in the two considered groups. Conversely, PS was present at similar levels in the investigated groups. Finally, differences in AP levels could not be explained by a variation in CSF albumin content. These findings indicate that AP is defective in the CSF of patients affected by SE. This phenomenon was not dependent on carriers for steroids, such as albumin.

KEY WORDS: Allopregnanolone, Cerebrospinal fluid, LC-MS/MS, Pregnenolone sulfate, Status epilepticus.

In the central nervous system (CNS), allopregnanolone (AP) behaves as a positive modulator of γ-aminobutyric acid type A ($GABA_A$) receptor, and it definitely enhances both tonic and phasic $GABA_A$-dependent inhibitory currents.[1–3] This biologic property has been investigated in different models of epilepsy, in which it was consistently demonstrated that AP is neuroprotective and anticonvulsant.[4] In contrast, sulfated neurosteroids such as pregnenolone sulfate (PS) negatively modulate the $GABA_A$-dependent inhibition.[2] Specifically, PS was shown to induce seizures when injected into the brain.[5]

Despite this evidence, few data are currently available on the possible role of AP and PS in human epileptic disorders.[6] One of the reasons for this still incomplete knowledge is the difficulty in quantifying with high specificity the neuroactive steroids by currently available methods.[7] To address this question, Rustichelli et al.[8] recently set a method based on the removal of interfering phospholipids from samples; PS was clearly detected and measured using this method. On the other hand, AP was probably incompletely separated from other steroids, such as the cognate molecule pregnanolone (PREG), which was not previously considered as a possible co-eluting isomer. Although PREG is also a positive modulator of $GABA_A$ currents, with slightly lower potency in comparison to AP, a correct identification of PREG and AP is required to clarify which step in their processing could be definitely altered.[1]

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For these reasons, we aimed at further improving our protocol by setting up a procedure to clearly separate PREG from AP. Then we applied the new procedure to evaluate the role of AP and PS in a serious neurologic disorder such as status epilepticus (SE). The role of neurosteroids appears to be particularly relevant since, recently, administration of AP has been proposed as possible therapeutic innovation to overcome the early refractoriness to benzodiazepines observed during SE.[9,10]

Materials and Methods

Chemicals and reagents

Amplifex-Keto Reagent Kit, human albumin (≥96%), AP (3α-hydroxy-5α-pregnan-20-one), PREG (3α-hydroxy-5β-pregnan-20-one), and PS (5-pregnen-3β-ol-20-one sulfate) were from Sigma-Fluka (St. Louis, MO, U.S.A.). The internal standards (ISs) sodium pregnenolone-17α,21,21,21-D_4 sulfate (PS-D_4) and 5α-pregnan-3α-ol-20-one-17α,21,21,21-D_4 (AP-D_4) were from CDN Isotopes (Quebec, Canada). The 3β isomers of AP and PREG, respectively, epiallopregnanolone (epiAP) and epipregnanolone (epiPREG) (Steraloids Inc., Newport, RI, U.S.A.), were used to monitor their retention times. Liquid chromatography-mass spectrometry (LC-MS) purity grade acetonitrile, methanol, formic acid, and ammonium formate were from Sigma-Fluka; ultra-pure water was purified by a Milli-Q Plus185 system from Millipore (Milford, MA, U.S.A.). Phree Phospholipid Removal Tubes (1.0 ml) were supplied by Phenomenex (Torrance, CA, U.S.A.).

High-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

See the Supporting Information Data S1.

Sample processing

Serum and cerebrospinal fluid (CSF) samples (200 μl) were spiked with 50 μl of the IS solution, vortexed (90 s), and added with 1,000 μl of acetonitrile/methanol (70/30; +1.0% formic acid). The samples were then sonicated (10 min, 4°C), centrifuged (27,627 g, 10 min, 10°C), and the supernatants were purified on Phree-SPE cartridges to remove endogenous phospholipids. Eluates were evaporated (Concentrator Plus #5305; Eppendorf AG, Hamburg, Germany) at 35°C and derivatized with 50 μl of Amplifex Keto Reagent for 1 h at room temperature. Subsequently, samples were added with 150 μl methanol/water (70/30), centrifuged (20,627 g, 10 min, 10°C), and transferred in autosampler vials for LC-MS/MS analysis. Injection volume: 10 μl.

Patients

We retrospectively considered records of patients admitted with SE between January 2007 and August 2015, who received lumbar puncture at SE onset or during SE. Serum and CSF samples were extracted from our CSF bank by considering patients for whom the clinical information and electroencephalography (EEG) were diagnostic for SE. SE was defined as ongoing seizures, or repetitive seizures without normalization of consciousness or return to baseline for at least 30 min.

