0021-972X/05/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 90(1):395–403

doi: 10.1210/jc.2004-0444

Neuroactive Pregnanolone Isomers during Pregnancy

Antonín Pařízek, Martin Hill, Radmila Kancheva, Helena Havlíková, Lyudmila Kancheva, Josef Cindr, Andrea Pašková, Vladimír Pouzar, Ivan Černý, Pavel Drbohlav, Zdeněk Hájek, and Luboslav Stárka

Department of Gynecology and Obstetrics, First Medical Faculty, Charles University (A.Pař., A.Paš., P.D., Z.H.); Institute of Endocrinology (M.H., R.K., H.H., L.K., L.S.); Institute of Clinical and Experimental Medicine (J.C.); and Institute of Organic Chemistry and Biochemistry (V.P., I.C.), CZ 128 51 Prague, Czech Republic

The pregnanolone isomers (PI) allopregnanolone (3α-hydroxy-5α-pregnan-20-one), pregnanolone (3α-hydroxy-5β-pregnan-20-one), isopregnanolone (3β-hydroxy-5α-pregnan-20-one), epipregnanolone (3β-hydroxy-5β-pregnan-20-one), progesterone, and estradiol were measured in 138 pregnant women. The sampling was carried out from the first through the 10th month of pregnancy. Gas chromatography-mass spectrometry analysis and RIA were used for the measurement of steroid levels. The ratios of individual PI were similar to those found previously around parturition: about 25:10:7:1 for allopregnanolone, pregnanolone, isopregnanolone, and epipregnanolone, respectively. All the PI showed a significant increase during pregnancy, which was more pronounced in the 3α-steroids. The results indicated changing ratios between 3α- and 3β-PI and between 5α- and 5β-PI throughout pregnancy. The constant allopregnanolone/isopregnanolone ratio found through pregnancy weakened the hypothesis of the role of isopregnanolone in the onset of parturition. The ratio of estradiol (stimulating uterine activity) to 5α-PI and epipregnanolone exhibited significant changes during pregnancy in favor of estradiol up to the sixth or seventh month, in contrast to the constant estradiol/pregnanolone ratio. A pregnancy-stabilizing role of pregnanolone, counterbalancing the stimulating effect of estradiol on the onset of parturition, was suggested. (*J Clin Endocrinol Metab* 90: 395–403, 2005)

PARTURITION IS A multifactorial physiological process that involves multiple interconnected positive and negative feedback loops (1). In rodents and ruminants, a significant decrease in progesterone and an increase in estradiol levels have been found before the onset of parturition (1, 2). The association of estradiol synthesis (controlled by the fetal hypothalamo-pituitary-adrenal axis) with a cascade of processes resulting in the onset of parturition is well known (1–3). In contrast to rodents, the mechanism in humans is connected to an altered (and mutually correlated) expression of isoforms of both estradiol and progesterone receptors before parturition (4) without significant changes in the circulating levels of the steroids. Another mechanism proposed in humans is connected to the excessive production of placental CRH near term (5). Nevertheless, there are still a number of significant gaps in our knowledge, particularly with respect to the impulse for parturition.

The role of neuroactive steroids in relation to the onset of human parturition is still not clear. Neuroactive steroids are effective primarily as modulators of the neurotransmitter receptors influencing the permeability of the ion channels (6–13); some also act at the progesterone receptors (14, 15). The activating effect of pregnanolone isomers (PI) on the γ-aminobutyric acid receptors type A ($GABA_A$-r) can be reversed by their sulfation at position C-3 (16). As reported for a rat model (17), a decrease in the levels of PI [which are also produced by placenta (18, 19)] could trigger the production of oxytocin (20–22), resulting in a rapid delivery. This mechanism probably comes into play in rhesus monkeys as well (23). Oxytocin brings the $GABA_A$-r from a neurosteroid-sensitive mode toward a condition in which the receptors are not sensitive (24). During parturition, the $GABA_A$-r become insensitive to allopregnanolone due to a shift in the balance between the activities of endogenous Ser/Thr phosphatase and protein kinase C (24). Additionally, changes in $GABA_A$-r subunit composition throughout gestation have been reported (25). PI with a hydroxy group in the 3α-position are known to attenuate neuronal activity and probably to sustain pregnancy via the aforementioned mechanism. In contrast, PI hydroxylated in the 3β-position exert the opposite effect (26, 27). Moreover, sulfation, which counteracts the effect of 3α-hydroxylated isomers, also amplifies the $GABA_A$-r-inhibiting effect in 3β-PI. For instance, the $GABA_A$-r-inhibiting efficiency of isopregnanolone sulfate is comparable to the $GABA_A$-r-activating effectiveness of allopregnanolone, the concentration of which is more than 10 times lower in maternal plasma before labor than the concentration of isopregnanolone polar conjugates (16, 28, 29).

As noted above, estradiol is well known as a substance provoking the onset of parturition via several mechanisms (30–36). In this respect changes in the ratios of PI to estradiol could be of importance. 5α-PI are known as activators of *N*-methyl-D-aspartate receptors (NMDA-r), which are present in both the central nervous system and the periphery (37). Sulfated 5β-PI have the opposite effect on the NMDA-r (13, 37–40).

In terms of the different neuromodulating effects of indi-

First Published Online October 14, 2004

Abbreviations: $GABA_A$-r, γ-Aminobutyric acid receptor, type A; GC-MS, gas chromatography-mass spectrometry; NMDA-r, *N*-methyl-D-aspartate receptor; P3α,5α, allopregnanolone, 3α-hydroxy-5α-pregnan-20-one; P3α,5β, pregnanolone, 3α-hydroxy-5β-pregnan-20-one; P3β,5α, isopregnanolone, 3β-hydroxy-5α-pregnan-20-one; P3β,5β, epipregnanolone, 3β-hydroxy-5β-pregnan-20-one; PI, pregnanolone isomer(s).

