Relationships of circulating pregnanolone isomers and their polar conjugates to the status of sex, menstrual cycle, and pregnancy

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Abstract

Pregnanolone isomers (PIs) and their polar conjugates (PICs) modulate ionotropic receptors such as γ -aminobutyric acid or pregnane X receptors. Besides, brain synthesis, PI penetrates the blood–brain barrier. We evaluated the physiological importance of PI respecting the status of sex, menstrual cycle, and pregnancy. Accordingly, circulating levels of allopregnanolone (P3 α 5 α), isopregnanolone (P3 β 5 α), pregnanolone (P3 α 5 β), epipregnanolone (P3 β 5 β), their polar conjugates, and related steroids were measured in 15 men (M), 15 women in the follicular phase (F), 16 women in the luteal phase (L), and 30 women in the 36th week of gestation (P) using GC–MS. The steroid levels were similar in M and F, increased about thrice in L and escalated in P (38–410 times compared with F). The PICs were prevalent over the PIs (16–150 times). Higher ratios of 5 α -PIC to 5 α -PI

found in P indicate the more intensive conjugation of 5 α -PI during pregnancy. This mechanism probably provides for the elimination of neuroinhibitory P3 α 5 α in the maternal compartment. Additionally, our result points to a limited sulfation capacity for neuroinhibitory P3 α 5 β in P. In contrast to the situation in M, F, and L where the P3 α 5 β C is the most abundant PIC, and P3 α 5 β is present in minor quantities compared with the P3 α 5 α , P3 α 5 β may acquire physiological importance during pregnancy, contributing to the sustaining thereof. On the other hand, the declining formation of P3 α 5 β may participate in the initiation of parturition, given the relative abundance of the steroid, its potency to suppress the activity of oxytocin-producing cells and its effectiveness in uterine relaxation.

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Introduction

Reduced progesterone metabolites, including pregnanolone isomers (PIs) and their polar conjugates (PICs), are efficient neuromodulators. They are effective on neurotransmitter receptors influencing the permeability of ion channels (Majewska 1990). PIs with a hydroxy group in the 3 α -position shorten the period of paradoxical sleeping, attenuate the release of acetylcholine in the neocortex and hippocampus, suppress neurogenesis, and deteriorate spatial memory. These effects are provided via modulation of the receptors of type A γ -aminobutyric acid (GABA_A-R; Darnaudery *et al.* 1999). The 3 β -PI competes with 3 α -PI for the binding sites on GABA_A-R (Prince & Simmonds 1992, Wang *et al.* 2002, Lundgren *et al.* 2003). Conjugation counteracts the effect of 3 α -PI, and further amplifies the antagonistic effect of the 3 β -PI on GABA_A-R. For instance, the GABA_A-R inhibiting efficiency of 3 β -hydroxy-5 α -pregnane-20-one sulfate is comparable with the GABA_A-R activating effectiveness of allopregnanolone (P3 α 5 α). Some reports indicate that in addition to the brain's *in situ* synthesis, circulating PIs penetrate the blood–brain barrier (Bixo *et al.*

1997, Corpechot *et al.* 1997). Although several studies have brought information to light regarding the levels of PI in humans, only a small number have included data regarding the respective polar conjugates (Mickan & Zander 1979, Hill *et al.* 2000, Havlikova *et al.* 2006).

In addition to the action on GABA_A-R, the 5 β -PI block type T calcium channels in rat peripheral neurons; these channels play a significant role in pain perception (Todorovic *et al.* 2004). The 5 β -PI also binds to nuclear progesterone receptors (Putnam *et al.* 1991). It has recently been reported that 5 β -PI may act chronically through a mechanism mediated by pregnane X receptors to regulate uterine contractility (Mitchell *et al.* 2005). The 5 α - and 5 β -PIC are effective as positive and negative modulators of *N*-methyl-D-aspartate receptors (NMDA-R) respectively (Park-Chung *et al.* 1997, Weaver *et al.* 2000). The activation of NMDA-R in hypothalamic magnocellular neuroendocrine cells of the supraoptic nucleus may induce oxytocin production (Pak & Curras-Collazo 2002), resulting in onset of parturition.

