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Reinstatement of Serum Pregnanolone Isomers and Progesterone During Alcohol Detoxification Therapy in Premenopausal Women

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Background: Alcohol abuse is associated with menstrual irregularities related to the inhibition of progesterone secretion involved in regulation of the menstrual cycle. Reduced progesterone metabolites, including pregnanolone isomers (PIs), are efficient neuromodulators. The authors attempted to evaluate whether levels of PIs reflect impairment in progesterone biosynthesis in premenopausal women treated for alcohol addiction and whether alcohol detoxification therapy contributes to the restoration of their reproductive functions and psychosomatic stability by influencing steroid biosynthesis.

Methods: Serum allopregnanolone (3α-hydroxy-5α-pregnan-20-one; P3α5α), pregnanolone (P3α5β), isopregnanolone (P3β5α), epipregnanolone (P3β5β), progesterone, pregnanolone sulfate (PregS), pregnanolone, and estradiol were measured in 20 women during therapy (at start, three days, 14 days, one month, and four months) by gas chromatography–mass spectrometry or radioimmunoassay. The results were evaluated by a linear mixed model for longitudinal data, with stage of the treatment and subject as categorical factors, phase of the menstrual cycle as a time-varying covariate, and age of the subject as a covariate and by regression in individual stages of the menstrual cycle.

Results: During detoxification treatment, progesterone increased in the luteal phase. P3α5α, P3β5α, and P3β5β rose in both phases of the menstrual cycle.

Discussion: Given the similar mechanism in the effects of alcohol and steroids in activating γ-aminobutyric acid $_A$ receptors, the restoration of progesterone and PIs during therapy could be explained by an adaptation to increasing requests for γ-aminobutyric acid $_A$–receptor activating substances owing to the cessation of alcohol intake or by the regeneration of progesterone formation. In conclusion, the reinstatement of progesterone, P3α5α, and P3β5β serum levels demonstrates the favorable effect of detoxification therapy on both reproductive functions and the psychosomatic stability of premenopausal women treated for alcohol addiction.

Key Words: Pregnanolone isomers, Progesterone, Alcohol detoxification therapy, Premenopausal women, Neuroactive steroids.

IN ADDITION TO various other adverse effects, alcohol abuse is associated with menstrual irregularities, including anovulation, luteal phase dysfunction, recurrent amenorrhea, and early menopause (Hugues et al., 1980). Most studies to date have demonstrated no effect of alcohol intake on gonadotropin synthesis (McNamee et al., 1979; Mendelson et al., 1987; Mendelson et al., 1989; Teoh et al., 1988; Valimaki et al., 1983). On the other hand, prolactin levels are stimulated at the beginning of intoxication (Mendelson et al., 1987). In terms of the sex steroids in premenopausal women, estradiol levels are elevated by acute alcohol intake (Mendelson et al., 1987; Mendelson et al., 1989; Sarkola et al., 1999; Teoh et al., 1988; Valimaki et al., 1983). Several studies have reported no influence of alcohol intake on basal progesterone (McNamee et al., 1979; Mendelson et al., 1987; Mendelson et al., 1989; Teoh et al., 1988; Valimaki et al., 1983); most of these, however, included low numbers of subjects, resulting in the low power of the corresponding statistical tests. The more recent study by Sarkola et al., (1999), evaluating more subjects separated into well-characterized groups, has reported a suppression of progesterone levels and an elevation of estradiol levels after acute low-dose alcohol intake, particularly during the luteal phase of the menstrual cycle (MC), al-

