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Serum Profiles of Free and Conjugated Neuroactive Pregnanolone Isomers in Nonpregnant Women of Fertile Age

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Background: Pregnanolone isomers (PI) with a hydroxy group in the 3α-position are neuroinhibitors operating via positive modulation of $GABA_A$ receptors. The 3β-PI and sulfates of PI and pregnenolone exert the opposite effect. In addition to the brain's *in situ* synthesis, some circulating steroids can penetrate the blood-brain barrier.

Methods: To assess the physiological impact of peripheral endogenous neuroactive pregnanolone isomers and their polar conjugates in women, serum allopregnanolone (P3α5α), isopregnanolone (P3β5α), pregnanolone (P3α5β), epipregnanolone (P3β5β), pregnenolone, estradiol (including their polar conjugates), and additional steroids were measured in 16 women in the follicular and luteal phases of the menstrual cycle using gas chromatography/mass spectrometry and RIA for the analysis. Linear models and Spearman's correlations were used for data evaluation.

Results and Discussion: The levels of conjugated PI were from one to almost three orders of magnitude higher in comparison with the free steroids. The results indicate that a substantial proportion of the progesterone is metabolized in the sequence progesterone→5β-dihydroprogesterone→P3α5β→conjugated P3α5β. The sulfation of PI and particularly of P3α5β moderates the levels of free PI and restrains estradiol biosynthesis via progesterone degradation. PI including the conjugates reflected changing progesterone formation during the menstrual cycle. In the follicular phase, the positive correlation with conjugated pregnenolone, the independence of progesterone, and the negative age relationships of PI indicate their adrenal origin. The dependence on progesterone and the independence of conjugated pregnenolone suggest a gonadal source of PI in the luteal phase. The neuroactivating PI prevailed over neuroinhibiting PI. **(*J Clin Endocrinol Metab* 91: 3092–3099, 2006)**

THE AIM OF THIS STUDY was to assess the physiological impact of endogenous neuroactive pregnanolone isomers (PI) and their polar conjugates in women, based on their serum levels and the known neuromodulating activities as reported in the literature. Another question was whether the subsequent metabolism of the PI could affect the balance between serum neuroactivating and neuroinhibiting steroids in the human organism and/or the production of estrogens. The levels of most of the PI were measured here for the first time in nonpregnant women, although their physiological effects are generally known. One of these substances, pregnanolone (P3α5β), is even recognized to be an efficient short-term anesthetic, eltanolone (1).

Reduced progesterone metabolites, including PI, are known to be efficient neuroactive steroids. They are primarily effective as modulators of neurotransmitter receptors influencing the permeability of ion channels (2). Although PI with a hydroxy group in the 3α-position are known to attenuate neuronal activity (2–4) via the positive allosteric modulation of γ-aminobutyric acid receptors of type A ($GABA_A$-r), a PI hydroxy group in the 3β-position exerts the opposite effect, reducing chloride uptake stimulation by 3α-PI (5). Moreover, sulfation, which counteracts the effect of 3α-hydroxy-group isomers, further amplifies the $GABA_A$-r, inhibiting effect in the 3β-PI. The $GABA_A$-r-inhibiting efficiency of 3β-hydroxy-5α-pregnane-20-one sulfate, for example, is comparable with the $GABA_A$-r activating effectiveness of allopregnanolone (P3α5α), the concentration of which is more than 10 times lower in the maternal plasma before labor (6, 7). Some reports indicate that in addition to the brain's *in situ* synthesis, circulating PI penetrate the blood-brain barrier (8, 9).

PI are regarded as progesterone metabolites originating through the action of ubiquitous 5α-reductase and liver 5β-reductase producing 5α- and 5β-dihydroprogesterone, respectively. The subsequent metabolism of dihydroprogesterone epimers to individual PI is catalyzed by stereospecific 3α- or 3β-hydroxysteroid oxidoreductases (9–11). The latter enzyme may be identical to the 3β-hydroxysteroid dehydrogenase as indicated in the human placenta study (12). To date, no study has reported on all of the PI and their polar conjugates in the serum of nonpregnant women. It is for this reason that the authors have measured the circulating levels of all PI, such as P3α5α, isopregnanolone (P3β5α, epiallopregnanolone, isoallopregnanolone), P3α5β, epipreg-

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Abbreviations: C/F, Ratios of conjugates to free steroids; FP, follicular phase; $GABA_A$-r, γ-aminobutyric acid receptors, type A; GC-MS, gas chromatography/mass spectrometry; LP, luteal phase; MC, menstrual cycle; P3α5α, allopregnanolone, 3α-hydroxy-5α-pregnan-20-one; P3α5αC, polar conjugates of allopregnanolone; P3α5β, pregnanolone, 3α-hydroxy-5β-pregnan-20-one; P3α5βC, polar conjugates of pregnanolone; P3β5α, isopregnanolone, 3β-hydroxy-5α-pregnan-20-one (epiallopregnanolone); P3β5αC, polar conjugates of isopregnanolone; P3β5β, epipregnanolone, 3β-hydroxy-5β-pregnan-20-one; P3β5βC, polar conjugates of epipregnanolone; PI, pregnanolone isomers.