PIs originate from progesterone (Prog) through the action of the ubiquitous 5 α - and 5 β -reductase, which is most active in the liver (Ingelman-Sundberg 1976). Both enzymes show

significant activity in the tissues associated with pregnancy (Lisboa & Holtermann 1976, Milewich *et al.* 1979, Sheehan *et al.* 2005); they catalyze the formation of 5α - and 5β -dihydroprogesterone ($P5\alpha$ and $P5\beta$) respectively. The subsequent metabolism of dihydroprogesterone epimers to individual PI is catalyzed by stereospecific 3α - or 3β -hydroxysteroid oxidoreductases (Okuda & Okuda 1984, Melcangi *et al.* 1994, Corpechot *et al.* 1997, Ottander *et al.* 2005). The latter enzyme may be identical to the 3β -hydroxysteroid dehydrogenase, as indicated in the human placenta study (Dombroski *et al.* 1997).

PIs, and particularly their polar conjugates, are synthesized in large quantities in the human fetoplacental unit from Prog (Fig. 1; Milewich *et al.* 1979, Dombroski *et al.* 1997). As has been documented for 5α -PI, the steroids are metabolized to respective 3-oxo-derivatives ($P5\alpha/\beta$), which are then

transported to the maternal compartment via the placenta – where they are again converted into PI (Dombroski *et al.* 1997). The levels of all PI in pregnancy are strikingly higher in comparison with the situation in non-pregnant women (Genazzani *et al.* 1998, Hill *et al.* 2000, Luisi *et al.* 2000, Pearson Murphy *et al.* 2001, Havlikova *et al.* 2006).

The aim of this study was to evaluate the physiological impact of endogenous neuroactive PI and PIC in humans, respecting the status of sex, menstrual cycle, and pregnancy. The evaluation was based on the steroid circulating levels and steroid neuromodulating activities reported in the literature. A further objective was to assess inter-group differences in the biosynthesis and metabolism of PI. For these reasons, we measured the circulating levels of all PIs, such as $P3\alpha5\alpha$, isopregnanolone ($P3\beta5\alpha$, epiallopregnanolone, isoallopregnanolone), $P3\alpha5\beta$, epipregnanolone ($P3\beta5\beta$), and PIC as

Figure 1 The biosynthesis of pregnanolone isomers.

conjugates of P3 α 5 α (P3 α 5 α C), P3 β 5 α (P3 β 5 α C), P3 α 5 β (P3 α 5 β C), and P3 β 5 β (P3 β 5 β C) in adult men (M), in women in the follicular (F) and luteal phases (L) of the menstrual cycle (MC), and in women in the 36th week of gestation (P), using GC–MS. In addition, the levels of the respective precursors, free pregnenolone (Preg) and conjugated pregnenolone (PregC), Prog, P5 α , and P5 β were also quantified.

Materials and Methods

The circulating levels of all PICs, Preg and PregC were measured in all groups. The levels of all PIs and Prog were measured in F, L, and P. The quantification of P5 α and P5 β was limited to P.

Subjects

The subjects in all groups were Caucasian. The M group consisted of 15 healthy men (16–62 years of age) participating in a study on iodine deficiency in the Czech Republic. The blood from non-pregnant women was collected on the 5th and 22nd days of the MC for the F ($n=15$) and L ($n=16$) groups respectively. Eleven women were followed in both phases. The 30 pregnant volunteers in the P group entered the study protocol according to the inclusion and exclusion criteria. The volunteers were enrolled into the study at a single institution between January 2004 and September 2005, according to the following inclusion criteria: age between 18 and 30 years, physiological pregnancy, cephalic presentation of the fetus. The exclusion criteria were as follows: manifestation of pre-eclampsia, chronic medication, anemia, any chronic disease or medication that might influence steroid levels or delivery, premature rupture of membranes, labor induction, or operative delivery in a previous pregnancy. The subjects in all groups used no hormonal treatment for at least 3 months prior to, and during, the trial. All patients enrolled in the study gave signed consent to participate in the study after a full explanation of the purpose and the nature of all the procedures used. The local Ethical Committees of the Institute of Endocrinology and the General Faculty Hospital (both in Prague, Czech Republic) approved the protocol for the study.

Sample collection

After signing written informed consent, the patients underwent blood sampling from the cubital vein. All blood samples were taken at the same time of day (0900–1200 h, at least 2 h after waking), under standard conditions (elimination of stress factors, after 15 min of resting).

Cooled plastic tubes containing 100 μ l of 5% EDTA and 50 μ l aprotinin (Antilysin from Spofa, Prague, Czech Republic) were used for blood sampling. Plasma was obtained

after centrifugation for 5 min at 2000 g at 0 °C. The plasma samples were stored at –20 °C until analysis.