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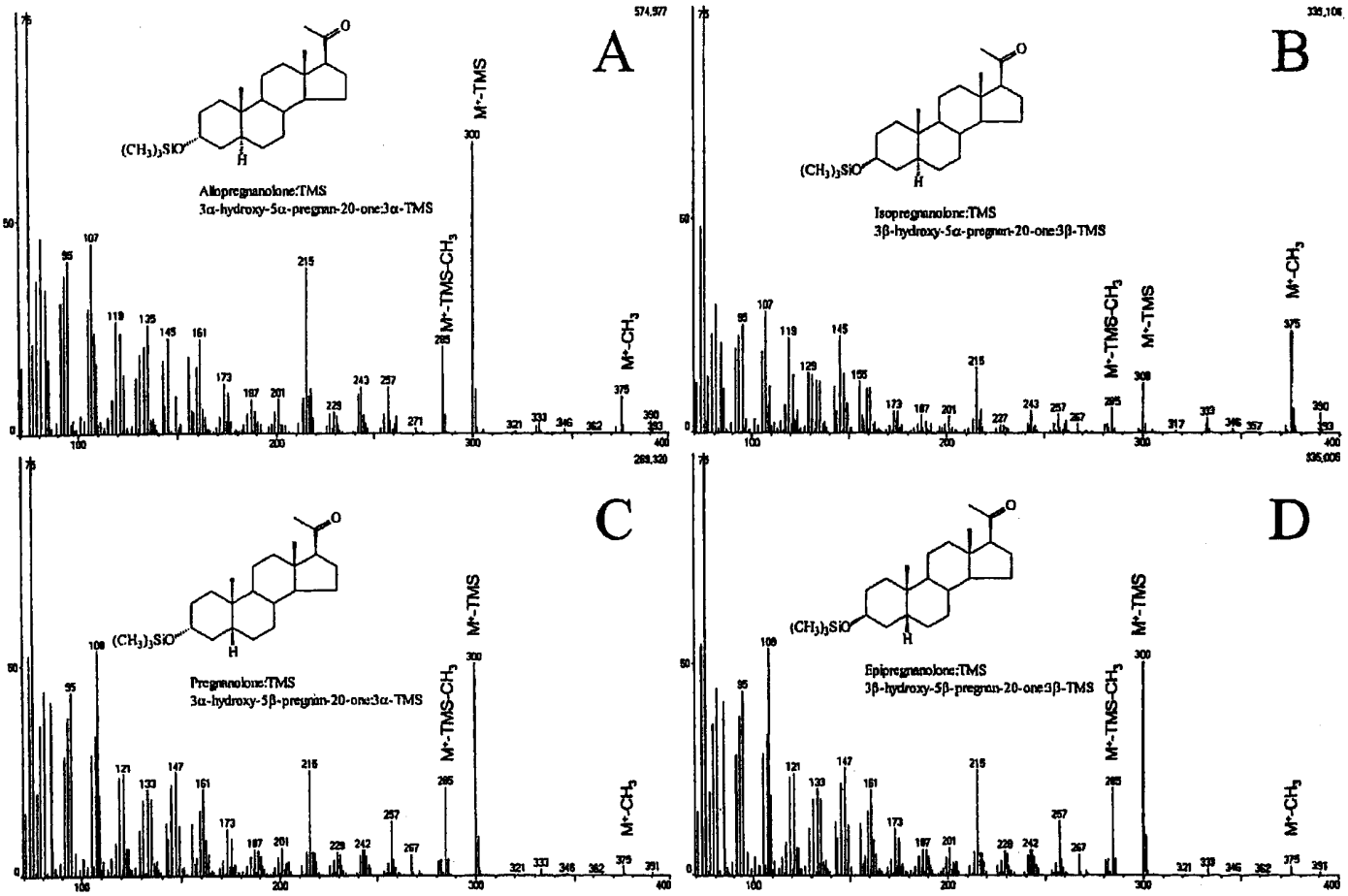

Fig. 1. Mass spectra of pregnanolone isomers. A QP 5050 A quadrupole electron-impact detector from Shimadzu with a fixed electron voltage of 70 eV was used for measurement.

though the effect was not dose dependent. The authors of the latter study assumed that the elevation of the estradiol-estrone ratio and the suppression of progesterone levels was connected to alcohol-induced changes in the redox state of the liver enzymes, providing reversible conversions of estradiol to estrone and of progesterone to 20α-dihydroprogesterone, and that both systems are catalyzed by the same isoenzyme (17β-hydroxysteroid dehydrogenase type 2), sharing the same cofactor (NAD) as alcohol dehydrogenase (Andersson and Moghrabi, 1997). Besides the latter study, earlier studies also reported similar progesterone effects but only during stimulatory conditions among premenopausal women not using oral contraceptives (Saxena et al., 1990; Teoh et al., 1990). On the other hand, contradictory results were obtained in female adolescents in whom acute alcohol intoxication increased the levels of the most abundant reduced progesterone metabolite, allopregnanolone (3α-hydroxy-5α-pregnan-20-one; P3α5α) (Torres and Ortega, 2003). Concerning chronic alcohol intake, higher estradiol (Garcia-Closas et al., 2002; Martin et al., 1999) and higher progesterone (Garcia-Closas et al., 2002) levels during the luteal phase have been reported in a group of light to moderate drinkers compared with nondrinkers.

