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Neuroactive steroids, their precursors, and polar conjugates during parturition and postpartum in maternal and umbilical blood: 1. identification and simultaneous determination of pregnanolone isomers

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Abstract

A rapid method for the identification and measurement of four pregnanolone isomers and their polar conjugates in human plasma was developed using a simple quadrupole GC/MS system with electron impact ionization. Steroid levels were measured in the plasma of 13 and three women at delivery with subarachnoidal and epidural analgesia, respectively, and in corresponding samples of umbilical plasma. A good correlation ($r = 0.94$, $P < 0.001$, $n = 8$) was found between the allopregnanolone in maternal plasma determined by GC/MS and that measured by RIA. Epipregnanolone (3β-hydroxy-5β-pregnan-20-one) was identified and measured for the first time in human plasma; its concentration in both maternal and umbilical plasma was much lower than that of other pregnanolone isomers. The levels of 3β-hydroxy-pregnanolone isomers were significantly higher in the umbilical plasma than in the maternal plasma, while the differences in 3α-hydroxy-isomers were insignificant. The differences in conjugates were insignificant except in the case of allopregnanolone, the levels of which were lower in umbilical plasma. In all of the pregnanolone isomers, a significantly lower conjugated/unconjugated steroid ratio was found in the umbilical plasma than in the maternal plasma. The possible role of the sulfatation of pregnanolone isomers around parturition is discussed.

Keywords: Pregnanolone isomers; Epipregnanolone; Allopregnanolone; Polar conjugates; Human parturition; GC/MS; Maternal serum; Umbilical serum

1. Introduction

Neuroactive steroids have recently been the subject of increasing interest among biochemists and physicians. These steroids regulate neuronal activity primarily as modulators of neurotransmitter receptors influencing the permeability of ion channels [1–7]; in addition, they act at progesterone receptors [8,9]. Pregnenolone sulfate, found in body fluids in relatively high concentrations, promotes the influx and rapid activation of calcium into the neuron through the activation

Abbreviations: AlloPal, allopregnanolone, 3α-hydroxy-5α-pregnan-20-one; AlloPalC, conjugated allopregnanolone; EpiPal, epipregnanolone, 3β-hydroxy-5β-pregnan-20-one; EpiPalC, conjugated epipregnanolone; GABA, γ-aminobutyric acid; GC/MS, gas chromatography/mass spectrometry; HPLC, high performance liquid chromatography; NMDA, *N*-methyl-D-aspartate; Pal, pregnanolone, 3α-hydroxy-5β-pregnan-20-one; PalC, conjugated pregnanolone; IsoPal, isopregnanolone, 3β-hydroxy-5α-pregnan-20-one; IsoPalC, conjugated isopregnanolone; RIA, radioimmunoassay; SIM, selected ion monitoring.

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of membrane *N*-methyl-D-aspartate (NMDA) receptors [1,2,10]. Epipregnanolone sulfate has been reported in *Xenopus laevis* oocytes as a competitor of pregnenolone sulfate known to act in an opposite manner [10]. NMDA receptors are present not only in CNS but also in the periphery [11,12]. Some steroid sulfates act as negative GABA receptor modulators [4–7,13], in contrast to their non-conjugated analogues [3,8,14,15].

The course of the action of pregnanolone isomers can even be reversed by their sulfatation at position C-3