Steroids and chemicals

The steroids were purchased from Steraloids (Wilton, NH, USA). The solvents for the extraction and high performance liquid chromatography (HPLC) were of analytical grade, sourced from Merck. The derivatization agent Sylon BFT (bis(trimethylsilyl)-trifluoroacetamide 99% and trimethylchlorosilane) was purchased from Supelco (Bellefonte, PA, USA).

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 a QP 5050A quadrupole electron-impact detector with a fixed electron voltage of 70 eV. A Zebtron ZB-50 medium polarity capillary column (50% phenyl/50% methylpolysiloxane) from Phenomenex (St Torrance, CA, USA) was used for the analysis. The length of the column was 15 m, the internal diameter was 0.25 mm, and the film thickness was 15 μ m.

Preparation of the plasma samples for GC–MS free steroids analysis

Frozen samples were thawed and 1 ml sample was spiked with 17 α -estradiol as an internal standard to attain a concentration of 1 μ g/ml. The spiked sample was extracted with 3 ml diethyl ether. The water phase was kept frozen in a mixture of solid carbon dioxide and ethanol, and the organic phase was decanted into glass tubes and evaporated to dryness. The dry organic phase residue was used for the determination of free steroids. The dry residue was partitioned between 1 ml of 80% methanol with water and 1 ml n -pentane to eliminate the lipids and sterols. The n -pentane phase was discarded, while the methanol/water phase containing steroids for analysis was evaporated in a vacuum centrifuge. The samples were prepared twice for further processing using two different derivatization techniques. The first was used for the preparation of steroids with hydroxy groups modified to trimethylsilyl (TMS) derivatives and with intact oxo groups. The second technique produced derivatives with hydroxy groups modified as in the former case but, in addition, with the oxo groups modified by methoxylamine (MOX–TMS derivatives).

The first derivatization technique was used for the quantification of Preg and PIs. The second technique was applied for the measurement of Prog, P5 α , and P5 β .

Sample preparation for the GC–MS analysis of steroid polar conjugates

The frozen water phase in glass tubes was thawed and mixed with 1 ml methanol. The tubes were centrifuged and

the 1 ml aliquot of the supernatant was transferred into a glass tube and evaporated in a vacuum centrifuge. The steroid sulfates were hydrolyzed using a method described elsewhere (Dehennin *et al.* 1996). The hydrolyzed sample was evaporated in a vacuum centrifuge; the dry residue was spiked with 17 α -estradiol as an internal standard to attain a concentration of 1 μ g/ml and further processed in the same way as the free steroids.

Derivatization

The TMS derivatives of the steroids were prepared using the modified method of Hill *et al.* (2000), with some of the modifications reported recently (Havlikova *et al.* 2006). In summary, Sylon B (99% bis (trimethylsilyl)-trifluoroacetamide (BTSFA) + 1% trimethylchlorosilane (TMCS); 50 μ l) was added to the dry residues from plasma, mixed briefly and heated at 90 °C for 45 min. The derivatization agent was evaporated under a stream of nitrogen. The dry residue was rinsed down with isooctane (50 μ l) and the mixture was evaporated again. Finally, steroid derivatives were dissolved into 20 μ l isooctane, and 4 μ l portions were injected into the GC–MS system.

The MOX–TMS derivatives were prepared as follows: 50 μ l of 2% solution of MOX–hydrochloride in pyridine were added to the dry residues from plasma, mixed briefly, and heated at 60 °C for 2 h. The mixture was then evaporated under a stream of nitrogen, before further TMS derivatization proceeded as described previously.

Temperature and pressure gradients for the GC–MS analysis of TMS derivatives

For the GC–MS analysis of TMS derivatives, the temperature and pressure gradients used were as follows: 1-min high–pressure

injection at 120 °C and 100 kPa followed by a pressure release to 30 kPa and a rapid linear gradient of 40 °C/min and 8.5 kPa/min up to 200 °C and 49.3 kPa, then a slow linear gradient of 2.9 °C/min and 0.5 kPa/min up to 220 °C and 52.7 kPa, a medium linear gradient of 20 °C/min and 8 kPa/min a up to 265 °C and 70 kPa, and a rapid linear gradient of 40 °C/min and 10.0 kPa/min up to 310 °C and 80.7 kPa, followed by a 2-min delay. The overall time taken for the analysis was 15.3 min.