In contrast, premenopausal women with a history of early alcohol abuse show lower estradiol, progesterone, androstenedione, and sex hormone–biding globulin levels and higher levels of free testosterone, which suggest a hyperandrogenic status in this group when compared with randomly selected controls of the same age (Pettersson et al., 1990). The decrease in progesterone levels may be related to alcohol inhibition of epidermal growth factor–stimulated progesterone secretion from human granulosa cells (Saxena et al., 1990; Wimalasena et al., 1993). The reduced metabolites of progesterone involving pregnanolone isomers (PIs) as well as one of the progesterone precursors, pregnanolone sulfate (PregS), are known as neuroactive steroids. Neuroactive steroids are effective primarily as modulators of the neurotransmitter receptors influencing the permeability of the ion channels (Hawkinson et al., 1996; Irwin et al., 1992; Majewska, 1990; Majewska et al., 1990; Park-Chung et al., 1997; Poisbeau et al., 1997; Wu et al., 1991), and some also act at progesterone receptors (Putnam et al., 1991; Rupprecht et al., 1996).

PIs with a hydroxy group in the 3α-position are known to attenuate neuronal activity (Gerak et al., 2004; Majewska, 1990) via positive allosteric modulation of γ-aminobutyric acid receptors, type A ($GABA_A$). On the other hand, PIs

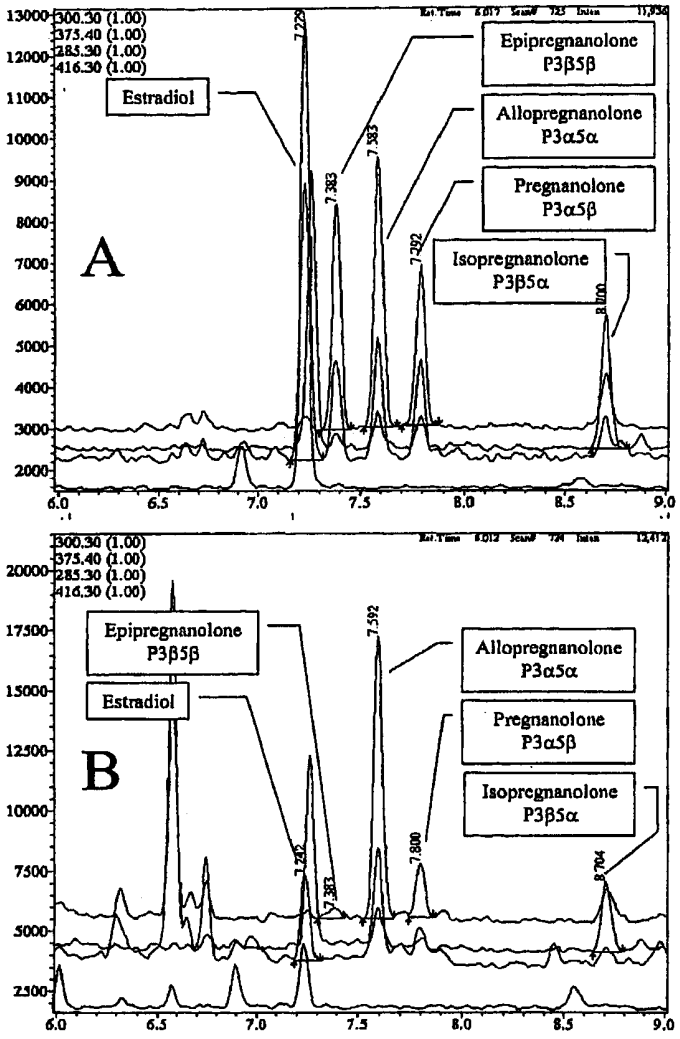

Fig. 2. Comparison between a sample of female serum and standard solution. (A) Response of a standard solution (4 μl) containing 4 ng of each of the steroids under study: estradiol, P3β5β, P3α5α, P3α5β, and P3β5α. The numbers in the upper-left corner indicate the effective masses of the fragments (mass-to-charge ratio), and the numbers in parentheses denote multiples of the original responses. (B) Response of a sample (4 μl) corresponding to 200 μl of serum from a woman in the luteal phase of the MC.