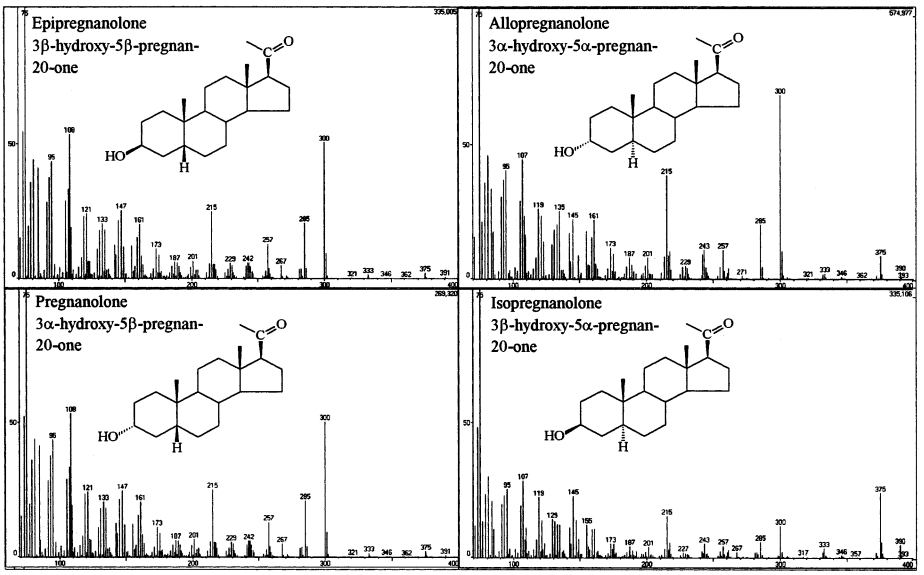

Fig. 1. Mass spectra of pregnanolone isomers. A QP 5050 A quadrupole electron-impact detector from Shimadzu with a fixed electron voltage of 70 eV was used for the measurement.

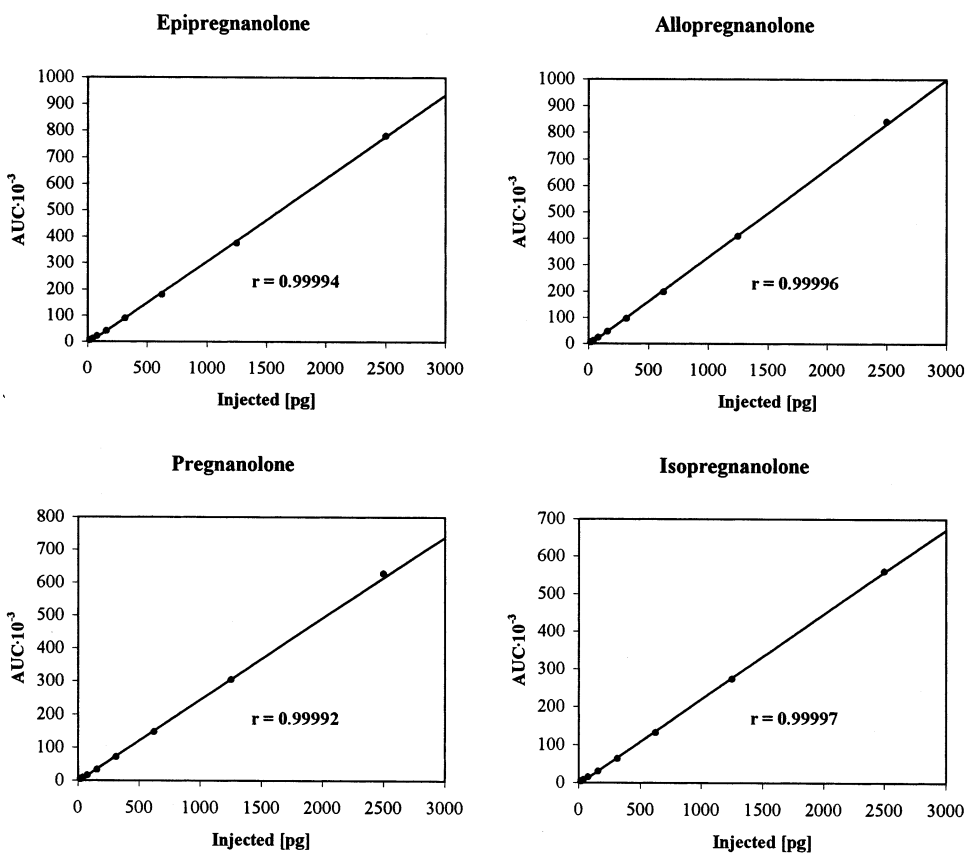

Fig. 2. Linearity of the response of the GC/MS system to pregnanolone isomers in SIM mode. The peak area is represented by AUC.