For the analysis of TMS–MOX derivatives, the following temperature and pressure gradient was used: one minute high–pressure injection at 120 °C and 100 kPa followed by a pressure release to 30 kPa and a rapid linear gradient of 40 °C/min and 8.5 kPa/min up to 220 °C and 51.0 kPa, then a slow linear gradient of 2.9 °C/min and 0.5 kPa/min up to 240 °C and 54.5 kPa, and a rapid linear gradient of 40 °C/min and 9.0 kPa/min up to 310 °C and 70 kPa, followed by a 3-min delay. The overall time taken for the analysis was 15.2 min.

Retention times and effective masses used for the determination of steroids

To exploit the samples, the individual samples were applied three times in independent courses, in each case employing a part of the steroids under investigation. The choices of the steroids measured within the individual courses, as well as the effective masses used for the measurement, were optimized to attain maximum sensitivity at sufficient selectivity. The types of gradients, effective masses for determination and quantification, order numbers of injection, and retention times for the individual steroids are shown in Table 1.

Table 1 Analytical criteria of the method for the multi-component quantification of neuroactive pregnanolone isomers and related steroids

Steroid		Form	Gradient/ derivatization	Injection	Retention time (min)	Effective mass (m/z)	Detection limit (nmol/l) (mean $\pm$ S.E.M., n = 5)
No							
1	Pregnenolone	*F, C	⁺ S	3	11.592	298, 388	0.058 $\pm$ 0.006
2	Progesterone	F	MS	1	11.250, 11.392	100, 341, 372	0.033 $\pm$ 0.004
3	3 α -Dihydroprogesterone (P3 α)	F	MS	1	10.975, 11.008	288, 343	0.020 $\pm$ 0.002
4	3 β -Dihydroprogesterone (P3 β)	F	MS	1	10.396, 10.475	288, 343	0.054 $\pm$ 0.010
5	Allopregnanolone (P3 α 5 α)	F, C	S	1	10.758	285, 300, 375	0.028 $\pm$ 0.004
6	Isopregnanolone (P3 β 5 α)	F, C	S	1	11.563	285, 300, 375	0.030 $\pm$ 0.004
7	Pregnanolone (P3 α 5 β)	F, C	S	1	10.950	285, 300, 375	0.043 $\pm$ 0.006
8	Epipregnanolone (P3 α 5 α)	F, C	S	1	10.550	285, 300, 375	0.032 $\pm$ 0.005
9	17 α -Estradiol (internal standard)	–	S, (MS)	1–4	9.863	285, 416	0.026 $\pm$ 0.003

*F, free steroid, C, conjugated steroid; ⁺S, trimethylsilyl derivatives, MS, methoxyamine-trimethylsilyl derivatives.

Statistical analysis of the data

The data showed mostly non-Gaussian distribution and non-constant variance. To evaluate changes in the steroid levels and steroid ratios, a robust Kruskal–Wallis ANOVA was used with group as the factor. Multiple testing was handled by Kruskal–Wallis multiple comparisons to evaluate differences between the individual groups. The relationships between the steroids were evaluated using Spearman's correlations and partial Spearman's correlations. Given the sufficient number of pairs, the partial Spearman's correlations in pregnant women were adjusted to constant levels of all steroids included in a correlation matrix, except the pair under investigation. Statistical computations were performed using NCSS 2002 statistical software from Number Cruncher Statistical Systems (Kaysville, UT, USA).

Results

Identification and quantification of the steroids

The steroids separated well from each other and from the background. As demonstrated in Table 1, the selectivity and sensitivity were sufficient for quantification of all of the investigated steroids. In terms of reproducibility, the inter-assay coefficient of variance did not exceed 10% for any steroid.

Steroid levels

The levels of free and conjugated steroids are shown in Figs 2–4. The levels of P3 α 5 α (Fig. 3A) increased in the sequence M, F, L, and P. All inter-group differences except the differences between M and F were significant. A similar situation was found in all PIs except the missing data for the M group in 5 β -PI. The PIC showed a similar trend. The levels in both PI and PIC in P were about two orders of magnitude higher than those found in F or M (Figs 3 and 4).

Ratios of conjugates to free steroids

Both 5 α -PI exhibited higher conjugate to free steroid ratios for M and P (which did not differ from each other) when compared with F and L (Fig. 5A and B). In the ratios of P3 β 5 α C to P3 β 5 α , however, the differences between M and the groups of non-pregnant women did not reach significance (Fig. 5B). In contrast to the situation in 5 α -PI, the ratio of P3 α 5 β C to P3 α 5 β showed decreasing trend in the sequence F, L, and P. Significantly lower value of the ratio was found in P when compared with the values in F and L (Fig. 5C). The ratios of P3 β 5 β C to P3 β 5 β did not exhibit any between-group difference (Fig. 5D).