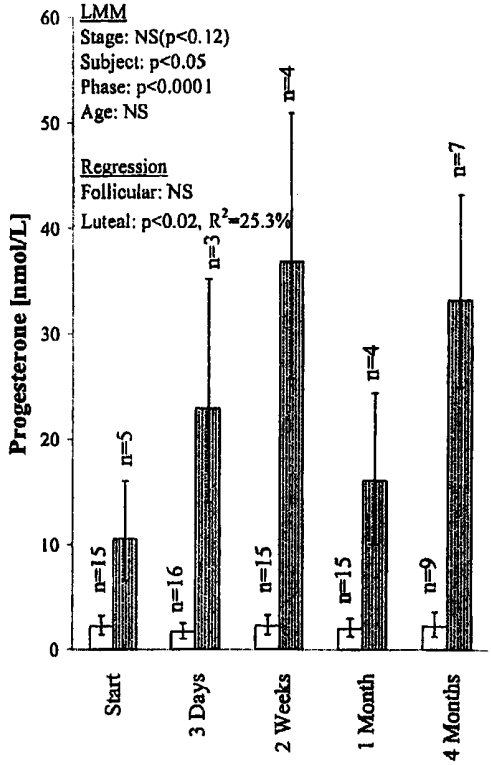

Fig. 3. Changes in levels of progesterone in female serum during alcohol detoxification treatment. The empty and dotted bars, with error bars, represent the retransformed mean values with their 95% confidence intervals in the follicular and luteal phases, respectively; R^2 is the squared correlation coefficient of regression expressing the percentage of the total progesterone variability explained by the model. LMM is the linear mixed model, which was used for evaluation (for details, see statistical analysis).

hydroxylated in the 3β-position exert the opposite effect, reducing the chloride uptake induced by 3α-PI (Lundgren et al., 2003; Prince and Simmonds, 1992). A strong neuroactivating effect has been reported in PregS via the positive modulation of *N*-methyl-D-aspartate receptors. In terms of the different neuromodulating effects of individual steroids, it is interesting to trace the proportional changes between 3α- and 3β-PIs, between 5α- and 5β-PIs, between individual PIs and progesterone, and between neuroinhibiting and neuro-activating steroids. Accordingly, in this study, changes were evaluated in the serum levels of some reduced progesterone metabolites involving all neuroactive PIs, i.e., P3α5α, pregnanolone (P3α5β), isopregnanolone (P3β5α), and epipregnanolone (P3β5β), in 20 premenopausal women during alcohol detoxification therapy. In addition, levels of the corresponding precursors, i.e., progesterone, PregS, P3α5β, and estradiol, were also traced. The effect of the MC was considered for all of the steroids.

The authors hypothesized that (particularly in the luteal phase) PI deficiency reflects impaired progesterone biosynthesis in women of fertile age treated for severe alcohol addiction. There was also speculation as to whether treatment could rectify the deficit in the formation of progesterone and its neuroactive reduced metabolites. In addition, the question was addressed as to whether the activities of the enzymes responsible for the biosynthesis of the progesterone metabolites could be influenced by alcohol detoxification therapy.

METHODS

Subjects

The patient group comprised 20 women during alcohol detoxification therapy at 5 stages (start, 3 days, 14 days, 1 month, and 4 months after termination of therapy). In all of the subjects, the effect of the MC was considered. For practical reasons, the individual patients started therapy at various stages of the MC. Nevertheless, the day of the MC was known at the beginning of and during treatment. Determination of the menstrual phase was based on self-reported information. The presence of the ovulatory cycle was checked by progesterone assay, with a level >6 nmol/liter indicating the luteal phase. The median age of the patients was 36.5 years with a minimum and maximum of 23 and 48 years, respectively, and a lower and upper quartile 30 and 43.5 years. Seventy percent of the patients