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nanolone (P3β5β), pregnenolone, estradiol and their polar conjugates, progesterone, 17-hydroxyprogesterone, 17-hydroxypregnenolone, and dehydroepiandrosterone, in women in the follicular phase (FP) and luteal phase (LP) of the menstrual cycle (MC), using gas chromatography/mass spectrometry (GC-MS) and RIA.

The first aim was to estimate the main source of PI in individual phases of the MC. Another question was whether sulfation could significantly influence the circulating levels of free PI, progesterone, estradiol, and/or estradiol precursors. We were also interested whether the serum 3α-PI closely correlate with the 3β-PI in free steroids and conjugates and whether there is a possibility that neuroinhibiting neuroactive steroids are promptly converted into their antagonists. Finally, we investigated whether the circulating levels of PI are age dependent within the fertile period of the women.

Subjects and Methods

Subjects

The patient group consisted of healthy premenopausal volunteers (22–45 yr of age). The blood was collected on the fifth and 22nd day of the MC for the FP (n = 15) and LP (n = 16), respectively. Eleven women were followed in both phases. The subjects used no hormonal contraception for at least 3 months before and during the trial. The local ethics committee approved the study. After signing written informed consent, the patients underwent blood sampling from the cubital vein.

Sample collection

The serum was obtained after centrifugation for 5 min at 2000 $\times$ *g* at 0 C. The serum samples were stored at −20 C until analyzed.

Steroids and chemicals

The steroids were from Steraloids (Wilton, NH). The solvents for the extraction were of analytical grade from Merck (Darmstadt, Germany). The derivatization agents Sylon BFT and TMCS were purchased from Supelco (Bellefonte, PA).

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.

Preparation of the serum samples for GC-MS free steroids analysis

Frozen samples were thawed, and 1 ml of the sample was spiked with trideuterated dehydroepiandrosterone as an internal standard to attain a concentration of 1 μg/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 pregnenolone, dehydroepiandrosterone, estradiol, and the four PI using a method published previously (6) with some modifications reported recently (13).

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 (14). The hydrolyzed sample was evaporated in a vacuum centrifuge; the dry residue was spiked with trideuterated dehydroepiandrosterone as an internal standard to attain a concentration of 1 μg/ml and further processed in the same way as in the free steroids.

Determination of steroids by RIA

17-Hydroxypregnenolone and progesterone were measured using in-house methods described elsewhere (15, 16). 17-Hydroxyprogesterone was measured using a commercial RIA kit (Immunotech, Marseilles, France) with intra- and interassay coefficients of variation of 7.8 and 15.7%, respectively, and a measurement range of 0.14–149 nmol/liter.

Statistical data analysis

To evaluate the differences between phases of the MC, results were evaluated using both the Mann-Whitney *U* test of the difference between means and Wilcoxon's paired test of the mean difference.

To investigate the age dependence of the steroids, a polynomial regression model was applied. The minimum of the mean error of prediction was used as a criterion for finding the best degree of the polynomial. In all cases, simple two-parameter linear regression was the best model. Given departures from a Gaussian distribution and the nonconstant variance, the regression diagnostics, and where necessary data transformations, were carried out as described previously (17). In addition to regression models, Spearman's correlations were applied to find relationships between the steroids. To avoid problems with univariate homogeneity and distributional symmetry, Spearman's robust correlations were applied. For graphical demonstration, the data were treated as follows. 1) The individual variables were transformed by power transformations to maximum conformity with a Gaussian distribution using linear regression with the actual fractiles *vs.* theoretical fractiles from a Gaussian distribution. The minimum value of the mean error of prediction was used as a criterion for finding the best transformation parameter. 2) The transformed variables were used for a calculation of Pearson's correlation with a 95% confidence ellipsoid and principal axis. 3) The 95% confidence ellipsoid and principal axis were retransformed to the original scale and used for a graphical demonstration, together with the original nontransformed data.

Statgraphics Plus version 5.1 from Manugistics (Rockville, MD) and NCSS 2000 from Number Cruncher Statistical Systems (Kaysville, UT) were used for the calculations.