Ratios of 3 α - to 3 β -isomers

As demonstrated in Fig. 6A, the F and L groups showed more than two times lower values for the ratio than was the case for the P group. The respective differences in the conjugates were much less pronounced (Fig. 6B); nevertheless, the ratios for P still exhibited the highest values.

Ratios of the 5 α - to 5 β -isomers

The ratios of 5 α -PI to 5 β -PI showed higher values for F and L groups when compared with P group (Fig. 7A). The situation was different in PIC, where the ratios were significantly higher in L and even more elevated in the P group when compared with the values found in M and F groups that did not differ from each other (Fig. 7B).

Correlations between conjugated and unconjugated steroids in women

In the F group, the only significant correlation between conjugated and free PI was found for the P3 β 5 β ($r=0.536$, $P=0.040$, $n=15$). The symbols r , P , and n symbolize the Spearman's correlation coefficient, its statistical significance, and number of pairs under investigation respectively. In L group, the P3 α 5 α significantly correlated with the P3 α 5 α C ($r=0.753$, $P=0.001$, $n=16$) and P3 β 5 α with P3 β 5 α C ($r=0.585$, $P=0.017$, $n=16$). The respective correlations for the P3 α 5 β ($r=0.409$, $P=0.116$, $n=16$) and P3 β 5 β ($r=0.338$, $P=0.200$, $n=16$) did not reach significance. In P group, the correlations between conjugated and free PI reached significance for the P3 β 5 α ($r=0.528$, $P=0.004$, $n=28$) and for the P3 β 5 β ($r=0.431$, $P=0.022$, $n=28$).

Correlations between 3 α - and 3 β -isomers

Significant correlations between 3 α - and 3 β -PI were mostly found within all groups for both 5 α - and 5 β -PI, except for the missing data for 5 β -PI in men. P3 α 5 α and P3 β 5 α significantly correlated in F ($r=0.693$, $P=0.040$, $n=15$), L ($r=0.965$, $P<0.001$, $n=16$), and P ($r=0.695$, $P<0.001$, $n=30$). The correlation between the P3 α 5 α C and P3 β 5 α C did not reach significance in the F group ($r=0.275$, $P=0.321$, $n=15$) but strongly correlated in L ($r=0.918$, $P<0.001$, $n=16$) and P ($r=0.660$, $P<0.001$, $n=28$). P3 α 5 β and P3 β 5 β significantly correlated in F ($r=0.764$, $P=0.001$, $n=15$), L ($r=0.594$, $P=0.015$, $n=16$), and P ($r=0.649$, $P<0.001$, $n=30$). Similarly, P3 α 5 β C and P3 β 5 β C significantly correlated in F ($r=0.543$, $P=0.037$, $n=15$), L ($r=0.600$, $P=0.014$, $n=16$), and P ($r=0.812$, $P<0.001$, $n=28$).

Correlations between PI and progesterone

The correlation between P3 α 5 α and Prog did not reach significance in F ($r=0.434$, $P=0.106$, $n=15$) but the steroids

Figure 2 Levels of polar conjugates of pregnenolone (A), unconjugated pregnenolone (B), and progesterone (C) in 15 adult men (M), 15 women in follicular phase of menstrual cycle (F), 16 women in the luteal phase (L), and 30 women in the 36th week of gestation (P). Bars with error bars represent group means with S.D.s. Owing to skewed data distribution and non-constant variance, the between-group were evaluated using robust Kruskal–Wallis ANOVA followed by Kruskal–Wallis multiple comparisons. The significance of ANOVA model is shown in the first line of the embedded table, while significant inter-group differences as found by the multiple comparisons ($P < 0.05$) are shown in the further lines of this table (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

strongly correlated in L ($r = 0.744$, $P < 0.001$, $n = 16$) and P ($r = 0.771$, $P < 0.001$, $n = 29$). $P3\beta5\alpha$ and Prog significantly correlated in F ($r = 0.523$, $P = 0.045$, $n = 15$), L ($r = 0.788$, $P < 0.001$, $n = 16$), and a weaker but still significant correlation was found in P ($r = 0.399$, $P = 0.032$, $n = 16$). The $P3\alpha5\beta$ and Prog strongly correlated in L ($r = 0.818$, $P < 0.001$, $n = 16$) but not in F ($r = -0.183$, $P = 0.514$, $n = 15$) and P ($r = 0.348$, $P = 0.434$, $n = 29$). Like the $P3\alpha5\beta$, $P3\beta5\beta$ did not correlate with Prog in F ($r = -0.219$, $P = 0.065$, $n = 15$), but the significant correlations were found in L ($r = 0.500$, $P = 0.049$, $n = 16$) and P ($r = 0.710$, $P < 0.001$, $n = 29$).

