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Glucocorticoid Metabolites Inhibit the Metabolism of Androstenedione in Red Blood Cells of Ruminants

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With 4 figures

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Summary

The present work aimed to confirm that erythrocytes of ruminants, in general, are capable of converting 17-oxo to 17-hydroxysteroids. Special attention was given to 11-oxoaetiocholanolone (a cortisol metabolite) and its possible interaction with androstenedione as substrates of 17-hydroxysteroid dehydrogenases (17-OH SDH). Blood samples were taken from cattle, sheep and goats ($n = 3$). Aliquots (100 or 300 μl) of washed red blood cell (RBC) suspensions were incubated in triplicates with Ringer's/glucose solution (1 ml) containing either androstenedione (10 ng) or 11-oxoaetiocholanolone (100 ng) or a mixture of 10 ng of each. Incubations were performed on a shaker at 38°C for 10, 20, 40 or 80 min, respectively. After centrifugation the supernatants were stored at −24°C until analysis. Concentrations of added steroids were measured with enzyme-immunoassays to monitor their decrease. The 17-OH SDH activity of RBC was highest in cattle followed by goats and sheep, and 11-oxoaetiocholanolone was a better substrate than androstenedione. Concentrations of the latter decreased more pronounced, if incubated alone. High performance liquid chromatography separations of the metabolites of 17-oxosteroids revealed the presence of both, a 17β- and 17α-hydroxylated product formed by erythrocytes of sheep and goats, but only the latter in cattle. The results demonstrated that 11-oxoaetiocholanolone was also a substrate of RBC 17-OH SDH and inhibited the metabolism of androstenedione. Therefore, in ruminants, there might be an interaction between cortisol metabolites and gonadal steroids on the level of peripheral steroid metabolism.

Introduction

In erythrocytes of cattle and sheep the presence of enzymes transforming gonadal steroids (e.g. 17α/β- and 20α-hydroxysteroid dehydrogenases) has been reported (Lindner, 1961, 1965; Tsang, 1976; Choi et al., 1989). The main metabolite of oestrone or androstenedione in cattle is oestradiol-17α or epitestosterone, respectively (Lunaas and Velle, 1960; Lindner, 1961; Möstl et al., 1980), whereas in sheep the 17β-epimer was formed in addition to both cases (Lindner, 1965; Tsang, 1976). To our knowledge experiments in goats have not been reported so far.

The response to some stressors leads to elevated levels of glucocorticoids in the blood (Axelrod and Reisine, 1984). Steroid hormones are quickly eliminated from the blood (Palme et al., 1996). They are mainly metabolized by the liver and excreted via the urine and bile as conjugates (Taylor, 1971). In the gut most of them are deconjugated by bacteria and partially reabsorbed. This enterohepatic recirculation of cortisol metabolites was demonstrated in sheep (Lindner, 1972). Steroid metabolites, which are not reabsorbed, are eliminated via the faeces (Taylor, 1971; Lindner, 1972; MacDonald et al., 1983; Palme et al., 1996). As glucocorticoids are heavily metabolized in the gut of ruminants, cortisol or corticosterone are not present in the faeces (Palme and Möstl, 1997). The same authors established and validated a 11-oxoaetiocholanolone enzyme immunoassay (EIA) for measuring faecal 11,17-dioxoandrostanes (11,17-DOA), a group of cortisol metabolites, formed from cortisol by side chain cleavage (Palme and Möstl, 1997; Palme et al., 1999).

The present work aimed to confirm that erythrocytes of ruminants in general are capable of converting 17-oxo to 17-hydroxysteroids. Special attention was given to 11-oxoaetiocholanolone, which is present in the blood after reabsorption from the gut. If this metabolite is a preferred substrate of the 17-hydroxysteroid dehydrogenases (17-OH SDH), this might result in an interaction between the metabolism of glucocorticoids and gonadal steroids and thus between stress and reproduction.