The treatment protocol for SE was similar in all the patients and followed the guidelines of the Italian League Against Epilepsy.[11] If seizures persisted after antiepileptic drug (AED) treatment, the SE was considered refractory or superrefractory, and patients were treated with one or multiple third-line agents.[12]

Accordingly, 41 patients with SE (63% female; 37% male) with a mean age of 55.8 years (ranging from 11 to 75 years) were investigated (see Table S1). Serum and CSF samples were acquired simultaneously from a few hours after admission to a maximum of 20 days (median of 4 days). Only one patient had previous epilepsy, whereas about half of the patients had an acute symptomatic SE episode and the 17% had a de novo SE (without definite etiology). More than 80% of the population had a nonconvulsive or a partial complex SE. Resolution after AEDs was reported in 56% of patients, whereas third-line treatments were necessary for the others. In these latter ones, 30-day mortality after SE was 15%.

The SE group was compared with patients who underwent a lumbar puncture for suspected idiopathic intracranial hypertension, CNS infection, or inflammatory disease between 2007 and 2015. Fifty subjects who had negative results at the end of the diagnostic workup were considered as nonepileptic control population. Nine patients were excluded from this initial group due to pathologic findings in CSF parameters (high intracranial pressure, high cell counts, and positivity for oligoclonal bands). The final control group consisted of 41 subjects (see Table S2). The mean age was 45.6 years (range 16–80); 28 were female (68%) and 13 were male (32%).

The health service (AUSL-Modena) Institutional Review Board approved the research protocol according to local regulations in accordance with the current revision of the Helsinki Declaration, and informed written consent was obtained from patients or their relatives.

Statistics

Data were compared using the Fisher's exact test or the Mann-Whitney test (Sigmaplot 11; Systat Software, San Jose, CA, U.S.A.). Results are presented as median and interquartile range (IQR), and they were regarded significantly different at $p < 0.05$.

Results

Steroids were derivatized as indicated in Figure S1,[13] and calibration curves were obtained for each analyte (Fig. S2). Elution ensured the complete separation of PREG and AP

and prevented any interference with other analytes (Fig. S3). Multiple reaction monitoring (MRM) ratios (Table S3) confirmed that interference from the biologic matrix was prevented (Fig. S4). Our method was validated (Tables S4 and S5) and allowed an accurate and precise detection of AP, PREG, and PS (Data S1). Each analyte level was determined via regression curve of the area ratios of the analyte to the corresponding IS.

In CSF obtained from both patients affected by SE and controls, AP was clearly detectable in most but not all samples (Fig. 1). Specifically, AP was below the limit of quantification in 15 of 40 controls (one sample was missing), and in 28 of 41 patients affected by SE ($p < 0.01$, Fisher's exact test). Then, we compared AP values by excluding subjects in which the analyte was undetectable, and found significantly lower levels (−30%; $p < 0.05$, Mann-Whitney test) in patients affected by SE (Fig. 1A). This difference was unaffected by excluding outliers from controls. At variance, AP was detectable in all serum samples, but no difference was found by comparing patients affected by SE with controls (Fig. 1B).

Changes similar to those found for AP were demonstrated also for PREG (not shown). Specifically, PREG was measured in CSF of 32 controls (0.022 ng/ml, IQR 0.016–0.025) and 34 patients (0.018 ng/ml, IQR 0.014–0.020; $p < 0.05$). No differences were instead found for serum levels (controls: 0.030 ng/ml, IQR 0.018–0.064; patients: 0.028 ng/ml, IQR 0.018–0.063).

Peaks corresponding to PS were detected in all sera, whereas 34 of 41 patients affected by SE and 36 of 40 controls presented detectable PS in CSF. As shown in Figure 2A, PS levels were not significantly different in CSF. Similarly, quantification of PS in serum of both groups did not reveal significant differences (Fig. 2B).

Finally, we considered the possibility that the alterations found in AP and PREG levels could be explained by CSF albumin content or, additionally, could be influenced by sex differences. However, albumin was present at similar concentration in CFS of both groups (SE patients: 19.1 mg/dl, IQR 12.8–25.1; controls: 17.9 mg/dl, IQR 11.2–22.7), and similar levels were observed for all analytes in both males and females of controls (Table S6) and patients (Table S7).