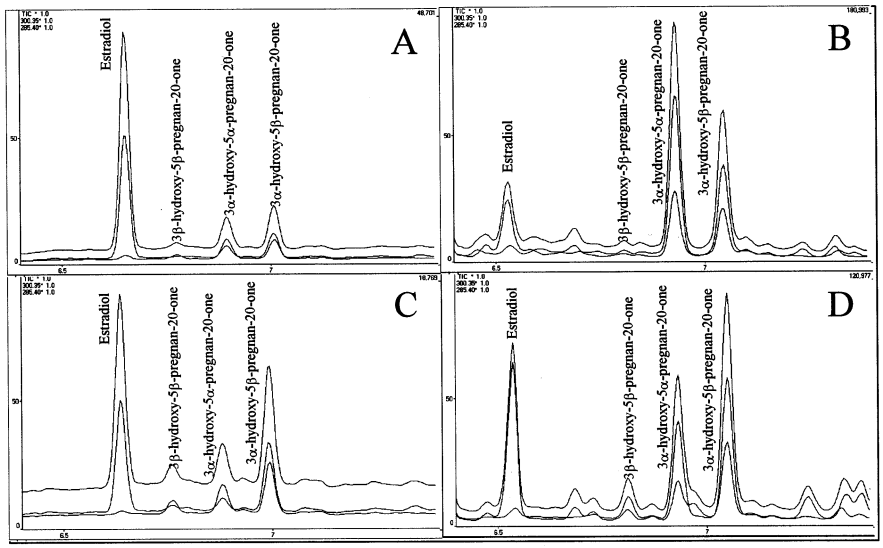

Fig. 3. Chromatograms of maternal and umbilical blood at delivery, measured using a GC/MS system in SIM mode: (A) unconjugated steroids in maternal plasma; (B) conjugated steroids in maternal plasma; (C) unconjugated steroids in umbilical plasma; (D) conjugated steroids in umbilical plasma. The isopregnanolone peak is not shown because of the notable difference in retention time from the other pregnanolone isomers.

Table 1
Levels of unconjugated pregnanolone isomers (nmol/l) in maternal and umbilical plasma at delivery ($n = 16$)[a]

Plasma	Epipregnanolone (5β,3α-OH)		Allopregnanolone (5α,3α-OH)		Pregnanolone (5β,3α-OH)		Isopregnanolone (5α,3β-OH)	
	M	U	M	U	M	U	M	U
Mean	2.32	5.95	28.78	26.99	18.92	32.82	8.31	19.87
S.E.M.	0.44	1.02	6.62	5.56	4.37	9.17	1.72	4.01
Median	1.87	4.78	20.37	16.11	12.08	17.26	6.06	17.72
25% percentile	1.01	3.65	9.96	11.55	6.58	9.85	3.68	6.44
75% percentile	3.41	7.13	36.75	32.39	30.34	39.33	10.81	29.85
Significance of the differences	$P < 0.0001$		NS		NS		$P < 0.05$	

[a] The differences between materal (M) and umbilical (U) plasma were evaluated using Student's t-test.

Table 2
Levels of conjugated pregnanolone isomers (nmol/l) in maternal and umbilical plasma at delivery ($n = 8$)[a]

Plasma	Epipregnanolone (5β,3α-OH)		Allopregnanolone (5α,3α-OH)		Pregnanolone (5β,3α-OH)		Isopregnanolone (5α,3β-OH)	
	M	U	M	U	M	U	M	U
Mean	15.8	14.7	358	141	308	175	269	146
S.E.M.	2.4	4.6	66	17	79	20	61	40
Median	15.3	11.9	293	139	266	182	194	117
25% percentile	10.4	9.7	233	121	135	146	141	69
75% percentile	19.2	13.9	496	182	403	196	418	188
Significance of the differences	NS		$P < 0.01$		NS		NS	

[a] The differences between materal (M) and umbilical (U) plasma were evaluated using Student's t-test.

[16]. A precursor of pregnanolone isomers, progesterone, decreases near parturition. As Leng and Russell [22] have previously suggested, a decrease in the levels of pregnanolone isomers (which are also produced by placenta [17,18]) could trigger the production of oxytocin [19–21], thus resulting in a rapid delivery.