Materials and Methods

Animals and preparation of erythrocyte suspensions

Heparinized blood samples of three ruminant species (cattle, sheep and goat) were taken. Samples from a non-ruminant species (horse) and Ringer's/glucose solution alone served as a control. A total of three mature, healthy animals (two females and one male) of each species were used. In order to incubate comparable volumes of erythrocytes, standardization according to 'Packed Cell Volume' (PCV) was performed. Therefore, 6 ml of heparinized whole blood was centrifuged (2500 ***g***; 15 min) and the plasma and buffy coat were removed. Afterwards Ringer's/glucose solution (containing 2.5% glucose) was added in order to give a suspension containing 30% erythrocytes.

In vitro incubation and steroid analysis

For the incubations, androstenedione (4-androstene-3,17-dione) and 11-oxoaetiocholanolone (5β-androstane-3α-ol-11,17-dione; both obtained from Steraloids, Wilton, NH, USA) were used. The incubation of the samples from each animal (in triplicate) occured in Eppendorf cups (1.5 ml) on a shaker

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(Thermomixer 5436; stage 10, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) at 38°C for 10, 20, 40 and 80 min, respectively. A total of 100 μl (in cattle) or 300 μl (in sheep, goats and the horse) erythrocyte suspension (30% PCV) was incubated with 1 ml Ringer's/glucose solution containing 11-oxoaetiocholanolone (100 ng) or androstenedione (10 ng) or a mixture of 10 ng of each. These concentrations were chosen for all incubations after preliminary incubations with 100 ng of androstenedione, 10 ng of 11-oxoaetiocholanolone and different mixtures (50 + 50, 5 + 5 and 10 + 90 ng) were tested. Glucose was used in the medium as its presence was found to be essential for the reduction of androstenedione to epitestosterone in red blood cells (RBCs) (Lindner, 1965).

Following incubation, the cups were centrifuged (9000 ***g***; 1 min) in an Eppendorf centrifuge, the supernatant transferred into another cup and stored immediately at −24°C until analysis. After a 1 : 10 dilution, the concentrations of added steroids in the incubations were determined using EIAs without further extraction. Incubations of 5β-androstane-3α-ol-11,17-dione were analysed with an 11-oxoaetiocholanolone EIA (measuring 11,17-dioxoandrostanes; Palme and Möstl, 1997) and that of androstenedione with an EIA described by Palme and Möstl (1994). In the latter instead of epiandrosterone, androstenedione was used as standard and the crossreactivity of epitestosterone (4-androstene-3-one-17α-ol) in this EIA was evaluated (< 0.1%). Coefficients of variation of pool samples were found to be between 9.5 and 15.8%. The detection limits of the EIAs were 1 and 34 fmol/well, respectively (Palme and Möstl, 1994, 1997).

High-performance liquid chromatography separations

To characterize products of the 17-OH SDHs, metabolites formed by the RBC were separated by straight phase high-performance liquid chromatography (HPLC). Samples ($n = 3$), which were incubated for 80 min with androstenedione or 11-oxoaetiocholanolone, respectively, were used. Each sample (1 ml) was extracted twice with 5 ml diethylether. The combined extracts were evaporated, redissolved in 0.5 ml methanol and stored until analysis at −24°C. In addition, 50 ml heparinized plasma (divided into 1 ml portions) of one cow, goat and sheep were extracted in a similar way immediately (10 min) after collection.

Extracted steroids from androstenedione incubations were chromatographed on a Lichrosorb Si 60 column (10 μm, 25 × 0.4 cm; Austrian Research Centers, Seibersdorf, Austria) as described by Palme and Möstl (1994). Separations were performed with *n*-hexane/chloroform (70/30; isocratic) at a flow rate of 4 ml/min. In case of 11-oxoaetiocholanolone metabolites and plasma extracts, the same column and eluent was used. However, the flow rate was 2 ml/min and a linear methanol gradient was applied (start: 0%; 30 min: 6%; 35 min: 6% and 40 min: 10%; Palme and Möstl, 1997).

