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CYP26A1-specific antagonist influence on embryonic implantation, gene expression and endogenous retinoid concentration in rats

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ABSTRACT

Retinoids are essential in vertebrate reproduction and embryonic development. All-*trans*-retinoic acid (ATRA) is tightly regulated during these processes. CYP26A1 is mainly responsible for its degradation. To study the role of CYP26A1 during implantation, we applied R115866, a CYP26A1-specific antagonist, to rats during early gestation days (GD). On GD 6.5 and 12 samples were collected and the number of embryos was evaluated. ATRA concentration increased in uterus and serum, mRNA expression of CYP26A1 and CRABP2 increased in the liver, but not in the uterus. Uterine COX1 and 17βHSD mRNA expression was decreased. The number of embryos on GD 12 was not altered in this setting. It can be concluded that uterine expression of the analyzed retinoid-response genes during early gestation is not altered by this R115866 treatment and instead indirectly *via* ATRA. From our experiment we cannot confirm that ATRA obtains a major influencing role in the regulation of embryonic implantation.

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1. Introduction

Vitamin A alcohol (retinol, ROL) is essential for reproduction (reviewed in [1]). The endogenous ligand for the retinoic acid receptors all-*trans*-retinoic acid (ATRA) is generated by the sequential oxidation of ROL [2,3]. Retinol sustains all vitamin A functions and ATRA maintains differentiation and growth of the adult organism, but is insufficient to support gestation (reviewed in [4]). All-*trans*-retinoic acid (ATRA) ligates the retinoic acid receptors (RAR α, β and γ) while its 9-*cis* analog mainly activates the retinoid X receptors (RXRs) [5]. ATRA can be further oxidized and inactivated by CYP26A1 [6,7]. ATRA and several synthetic retinoids are used in cancer therapy and for the treatment of dermatological disorders [8]. The clinical use of ATRA is hampered by the induction of resistance, caused partly by increased metabolism by various CYP450 enzymes [9], therefore retinoic acid blocking agents (RAMBAs) including this derivative (R115866, also called talarozole or rambazole) and various others belonging to the substance class RAMBAs are successfully used and under development for various dermatological and oncological therapies [10–16].

In addition, ATRA exhibits a strong teratogenic potential [17], depending on time of treatment and dose various specific abnormalities will be exhibit [18]. CYP26A1 expression is stimulated by RA and various other retinoids [19], however, uterine CYP26A1 expression has been described as mainly regulated by gestagenes [20,21]. RA-synthesizing enzymes, the retinal dehydrogenases (RALDH1 and RALDH2), as well as the RARs and RXRs exhibit a distinct expression pattern within the female reproductive organs [22,23]. In addition to cellular retinoic acid binding protein 2 (CRABP2) which was investigated only in the rat uterus [24], it has been shown that estrogen can also induce RALDH2 expression in rats, mice and humans [22,25]. There are different genes known to be necessary and essential for the process of implantation (reviewed in [26–28]), including different RA-responsive and regulated genes. Additionally retinoids, mainly retinoic acid [29–31] and oxo-retinoids [32] have also been shown to be important in the developing blastocyst/embryonic stem cells.

Various RA-response genes like CRABP2 [33], TG2 [34] and CYP26A1 [35,36] are excellent markers for RA-mediated signal-

Abbreviations: ATRA, all-*trans*-retinoic acid; COX, cyclooxygenase; CRABP2, cellular retinoic acid binding protein 2; QRT-PCR, semi-quantitative real time PCR; TG2, tissue transglutaminase 2; 17βHSD, 17beta-hydroxysteroid dehydrogenase.