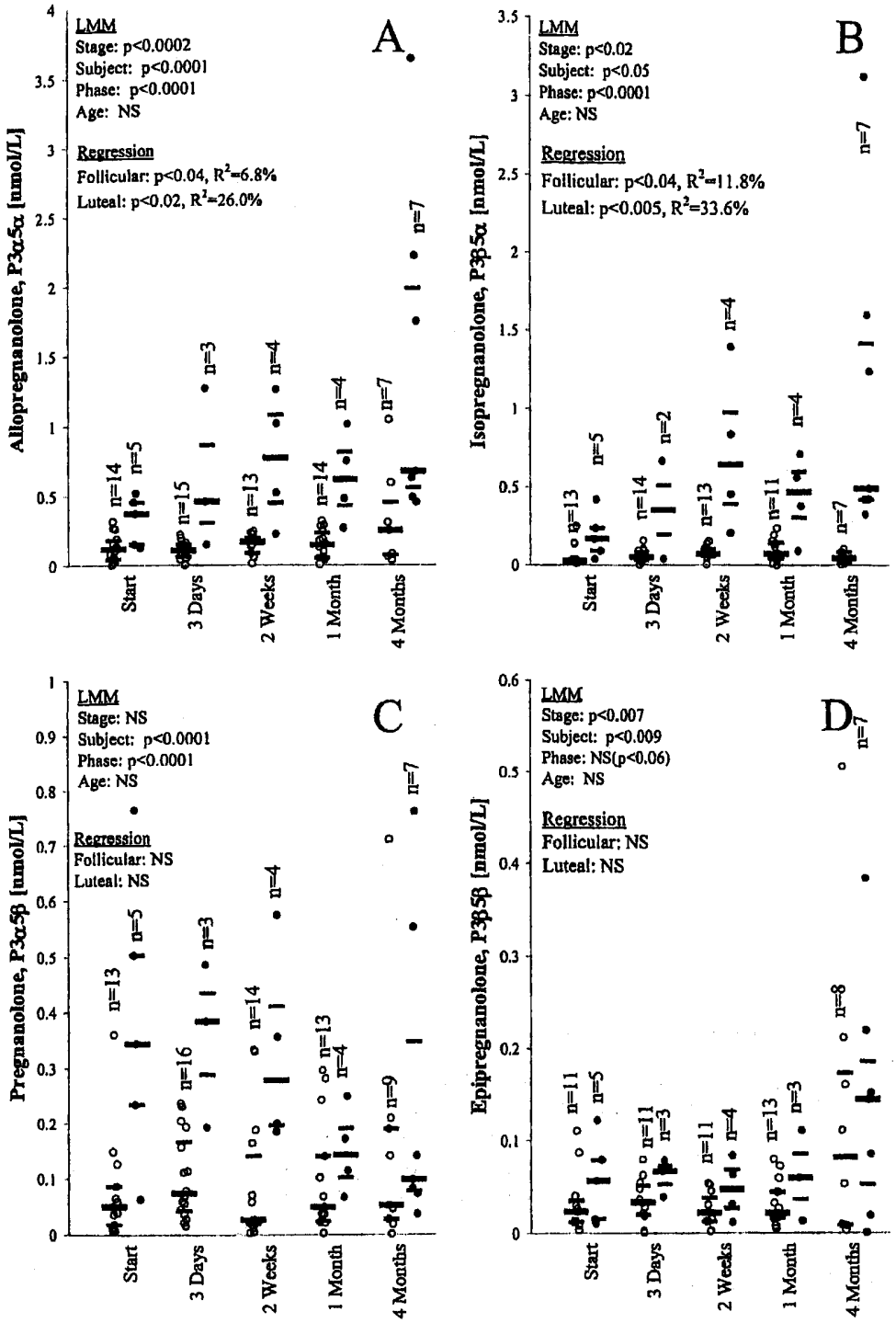

Fig. 4. Changes in the levels of pregnanolone isomers in female serum during alcohol detoxification treatment. Empty and full circles indicate the individual subjects in the follicular and luteal phases of the MC, respectively; bold and thin bars correspond to group medians and quartiles, respectively. The remaining symbols are the same as for Fig. 3.

were smokers. All patients were not using hormonal contraceptives or taking medication that might have affected hormonal levels. The local ethics committee approved the protocol for the study. After signing written, informed consent, the patients underwent blood sampling from the cubital vein.

Sample Collection

Serum was obtained after centrifugation for 5 min at 2000 × *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 extraction and high-performance liquid chromatography and pyridine were analytical grade from Merck (Darmstadt, Germany). The derivatization agent (Sylon BFT) was purchased from Supelco (Bellefonte, PA).