In all separations, fractions (2/min) were collected and an aliquot tested in the different EIAs to determine the presence of formed metabolites. For the androstenedione incubations an androstenedione-, epitestosterone- and testosterone-EIA were used (Palme and Möstl, 1994). As the 11-oxoaetiocholanolone EIA, which was applied for measuring levels in the incubation experiments, only reacts with 11-oxoaetiocholanolone, but not with the 17-hydroxy metabolites, another EIA had to be utilized. In this latter 11-oxoaetiocholanolone EIA, an antibody produced against 11-oxoaetiocholanolone-17-CMO:BSA was used (Möstl et al., 2002). Due to its cross-reactions it was possible to measure the mother substance (11-oxoaetiocholanolone) and both 17-hydroxy epimers in the respective fractions.

Statistical analyses

Amounts of measured steroids, present after a given time, were expressed as a percentage of added steroids. To examine an effect of incubation time on determined steroid concentrations a repeated measures ANOVA was used. To isolate the group(s) that differed from the others, a multiple comparison procedure (Tukey test) was applied afterwards. To test if the presence of 11-oxoaetiocholanolone had an influence on androstenedione concentrations, respective groups at a given time point were compared with a *t*-test. All tests were performed with the software package SIGMASTAT© (SPSS Inc., Chicago, IL, USA).

Results

In vitro incubations

In the incubations of RBC of cattle, goats and sheep a dramatical decrease ($P < 0.001$) in the concentrations of added 11-oxoaetiocholanolone (100 ng) was observed. In cattle < 60% of the starting levels were reached after 10 min. After 80 min almost no 11-oxoaetiocholanolone could be detected (Fig. 1). The same pattern was found in goats and sheep, but the erythrocytes were less active in converting the steroid (Fig. 1). There was some individual variation between the three animals (of a species) concerning time course and amount of the decrease. As incubating erythrocytes with 100 ng androstenedione resulted only in an slight decrease (e.g. reaching levels of about 60% after 80 min in cattle) in the three ruminant species, smaller amounts (10 ng) were incubated. In cattle, goats and sheep a decrease in androstenedione (10 ng) concentrations was more pronounced, if incubated alone ($P < 0.001$). When androstenedione was incubated together with 11-oxoaetiocholanolone (10 ng each), this decrease was delayed (but still highly significant; $P < 0.001$), reflected by significantly higher concentrations of androstenedione in these samples ($P < 0.05$; except after incubations for 80 min in all

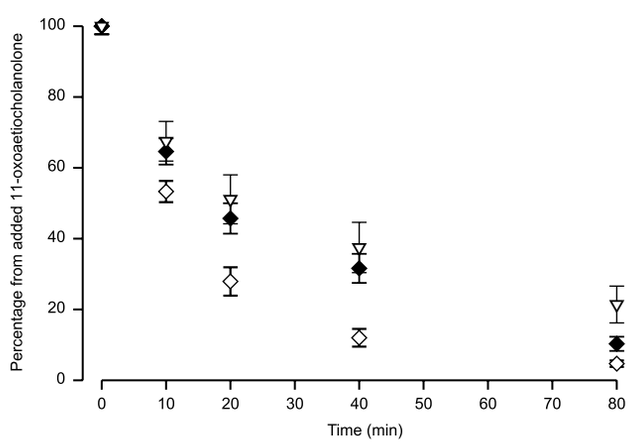

Fig. 1. Metabolism of 11-oxoaetiocholanolone (100 ng) by 100 μl (or 300 μl, respectively in sheep and goats) of suspended RBC (30% PCV) of cattle (◇), goats (◆) and sheep (▽; $n = 9$, $x \pm$ SE).