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ing. CRABP2, which is mainly involved in RA-shuttling [37] and RAR-mediated signaling [38], exhibits a dynamic expression pattern during the uterine cycle and early gestation in human and mouse endometrial cells [39,40]. CYP26A1 is mainly expressed during early murine gestation in luminal and glandular cells [25]. Also, tissue transglutaminase 2 (TG2) mediates apoptosis-signaling [41] and is mainly expressed during the secretory phase [22]. Besides RA-mediated regulation, estradiol also induces the expression of TG2 [22]. Cyclooxygenases (COX) 1 and 2 [42,43], transforming growth factor β2 (TGFβ2) [44], leukemia inhibitory factor (LIF), LIF-receptor (LIF-R) [28], tumor necrosis factor α 1 (TNFα1) [45], calcitonin [46] and 17beta-hydroxysteroid dehydrogenase type 1 (17βHSD1) [47] are all involved in blastocyst implantation and are excellent markers for this process. 17βHSD1 is an enzyme involved in steroid metabolism [48], while enzymes responsible for prostaglandine synthesis are the COX1 and 2, which are essential for blastocyst implantation and decidualization [43,49]. LIF, LIF-R, calcitonin, TGFβ2 and TNFα1 are highly expressed in the maternal endometrium and are associated with enabling embryonic implantation [28,32,44–46,50,51].

CYP26A1 is strongly expressed in the endometrium during implantation and might be regulating endogenous ATRA concentration during the implantation process. The derivative R115866 was found to be a selective inhibitor of CYP26A1-mediated RA oxidation [52]. Further studies have shown that RA-induced blastocyst apoptosis [53] and RA-induced suppression of decidualization, differentiation and development of the blastocyst [53,54]. The aim of our study was to determine the effects of CYP26A1-inhibitor R115886 on endogenous ATRA concentrations, gene expression and blastocyst implantation.

2. Material and methods

2.1. Animals

Female Wistar rats (∼200 g; Charles River, Bad Salzuflen, D) were kept at room temperature (21 ± 1 °C), a light–dark-regime of 12 h and relative humidity of 55 ± 5%. Animals had free access to food (Ssniff-Spezialdiäten, Soest, D) and water. Female rats (30 animals) were mated during the proestrous phase with adult males; the presence of a vaginal plug on the morning after mating was set as day 1 of pregnancy (GD 1 pc). Overall 27 pregnant rats were used for the investigation. From GD 3 to day 6 pc, the animals received either the vehicle substance (2.5 ml/kg), groups A and C; a mixture of Myrj 53 (85 mg/100 ml NaCl 0.9%) no. A-8565, ICI Speciality Chemicals Essen, or R115886 (2.5 ml/kg, groups B and D; solved in vehicle substance) at 2 p.m. daily by oral gavage. The dose of R115866 is similar to the dose reported by Stoppie et al. [52]. R115866 was a generous gift from J. van Wauwe of the Janssen Research foundation, Breese, B. On day 6 pc, the animals received either vehicle or the R115866 solution at 8 a.m. On the same afternoon (GD 6.5 pc), animals from groups A and B were sacrificed under yellow dimmed light, to prevent retinoid degradation and isomerization. Number of animals per group—A: $n = 6$ and B, C and D: $n = 7$. The weights of uteri were measured; liver and uteri were collected and immediately frozen in liquid nitrogen and stored at −80 °C. Blood was collected when animals were killed; serum was harvested and stored at −20 °C until retinoid examination. Endogenous estrogen and progesteron levels were examined on blood samples by radio immuno-assay on day 6.5 pc and 12. On day 12, animals from groups C and D were sacrificed under the same conditions and the number of implanted embryos was examined. All animal experiments were approved by the respective ethical authorities from Land Berlin, D.