Instruments

The gas chromatography–mass spectrometry system was supplied by Shimadzu (Kyoto, Japan). The system comprised a GC17A gas chromatograph equipped with automatic flow control and an AOC-20 autosampler, and for mass spectrometry, a QP 5050A quadrupole electron-impact detector with a fixed electron voltage of 70 eV. The liquid scintillation spectrometer was supplied by Beckman Instruments (Fullerton, CA).

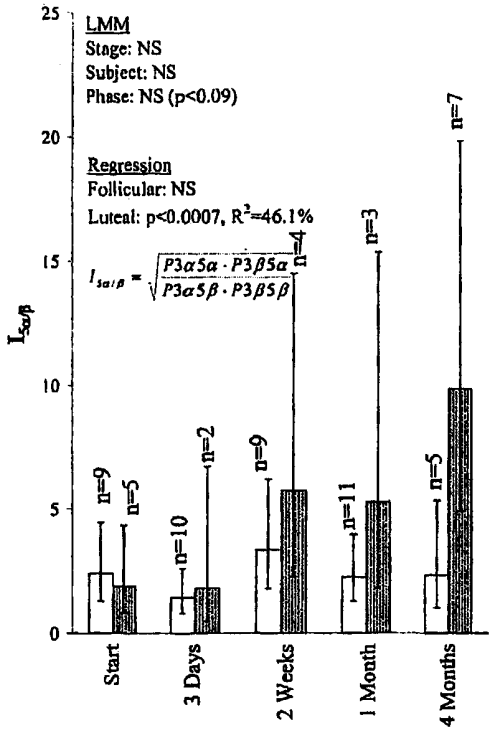

Fig. 5. Changes in the $I_{5\alpha/\beta}$ index characterizing the overall conjugated/free isomer ratio of serum pregnanolone isomers (defined as the square root of the ratio of product of the 5α- to 5β-isomers) during alcohol detoxification treatment. The drawings and symbols are the same as for Fig. 3.

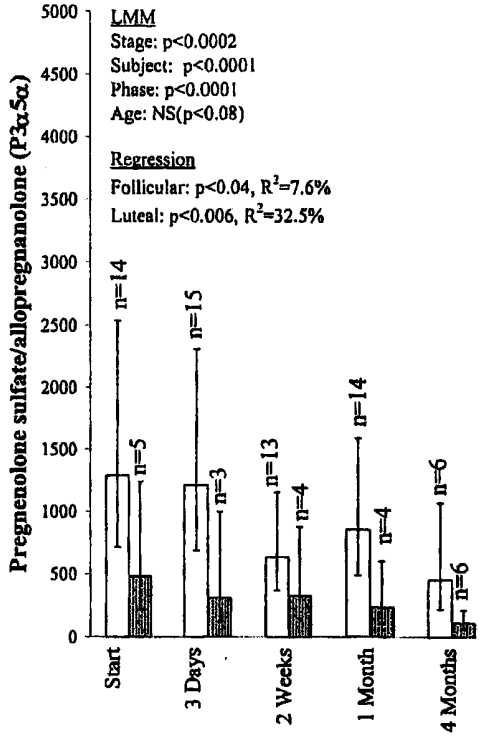

Fig. 6. Changes in the ratio of neuro-activating PregS to neuro-inhibiting P3α5α during alcohol detoxification treatment. The drawings and symbols are the same as for Fig. 3.

Analytical Methods

The PIs were measured with a modified method published previously (Hill et al., 2000). 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, a rapid linear gradient of 40°C and 8.5 kPa up to 220°C and 51 kPa, then a slow linear gradient of 2.9°C and 0.5 kPa up to 240°C and 54.5 kPa and finally a rapid linear gradient of 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 (DHEA) added to the standard solution or to the sample in 1 ng/μl increments and recorded at an effective mass of 307. The third change was the addition of pregnanolone measurement. The pregnanolone was recorded at effective masses of 298 and 398, and the former was used for further processing. The overall time taken for the analysis was 14.2 min. The retention times were 7.10, 7.25, 7.40, 7.60, 7.80, 8.71, and 8.73 min for trideuterated DHEA, estradiol, P3β5β, P3α5α, P3α5β, P3α5β, and pregnanolone, respectively. The last change represented the substitution of micro-extraction in the vials by more rapid drying of the derivatization agent under a stream of nitrogen.

PregS was measured by using the authors' specific radioimmunoassay as described elsewhere (Hill et al., 2002). Progesterone was estimated by the method of Langer et al. (Langer et al., 1978).