Results

Serum levels of free and conjugated PI in the FP and LP of the MC

The circulating levels of free and conjugated PI are shown in Table 1. As expected, the levels of unconjugated PI in the FP were low, with median values of 0.51, 0.27, 0.134, and 0.062 nmol/liter for P3α5α, P3β5α, P3α5β, and P3β5β, respectively. The levels in the conjugates were markedly higher, with medians of 7.6, 10.0, 20.3, and 3.13 nmol/liter for P3α5α, P3β5α, P3α5β, and P3β5β, respectively.

Compared with the FP, the PI levels in the LP were conspicuously elevated in both the free and conjugated steroids, with median values of 1.89, 1.12, 0.428, and 0.284 nmol/liter for P3α5α, P3β5α, P3α5β, and P3β5β, respectively, in the free PI and medians of 28.8, 37.2, 51.2, and 6.5 nmol/liter for P3α5α, P3β5α, P3α5β, and P3β5β, respectively, in the conjugates.

Differences between the FP and LP of the MC

The ratios of PI in the LP compared with those in the FP were evaluated using a linear model with the ratio as a dependent variable. This model also contained the steroid status (P3α5α, P3β5α, P3α5β, and P3β5β) as the first factor

TABLE 1. Circulating levels of neuroactive free and conjugated PI (nmol/liter) and related steroids in premenopausal women

Substance	FP (n = 15)					LP (n = 16)					Difference (LP-FP) (n = 11)					Difference between means (*P*)	Mean difference (*P*)
	Mean	SD	Median	25th percentile	75th percentile	Mean	SD	Median	25th percentile	75th percentile	Mean	SD	Median	25th percentile	75th percentile	Mann-Whitney *U* test	Wilcoxon's paired test
Preg	4.25	1.74	4.21	2.50	5.62	6.59	3.02	5.85	4.09	8.77	2.32	2.95	1.80	−0.30	4.09	<0.03	<0.05
Prog	1.4	0.8	1.3	0.6	2.0	32.7	19.3	36.2	15.8	47.1	36.1	16.5	43.4	18.4	45.3	$<2.10^{-6}$	<0.004
Prog17	4.33	2.80	4.21	1.88	5.63	8.47	6.70	6.50	4.78	8.86	5.44	6.66	4.57	0.68	6.82	<0.03	<0.006
P3α5α	0.53	0.31	0.51	0.36	0.54	2.14	1.89	1.59	0.87	2.75	2.03	2.17	1.63	0.45	3.41	<0.0002	<0.009
P3β5α	0.29	0.17	0.27	0.13	0.35	1.23	1.12	0.90	0.44	1.54	1.20	1.29	0.74	0.23	1.77	<0.0001	<0.004
P3α5β	0.167	0.186	0.134	0.067	0.186	0.523	0.428	0.375	0.204	0.804	0.526	0.414	0.358	0.150	0.873	<0.003	<0.004
P3β5β	0.089	0.088	0.062	0.031	0.099	0.260	0.284	0.168	0.113	0.261	0.212	0.331	0.106	0.000	0.247	<0.01	<0.04
PregC	205	125	157	134	284	196	87	188	125	266	31	63	18	−22	79	NS	NS
P3α5αC	8.3	3.3	7.6	5.6	10.3	33.2	17.0	28.8	20.9	46.7	29.5	18.0	29.7	12.5	49.7	$<2.10^{-6}$	<0.004
P3β5αC	11.9	7.3	10.0	7.1	16.1	40.1	22.5	37.2	24.1	57.5	32.0	26.2	28.0	14.2	57.1	<0.0003	<0.009
P3α5βC	24.8	15.0	20.3	13.1	33.1	47.4	16.8	51.2	41.0	54.9	31.2	13.5	30.5	22.3	38.8	<0.002	<0.004
P3β5βC	3.82	3.08	3.13	1.50	4.54	8.35	5.82	6.50	5.70	8.27	4.94	5.14	3.39	1.57	5.66	<0.0008	<0.009

Preg, Pregnenolone; Prog, progesterone; Prog17, 17-OH-progesterone; C, polar conjugates of the corresponding steroid; NS, not significant

and conjugation status as the second and also included interfactor interaction and age as a covariate. The factors and the interfactor interaction were insignificant. Only the covariate age showed a borderline negative correlation ($P < 0.03$) with the ratio (data not shown).