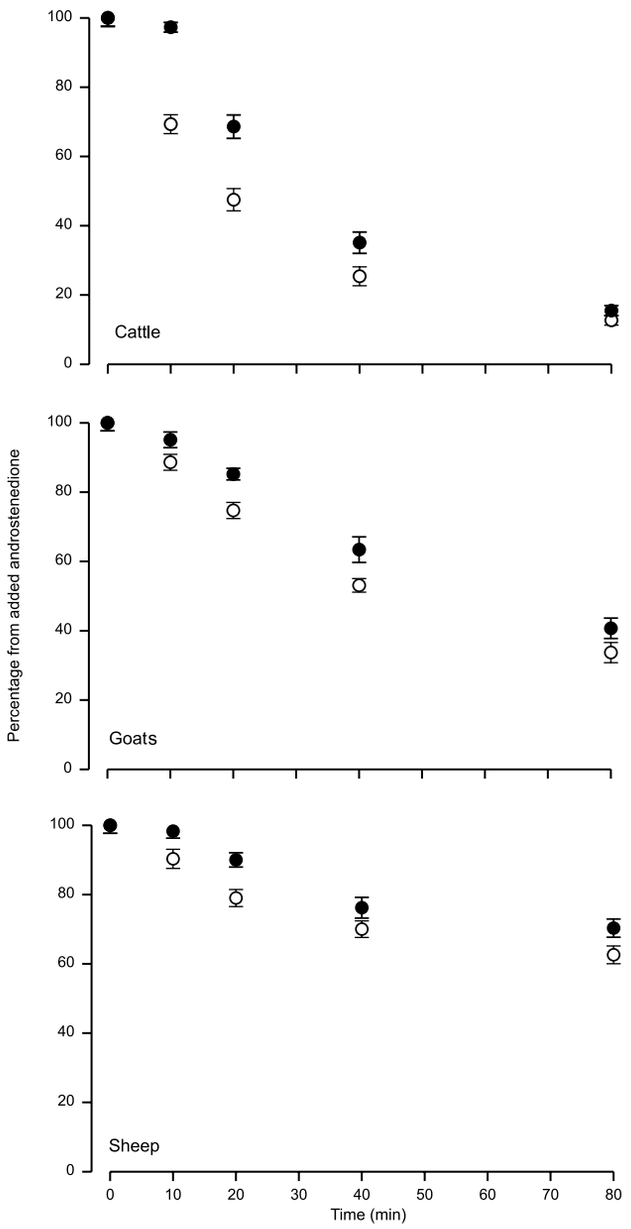

Fig. 2. Metabolism of androstenedione (10 ng) by 100 μl (or 300 μl, respectively) of suspended RBC (30% PCV) of cattle, goats or sheep, alone (○) and in the presence of 11-oxoaetiocholanolone (10 ng; ●, $n = 9$, mean ± SE).

species and 40 min in sheep). As in case of 11-oxoaetiocholanolone, RBC of cattle showed the highest enzymatic activity, followed by those of goats and sheep (Figs 1 and 2). For comparison, 10 ng of 11-oxoaetiocholanolone was also incubated with RBC of a single cow, reaching concentrations below the detection limit after 20 min of incubation (Fig. 3). Incubations with erythrocytes of a horse or Ringer's/glucose solution alone showed no significant changes in steroid concentrations.

HPLC separations

In the HPLC fractions of the separated metabolites from the 11-oxoaetiocholanolone incubations of all three species stud-

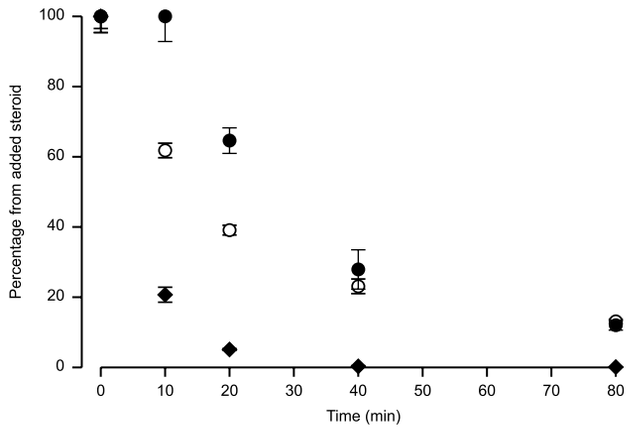

Fig. 3. Metabolism of 11-oxoaetiocholanolone (10 ng; ◆) by 100 μl of suspended RBC (30% PCV) of a single cow. To enable comparison, results of androstenedione incubated alone (10 ng; ○) and in the presence of 11-oxoaetiocholanolone (10 ng; ●) are also shown ($n = 3$, mean ± SE).