In this study, the authors investigated all of the isomers of pregnanolone, i.e. epipregnanolone (3β-hydroxy-5β-pregnan-20-one), allopregnanolone (3α-hydroxy-5α-pregnan-20-one), pregnanolone (3α-hydroxy-5β-pregnan-20-one), and isopregnanolone (3β-hydroxy-5α-pregnan-20-one). The primary aim was to develop a

simple, rapid method for the simultaneous measurement of pregnanolone isomers-including their polar conjugates-in maternal and umbilical plasma at delivery. Epipregnanolone and its sulfate were identified for this purpose, and measured in humans for the first time. Given the differences in steroid metabolism between mother and fetus, attempts were made to evaluate the differences in the levels of individual pregnanolone isomers and their polar conjugates at delivery. The following questions were addressed:

1. How do the concentrations of unconjugated pregnanolone isomers in maternal and umbilical plasma differ?
2. How do the concentrations of conjugated pregnanolone isomers in maternal and umbilical plasma differ?
3. For these steroids, what is the difference in the conjugated/unconjugated steroid ratio between maternal and umbilical plasma?

2. Experimental method

2.1. Subjects

The patient group consisted of 16 women at delivery, of whom 13 were treated with subarachnoidal and three with epidural analgesia. All of the women in the study were primiparous, 20–25 years old, with heights varying within the range 160–175cm and body weights within the range 55–70 kg. The body-mass increments in pregnancy did not exceed 12 kg, and no anamnestic stresses were detected in the subjects. All of the parturitions were in term and spontaneous between 6 a.m. and 6 p.m., with a usual presentation of the fetus.

In the cases of epidural analgesia, the anesthetic was applied using only equipment from B. Braun (Perifix 421) (Melsungen, Germany). Bupivacain local anesthetic from Marcain (Astra, Stockholm, Sweden) was diluted by saline to 0.125%, and 10 μg of sufentanil Sufenta from Janssen-Cillag (Zürrich, Switzerland) were added to the solution. The total dose did not exceed 30 μg of sufentanil. The method of subarachnoidal analgesia was similar, except that the first dose of sufentanil was introduced directly into the subarachnoidal space.

Informed written consent was obtained from all of the subjects both for the collection and utilization of the samples.

2.2. Sample collection

Samples of maternal blood were collected when the cervical dilatation reached a diameter of 10–11 cm. A sample of umbilical blood was withdrawn immediately after the separation of the newborn from the umbilical cord. Each sample was collected into a cooled plastic tube containing 100 μl of 5% EDTA and 50 pl of aprotinin (Antilysin from Spofa, Prague, Czech Republic). The plasma was obtained using centrifugation for 5 min at 2000 *g* at 0°C. The plasma samples were stored at −20°C until analyzed.

2.3. Steroids and chemicals

The non-radioactive steroids and their conjugates were from Steraloids (Wilton, NH). The solvents for extraction and HPLC, and pyridine were of analytical grade and were from Merck (Darmstadt, Germany). The derivatization agent Sylon BFT was purchased from Supelco (Bellefonte, PA).

2.4. Instruments

The GC/MS system was supplied by Shimadzu (Kyoto, Japan). The system consisted of a GC 17A gas chromatograph equipped with automatic flow control, AOC-20 autosampler and for the MS QP 5050A quadrupole electron-impact detector with a fixed electron voltage of 70 eV. The liquid scintillation spectrom-

Table 3
Comparison of the conjugated/unconjugated steroid ratios of pregnanolone isomers in maternal umbilical plasma at delivery ($n = 8$)[a]

Plasma	Epipregnanolone (5β,3α-OH)		Allopregnanolone (5α,3α-OH)		Pregnanolone (5β,3α-OH)		Isopregnanolone (5α,3β-OH)	
	M	U	M	U	M	U	M	U
Mean	8.9	2.8	24.4	8.4	26.5	8.5	59.1	8.8
SEM	1.6	0.6	7.1	1.4	7.3	2.2	19.0	2.3
Median	9.6	2.4	23.0	8.0	24.3	8.7	39.9	6.3
25% percentile	5.1	1.8	9.0	4.9	9.2	3.1	21.7	4.1
75% percentile	12.6	4.1	28.7	12.4	43.3	11.7	89.6	14.2
Significance of the differences	$P<0.01$		$P<0.05$		$P<0.05$		$P<0.01$	

[a] The differences between materal (M) and umbilical (U) plasma were evaluated using Student's *t*-test.

eter was supplied by Beckmann Instruments, (Fullerton, CA).