Relationships between steroids

PregC negatively correlated with the 5α -PI in men, attaining significance for $P3\alpha5\alpha$ ($r = -0.709$, $P < 0.022$, $n = 10$) but not for $P3\beta5\alpha$ ($r = -0.503$, $P < 0.14$, $n = 10$). The subsequent partial correlations confirmed a negative borderline association between PregC and $P3\alpha5\alpha$ ($r = -0.633$, $P < 0.1$, $n = 10$, adjusted to constant Preg and $P3\beta5\alpha$), but showed no

direct relationship between PregC and $P3\beta5\alpha$ ($r = 0.260$, $P = 0.504$, $n = 10$, adjusted to constant Preg and $P3\alpha5\alpha$). Both pair correlations and partial correlations identified a strong positive relationship between $P3\alpha5\alpha$ and $P3\beta5\alpha$ ($r = 0.707$, $P < 0.003$, $n = 15$) and ($r = 0.758$, $P < 0.003$, $n = 15$, adjusted to constant PregC and Preg).

Discussion

The levels of Preg, PregC, all PIs, and PICs in women in the 36th week of gestation and in non-pregnant women in both phases of the MC were quantified. In addition, the levels of the Preg, PregC, 5α -PI as well as the concentrations of all PICs were measured in men (Figs 2–4). The results clearly showed the need to differentiate among M, F, L, and P groups when evaluating changes of circulating PI in connection with various pathologies. The distinctive effects of PI are to be expected in pregnancy due to persistently elevated levels of 3α -PI, resulting in a decreased affinity of $GABA_A$ -R for

Figure 3 Levels of 5 α -pregnanolone isomers (A and B) and their polar conjugates (C and D), in 15 adult men (M) (only ten men were examined for conjugated steroids), 15 women in follicular phase of menstrual cycle (F), 16 women in the luteal phase (L), and 30 women in the 36th week of gestation (P). The drawings and symbols are the same as for Fig. 2.

these neuroactive steroids either due to the changed expression of the receptor subunits and/or as a result of the changed phosphorylation status of the specific sites on the GABA_A-R (Leng & Russell 1999, Brussaard *et al.* 2000, Koksmä *et al.* 2003).

Figure 4 Levels of 5 β -pregnanolone isomers (A and B) and their polar conjugates (C and D), in 15 adult men (M) (only ten men were examined for conjugated steroids), 15 women in follicular phase of menstrual cycle (F), 16 women in the luteal phase (L), and 30 women in the 36th week of gestation (P). The drawings and symbols are the same as for Fig. 2.

In men and to a large extent in women in the follicular phase, the most important role of adrenal activity and *in situ* brain synthesis of PI can be expected in the overall balance of PI (Fig. 8A and B). Our data suggest that the production of PI in men may depend on sulfatase activity. In M group, PregC

Figure 5 Ratios of polar conjugates to unconjugated steroids for allopregnanolone (P3 α 5 α ; A), isopregnanolone (P3 β 5 α ; B), pregnanolone (P3 α 5 α ; C), and epipregnanolone (P3 β 5 β ; D), in the groups of 15 women in the follicular (F) and 16 women in the luteal (L) phases of the menstrual cycle, and 30 women in the 36th week of gestation (P). The letter C at the end of the aforementioned abbreviations denotes conjugated steroids. The remaining drawings and symbols are the same as for Fig. 2.

correlated with P3 α 5 α . This finding, like the increased ratios of PIC to PI, may indicate that lower sulfatase activity could be associated with a lower amount of Preg as the primary substrate for enzymes catalyzing the formation of Prog and PI.

Figure 6 Ratios of the sum of unconjugated pregnenolone isomers with a hydroxy group in the 3 α -position (3 α -PI/3 β -PI; A) to the sum of unconjugated pregnenolone isomers with a hydroxy group in the 3 β -position and the ratio of the steroid polar conjugates (3 α -PIC/3 β -PIC; B), in the groups of adult men (M), women in the follicular (F) and luteal (L) phases of the menstrual cycle, and women in the 36th week of gestation (P). The drawing and symbols are the same as for Fig. 2.