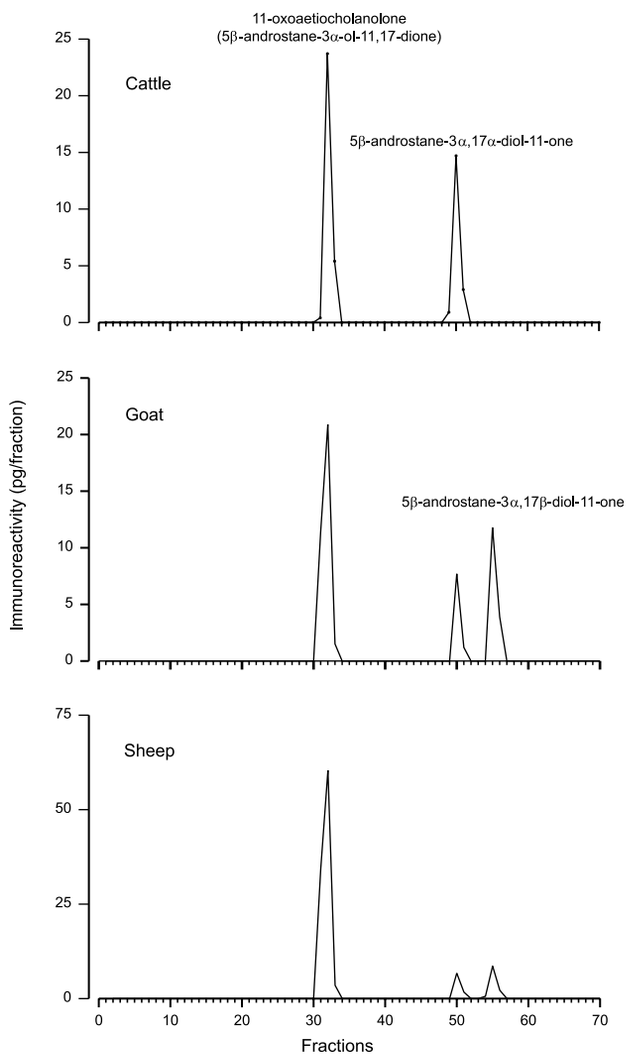

Fig. 4. The HPLC separation of metabolites of 11-oxoaetiocholanolone (100 ng) formed after 80 min by incubating standardized amounts of erythrocytes (30% PCV) of cattle (100 μl), goat and sheep (both 300 μl). Injected 20α-dihydroprogesterone and cortisol eluted around fractions 20 and 80, respectively.

ied, one immunoreactive peak, eluting around fraction 32, was detected (Fig. 4). It behaved chromatographically as the unchanged mother substance 11-oxoaetiocholanolone. This peak showed comparable immunoreactivity in both 11-oxoaetiocholanolone EIAs applied. Further peaks were only detected using the EIA described by Möstl et al. (2002). In case of sheep and goat two more polar peaks were present (fractions 50 and 55). In incubations of cattle only the former was deteced (Fig. 4). No differences in the proportion of formed metabolites were present, independent if 100 or 10 ng of 11-oxoaetiocholanolone were incubated.

The same number and elution order of separated metabolites was found in case of androstenedione incubations (i.e. the added mother steroid followed by one metabolite in cattle and an additional one in sheep and goat). The steroids were identified by their elution position, which was the same as the respective standards (epitestosterone and testosterone). After HPLC of plasma samples, a number of small peaks was detected with the 11-oxoaetiocholanolone EIAs, some of which corresponded with the metabolites formed after incubation of 11-oxoaetiocholanolone.