Differences in the ratios of conjugated to free steroids (C/F)

The differences in the C/F values in dehydroepiandrosterone, estradiol, pregnenolone, and individual PI (Fig. 1) were evaluated using a linear model with the C/F as a dependent variable, the steroid status (dehydroepiandrosterone, estradiol, pregnenolone, P3α5α, P3β5α, P3α5β, and P3β5β) as the first and MC status as the second factors, age as a quantitative factor, and all the interfactor interactions of the second order. The model indicated highly significant differences between individual steroids ($P < 0.0005$), and C/F values rose to a greater or lesser degree in the FP ($P < 0.007$). Of the interactions, phase of the MC × age reached significance ($P < 0.02$), indicating differences between younger and older subjects in respect of the factor phase of the MC. As illustrated on Fig. 1, the dehydroepiandrosterone showed the highest C/F (around 400). The steroid with the

FIG. 1. Ratios of polar conjugates to free steroids in dehydroepiandrosterone (DHEA), estradiol, pregnenolone, and PI (P3α5α allopregnanolone, P3β5α isopregnanolone, P3α5β pregnanolone, P3β5β epipregnanolone) in the circulation of nonpregnant women. A general linear model with the steroid and phase of the menstrual cycle as the main factors, age as a covariate, and all combinations of second-order interactions was used to evaluate the effect of the factors and covariate. The differences between individual subgroups were evaluated using Bonferroni multiple comparisons. *White* and *dotted bars* with *error bars* represent retransformed mean values with 95% confidence intervals in the FP and LP, respectively. For details, see *Statistical data analysis* in *Subjects and Methods*.

second highest C/F values (around 150) was, surprisingly, P3α5β, this markedly differing from the remaining PI. The group of steroids containing a hydroxy group in the 3β-position followed (pregnenolone, P3β5α, and P3β5β). These substances did not differ from each other in the C/F, with values close to 40. The P3α5α showed the lowest C/F values from among the PI (around 15).

Correlations of free and conjugated PI to their precursors progesterone, pregnenolone, and pregnenolone sulfate

In the FP, the correlations between PI and their precursors were insignificant, with the exception of the borderline correlation between P3β5α and progesterone. The opposite situation was found in the LP, where strong and significant or medium and borderline correlations were recorded between progesterone and PI. The correlations of pregnenolone and PI did not reach significance, with the exception of P3β5β, which significantly correlated with pregnenolone (Table 2).

In contrast to conjugated 3α-isomers significantly correlating with pregnenolone polar conjugates in the FP, conjugated 3β-PI did not (Table 2). The correlations of polar conjugates of pregnenolone with all the conjugated PI in the LP were insignificant (Table 2).

Correlations between 3α- and 3β-PI in free steroids

The correlations between free 3α-PI and corresponding free 3β-PI with a hydrogen atom in the same position at the C5-carbon were strong and significant in both phases of the MC (Table 2).

The influence of sulfation in PI on estradiol biosynthesis

As demonstrated in Fig. 2, the ratio of total conjugated PI to total free PI in the LP negatively correlated with the C21 3-oxo-4-en steroids and estradiol, reaching significance in 17-OH-progesterone and estradiol. The correlations with 3β-hydroxy-5-en steroids were insignificant (data not shown).

Age relationships in circulating PI

Of the free and conjugated PI, only the P3α5βC showed a significant age relationship, with decreasing values of the conjugate accompanying increasing age in the FP ($R = -0.602$; $P = 0.018$; $n = 15$). In terms of steroid ratios reflecting PI metabolism, the P3α5αC/P3α5α ratio negatively correlated with age in the FP (Fig. 3). In the FP, 3α-PI significantly increased with age, as did the ratio of 3α- to 3β-PI (Fig. 4, A and C). An opposite borderline trend was observed in 3β-PI (Fig. 4B). None of the aforementioned steroids and steroid ratios showed any significant age dependence in the LP.

Discussion

A number of reports have appeared concerning the pharmacological effects of neuroactive steroids. Most of these studies were conducted using laboratory animals in which the biosynthesis of neuroactive steroids differs substantially from that in humans. On the other hand, the information concerning physiological levels of the substances in humans and particularly in nonpregnant women of fertile age is limited. Reports have concentrated predominantly on the most abundant of the pregnane steroids, P3α5α (18–20). A limited number of studies have dealt with other endogenous pregnane derivatives (13, 21, 22), and none have addressed the polar conjugates of PI. As noted above, the sulfation of originally neuroactive substances can not only eliminate but can also even reverse their effects (7), and analogous results may be expected in terms of the influence of enzymes participat-

TABLE 2. Spearman's correlations among circulating neuroactive steroids and their precursors and metabolites in 15 and 16 premenopausal women in the FP and LP of the menstrual cycle, respectively