2.5. Analytical methods

2.5.1. GC/MS identification and determination of pregnanolone isomers

The pregnanolone isomers epipregnanolone (EpiPal), allopregnanolone (AlloPal), pregnanolone (Pal), and isopregnanolone (IsoPal), and their polar conjugated analogues EpiPalC, AlloPalC, PalC, and IsoPalC, were measured using the GC/MS system as follows.

2.5.1.1. Extraction and hydrolysis. Plasma (500 μl) was extracted with 1.5 ml of diethyl ether. The polar phase was frozen in a mixture of ethanol and solid carbon dioxide, and stored until further treatment.

The organic phase was separated and evaporated in a vacuum centrifuge. To eliminate the majority of lipids and sterols, the dry residue was partitioned between 1 ml of 80% methanol with water and 1 ml of petroleum ether. The upper petroleum ether phase was discarded, while the methanol–water phase containing free steroids was evaporated in a vacuum centrifuge, and the dry residue derivatized as described below.

Methanol (500 μl) was added to the polar phase, and the mixture was centrifuged at 2000 g for 5 min. Supernatant was decanted from the pellet, and five drops of concentrated HCl were then added to it. The mixture was heated at 80°C for 24 h in a tightly closed vial and then left for a further 16 h at room temperature. After the addition of an excess of sodium bicarbonate, the mixture was extracted with 3 ml of diethyl ether. The extract was evaporated in a vacuum centrifuge, and the dry residue was partitioned between 1 ml of 80% methanol and petroleum ether (1 ml) to eliminate the hydrolyzed sterol conjugates. The petroleum ether phase was discarded, while an 800 μl aliquot of the methanol–water phase was evaporated in a vacuum centrifuge and then derivatized.

2.5.1.2. Derivatization. The dry residue of treated sample diluted in 100 μl of acetonitrile was evaporated under nitrogen; 15 μl of pyridine and 15 μl of Sylon B (99% BTSFA + 1% TMCS) were added to the dry residue with brief mixing, and the resultant mixture was heated at 65°C for 30 min. Subsequently, 25 μl of acetonitrile and 100 μl of isooctane were added and shaken for 1 min. The isooctane phase was separated and dried under nitrogen. A further 30 μl of isooctane were then added with brief mixing to rinse down the dry residue; the mixture was evaporated again, 10 μl of isooctane was added with brief mixing, and 2 μl of the mixture were injected into the GC/MS system.

2.5.1.3. GC/MS analysis. GC separation was carried out using a ZEBRON ZB-50, 15 m × 0.25 mm column, and 0.15 μm film thickness (cat. No. 7EG-G004-05). The temperature of the injection port was 300°C.

The following protocol was used:

Splitless high-pressure injection for 1 min at 100 kPa, 1 min delay at 120°C and 30 kPa, then 40°C/min and 10 kPa/min to 220°C and 55 kPa, followed by 8°C/min and 2 kPa/min to 300°C and 75 kPa. The duration of the analysis was 13.5 min.

The MS program in SIM mode was as follows:

0–6 min, $m/z = 305$, 344 (androstenediol RT = 5.77 min)

6–6.4 min, $m/z = 253$, 268 (17α-methyl-androstenediol RT = 6.22 min, internal standard)

6.4–7.4 min, $m/z = 285$, 416 (estradiol RT = 6.66 min) and $m/z = 300$, 285 (EpiPal RT = 6.78 min, AlloPal RT = 6.90 min, Pal, RT = 7.02 min)

7.7–8.2 min, $m/z = 375$, 300 (IsoPal RT = 6.66 min) and $m/z = 298$, 241 (pregnenolone RT = 7.575 min) and $m/z = 504.5$ (estriol RT = 7.7 min).