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

vidual PI, it is interesting to trace the proportional changes between the 3α- and 3β-PI and between the 5α- and 5β-PI, as well as between individual PI, estradiol, and progesterone during pregnancy. Taking the PI actions into account, the occurrence of various disturbances connected with an imbalance of PI during pregnancy and postpartum as antenatal or puerperal psychiatric events might also be expected. A knowledge of the normal physiological range of PI in pregnant women could be helpful for the diagnosis of these disorders. In this study the researchers investigated the changes in all four isomers of pregnanolone, *i.e.* allopregnanolone [3α-hydroxy-5α-pregnan-20-one (P3α,5α)], pregnanolone [3α-hydroxy-5β-pregnan-20-one (P3α,5β)], isopregnanolone [3β-hydroxy-5α-pregnan-20-one (P3β,5α)], and epipregnanolone [3β-hydroxy-5β-pregnan-20-one (P3β,5β)] as well as changes in progesterone and estradiol in maternal plasma during pregnancy. The differences in the production of the individual PI and their profiles during parturition were investigated. The following questions were addressed. 1) What are the changes in plasma levels of PI during pregnancy? 2) Are there any changes in the ratios of the 3α- and 3β-isomers? 3) Are there any changes in the ratio of the 5α- and 5β-isomers? 4) What are the changes in the ratios of PI to their precursor progesterone? 5) What are the changes in the ratios of PI to estradiol? 6) Could the balance between neuroinhibiting allopregnanolone and neuroactivating isopregnanolone influence the timing of parturition? 7) What physiological role could be expected for individual PI with respect to their presumed changes during pregnancy?

Subjects and Methods

Subjects

The patient group consisted of 138 pregnant women from the first to 10th months of pregnancy. The local ethical committees of the Institute of Endocrinology and Charles University Teaching Hospital approved the protocol for the study. After signing written, informed consent, the patients underwent blood sampling from the cubital vein.

Sample collection

Cooled plastic tubes containing 100 μl 5% EDTA and 50 pl aprotinin (Antilysin, Spofa, Prague, Czech Republic) were used for blood sampling. The plasma was obtained after centrifugation for 5 min at 2000 × *g* at 0 C. The plasma samples were stored at −20 C until analyzed.

Steroids and chemicals

The steroids were obtained from Steraloids (Wilton, NH). The solvents for extraction and HPLC were of analytical grade and were purchased from Merck & Co. (Darmstadt, Germany). The derivatization agent Sylon BFT was purchased from Supelco (Bellefonte, PA).

Instruments

The gas chromatography-mass spectrometry (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. The liquid scintillation spectrometer was supplied by Beckmann Coulter (Fullerton, CA).

Analytical methods

The PI were measured using a modified method of Hill *et al.* (28). The first modification of the method was the use of less steep temperature and pressure gradients, 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 40 C and 8.5 kPa up to 220 C and 51 kPa, then a slow linear gradient at 2.9 C and 0.5 kPa up to 240 C and 54.5 kPa, and finally a rapid linear gradient at 40 C and 9 kPa up to 310 C and 70 kPa with a 2-min delay. The second modification was the substitution of 17α-methyl-3β,17β-androstenediol as an internal standard for trideuterated dehydroepiandrosterone, added to the standard solution or to the sample in a 1 ng/μl concentration and recorded on an effective mass of 307. The overall time taken for the analysis was 14.2 min. The retention times were 7.104, 7.246, 7.404, 7.604, 7.808, and 8.721 min for trideuterated dehydroepiandrosterone, estradiol, epipregnanolone, allopregnanolone, pregnanolone, and isopregnanolone, respectively. The last change represented the substitution of microextraction in the vials by rapid drying of the derivatization agent under a stream of nitrogen.

Statistical analysis of the data

For evaluation of the changes in steroid and steroid ratios, one-way ANOVA (Kruskal-Wallis test) was used, with gestational age as a factor.

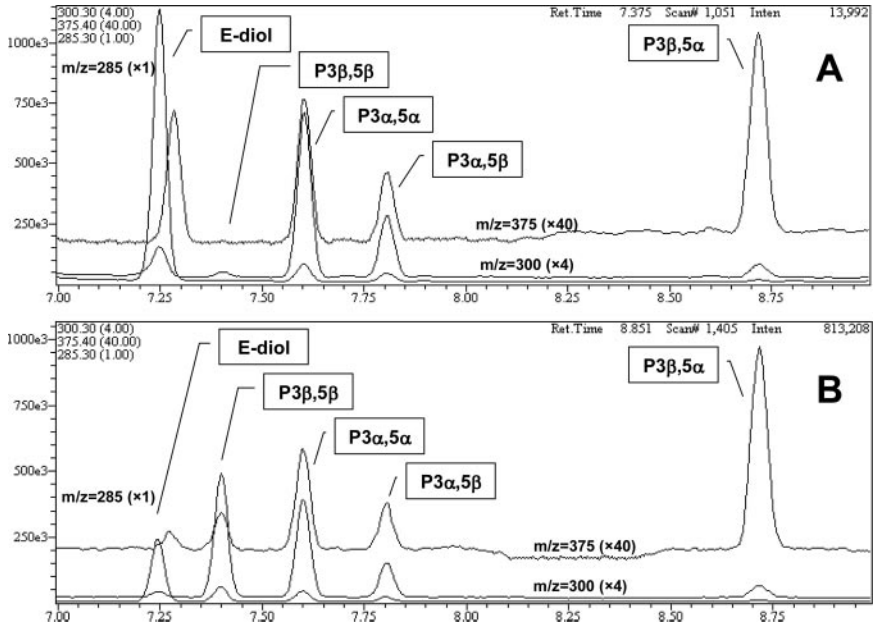

FIG. 1. Comparison between a sample of maternal plasma from a woman in the eighth month of pregnancy and standard solution. A, Response of a sample (4 μl) corresponding to 200 μl plasma from a woman in the eighth month of pregnancy. B, Response of a standard solution (4 μl) containing 4 ng of each of the steroids under study. E-diol, Estradiol; P3β,5β, epipregnanolone; P3α,5α, allopregnanolone; P3α,5β, pregnanolone; P3β,5α, isopregnanolone; m/z, effective masses of the fragments. The *numbers in parentheses* denote multiples of the original responses.