The partial correlations confirmed the negative borderline association between PregC and P3 α 5 α but showed no relationship between PregC and P3 β 5 α . As expected, both pair and partial correlations found a strong positive relationship between P3 α 5 α and P3 β 5 α . Given the aforementioned results, the major metabolic pathway in adult men may be expected in the sequence PregC $\rightarrow$ Preg $\rightarrow$ Prog $\rightarrow$ P5 α $\rightarrow$ P3 α 5 α $\rightarrow$ P3 β 5 α .

Women in the luteal phase and early pregnancy produce the bulk of the PI in the corpus luteum (Ottander *et al.* 2005) PI (Fig. 8C).

After the luteo-placental shift, the primary precursor of the PI, PregS, originates almost entirely from the fetal zone of the fetal adrenal (Fig. 8D). PregS is transported into the placenta by circulation, being converted to Preg, Prog, and P5 α / β , in sequence. The P5 α and possibly also the P5 β penetrate into the maternal compartment, being further metabolized to PI and PIC (Dombroski *et al.* 1997).

While the 5 α -PI showed higher ratios of conjugates to the free steroids (Fig. 5A and B) in P group, the respective ratios were similar in F, L, and P groups for P3 β 5 β (Fig. 5D) and showed a significantly decreasing trend for P3 α 5 β in sequence F, L, and P (Fig. 5C) groups. It is evident that the groups with low Prog production exhibited very low concentrations of unconjugated P3 α 5 β . In these subjects, the P3 α 5 β may be

Figure 7 Ratios of the sum of unjugated pregnenolone isomers with hydrogen in the 5 α -position (5 α -PI/5 β -PI; A) to the sum of unjugated pregnenolone isomers with hydrogen in the 5 β -position and the ratio of the steroid polar conjugates (5 α -PIC/5 β -PIC; B), in the groups of adult men (M), women in the follicular (F) and luteal (L) phases of the menstrual cycle, and women in the 36th week of gestation (P). The drawings and symbols are the same as for Fig. 2.

rapidly metabolized to its conjugates. Alternatively, in L and P groups, the conjugation capacity for P3 α 5 β appears to be limited. The increased conjugation of 5 α -PI probably diminishes the difference between the P3 α 5 α and P3 α 5 β levels in pregnant women and may also regulate the proportions between neuroinhibiting P3 α 5 α and counter-acting 5 α -PIC. On the GABA_A-R, the P3 β 5 α C exerts about 10% efficiency when compared with the effect of P3 α 5 α . However, P3 β 5 α C operates in the opposite way (Park-Chung *et al.* 1999). In all groups, the levels of all PICs exceed the levels of PI from one to more than two orders of magnitude. The situation in the proximity of the active sites may not necessarily reflect the conditions in the circulation, however. The conjugation may also hinder the transport of PI across the blood–brain barrier, but in the periphery, it probably facilitates the transport of significant amounts of neuroactive steroids by circulation to the sites where they take effect.

To evaluate the inter-group differences in the relative amount of 3 α -PI positively modulating GABA_A-R and 3 β -PI being inactive but competing with the former PI on the receptors, the ratios of the sum of the 3 α -PI to the sum of 3 β -PI were evaluated. As demonstrated in Fig. 6A, the F and L groups displayed more than two times lower values for the

ratio than the P group. This means that as regards the free steroids, the circulating positive GABA_A-R modulators are more manifestly prevalent in pregnancy over their inactive competitors on the GABA_A-R. The respective differences in conjugates were much less pronounced (Fig. 6B).

Strong correlations between 3 α - and 3 β -PI that were found within all groups illustrate an uncomplicated, reversible oxidoreductive switch between the 3 α - and 3 β -PI via the 3-oxo-intermediate product in all groups. These metabolic steps converting neuroinhibiting 3 α -PI to 3 β -PI which competes with the 3 α -PI on GABA_A-R, as well as the sulfoconjugation of the PI-forming products operating on the GABA_A-R in an opposite way to free 3 α -PI, may be of great importance. It is known that P3 α 5 α dosage in animals or humans usually results in a weaker response than expected in consideration of the results of *in vitro* experiments. P3 α 5 α dosage eventuates in a bell-shaped response. At lower concentrations, P3 α 5 α paradoxically acts as neurostimulant showing a neuroinhibiting effect as late as at levels comparable with the concentrations common in pregnancy (Backstrom *et al.* 2003). Moreover, one of the neuroinhibiting PIs, P3 α 5 β is an unstable substance with a short half-life in the human organism (Carl *et al.* 1994). P3 α 5 β is identical to a short-term central anesthetic eltanolone that has previously been tested in pharmacological studies (Hering *et al.* 1996). Given the data from this study, it seems likely that the instability of P3 α 5 β is strongly associated with the reversible metabolic steps outlined above.