The detector voltage was 1.2 kV, and the sampling rate was 0.25 s. The temperature of the interface was 310°C. The first of the pair of effective masses (m/z) was used for the calculation of concentration, while the second value was measured to ensure the correct identification of the substance. Besides agreement in the retention times, the identity of substances with the standards was confirmed using the effective masses characteristic of TMS derivatives of pregnanolone isomers ($m/z = 285$, 300 and 375), and for the TMS derivative of pregnenolone ($m/z = 298$, 241 and 288).

Standard mixtures, used for calibration with an external standard method, were derivatized in the same manner as the samples. They contained androstenediol, 17α-methylandrostenediol, estradiol, epipregnanolone, allopregnanolone, 5β-pregnan-3α-ol-20 one, 5α-pregnan-3β-ol-20 one, pregnenolone, and estriol in three concentrations: 1000, 100 and 10 pg/μl. To check the reproducibility of injection, 17α-methylandrostenediol (10 000 pg) was added to each sample prior to derivatization. The efficiency of both extraction steps for each steroid was determined using HPLC separation of the standards extracted in the same way as for the samples, but in amounts sufficient for UV detection. The recovery of steroids that underwent all of the separation steps was 0.607, 0.652, 0.644, and 0.642 for EpiPal, AlloPal, Pal, and IsoPal, respectively. The losses in individual steroids were similar irrespective of their different polarity.

2.5.2. RIA of allopregnanolone

In addition to the use of GC/MS, levels of AlloPal were also measured using RIA following the permanganate oxidation of unsaturated steroids and microcolumn chromatography as described elsewhere [23].

2.6. Statistical evaluation of the data

Due to the skewed distribution of the data, medians were used instead of means to demonstrate differences. Prior to the performance of statistical tests, the data underwent power transformations to obtain an approximation of Gaussian distribution, which is the prerequisite for the correct use of the t-test. Mean values calculated from the transformed data with lower and upper limits of confidence intervals were re-transformed to the original scale. The re-transformed mean values were close to medians, and their confidence intervals were more or less asymmetrical, reflecting the skewed nature of the original data. Student's t-test was used for the comparison of mean values in maternal and umbilical plasma at delivery

3. Results

3.1. Analytical criteria

The mass spectra of pregnanolone isomers are shown in Fig. 1. The response of the GC/MS system was linear from 10 to 10 000 pg of injected sample in all of the pregnanolone isomers and in pregnenolone. The correlation coefficients of the response versus mass of injected sample were 0.99994, 0.99996, 0.99992 and 0.99997 for EpiPal, AlloPal, Pal and IsoPal, respectively (Fig. 2). The intra-assay coefficients of variance, tested on eight samples from pooled umbilical plasma, were 7.1, 5.5, 6.3 and 9.2 for EpiPal, AlloPal, Pal and IsoPal, respectively. The inter-assay coefficients of variance, tested on six samples from pooled umbilical plasma, were 9.3, 7.1, 9.8 and 10.3 for EpiPal, AlloPal, Pal and IsoPal, respectively. The sensitivities of the assay were 0.16, 0.034, 0.61 and 0.673 pg for EpiPal, AlloPal, Pal and IsoPal, respectively. A good correlation was found between allopregnanolone in maternal plasma measured by RIA and measured by GC/MS ($r = 0.940$, $P < 0.001$, $n = 8$). The chromatograms of three pregnanolone isomers and estradiol (unconjugated steroids and their conjugates) in maternal and umbilical plasma are shown in Fig. 3. The isopregnanolone peak is not shown because of the notable difference in retention time from the other pregnanolone isomers.

3.2. Levels of pregnanolone isomers in maternal and umbilical plasma at delivery

3.2.1. Unconjugated pregnanolone isomers

The levels of unconjugated pregnanolone isomers in maternal and umbilical plasma at delivery are shown in Table 1. The least abundant pregnenolone isomer in both maternal and umbilical blood was EpiPal (median concentration at delivery being 1.87 and 4.78 nmol/l in maternal and umbilical plasmas, respectively). The proportions of median concentrations of pregnanolone isomers in maternal plasma at delivery were 1:11.1:7.3:2.2 (EpiPal:AlloPal:Pal:IsoPal). The proportion of pregnanolone isomers in umbilical plasma was 1:3.4:3.6:3.7 (EpiPal:AlloPal:Pal:IsoPal).