	Preg	Prog	Prog17	P3α5α	P3β5α	P3α5β	P3β5β	PregC	P3α5αC	P3β5αC	P3α5βC	P3β5βC
Preg		0.258	0.361	0.207	0.446	0.304	0.321	0.329	0.221	0.232	0.471	0.286
		0.334	0.177	0.438	0.095	0.256	0.229	0.219	0.407	0.385	0.078	0.285
Prog	0.215		0.617	0.434	0.523	−0.183	−0.219	0.194	0.186	−0.129	−0.047	0.072
	0.406		0.021	0.105	0.050	0.494	0.413	0.469	0.486	0.629	0.862	0.789
Prog17	−0.062	0.579		0.239	0.450	0.096	−0.061	0.000	−0.011	0.414	0.093	0.361
	0.811	0.025		0.371	0.092	0.718	0.820	1.000	0.968	0.121	0.728	0.177
P3α5α	0.327	0.744	0.477		0.693	0.204	0.136	−0.068	0.229	−0.336	−0.071	0.243
	0.206	0.004	0.065		0.010	0.446	0.612	0.800	0.392	0.209	0.789	0.364
P3β5α	0.353	0.788	0.465	0.965		0.425	0.289	0.254	0.211	−0.014	0.125	0.343
	0.172	0.002	0.072	0.000		0.112	0.279	0.343	0.430	0.957	0.640	0.200
P3α5β	0.191	0.818	0.509	0.915	0.906		0.764	0.289	0.268	0.511	0.246	0.539
	0.459	0.002	0.049	0.000	0.001		0.004	0.279	0.316	0.056	0.357	0.044
P3β5β	0.632	0.500	0.309	0.677	0.688	0.594		0.336	0.368	0.264	0.446	0.536
	0.014	0.053	0.232	0.009	0.008	0.021		0.209	0.169	0.323	0.095	0.045
PregC	0.074	−0.027	0.068	−0.047	−0.032	−0.141	−0.185		0.793	0.339	0.750	0.271
	0.776	0.918	0.793	0.855	0.900	0.585	0.473		0.003	0.204	0.005	0.310
P3α5αC	0.291	0.688	0.241	0.753	0.741	0.777	0.450	0.362		0.275	0.664	0.496
	0.259	0.008	0.350	0.004	0.004	0.003	0.081	0.161		0.304	0.013	0.063
P3β5αC	0.441	0.550	0.124	0.597	0.585	0.562	0.415	0.427	0.918		0.332	0.418
	0.088	0.033	0.632	0.021	0.023	0.030	0.108	0.099	0.000		0.214	0.118
P3α5βC	0.071	0.491	−0.035	0.274	0.271	0.409	0.050	0.359	0.679	0.668		0.543
	0.785	0.057	0.891	0.289	0.295	0.113	0.846	0.165	0.009	0.010		0.042
P3β5βC	0.144	0.644	0.365	0.397	0.329	0.435	0.338	−0.056	0.409	0.435	0.600	
	0.577	0.013	0.158	0.124	0.202	0.092	0.190	0.829	0.113	0.092	0.020	

The correlation coefficients and their statistical significances are in the *upper* and *lower parts* of the cells, respectively. The data for the FP and LP are *above* and *below the diagonal*, respectively. Preg, Pregnenolone; Prog, progesterone.