2.2. QRT-PCR

Collected liver and uterus samples were homogenized by mortar and pestle in liquid nitrogen, RNA was isolated, reverse transcribed (please refer to [55,56]) and amplified as follows: primers were designed with Primer Express-Software and ordered from Invitrogen (UK). PCR conditions were: 95 °C for 10 min; 95 °C for 15 s and 60 °C for 1 min, 40 cycles in the ABI Prism SDS7700 (Perkin-Elmer Applied Biosystems, Foster City, CA). Validation of PCR efficiency for target and reference was performed and the relative semi-quantitative expression of the mRNA (increasing fluorescence signal) was detected. The relative amount of target was normalised to cyclophillin, an endogenous control that was run in the same PCR reaction, and calculated with the formula $2^{-\Delta Ct}$ (ΔCt defined as $\Delta Ct = Ct_{sample} - Ct_{cyclophillin}$) using the ABI PRISM 7700 Sequence Detection System. The PCR products have been controlled for unspecific amplifications by performance of dissociation curves. For each target gene and animal QRT-PCR was run three times in parallel, the mean of these runs was calculated and is represented as "×". Symbols in the figures indicate the mean of the analyzed target in the experimental group.

Table 1
Concentration of endogenous retinoids in serum (ng/ml), liver (ng/g) and uterus (ng/g) on GD 6.5.

GD 6.5	Vehicle group; A, mean ± SD; $n = 6$	R115866-treated group; B, mean ± SD; $n = 7$
Serum		
Retinol	249 ± 121	326 ± 92
Retinyl ester	125 ± 80	42 ± 16*
Liver		
Retinol	43,229 ± 4071	31,347 ± 6736**
Retinyl ester	324,331 ± 28,724	254,489 ± 52,031**
Uterus		
Retinol	88 ± 40	105 ± 57
Retinyl ester	235 ± 87	262 ± 135

Retinyl ester (retinyl-linoleat, -myristat, -oleat, -palmitat, -stearat).
* $p < 0.05$.
** $p < 0.01$.

2.3. HPLC-analysis of endogenous retinoids

Retinoid analysis was performed according to the procedure described in [57]. Uterine tissue samples were first homogenized by mortar and pestle in liquid nitrogen and further prepared as previously described [57].

2.4. Statistical analysis

Using SPSS software (10.0) (SPSS Inc., Chicago, USA) data were analyzed by Mann–Whitney *U*-test. A *p*-value of 0.05 was estimated as significant.

3. Results

3.1. Influence of R115866 application on endogenous retinoids

In response to treatment with R115866, serum ROL concentration was not significantly altered, while rats treated with R115866 showed significantly reduced serum retinyl ester concentrations (Table 1). The application of R115866 led to a significant increase of serum ATRA concentration of 0.6 ± 0.1 ng/ml in the vehicle treated group, and to a concentration of 1.8 ± 0.4 ng/ml in the R115866-treated group, respectively (Fig. 1). After application of R115866, liver ROL concentration on GD 6.5 was significantly reduced (Table 1, $p < 0.01$). Liver RA concentration was slightly, but not significantly, increased after the application of R115866 (from 7.8 ± 2.4 ng/g in the vehicle treated group to 11.1 ± 3.1 ng/g in the R115866-treated group). The application of R115866 did not change the uterine ROL concentration (Table 1). No significant changes could be observed in endogenous retinyl ester concentra-

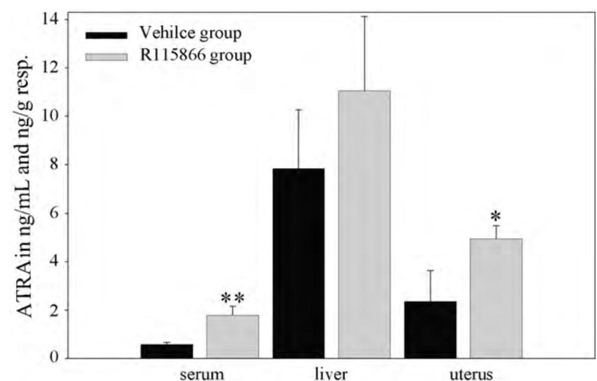

Fig. 1. Concentration of all-*trans*-retinoic acid in the examined tissues at GD 6.5, columns represent the mean of each group plus standard deviation; *$p < 0.005$, **$p < 0.001$.

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