The levels of 3β-hydroxy-steroids (EpiPal and IsoPal) were significantly higher in the umbilical than in the maternal plasma, while the differences in 3α-steroids (AlloPal and Pal) were insignificant. The proportions of umbilical to maternal plasma medians at delivery were 2.6, 0.79, 1.4 and 2.9 for EpiPal, AlloPal, Pal, and IsoPal, respectively. Significantly higher levels of EpiPal and IsoPal were found in umbilical in comparison to maternal plasma.

3.2.2. Conjugated pregnanolone isomers

The levels of conjugated pregnanolone isomers in maternal and umbilical plasma at delivery are shown in Table 2. As is the case for the unconjugated steroids, the least abundant pregnanolone isomer in both maternal and in umbilical plasma was EpiPalC (its median concentration being 15.3 and 11.9 nmol/l in maternal and umbilical plasma, respectively). The proportions of median concentrations of conjugated pregnanolone isomers in maternal plasma were 1:38:36:26 for EpiPalC:AlloPalC:PalC:IsoPalC, respectively. The proportions of pregnanolone isomer levels in umbilical plasma were 1:12:15:10 for EpiPalC:AlloPalC:PalC:IsoPalC, respectively.

No significant differences were observed between maternal and umbilical plasma in conjugated pregnanolone isomers, except that the levels of AlloPalC were significantly lower in umbilical plasma. The differences in the levels of PalC and IsoPalC did not reach statistical significance due to a broad variance, although the mean values in umbilical plasma were almost half those in maternal plasma. The proportions of the median umbilical/maternal plasma at delivery were 0.78, 0.47, 0.68 and 0.60 for EpiPalC, AlloPalC, PalC and IsoPalC, respectively.

3.3. Differences in proportions between conjugated and unconjugated steroids

The conjugated/unconjugated steroid ratio in pregnanolone isomers in maternal and umbilical plasmas at delivery is given in Table 3. In all instances, significantly lower values were found in umbilical plasma.

4. Discussion

The discovery of the effect of allopregnanolone downregulating the production of oxytocin during

gravidity [22,24–26], and the identification of changes in GABA receptor affinity to allopregnanolone during pregnancy and the lactation period [19,20], resulted in an increased interest on the part of investigators in role of neuroactive steroids in human reproduction. Recently, the importance of polar conjugates of pregnanolone isomers has grown with the discovery of the retrogression in the effect of GABA receptor activators by their sulfatation on the steroid C-3 hydroxyl [16]. In this connection, the investigation of changes in the levels of neuroactive steroids around parturition suggests itself. The current lack of available information is particularly obvious in respect of pregnanolone isomers and their conjugates. The authors' aim was to describe the differences in the levels of pregnanolone isomers in maternal and fetal plasma at delivery, and to suggest the possible physiological consequences of such differences. The identification and evaluation of epipregnanolone were of particular interest, as it had not previously been measured in human plasma.

The levels of epipregnanolone found in both umbilical and maternal plasma were lower than those for other pregnanolone isomers. The physiological importance of epipregnanolone and in particular of its sulfate, the allosteric inhibitor of both GABA [16] and NMDA [10] receptors, in human beings requires further investigation. The levels of both of the unconjugated 3β-hydroxy-pregnanolone isomers were more than doubled in umbilical plasma, while the differences in 3α-hydroxy-isomers were insignificant.

The level of AlloPalC was significantly higher in maternal plasma than in umbilical plasma, while the differences in the other conjugated pregnanolone isomers were insignificant. In all of the pregnanolone isomers, a higher conjugated/unconjugated steroid ratio was found in maternal plasma than in umbilical plasma. It is known that the sulfatation of 3α-pregnanolone isomers reverses the positive modulating effect on GABA receptor activity [16]; moreover, the role of GABA receptors in the timing of parturition in rats has been reported [19–22]. Taking these facts together with the new results of this study, it may be concluded that pregnanolone isomers and their conjugates do operate in the onset of labor in humans.

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