Evidence for the presence of endogenous 19-nortestosterone in the cow peripartum and in the neonatal calf

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Urine samples were collected from five Brown Swiss cows during the 18 days prior to and 11 days after parturition and were analysed for 19-nortestosterone using an enzyme immunoassay. Nortestosterone concentrations ranged from 70 to 130 nmol/l in all samples taken before parturition. The levels declined within two days, and 11 days post partum no nortestosterone was detectable. In urine from newborn calves, maximal nortestosterone concentrations were determined during the first day of life (10.9–120 nmol/l), declining below 7.3 nmol/l until day 3 in most animals and remaining below the detection limit (<3.6 nmol/l) after day 8 in all animals. There was no obvious difference between cows carrying a male or a female calf nor between newborn male or female calves. Using the combined methods high performance liquid chromatography/enzyme immunoassay and high performance liquid chromatography/gas chromatography/mass spectrometry, the immunoreactivity in urine was identified to be 19-nortestosterone-17α. Although there is unequivocal evidence for the endogenous production of nortestosterone in pregnant cows, its function for placenta physiology, pregnancy anabolism and parturition remains unclear. However, new threshold levels for residue control of nortestosterone need to be fixed in accordance with the endocrine status of the animals.

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19-Nortestosterone (NT) is a well known androgenic, anabolic steroid (1) and preparations containing NT are registered in many countries. In the European Community (EC) its use as a growth promoter for animal production is forbidden according to EC directive 86/649. Nevertheless, the illegal use of NT for veal production has been described frequently (2–4) and the efficient control of its misuse is based on the absence of NT in male and non-pregnant female cattle (5). The biosynthesis of NT within the biochemical pathways for estrogens has been described earlier (6) and there are several reports of NT in males with significant estrogen production, like stallions (7) or boars (8, 9), and in human (10) or equine (11) follicular fluid. However, the biological function of endogenous NT remains unclear.

The bovine placenta is a very efficient steroid-producing organ, but the progesterone synthesis seems to be limited to a few months after mid-pregnancy (12). The synthesis of estradiol increases over the whole gestational period, with maximal activity at parturition, and needs 19-androgens as substrate for the aromatase complex (13, 14). It is unknown whether NT is produced as an intermediate in the pregnant cow. In the present study urine and plasma samples were investigated for the elucidation of that problem in order to adjust the guidelines for residue control measures and to get some idea of the potential functions of NT.

Materials and methods

Animals and samples

Urine samples were collected from five Brown Swiss cows during 18 days ante partum until day 11 post partum and from 9 neonatal calves during the first 30 days. The middle uterine vein of six Black and White cows was cannulated (15, 16). Four blood samples (10 min intervals) were collected from each of two cows on days 30, 14 and 5 ante partum. Additionally, blood samples were obtained from five Brown Swiss cows before and after parturition, as indicated in the figures, via puncture of the jugular vein. EDTA to 6 mmol/l was added to the blood and plasma was obtained after centrifugation (20 min, 3600 × g. 4°C). All samples were stored at −25°C until analysed. With the exception of a single animal (Fig. 4, cow 10), all placenta were delivered within 4 h of parturition of the calf.

Enzyme immunoassay (EIA)

Urine samples (0.5 ml) were cleaned up by solid phase extraction and were analysed using EIA as described (17). Briefly, 17α-NT-glucuronide was hydrolysed with β-glucuronidase, for clean-up 17α-NT was adsorbed to octadecyl silica gel cartridges, washed in 3 ml methanol/
water (1/1), eluted in 1 ml methanol/water (4/1) and diluted in 1 ml water.

For the immunoassay, microtitre plates were coated with 1 μg affinity purified sheep IgG (anti rabbit IgG) per well; then the EIA was performed with an antibody raised in rabbits against NT-17β-hemissuccinate-BSA (code: NT 2 II, dilution 1: 180 000) and the tracer was NT-17α-glucuronide-alkaline phosphatase (14 ng/well). The assay allows the simultaneous detection of NT-17β (100% cross reaction), 19-norandrostendione (100%) and of the epimer NT-17α (80%), which is the major metabolite in urine (18). For urine samples the standard was prepared with 17α-NT (Schering, Berlin, Germany). The 17α-NT standard curve was prepared in 40% methanol, ranged from 1.4 fmol/well (90% rel. binding) to 364 fmol/well (10% rel. binding) and 50% rel. binding was obtained at 29 fmol/well; 20 μl standard or sample was pipetted per well and hence the theoretical detection limit amounted to 0.28 nmol NT/l urine. Sample blanks in urine from untreated animals were < 3.6 nmol NT-equivalents/l in calf urine and < 11 nmol/l in urine from non-pregnant adult cattle fed on the same feed as peri parturient cows; all variabilities were < 10% at levels > 3.6 nmol/l.

Plasma (1 ml) was extracted with 5 ml tert. butyl-methyl ether/petrol ether (3/7), the solvent evaporated, the residue dissolved in 200 μl 40% methanol and NT was measured by EIA using a 17β-NT (Sigma, Munich, Germany) standard curve (range 1.4 fmol to 364 fmol/well; 50% rel. binding at 23 fmol/well). The theoretical detection limit amounted to 0.014 nmol/l plasma and the assay variabilities were < 16%.