Statistical Analysis of the Data

The results were evaluated with a linear mixed model for longitudinal data, with stage of treatment and subject as categorical factors, phase of the MC as a time-varying covariate, and age of the subject as a covariate. The original data were transformed by a power transformation to attain a gaussian distribution and a constant variance of studentized residuals. The experimental points after transformation with absolute values of studentized residuals >3 were excluded from calculations; such points never accounted for >5% of the total. Besides the linear mixed model, the trends were evaluated separately in individual phases of the MC by using linear polynomial regressions of the first or second degree (straight line or parabola) with the logarithm of the day of treatment as an independent variable. The choice depended on the minimum value of the correlation coefficient of the multiple regression, adjusted for the degrees of freedom. Statgraphics Plus v.5.1 statistical software was used for the calculations.

RESULTS

Identification of Steroids

The mass spectra of the PIs are shown in Fig. 1. The steroids recorded on the chosen fragments in selected ion monitoring were well separated both from each other and from the background (Fig. 2). The sensitivity was sufficient for the quantification of all of investigated steroids.

Changes in PIs, P3α5β, PregS, Progesterone, and Estradiol During Treatment

Progesterone rose in the luteal phase during treatment (Fig. 3). The corresponding regression model was significant, explaining 25.3% of the total progesterone variability. P3α5α, P3β5α, and P3β5β increased in both phases of the MC (Fig. 4*A, B, and D*), whereas P3α5β (Fig. 4*C*), estradiol, pregnanolone, and PregS exhibited significant changes (data not shown) in either phase of the MC.

Changes in 3α-/3β-Isomer and 5α-/5β-Isomer Ratios

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

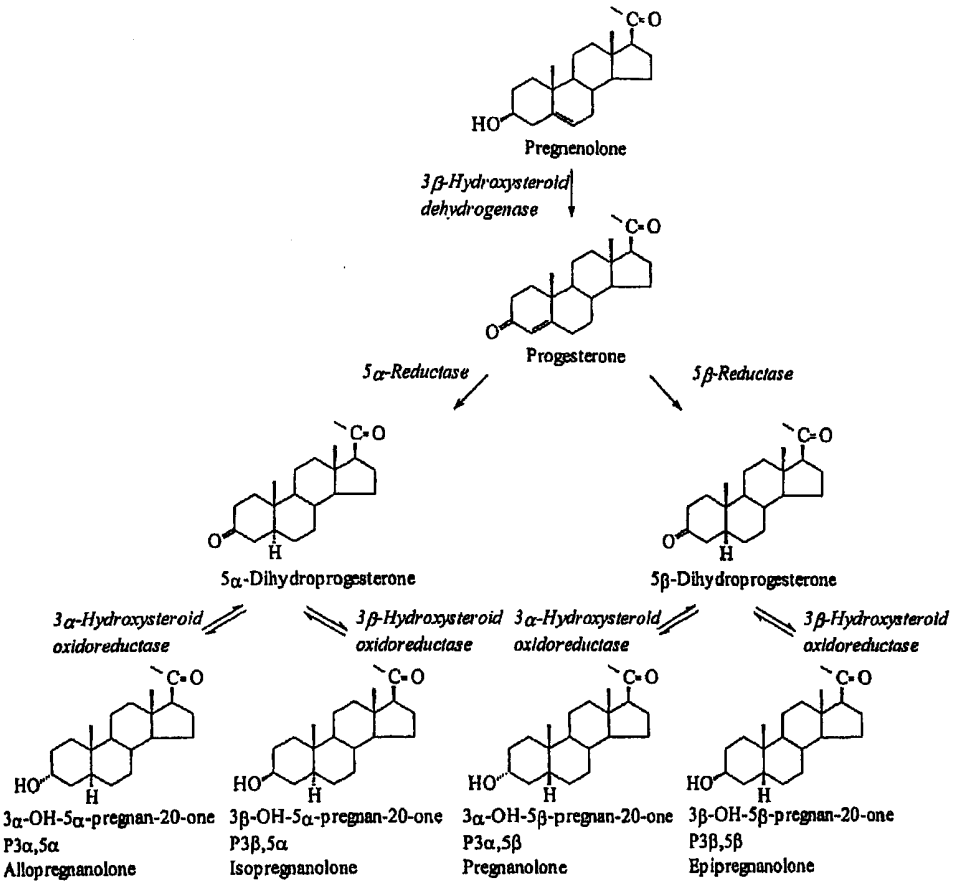

Fig. 7. Simplified scheme of the biosynthesis of P3α5β isomers.

products of the 3α- and 3β-isomers, did not change during treatment (data not shown). On the other hand, the overall change in the ratios of the 5α- to 5β-isomers ($I_{5\alpha}/^{5\beta}$) (expressed analogously as the square root of the ratio of products of the 5α- and 5β-isomers) showed a gradual increase in the luteal phase (Fig. 5).