Multiple testing was handled by nonparametric Kruskal-Wallis multiple comparisons to evaluate the differences between the individual groups. Statistical computations were performed using NCSS 2002 statistical software (Number Cruncher Statistical Systems, Kaysville, UT). The Mann-Whitney test was used for comparison between the first and second months of pregnancy in the P3β,5β/progesterone ratio.

Results

Identification of the steroids

The steroids were separated well from each other and from the background (Fig. 1). The sensitivity was sufficient for the quantification of all of the investigated steroids. Figure 2 shows the responses of the individual PI at the effective masses 300 and 375 for selected subjects from the second to the ninth month of parturition.

Changes in PI, progesterone, and estradiol

The changes in PI during pregnancy are shown in Fig. 3. The time profiles for progesterone, estradiol, and the estradiol/progesterone ratio are shown in Fig. 4. All PI, progesterone, and estradiol exhibited significantly increasing trends (by Kruskal-Wallis test).

In the 5α-isomers a plateau occurred within the first and the fifth months of pregnancy. For pregnanolone (Fig. 3C) and estradiol (Fig. 4B), an accelerating increase was found within the first and the seventh months of pregnancy. For epipregnanolone (Fig. 3D), a gradual increase was observed within the first and the sixth months; in this steroid, a significant increase was found between the first and the second months ($P < 0.05$, by Kruskal-Wallis multiple comparisons). A significant increase was observed from the fifth month in the 3α-isomers (Fig. 3, A and B; $P < 0.01$, by Kruskal-Wallis multiple comparisons). The changes in the 3α-isomers were more pronounced compared with 3β-PI (Fig. 3). Progesterone (Fig. 4A) showed a gradual increase from the first to the 10th months as expected.

Changes in the 3α-/3β-isomer ratio

The overall change in the ratios of 3α- to 3β-isomers ($I_{3\alpha/3\beta}$), expressed as a square root of the ratio of the products

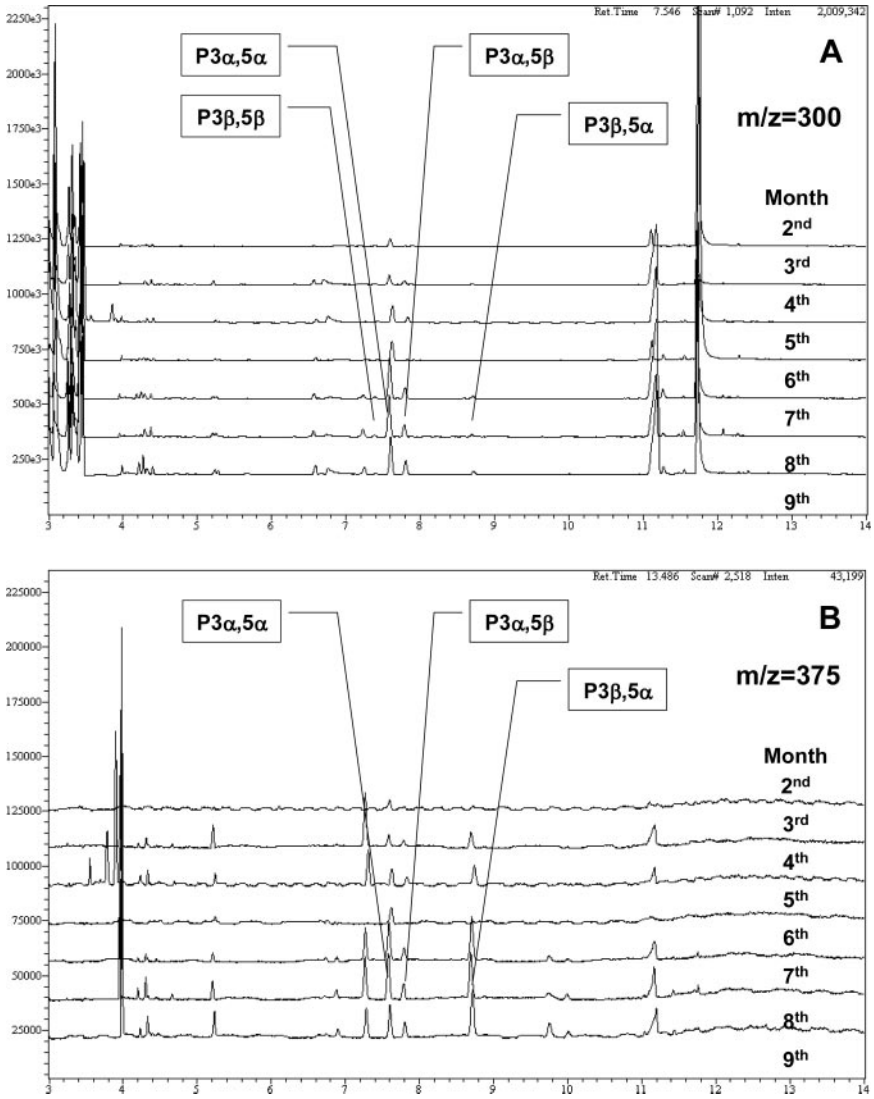

FIG. 2. Responses of all PI in selected maternal plasma from the second to ninth months of pregnancy (4-μl samples corresponding to 200 μl plasma were injected) recorded on effective masses of 300 (A) and 375 (B).