Discussion

We evaluated AP, PREG, and PS in CSF of patients affected by SE using a modified LC-MS/MS approach. Major achievements of this investigation were the following: (1) the possibility of separating and quantifying AP and

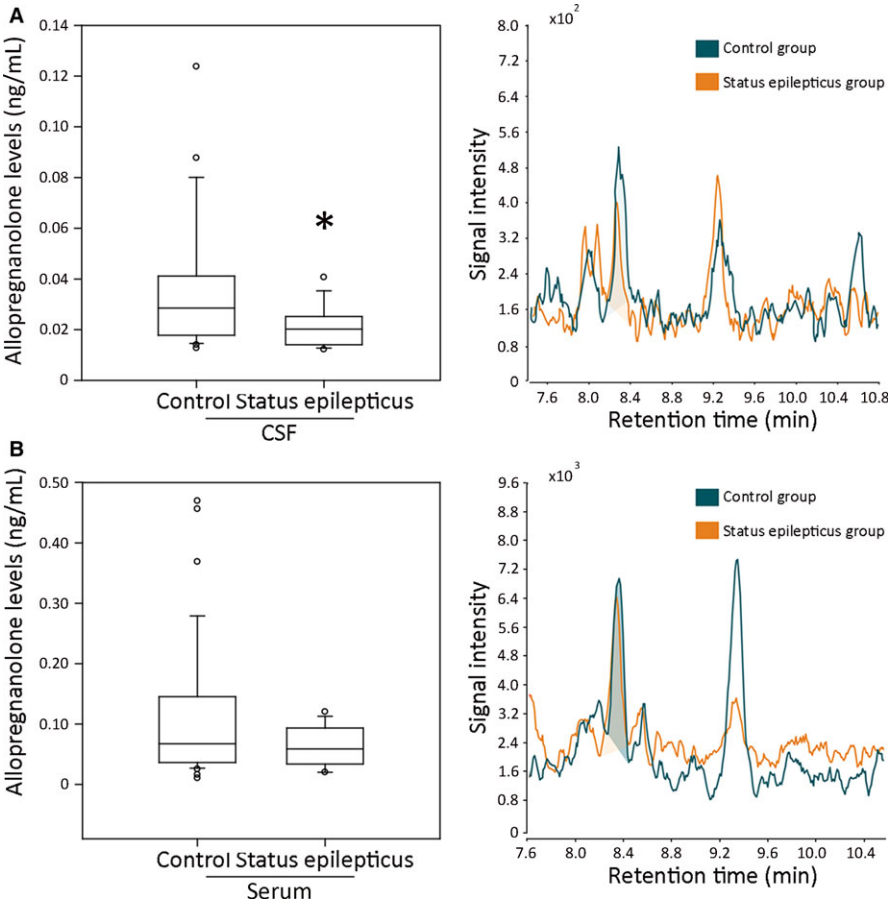

Figure 1.
Allopregnanolone (AP) levels measured in cerebrospinal fluid (CSF) and serum in the course of status epilepticus (SE). In (**A**), AP levels in CSF, illustrated in box plot, were significantly lower (*$p < 0.05$, Mann-Whitney test) in patients affected by SE compared with controls. Peak areas corresponding to the respective median values are illustrated on the right. In (**B**), AP levels in serum were not significantly different in patients affected by SE compared with controls. Peak areas corresponding to the respective median values are illustrated on the right.