Changes in the Ratio of Neuro-Activating PregS to Neuro-inhibiting P3α5α

The ratio of neuro-activating PregS to neuro-inhibiting P3α5α showed a regular decrease during therapy in both phases of the MC (Fig. 6).

DISCUSSION

The aim of this study was to evaluate whether levels of PIs reflect impairment in progesterone biosynthesis in premenopausal women treated for alcohol addiction (for better orientation, see Fig. 7) and whether alcohol detoxification therapy contributes to the restoration of their reproductive functions and psychosomatic stability by influencing steroid biosynthesis. Accordingly, the serum levels of all pregnanolone isomers, progesterone, P3α5β, and estradiol were evaluated during alcohol detoxification therapy (at start, 3 days, 14 days, 1 month, and 4 months).

The new results accorded well with a study by Sarkola et al. (1999) reporting the suppression of progesterone levels after acute low-dose alcohol intake during the luteal phase of the menstrual cycle in particular, as well as with the study by Pettersson et al. (1990) reporting lower progesterone levels in premenopausal women with a history of early alcohol abuse. An increasing trend was recorded in serum progesterone during alcohol detoxification therapy in the luteal phase ($p < 0.04$), as well as a rise in levels of P3α5α, P3β5α, and P3β5β in both phases, even though this was more pronounced in the luteal phase. By contrast, no significant changes in the levels of P3α5β, pregnanolone, and estradiol were recorded. The relatively constant P3α5β levels may be due to its rapid elimination characteristics (Carl et al., 1994; Hering et al., 1996).

The potential influence of smoking in the follicular phase on progesterone and estradiol levels as reported by Zumoff et al. (Zumoff et al., 1990) also should be taken into account, inasmuch as 70% of the patients were smokers. Nevertheless, the effect of smoking was constant during the trial, and we did not expect a substantial interference with the positive hormonal changes during detoxification treatment.

Given the similar mechanism in the effects of alcohol and steroid activators of $GABA_A$ receptors, the restoration of progesterone and particularly PIs to their physiological levels during alcohol detoxification therapy might be explained by adaptation of the organism to increasing demand for $GABA_A$-activating substances owing to the ces-

sation of alcohol intake or by the regeneration of progesterone formation impaired by alcohol abuse. The recovery of enzyme activities changed by alcohol intake as reported by Sarkola et al. (Sarkola et al., 1999) should also be taken into consideration.

Given the antistress and neuroprotective effects of P3α5α (Dazzi et al., 1996; Kajta et al., 1999; Lockhart et al., 2002), its increasing profile during therapy might contribute to improvement in the psychosomatic stability of the patients. On the other hand, because P3β5α and P3β5β levels exhibited concurrent increase with the levels of P3α5α, one might speculate as to their opposite effect on $GABA_A$. Nevertheless, the intensity of $GABA_A$-inhibiting actions of unconjugated 3β-PIs is probably less than the activating effect of P3α5α (Lundgren et al., 2003; Prince and Simmonds, 1992). Moreover, it has been reported that in the avian central nervous system, P3β5β acts in a similar way to P3α5α (Gravielle et al., 1998; Matsunaga et al., 2004; Pignataro and Fiszer de Plazas, 1997; Viapiano and Fiszer de Plazas, 1998).

Given the increasing ratio of 5α-PI to 5β-PI and the more pronounced profiles of 5α-PI when compared with progesterone, the restoration of overall activity of 5α-reductase is also likely. On the other hand, the relatively prominent profile of P3β5β supports the primary role of progesterone reinstatement.

In conclusion, the restoration of serum levels of progesterone, P3α5α, and perhaps also P3β5β demonstrates the favorable effect of detoxification therapy on both reproductive functions and the psychosomatic stability of premenopausal women treated for alcohol addiction.

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