The initiation of human parturition represents a complex system involving various factors and multiple, interconnected feedback loops (Nathanielsz 1998). Several mechanisms have been suggested to explain the primary impulse for parturition. Some of them involve significantly decreasing the placental synthesis of Prog, while at the same time increasing estradiol production before the onset of parturition (Nathanielsz 1998, Wu *et al.* 2004). In contrast to non-primate species, progesterone levels in human maternal serum do not change markedly around parturition, while estradiol levels escalate up to labor, as in other mammals (Mathur *et al.* 1980). Despite the number of mechanism suggested (McLean & Smith 2001, Patel *et al.* 2003, Condon *et al.* 2004, Mesiano 2004), our knowledge regarding the primary impulse for the human parturition remains inadequate. The role of neuro-active reduced progesterone metabolites – and particularly the most abundant of them, PIs – in the timing of human parturition is still unclear, despite studies reporting their major effects in other mammals (Brussaard *et al.* 1997, 2000). In rats, decreasing allopregnanolone levels just before labor induce a positive feedback loop in oxytocin production, resulting in a rapid delivery (Brussaard *et al.* 1997, 2000). The latter substance brings the GABA_A-R from a neurosteroid-sensitive mode toward a condition in which the receptors are not sensitive (Koksma *et al.* 2003), via a shift in the balance between the activities of endogenous Ser/Thr phosphatase and protein kinase C (Koksma *et al.* 2003). Given all the foregoing, it seems likely that changing the biosynthesis of PIs

Figure 8 Biosynthesis of pregnanolone isomers in men (A), women in the follicular and luteal phase of menstrual cycle respectively (B and C respectively) and in pregnant women (D).

could influence the onset of labor. Moreover, unconjugated 5 β -pregnane steroids cause rapid uterine relaxation through a pregnane receptor X-mediated mechanism (Mitchell *et al.* 2005). In this respect, the increased proportion of the 5 β -PI demonstrated in Fig. 7A allows speculation regarding the pregnancy sustaining role of pregnanolone. The weakening activity of 5 β -reductase (Sheehan *et al.* 2005) as well as the declining levels of reduced 5 β -pregnane steroids near term, further supports this hypothesis (Gilbert Evans *et al.* 2005). The results obtained in this study suggest that the sulfation of P3 β 5 α is an important metabolic step contributing to progesterone catabolism in the maternal compartment. Using partial correlations with adjustment to constant levels of all steroids in the correlation matrix, except for the pair under investigation, P3 β 5 α C in pregnant women negatively correlated with Prog ($r = -0.512$, $P = 0.043$, $n = 27$). The negative partial correlation between Prog and P3 α 5 β ($r = -0.548$, $P = 0.019$, $n = 29$) indicates that a significant amount of Prog may be also metabolized via the 5 β -pathway.

In conclusion, the circulating levels of PI and PIC showed similar values for the M and F while significantly higher levels of the steroids were found in L, and prominently higher concentrations were observed in P. The neuroactivating PICs were strikingly prevalent over the neuroinhibitory 3 α -PI in the circulations of all groups. Significantly higher ratios of 5 α -PIC to 5 α -PI found in P compared with F and L groups indicate the more intensive conjugation of 5 α -PI in pregnancy, including that of the most abundant neuroinhibiting steroid P3 α 5 α . This mechanism probably provides for the elimination of excessive amounts of neuroinhibiting P3 α 5 α in the maternal compartment. The opposite situation was observed in P3 α 5 β . This result points to a limited sulfation capacity for pregnanolone in pregnant women. In contrast to the situation in men and non-pregnant women, where the P3 α 5 β C is the most abundant PIC but P3 α 5 β is a minor substance compared with the remaining PI, P3 α 5 β may acquire physiological importance in pregnancy, contributing to the sustaining thereof. Quite the opposite effect, the declining formation of P3 α 5 β as reported recently (Gilbert Evans *et al.* 2005, Sheehan *et al.* 2005), may participate in the initiation of human parturition, given relative abundance of the steroid, its potency to suppress the activity of oxytocin-producing cells (Leng & Russell 1999, Brussaard *et al.* 2000, Koksmä *et al.* 2003) and its effectiveness in uterine relaxation (Mitchell *et al.* 2005).

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