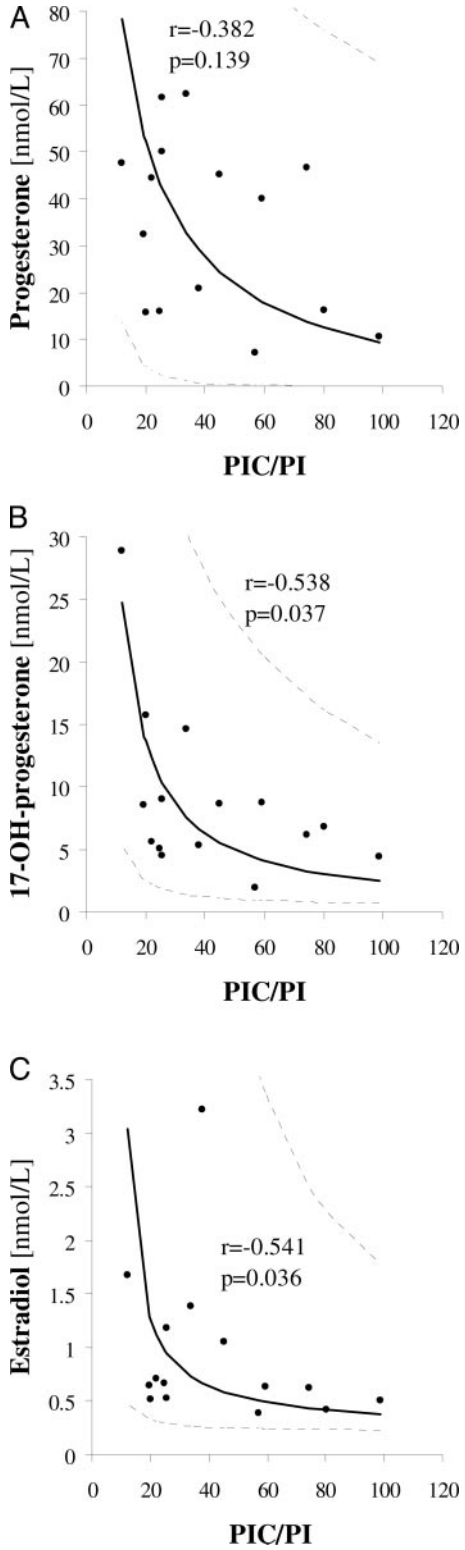

FIG. 2. Correlations between circulating progesterone, 17-OH-progesterone, estradiol, and the ratio of conjugates to free PI (PIC/PI) in the luteal phase of the menstrual cycle; r and p represent Spearman's correlation coefficient and its statistical significance. The *bold* and *dashed curves* demonstrate the retransformed principal axes and 95% confidence ellipsoids computed from the data after power transformation to symmetry and homoscedasticity. For details, see *Statistical data analysis* in *Subjects and Methods*.

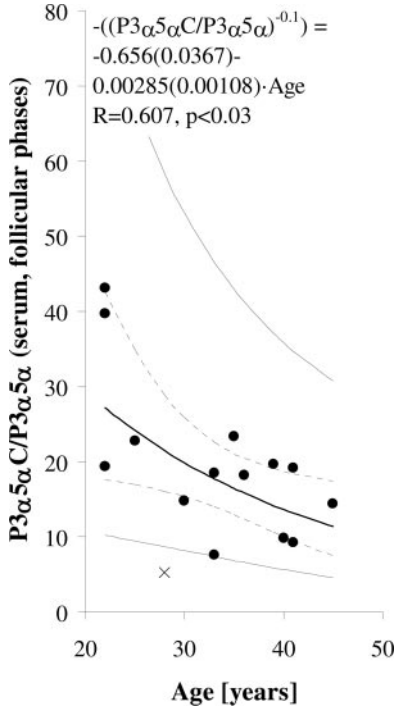

FIG. 3. Age dependence of the ratio of conjugated allopregnanolone (P3α5αC) to free allopregnanolone (P3α5α) in the FP of the MC; the *black circles* and *cross* represent experimental points and an outlier, respectively. The *bold solid curve* represents the retransformed regression line, the *thin dashed curves* symbolize the 95% confidence interval of the retransformed regression line, and the *thin solid line* denotes the region where 95% of the experimental points should theoretically occur. The *numbers in parentheses* in the regression equation represent SE of individual parameters determining the retransformed regression line. For details, see *Statistical data analysis* in *Subjects and Methods*.

ing in oxidoreductive interconversion between neuroinhibiting pregnane steroids with a hydroxy group in the 3α-position and their 3β-metabolites acting in the opposite way (5, 23–25).

The levels of all the PI including the conjugates strongly depended on the MC, reflecting changes in progesterone formation well (Tables 1 and 2). In considering the physiological impact of free PI in nonpregnant women, absolute levels of the steroids and the ratios of neuroactivating PI conjugates to neuroinhibiting 3α-PI should be taken into account. As documented in Table 1, in all the PI, the levels of conjugates were markedly higher than the free steroids. Another balance that requires evaluation is the proportion of neuroinhibiting 3α-PI positively modulating $GABA_A$-r and the 3β-PI reducing their uptake on the receptors. Here, the balance was shifted more toward the neuroinhibiting substances, but the differences were not particularly prominent. Given the foregoing, it is obvious that the circulating levels of neuroactivating PI are markedly prevalent over neuroinhibiting PI. On the other hand, the proportions in the circulating levels need not necessarily reflect steroid ratios at the sites where they take effect. It is likely that the pronounced excess of polar PI conjugates in the circulation is principally connected to their higher solubility in the circulation in comparison with their nonpolar free analogs. Other important