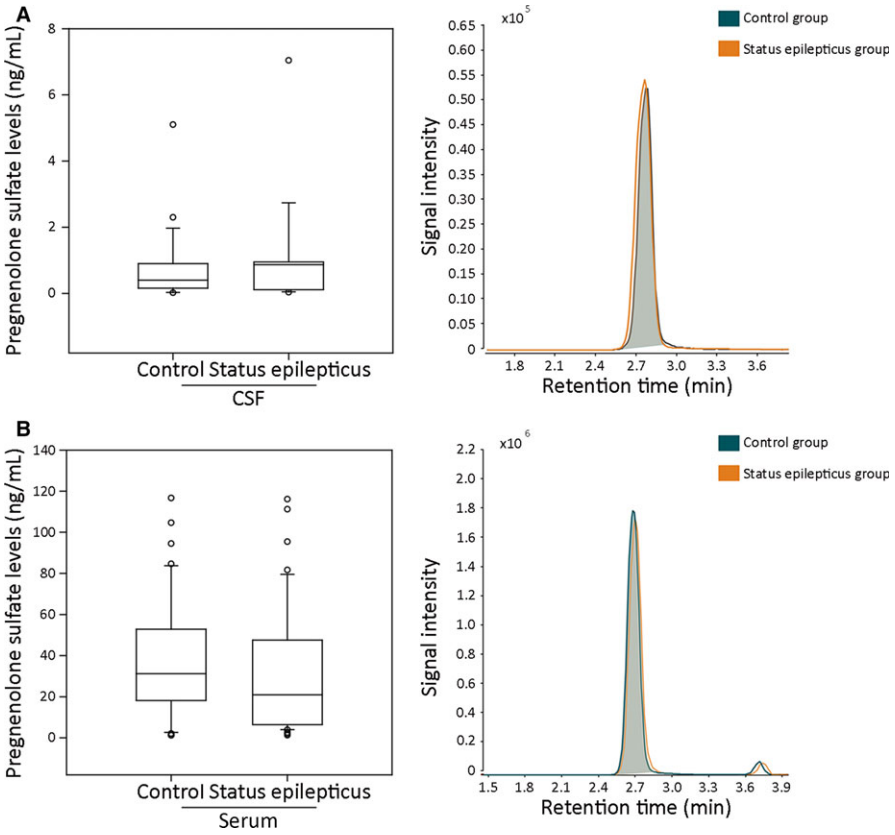

Figure 2.
Pregnenolone sulfate (PS) levels measured in cerebrospinal fluid (CSF) and serum in the course of status epilepticus (SE). In (**A**), PS levels in CSF, illustrated in boxplot, were significantly similar in patients affected by SE compared with controls. Peak areas corresponding to the respective median values are illustrated on the right . In (**B**), PS levels in serum were not significantly different in patients affected by SE compared with controls. Peak areas corresponding to the respective median values are illustrated on the right.

PREG, and (2) the observed significant reduction in CSF but not in serum of these steroids, in presence of (3) stable levels of PS. These results suggest that synthesis of endogenous anticonvulsants such AP and PREG was defective or that, alternatively, their catabolism was enhanced in patients in which seizures evolved into SE. Moreover, reduction in their levels may result in enhanced modulation by proconvulsant steroids such as PS.

The possibility of characterizing the role of neuroactive steroids in humans and animal models has been hampered by technical limitations, of which the "matrix effect" is the most important for LC-MS/MS.[14] Here we show that it is possible to overcome this specific problem by increasing the efficiency of phospholipid removal.[7] Using this strategy we obtained a specific identification and quantification of AP, PREG, and PS in human CSF and serum samples, leading to the observation of the selective reduction in CSF AP and PREG levels. Because no other data are available on these steroids in SE, we cannot exclude that the observed changes might have preceded the SE. The few available studies on peripheral AP in epileptic patients suggest the presence of reduced interictal levels[15] and biphasic changes following a seizure.[6] However, almost all of our patients had no epilepsy before developing SE.

Indeed, the role of AP has been investigated both in animal models, and humans and inhibition of 5α-reductase by finasteride consistently resulted in increased epileptic activity.[2,4,6,15] No information is instead available for 5β-reductase, which synthesizes PREG. Thus, it is possible that a reduced activity of 5α-reductase in SE could be responsible for lower AP availability in CSF. However, we found that also PREG was affected in a similar manner, suggesting that 5β-reductase and maybe other enzymes could be dysregulated in SE. Alternatively, a reduced availability of a common substrate of these enzymes, such as progesterone, may be the reason for the reduced availability of AP and PREG in SE. In such a case, restorative therapies could be taken into account.

Because of their lipophilic nature, steroids require a carrier to be solubilized in CSF. For this reason, we hypothesized that changes in CSF albumin could explain the differences observed in AP and PREG levels. However, albumin levels were similar in both groups of patients, suggesting that AP was less available under SE. In addition, no differences were found for the proconvulsant neurosteroid PS, for which the number of available samples was similar to that of PREG, so that we exclude that differences in albumin or other carriers could have affected our results.

In conclusion, an important consequence of our study is that AP and PREG reduction may contribute to the epileptic activity observed in SE. In such a case, to reestablish levels of these steroids may be an important therapeutic target in patients affected by SE. Indeed, the limited, but encouraging, evidence obtained in pediatric patients treated with AP

to stop superrefractory SE is in agreement with this hypothesis.[10]

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Disclosure of Conflict of Interest

SM has received personal compensation as scientific advisory board member for UCB and EISAI. All the other authors declare no conflict of interest. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. Chemical structures of the selected neurosteroids as quaternary aminooxy (QAO) derivatives.

Figure S2. Calibration curves for all investigated analytes.

Figure S3. Representative MRM chromatogram of a calibration sample showing the monitored transitions for the selected neurosteroids as QAO derivatives.

Figure S4. Representative MRM chromatogram showing the QAO neurosteroids in the serum of a control subject.

Table S1. Demographic and clinical features of the patients with SE.

Table S2. Demographic and clinical features of the control population.

Table S3. Experimental HPLC-MS/MS parameters for the target neurosteroids as QAO derivatives.

Table S4. Calibration curve parameters for the derivatized neurosteroids.

Table S5. Method validation data for the derivatized neurosteroids.

Table S6. Levels of analytes in subgroups of male and female controls.

Table S7. Levels of analytes in subgroups of male and female patients.