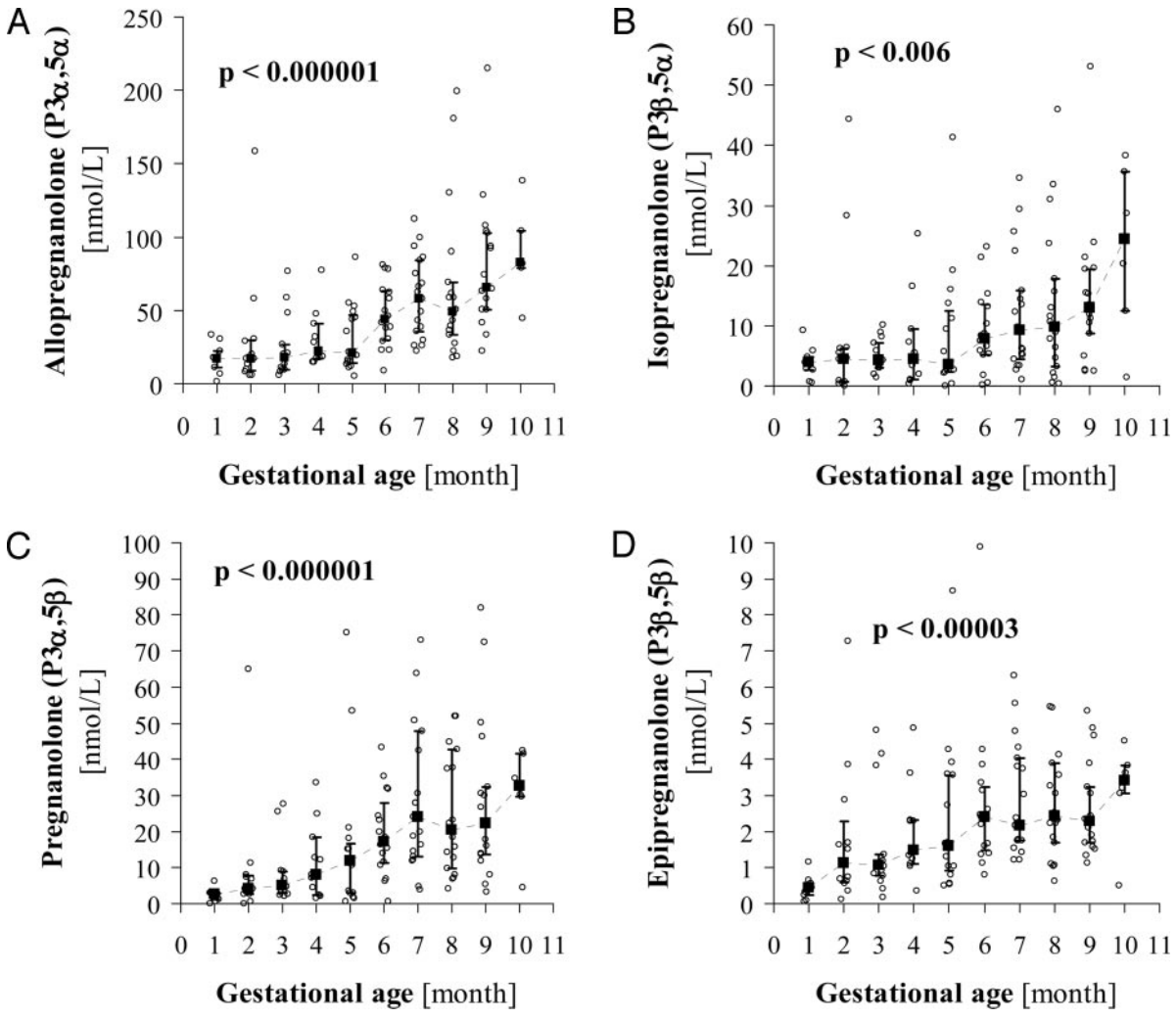

FIG. 3. Changes in the levels of PI in maternal plasma during pregnancy. ○, Individual subjects; ■ with error bars, group medians with quartiles. *P* values represent the statistical significance of the overall trend, as found by Kruskal-Wallis robust ANOVA. The numbers of subjects were 138, 137, 138, and 138 for allopregnanolone, isopregnanolone, pregnanolone, and epipregnanolone, respectively.

of the 3α- and 3β-isomers (Fig. 5), showed a gradual increase within the fifth and the eighth months, when it reached a maximum value. In contrast, the ratio of allopregnanolone (P3α,5α) to isopregnanolone (P3β,5α) was constant during pregnancy (data not shown).

Changes in the 5α-/5β-isomer ratio

The overall change in the ratios of 5α- to 5β-isomers ($I_{5\alpha/5\beta}$) was expressed analogously as in the case of the 3α-/3β isomer ratio as a square root of the ratio of products of the 5α- and 5β-isomers (Fig. 6). This index showed a significant decrease between the first and the second months, a plateau within the second and eight months, and a significant increase within the eighth and 10th months ($P < 0.05$, by Kruskal-Wallis multiple comparisons).

Changes in the ratios of pregnanolone isomer to progesterone

The ratio between 5α-PI and progesterone levels during pregnancy was constant (data not shown). The P3α,5β/progesterone ratio showed an increasing trend within the first and fifth months of pregnancy (Fig. 7; $P < 0.05$, by Kruskal-Wallis multiple comparisons). For the P3β,5β/progesterone ratio, Kruskal-Wallis multiple comparisons indicated a significant increase ($P < 0.02$) between the first month (median, 0.0039; 25% percentile, 0.0029; 75% percentile, 0.0053) and the second month (median, 0.0105; 25% percentile, 0.0063; 75% percentile, 0.0252), which was confirmed by Mann-Whitney testing ($P < 0.02$). No significant change was detected within the second and 10th months, nor did the overall trend evaluated by Kruskal-Wallis ANOVA reach significance.