**HPLC/EIA**

Urine samples were hydrolysed, cleaned up on octadecyl silica gel and steroids were separated using two HPLC systems with different selectivity (18). System I: UltraspHERE ODS, 5 μm, 250 × 4.6 mm column (Beckman, Munich, Germany) eluted with methanol/20 mmol/l TRIS-acetate buffer pH 7.2 (65/35) at 1 ml/min delivered by a Beckman 11 solvent delivery module; system II: Nucleosil C18, 5 μm, 125 × 4 mm column (Bischoff, Leonberg, Germany) eluted with acetonitrile/20 mmol/l TRIS-acetate buffer pH 7.2 (40/60) at 1 ml/min. Fractions were collected as indicated in the figures; the steroid content was measured by the EIA 1 as described above as well as by a second system (EIA 2) using an antibody raised against 17α-NT-3-CMO-BSA and the tracer was 17α-NT-3-CMO-horseradish peroxidase (19). The latter system detected 17α-NT (standard) but not 17β-NT and was aspecific for modifications at the A-Ring of the steroid (cross reaction: 17α-NT 100%, estradiol-17α 12.7%, 5α-estrane-3β,17α-diol 4.9%, testosterone-17α 1.7%, 17β-NT 0.03%).

**GC–MS**

Five millilitres urine was hydrolysed, cleaned up on octadecyl silica gel and fractionated by the HPLC-system I. The respective fractions of peaks 1, 2 and 3 (EIA 2) were combined separately and the buffer ions of each pool were removed by a second, similar passage over octadecyl-silica gel cartridges.

The solvent was evaporated to dryness; the residue was silylated (10 min. 25°C) with 100 μl of a mixture of MSTFA/TMSCl (100/1, v/v) and 1 μl was applied onto GC–MS equipment (Gaschromatographat 5890, fused silica capillary column HP5, mass specific detector 5970; Hewlett Packard, Bad Homburg, Germany) for analysis by electron-impact-ionization (EI) and SCAN respective SIM technique, as recently described (20). For identification of 17α-NT in biological samples the four diagnostic ions (215, 256, 331 and 346) as prescribed by the EC directive 87/410 were monitored and integrated after calibration with 17α-NT.

**Relative receptor binding affinities (RBA)**

An androgen receptor preparation that was prepared from calf uteri (after homogenization in a two fold volume of 10 mmol/l NaPi, 3 mmol/l DTT pH 7.5. the supernatant of a high speed centrifugation was used) (21) was incubated with 3H-dihydrotestosterone in the presence or absence of various unlabelled steroids for 2 h at 0°C. After treatment with charcoal (0.2% final) the remaining binding of 3H-sterol was measured by liquid scintillation counting.

**Results**

By screening the urine of cows during the late pregnancy with the EIA-1 NT was found at varying concentrations between 70 and 130 nmol/l (Table 1). There was no obvious difference between cows carrying a female or male calf. After parturition, levels declined immediately on day +1 and NT seems to be almost absent on day +11. The urine sample blanks from 12 additional post partum cows after day 11 were always < 11 nmol NT-equivalents/l. NT was also present in the first urine samples of female and male calves (Table 2), but the concentrations rapidly declined in all animals. A further 21 urine samples were collected from these nine calves on days 7 to 30. Only in one individual sample were 9.5 nmol NT-equivalents/l (animal 7, day 8) detected; in none of the other 120 samples was significant NT found (< 3.6 nmol/l).

<p>| Table 1. Total NT-equivalents (nmol/l) found in urine samples of five cows peripartum. |
|---------------------------------|-------|-------|-------|-------|-------|</p>
<table>
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<th>Days</th>
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<th>5(5)</th>
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Table 2. Total NT-equivalents (nmol/l) found in urine samples of nine calves during the first six days post partum.

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<td>2.2</td>
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For identification of the immunoreactive 19-norsteroid, the extracts from urine samples were fractionated after HPLC (system I) and measured using the different EIA systems 1 and 2. In samples from day 10 post partum no immunoreactive material was detected, whereas in cow samples ante partum and in the first samples from calves the majority of the immunoreactive material (EIA 1) was eluted in the position of 17α-NT (Fig. 1). In a few samples minor activity was additionally present in the position of 17β-NT at 12 min, but no immunological activity was detectable in the pooled samples before 11 min (results not presented) showing the absence of norandrostendione (~10 min) in these urine samples. The presence of 17α-NT was confirmed by EIA 2 (Fig. 2) with a different specificity (antigen: 17α-NT-3-CMO-BSA). The second peaks (NT-17α) are almost identical to the respective peaks of Fig. 1. The first and third peaks co-migrate with 17α-estradiol and 17α-testosterone, respectively; the antibody detects these substances with minor affinity. After HPLC-system II (different selectivity) and detection by EIA 1 again the immunoreactive material of the four samples migrated like 17α-NT (results not shown).