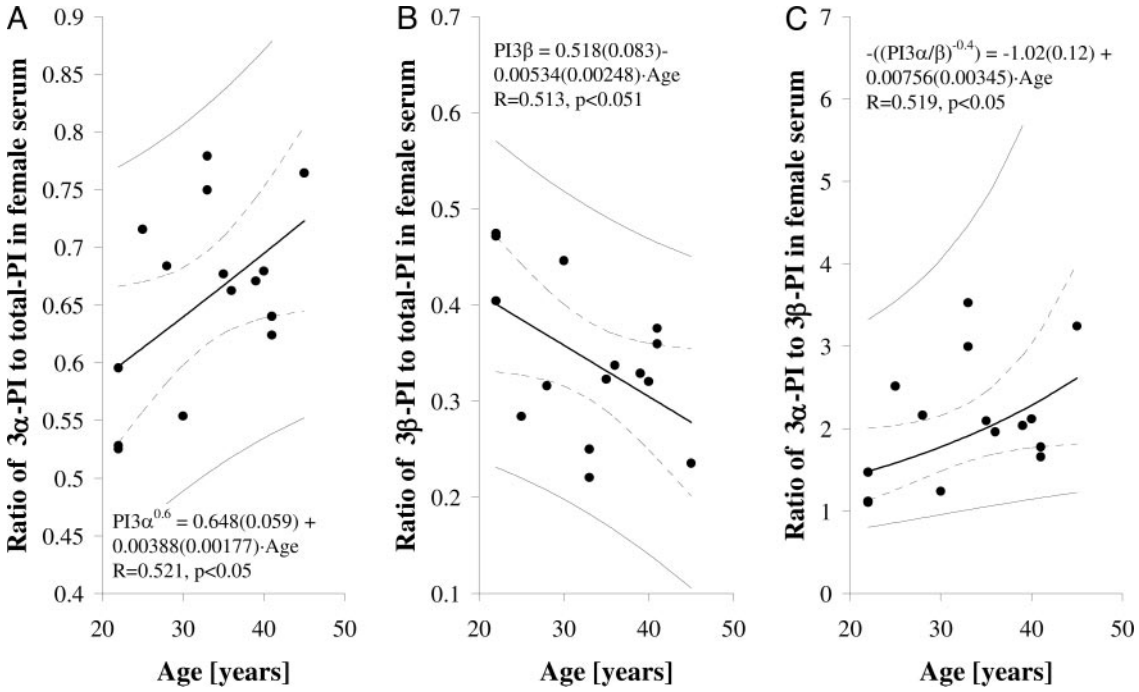

FIG. 4. Age dependence of the ratio of 3α-PI to total PI, the ratio of 3β-PI to total PI, and the ratio of 3α- to 3β-PI in the FP of the MC. The *lines* and *symbols* are the same as for Fig. 3.

circumstances that should be taken into account are the effect of the blood-brain barrier on the transport of neuroactive steroids from the circulation to the brain and the brain biosynthesis of neuroactive steroids *in situ*. As regards the former, the chances of overcoming the blood-brain barrier generally increase with the decreasing polarity of the substance (26). This means that the transport of free PI will be preferred over that of the conjugates, despite their striking excess as reported in a model focused on the transport of free and conjugated pregnenolone from the circulation into the brain in rats (27). The conjugation of PI is nevertheless of interest as an instrument for the transport of PI by circulation, as a mechanism regulating the proportion of neuroactivating to neuroinhibiting pregnane steroids, or at least, as a key metabolic step responsible for the elimination of neuroactive PI.

As has been reported for the guinea pig, stereospecific 3α- and 3β-steroid sulfotransferases catalyze the sulfation of PI (28). In this regard, similar C/F values might be expected among the 3α-isomers and analogously among the 3β-PI. This assumption was confirmed only for the 3β-isomers (Fig. 1). The values of the C/F were about 40 for both P3β5α and P3β5β, showing no significant differences from each other or from their common precursor with a hydroxy group in the 3β-position, pregnenolone. On the other hand, a striking difference was observed between the ratios in P3α5α (about 15) and in P3α5β (about 10 times higher). Although the ratio in P3α5α was about three times lower than the values found in the 3β-steroids, and the difference was significant, the values in P3β5α were by contrast about three times higher than in the 3β-steroids, and the difference was again significant. These data demonstrate that the concentrations of P3α5β in nonpregnant women are low because of excessive sulfation. Given the probable rapid and reversible interconversion between 3α- and 3β-PI, it is likely that a substantial proportion of progesterone is metabolized in the sequence progesterone→5β-dihydroprogesterone→P3α5β→P3α5βC. Moreover, the negative correlations of estradiol and its precursors in the 4-ene steroid metabolic pathway to the ratio of total conjugated PI to total free PI in the LP also support this idea (Fig. 2). This means that the sulfation of PI and particularly of P3α5β not only moderates the levels of free PI but also significantly restrains estradiol biosynthesis via the degradation of progesterone as a substrate.