Changes in the ratios of estradiol to PI

The changes of the ratios of estradiol to PI are shown in Fig. 8. With the exception of the estradiol/P3α,5β ratio being constant during pregnancy (Fig. 8C), all remaining ratios significantly increased up to the sixth or seventh month of pregnancy, with maximum significance for the estradiol/P3α,5α ratio (Fig. 8A).

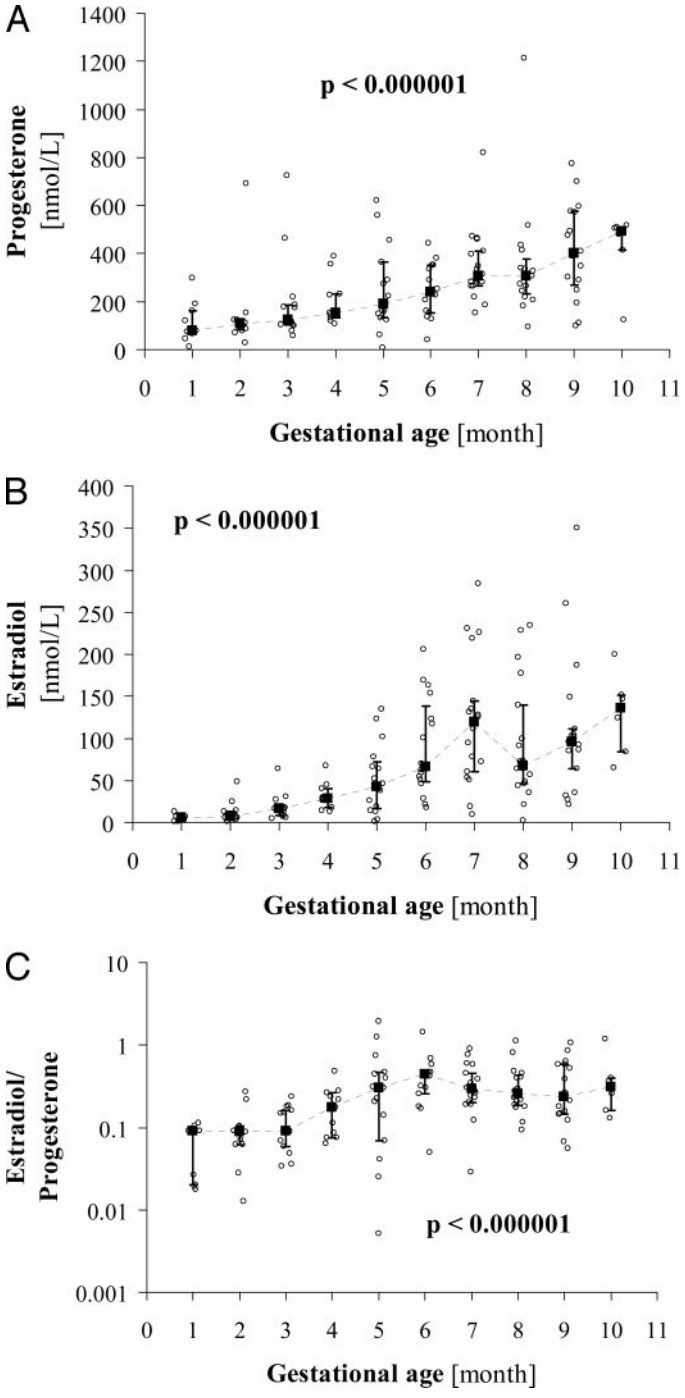

FIG. 4. Changes in the plasma levels of progesterone, estradiol, and estradiol/progesterone ratio in maternal plasma during pregnancy. The symbols are explained in Fig. 3. The numbers of subjects were 131, 138, and 130 for progesterone, estradiol, and estradiol/progesterone ratio, respectively.

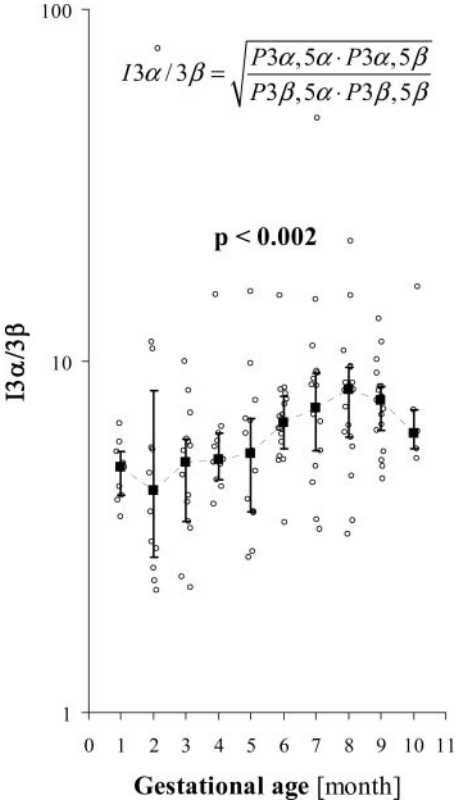

FIG. 5. Index reflecting the overall proportions between 3α- and 3β-PI in the plasma of 137 women during pregnancy. The symbols are explained in Fig. 3.

Discussion

Given the discovery of the effect of allopregnanolone in down-regulating the production of oxytocin during pregnancy (17, 41–43) in a rat model and the finding of changes in $GABA_A$ receptor affinity to allopregnanolone during pregnancy and lactation (20, 21), the changes in the plasma levels of PI were monitored during human pregnancy. For a better view of the results, a simplified scheme of the biosynthesis of the steroids under study was included (Fig. 9). As noted above, several hypotheses have been suggested regarding the mechanism of parturition initiation. We are aware that the timing of parturition in humans is a multifactorial process, so the actual physiological effects of PI cannot be identified without additional investigation. Nevertheless, an assessment of the possible physiological impact of PI on the course and timing of parturition requires that the time profiles of the circulating steroids during pregnancy be known.