Discussion

The presence of 17-OH SDH in RBC of cattle and sheep has been described (Lindner, 1961, 1965; Tsang, 1976; Möstl et al., 1980). The present, comparative work aimed to confirm the same in the three different ruminant species studied. In addition it was investigated if 11-oxoaetiocholanolone, which is present in the blood after reabsorption from the gut, is a preferred substrate of these enzymes and thus influences the metabolism of androstenedione.

A dramatic decrease in the concentrations of added 11-oxoaetiocholanolone was observed in cattle. In the incubations of goat and sheep RBC the same pattern was found, but less pronounced (stressed by the fact that 300 μl, instead of 100 μl, of standardized RBC was used). Similar results in case of incubated androstenedione indicated that erythrocytes of cattle were more active (followed by those of goat and sheep, which were less) in converting the 17-oxosteroids. Therefore, 17-OH SDH in RBC is more active or present in larger amounts in cattle than in sheep and goats and/or the transfer of the steroids and their metabolites through the membrane of cattle erythrocytes is quicker. Differences in the haematocrit could be excluded as it was adjusted to 30% after washing the erythrocytes. There were still some amounts of added steroids present after 80 min of incubation. However, this might just be the reflection of an equilibrium reached between both directions of enzymatic conversion.

In addition to differences in the activity of 17-OH SDH between ruminant species, there were also differences between the steroids incubated. 11-oxoaetiocholanolone was more quickly converted than androstenedione in the same species. This is in contrast to an impression stated by Lindner (1965). However, he could not perform accurate experiments due to the lack of sensitive determination methods. Our findings indicate a different substrate affinity of 17-OH SDH or a different transfer rate of the investigated steroids through the membrane of the erythrocytes. The latter may be due to different functional groups and thus polarity of the steroids (Reznikov, 1978). These results demonstrated that 11-oxoaetiocholanolone (a cortisol metabolite formed by side chain cleavage) is also a substrate for 17-OH SDH of RBC in all ruminants.

The extracted steroids from the different incubations and blood plasma of the three ruminant species were separated by straight phase HPLC and the eluent fractionized in order to get indications on the metabolites formed. In all incubations the same number and elution order of separated metabolites were found. That was the added steroid, followed by one metabolite in cattle and an additional one in sheep and goat. In case of 11-oxoaetiocholanolone there were no standards of the respective 17-hydroxy metabolites available. That is why the formed metabolites could not be identified definitely. However, due to the same elution order and pattern of the peaks in the straight-phase HPLC, there is evidence that they are the respective 17α- and 17β-epimers. This is also supported by results of Lindner (1965) and Tsang (1976) in sheep, who described the same pattern of formed metabolites. The separations revealed that both, 17β- and 17α-OH SDH, are present in sheep and goat erythrocytes, but only the latter (17α-OH SDH) is active in cattle blood.

In cattle, goat and sheep decrease in androstenedione (10 ng) concentrations was more pronounced, if incubated alone. In the presence of the same amount of 11-oxoaetiocholanolone, this decrease was delayed. These results demonstrate that 11,17-dioxoandrostanes are not only substrates of RBC 17-OH SDH but that they also inhibit the metabolism of androstenedione. As faecal steroids are reabsorbed from the gut and thus present in the blood (Lindner, 1972), competition will take place *in vivo*, too. HPLC separations of plasma samples indicated the presence of very small amounts of the respective metabolites. However, this may be due to a quick elimination by the liver.

It is well known that there is an interaction between stress and reproduction, which takes place at different levels, such as the hypothalamus (Knol, 1991), the pituitary (Rivier and Rivest, 1991) and the gonads (Rivier and Rivest, 1991; Liptrap, 1993). Our results indicate a possible interaction between cortisol metabolites and gonadal steroids on the level of peripheral steroid metabolism.

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