In order to provide unequivocal evidence of the production of endogenous NT in the pregnant cow, samples were also analysed by GC-MS. The SIM analysis of the urine sample from cow 1 (day 7 ante partum, HPLC system I peak 2, EIA 2) shows the presence of the four diagnostic ions originating from TMS derivatives of 17α-NT as generally found in the EI-mode at the GC-retention time of 17α-NT (Fig. 3). The separate integration of the four ions resulted in similar amounts of 17α-NT (ion 215~2.0 pmol; ion 256~2.2 pmol; ion 331~2.2 pmol; ion 346~2.0 pmol) proving the identity of the analyte with the calibration standard 17α-NT. In HPLC peaks 1 and 3 estradiol-17α and testosterone-17α were identified by GC-MS, respectively.

NT was measured in plasma from the uterine vein of six cows on different days before parturition and the
means from four samples of each cow were calculated (two cows, 30 days ante partum: 0.6 and 1.1 nmol/l; two cows, 14 days ante partum: 0.4 and 1.2 nmol/l; two cows 5 days ante partum: 1.1 and 4.4 nmol/l). In jugular vein plasma from five other cows, between 0.2 and 0.9 nmol/l was measured before parturition (Fig. 4), and levels dropped to plasma sample blank levels (approx. 0.15 nmol/l) of the assay in four cows with normal delivery of calf and placenta. The NT pattern was slightly different in a cow with a retained placenta; a single elevation of NT was monitored during its manual removal.

The potential biological activity of NT was estimated by measuring the relative binding affinities (RBA) to bovine androgen receptors (RBA values: Dihydrotestosterone = 1.0, Trenbolone-17β = 1.0, 19-Normethyltestosterone-17β = 0.7, Methyltestosterone = 0.5, Testosterone = 0.4, Estradiol = 0.07, Progesterone = 0.02).

Discussion

Methods

For the investigations documented here, three different methods were applied. First, the EIA, a sensitive detection and screening method; second, HPLC/EIA combinations, which allow the determination of NT and possibly indicate further metabolites; third, HPLC/GC-MS, as the most reliable reference method. The EIA is now a very useful method for sensitive and economic screening. It provides a good overview of the secretion patterns and the origin of NT and can be applied for the analysis of total NT in urine or plasma. The HPLC allows separation of the different steroid metabolites and enables the following selective detection by immunoassays. The combination of HPLC and two EIA methods with different selectivities provides strong evidence of the existence of NT in pregnant cows carrying a female or male calf as well as in newborn female and male calves. The HPLC/EIA results in negative urine samples also document the absence of NT in urine from untreated animals and in the reagents used. The combined method HPLC/GC-MS then provides unambiguous proof of the existence of NT in the urine of pregnant cows. In consideration of the results from this set of methods NT can no longer be considered as a xenobiotic compound in cattle.

Origin of NT

Our results indicate that NT may be produced by the bovine placenta. This is supported by the rapid decline in NT after the delivery of the calf and the placenta. The high levels in the urine of newborn calves may reflect the elimination after the accumulation during pregnancy. A similar situation was also found for estrogen metabolites (22), which are also accumulated in the fetal calf. The production of NT as an intermediate during estrogen synthesis via the C-19 decarboxylation pathway has been proposed earlier (6). It is possible that this pathway is also used in the bovine placenta for the estrogen synthesis. However, it is still unclear whether NT is originally synthesized in the bioactive 17β-form, with a following epimerization, or whether most of it is already produced in the inactive 17α-form. Canulation of the uterine vein and of fetal blood vessels would provide more detailed information on the production of NT and analysis by HPLC/EIA would permit distinction of the different NT metabolites.

Aspects for animal physiology

NT is a well known, effective anabolic steroid (1) and we have documented its strong binding to the uterine androgen receptor. NT-17β therefore might have several effects on the animal. First, the anabolic activity could be of importance for the growing calf as well as for the dam in an anabolic state during pregnancy. Second, it could be important for placenta physiology and maturation. In this respect it cannot be excluded that NT may be a signal, or even a prerequisite, for the changing production rates of progestins (12) or prostaglandins (23).

Consequences for residue monitoring

In earlier studies we documented that NT is absent in young calves between 2 and 16 weeks of age; later we could not find any NT metabolite in testes and ovariess from cattle, the major production sites of the sex hormones of non-pregnant animals (5). However, the new studies provide evidence that NT can be produced in cattle and that new threshold values in accordance with the animals' endocrine status are necessary. This will be important for several countries, e.g. EC countries, where the application of sex hormones for anabolic purposes is forbidden. We suggest that 18.2 nmol nortestosterone/l urine (5 ng/ml) could be a useful discriminatory level for treated male and non-pregnant female cattle. Establishing a threshold level for pregnant cows seems not...
possible at present because the maximal concentration of NT which might be found in the urine of animals at different stages of pregnancy demands further investigation.

Acknowledgments. This paper is dedicated to Wilhelm Stöckl, University of Vienna on the occasion of his 65th birthday.

References

Received August 23rd, 1991
Accepted January 2nd, 1992