We have recently reported data concerning the levels of PI and their polar conjugates in maternal and umbilical plasma at delivery (28) and in maternal plasma around parturition and postpartum (29). The levels of allopregnanolone during pregnancy and at delivery have also been measured by Luisi *et al.* (44). Pearson Murphy *et al.* (45, 46) measured the levels of the three PI in various physiological situations using HPLC separation, followed by nonspecific RIA. Although the latest results are in accord with those reported in both of the latter studies, the GS-MS method was extremely selective and sufficiently sensitive. It exhibited linearity in the response and enabled a simultaneous analysis of all PI (28); compared with the selective method of Pearson Murphy *et al.* (45, 46), this method was less laborious, involving only two liquid-liquid extraction steps, derivatization with 30-min simultaneous incubation of all samples, and 14.2-min separation on a gas chromatograph with instant mass-spectrometric detection. The time-consuming drying of HPLC fractions was avoided. In addition, the 3α,5β-isomer, preg-

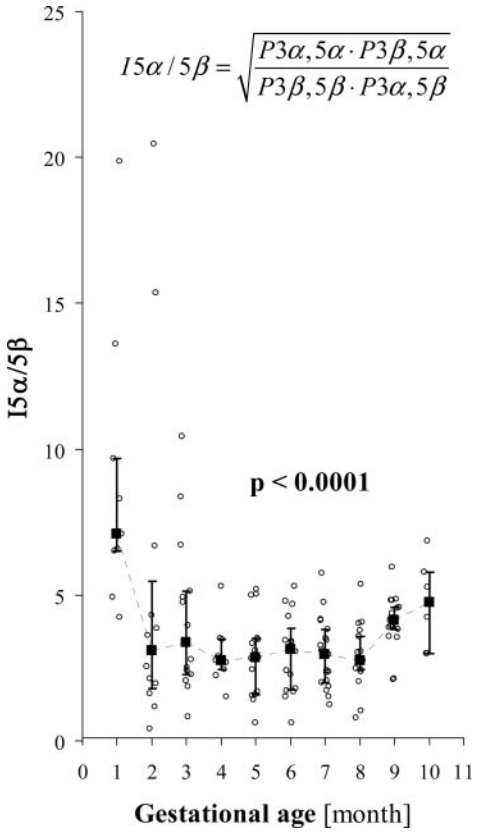

FIG. 6. Index reflecting the overall proportions between 5α- and 5β-PI in the plasma of 137 women during pregnancy. The symbols are explained in Fig. 3.

nanolone, which was not monitored in the aforementioned studies, is the second most abundant PI in the plasma of pregnant women (28, 29). Pregnanolone possesses a specific activating effect on $GABA_A$-r (11, 15). To the best of our knowledge, no study performing simultaneous measurement of all PI, their common precursor progesterone, and estradiol (as a labor inducing steroid) from the first to the 10th months of pregnancy with the use of a highly selective GC-MS method has previously been reported.

The proportions of the individual PI were similar to those found around parturition (28, 29). All PI exhibited a significantly increasing trend during pregnancy; this was more distinct in the 3α-steroids, which are known to attenuate neuronal activity and reduce the excitotoxicity induced by NMDA (47).

In contrast to 5β-PI, which showed an increase from the first month of pregnancy, 5α-PI exhibited a plateau from the first to the fifth months. In addition, the I5α/5β index showed a decrease between the first and second months, a plateau from the second to eighth months, and a significant increase from the eighth to 10th months ($P < 0.05$, by Kruskal-Wallis multiple comparisons). We speculate that these findings might be connected to the changing activities of 5α- and/or 5β-reductase during pregnancy. 5β-Reductase activity is absent in placenta (18); it is localized mainly in the liver (48, 49). In contrast, the overall 5α-reductase activity showed no alterations during gestation. It has been reported that during pregnancy, 5α-reductase operates predominantly in placental tissue (50), but it has also been found in fetal membranes, exhibiting a pronounced decrease at wk 33 compared with samples from early pregnancy (51). Given the increase in the ratios of 5β-PI to progesterone , particularly between the first and second months, and the constant overall activity of 5α-reductase during pregnancy (as documented above by the independence of the 5α-PI/progesterone ratios from gestational age), it is apparent that the 5β-isomers (unlike 5α-PI) did not simply follow the increasing concentrations of progesterone.

With reference to the timing of parturition, the researchers suggested a role for the changing ratio between allopregnanolone, which is known to inhibit neuronal activity by supporting chloride transport to neuronal cells via modulation of $GABA_A$-r, and isopregnanolone, which is the functional blocker of allopregnanolone (26). As stated above, no change in the ratio between the steroids was found during pregnancy. This knowledge weakened the hypothesis concerning the role of isopregnanolone in the onset of parturition.