As shown in Table 1, P3α5β levels in nonpregnant women are about four times lower than those of the most abundant PI, P3α5α. As previously reported, the ratio in pregnancy was about 2:1 (6, 29). In this case, P3α5β (operating on $GABA_A$-r in a similar way as P3α5α) was the second most abundant PI. These findings may indicate that the capacity of steroid 3α-sulfation may be limited in pregnancy, most likely because of the excessively increased levels of the substrates.

Mutual simple oxidoreductive conversion and uncomplicated sulfation may explain the bimodal effect of 3α-pregnane steroids on the circulation and the resulting neuroinhibiting activity reported in the study of Backstrom *et al.* (30). In lower concentrations, neuroinhibiting 3α-PI are readily metabolized into neuroactivating 3β- and sulfated PI, whereas in higher concentrations, the saturation of active sites by the corresponding enzymes may result in a shift of the balance away from the neuroactivating to the neuroinhibiting substances in the circulation and consequently at the target sites. In the present data, no dependence of the ratio of 3α- to 3β-PI on the MC or on the position of hydrogen on steroid carbon C5 was observed. The results also show strong

correlations between the 3α- and 3β-PI in both phases of the MC (Table 2). These findings indicate uncomplicated interconversion between neuroinhibiting 3α- and neuroactivating 3β-PI, all operating on $GABA_A$-r but in opposite manners. As regards explaining the U-shaped relationship between concentrations of circulating 3α-pregnane steroids and resultant neuroinhibiting activity, the aforementioned findings indicate sufficient capacity in the oxidoreductase system to convert the 3α- to 3β-PI and vice versa in nonpregnant women. On the other hand, limited sulfation capacity appears to be a more likely explanation.

In contrast to the proportions among individual PI being independent of the MC, significant differences between phases of the MC were observed in the correlations of PI to their precursors (Table 2 and Figs. 2–4). A strong correlation of polar conjugates of pregnenolone with P3α5α in the FP (Table 2) and its diminution in the LP (Table 2) and, alternatively, the absence of a correlation with progesterone in the FP (Table 2) and its presence in the LP (Table 2) probably adhere to the predominant importance of adrenal steroids in P3α5α biosynthesis in the FP and, conversely, with the rise of gonadal progesterone production in the LP. Pregnenolone sulfate and cortisol are mostly synthesized in the adrenal cortex zona fasciculata. Unlike gonadal steroids, pregnenolone sulfate, like cortisol, readily responds to ACTH stimulation (31–37). It appears that in the FP, the important metabolic step is just the adrenal formation of the primary steroid precursor, the conjugated pregnenolone sulfate, which in contrast to its free analog, is well soluble in the circulation and could be easily transported to various tissues and organs. The subsequent metabolic steps do not appear so critical in terms of the sufficiency of the unoccupied active sites in the respective enzymes responsible for the successive conversion of polar conjugates of pregnenolone via progesterone and dihydroprogesterones up to PI. The situation is quite the opposite in the LP; in this case, the critical step is gonadal progesterone formation, which is determinative for levels of circulating PI. This finding is in accordance with a recent study indicating the corpus luteum as a source of 3α-PI (38).

The last question addressed in this study was that of the age dependence of PI. In the FP, P3α5βC showed significant negative age dependence ($R = -0.602$; $P = 0.018$; $n = 15$), whereas the corresponding age relationship in the LP was insignificant (data not shown). The former finding may be linked to a gradual age-conditioned decrease in the adrenal production of sulfated 3β-hydroxy-5en steroids and particularly of pregnenolone sulfate as a substrate for the biosynthesis of progesterone and consequently the PI in the FP. No analogous decline appeared in the LP, where PI levels depended on the formation of gonadal steroids.

Positive age dependencies in the FP were found in the ratios of 3α-PI to total PI and 3α- to 3β-PI, whereas a negative dependency was detected in the ratio of 3β-PI to total PI (Fig. 4). Furthermore, a significant negative age dependence was recorded in the C/F for P3α5α (Fig. 3). Given the aforementioned results of the negative age relationship in P3α5βC, it appears that the proportion of circulating neuroinhibiting PI in women exhibits a growing tendency with increasing age in the FP but not in the LP.

In conclusion, the results indicate adrenal origin of PI in the FP and gonadal source of the steroids in the LP. The sulfation of PI and particularly of P3α5β not only moderates free PI levels but also significantly restrains estradiol biosynthesis via degradation of progesterone as a substrate. The conjugation of PI is of interest as an instrument for transport of PI by circulation, as a mechanism regulating the proportions of neuroactivating and neuroinhibiting pregnane steroids, and as a key metabolic step responsible for elimination of neuroactive pregnane steroids.

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