In contrast, the results showed that the ratio of estradiol, which is known to stimulate uterine activity (3, 30, 32–36), to allopregnanolone, which exhibits the opposite effect (17, 20–22), gradually changed in favor of estradiol up to the sixth or seventh month, reflecting the profile of the estradiol/progesterone ratio. The pregnancy-stabilizing effect of progesterone via progesterone receptors and the opposite effect of estradiol through estradiol receptors are both well known in humans, including the parturition-provoking changes in subunit expression in the both systems (4, 52). Alternatively, to date, the nongenomic effect of allopregnanolone with respect to its presumably stabilizing effect during gestation has been extensively reported in animals (17, 22, 24), but rarely in humans (48, 53). Regarding the second 3α-pregnanolone isomer, pregnanolone acts in a similar way on the $GABA_A$-r (39, 54, 55) as allopregnanolone; information on this is very limited even in animals (56). Our current data as well as those reported previously (28, 29) show that both 3α-PI (allopregnanolone and pregnanolone) are present in high concentrations in maternal plasma. One could speculate as to whether stabilization of pregnancy could be produced at least to some extent by increasing concentrations of circulating pregnanolone. Despite its very high clearance rate (57), its circulating levels in maternal blood are about 40% compared with those of allopregnanolone (28, 29), whereas the circulating levels of pregnanolone in nonpregnant women are considerably lower than those of allopregnanolone (45). Considering the very different profiles of allopregnanolone and estradiol and the significantly changing estradiol/allopregnanolone ratio and, at the same time, the similarity in the profiles of pregnanolone and estradiol together with the constant estradiol/pregnanolone ratio, such speculation is appropriate. Moreover, findings such as the rapid metabolism of pregnanolone together with its high production rate suggest the relatively dynamic balance between pregnanolone and estradiol to be one of the mechanisms that influences the timing of parturition.

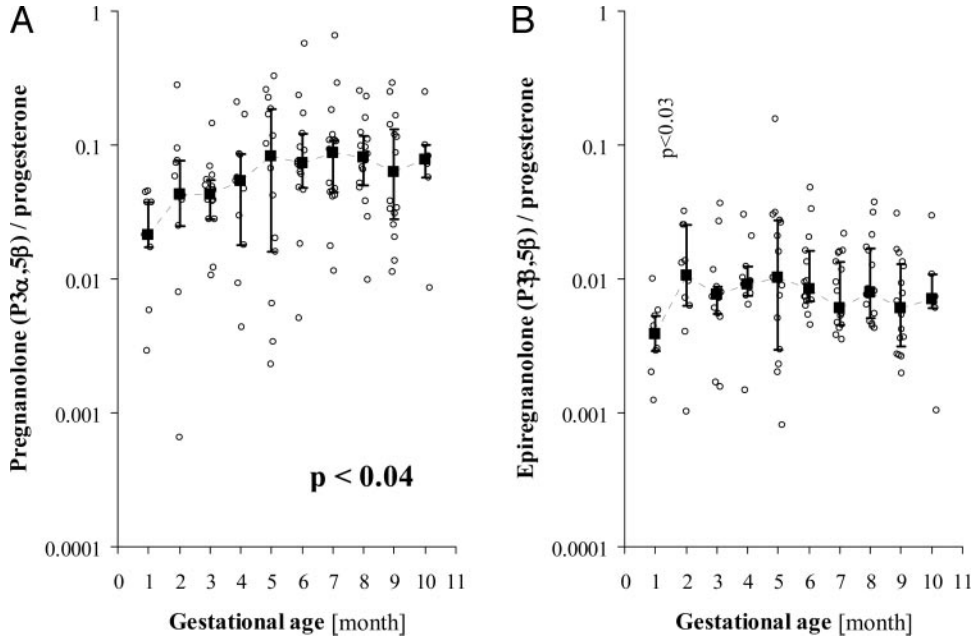

FIG. 7. Changes in the ratios reflecting the proportions between 5β-PI and progesterone in the plasma of 130 women during pregnancy. The symbols are explained in Fig. 3.

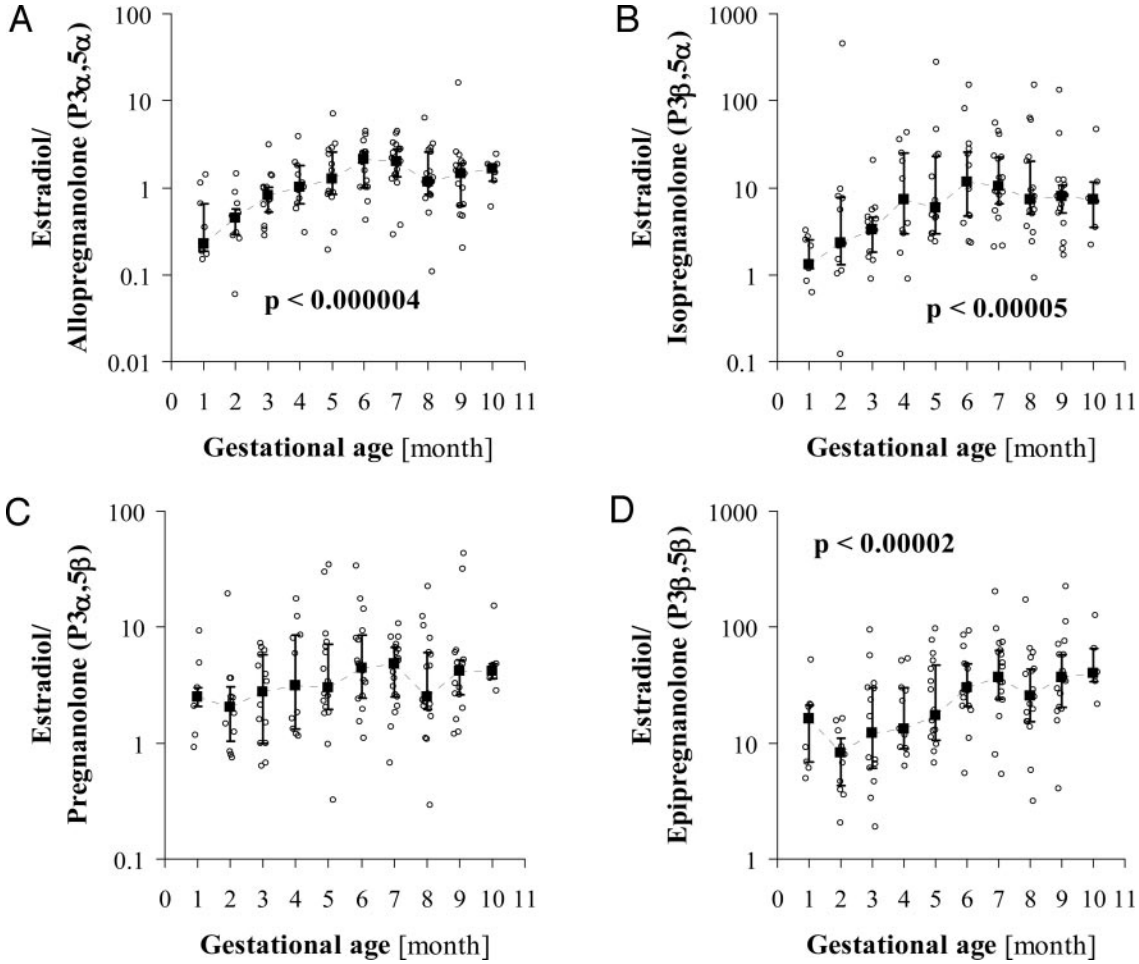

FIG. 8. Changes in the ratios between estradiol and PI in maternal plasma during pregnancy. The symbols are explained in Fig. 3. The numbers of subjects were 138, 137, 138, and 138 for estradiol to allopregnanolone, isopregnanolone, pregnanolone, and epipregnanolone ratios, respectively.

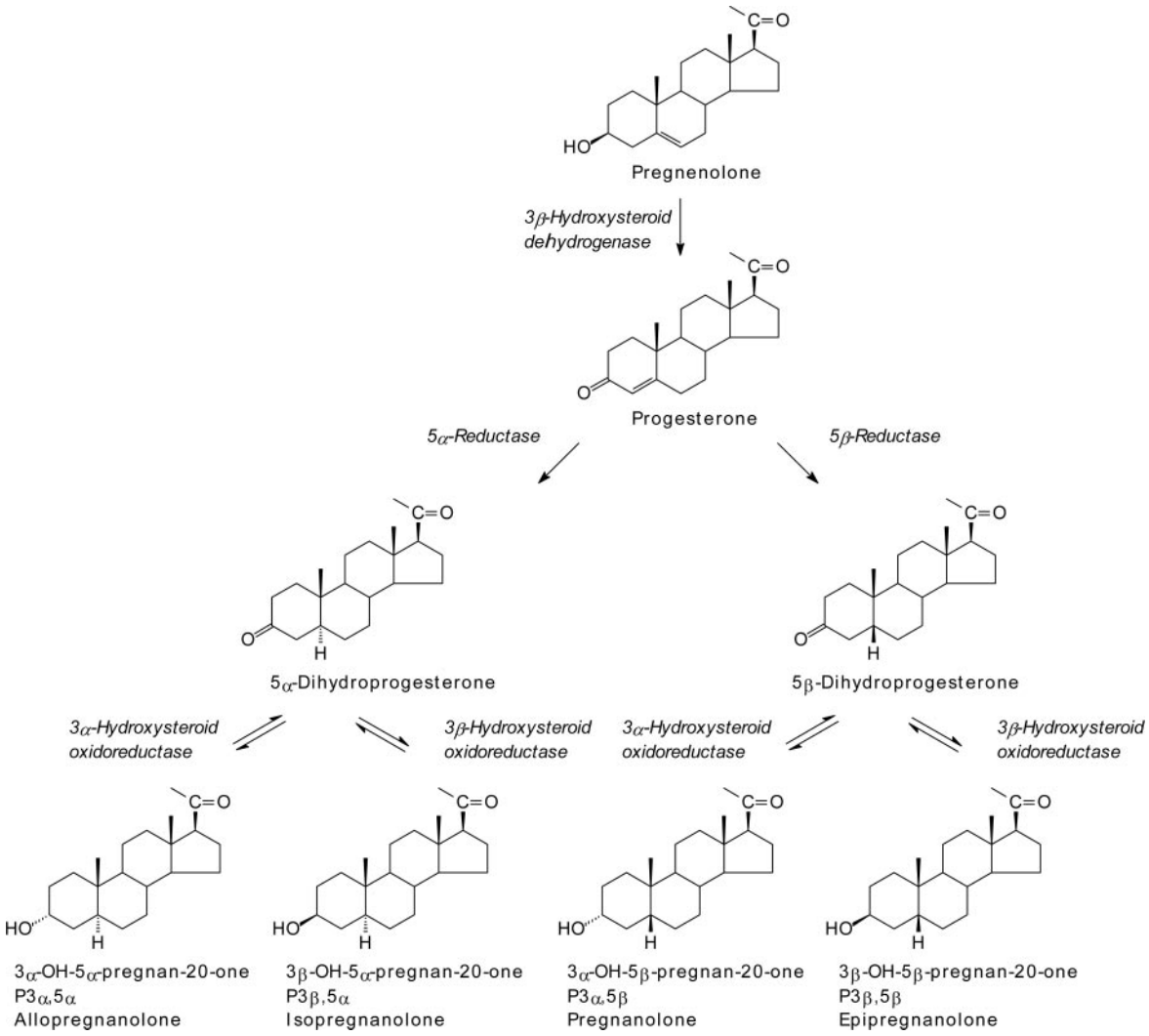

FIG. 9. Simplified neurosteroid biosynthesis scheme.

Acknowledgments

The excellent technical assistance of Mrs. Ivona Králová is gratefully acknowledged.

Received March 5, 2004. Accepted September 24, 2004.

Address all correspondence and requests for reprints to: Dr. Martin Hill, Institute of Endocrinology, Národní Třída 8, CZ 116 94 Prague 1, Czech Republic. E-mail: mhill@endo.cz.

This work was supported by Grant NB/7070-3 from the Internal Grant Agency of the Czech Ministry of Health.

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