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Influence of the antiandrogen flutamide on the androgen receptor gene expression in the placenta and umbilical cord during pregnancy in the pig

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ABSTRACT

The aim of this study was to determine the immunolocalization and expression of the androgen receptor (AR) in the pig placenta and umbilical cord during pregnancy following exposure to flutamide, a non-steroidal antiandrogen, at its various stages. Pregnant pigs were injected with flutamide at a daily dose of 50 mg/kg body weight at different stages of pregnancy: from day 83–89 ($n = 2$); from day 101–107 ($n = 2$). They were sacrificed and tissues collected one day after the last injection. Control animals, two for each experimental point, were injected only with the vehicle (corn oil). Collected tissue samples were fixed for immunohistochemistry or frozen for protein isolation. AR protein was detected in the nucleus of trophoblast cells forming the structure of ridges and in maternal endothelial cells, which are involved in the placental barrier formation. It was also localized in the nuclei of cells forming umbilical cord components: allantoic duct epithelium, amniotic epithelium, Wharton's jelly and the muscular layer of the umbilical cord vein and arteries. Relative optical density analysis showed increased expression in the material derived from animals treated with flutamide. The presence of AR in the placental barrier and in the umbilical cord components suggests a role of androgen in those temporary organs. Flutamide could impact on the levels of the AR protein in the reproductive tracts during pregnancy in sows.

Introduction

Androgens were originally considered to be solely male sex hormones, but they are also secreted and perform regulatory functions in females (Palacios, 2007). The female reproductive system is a target for many biological compounds including steroid hormones such as estrogens, progesterone and also androgens.

In female pigs (sows), the most important sources of androgens are the ovaries and to a lesser extent the adrenal glands, however, during pregnancy there are two additional sources of androgens: elongating blastocysts and the placenta (Drews et al., 2001; Slomczynska et al., 2008). Androgens exert various effects in tissues that express the androgen receptor (AR) protein, which belongs to the superfamily of nuclear receptors and works as a transcription factor. Binding of the two biologically active androgens: testosterone (T) and dihydrotestosterone (DHT) results in some conformational changes including: heat shock protein (HSP) disaggregation, dimerization, translocation into the nucleus and binding to the androgen response element (ARE) in the DNA that allows running of the transcriptional machinery (Brinkmann et al., 1999).

AR has been detected in the female reproductive tract in various mammalian species: rat, mouse, cattle, pig and primates including humans (Dai et al., 1996; Cheng et al., 2002; Aflatoonian and Moore, 2006; Burek et al., 2007; Edmondson et al., 2002). Our previous work demonstrated AR expression in the porcine uterus throughout pregnancy (Slomczynska et al., 2008). To evaluate the role of AR in uterine physiology, the presence of AR mRNA and protein localization in the porcine uterus from day 10 to 90 of pregnancy were studied. AR protein was detectable in the glandular epithelium, stromal cells and myometrium on all investigated days of pregnancy. However, the intensity of staining declined. The data obtained from experiments conducted at early pregnancy up to day 18, indicated the presence of AR in the porcine endometrium of pregnancy and in the smooth muscle layer myometrium (Cardenas et al., 2002; Cardenas and Pope, 2002).

To determine the role of androgens in the regulation of androgen-dependent genes during pregnancy, AR was blocked with flutamide. Flutamide was the first discovered non-steroidal antiandrogen compound, which binds to the AR and inhibits its activity (Gao et al., 2006).

Flutamide can cross the placental barrier and thus can have an impact on fetal development as shown in our previous paper (Durlej et al., 2011). So far there are no data on the possible effects of antiandrogens on the expression of the AR in the placenta and umbilical cord in sows.

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In animals several fundamental types of placentas can be distinguished. Their classification is based on differences in shape and the level of complexity of the connection between the maternal endometrium and chorionic epithelium of the fetus. According to these assumptions the pig placenta is classified as a diffuse one (the whole allantochorionic surface is involved in the formation of the placenta) and epitheliochorial (three maternal layers: endometrial epithelium, connective tissue, uterine endothelium are distinguished in the placenta) (Johansson et al., 2001).

In late gestation when placentation is advanced, some characteristic structures can be seen such as: ridges from the maternal and fetal side, numerous folds, bulbous protrusions, regular and irregular areoles (Friess et al., 1980). The umbilical cord is another specific structure that appears during pregnancy connecting the fetus with the body of its mother. The umbilical cord is covered by the amniotic epithelium, filled with Wharton's jelly (mucous connective tissue) in which two arteries, one vein and the allantoic duct are embedded (Chrusciel et al., 2011). The proper development of this organ, as well as that of the placenta, is critical for normal development and success rate of pregnancy.

The objective of our study was to characterize possible changes in the immunolocalization and AR expression after exposure to flutamide during late pregnancy. The current study has focused on the transient appearing structures: the feto-maternal compartment and the umbilical cord.

Materials and methods

Animals

All procedures were performed in accordance with the Polish legal requirements, under a license given by the Local Ethics Committee at the Jagiellonian University, Krakow, Poland (No. 122/2009).

To obtain material for investigation, pregnant crossbred pigs (Large White × Polish Landrace) were bred and they were divided into two groups: control and experimental one. Additionally the experimental group was divided into two subgroups treated with flutamide (2-methyl-N-[4-nitro-3′-(trifluoromethyl)-phenyl]propamide) (Sigma–Aldrich, St. Louis, MO, USA). In the first experimental group two pregnant pigs were treated subcutaneously with flutamide at the daily dose of 50 mg/kg body weight, from day 83 to 89 of pregnancy. Similarly, two pigs from the second experimental group were flutamide treated from day 101 to 107. Animals from control groups were treated only with vehicle (corn oil) under identical conditions. The uteri, placentas and umbilical cord fragments were collected one day after the last injection i.e. day 90 or 108 of pregnancy. This schedule of gestational days was chosen on the basis of literature data and our previous studies.

AR immunolocalization

Samples collected for immunohistochemical localization of the AR were fixed in 10% buffered formalin overnight. Next, samples were dehydrated in an increasing gradient of ethanol and equilibrated with xylene prior to embedding in Paraplast® wax (Monoject Scientific Division of Sherwood Medical, St. Louis, MO, USA). 5 μm thick wax sections were cut on a microtome and mounted on slides, deparaffinized in xylene, rehydrated in decreasing concentration of alcohol. To retrieve antigens a microwave technique with 0.01 M citrate buffer (pH 6.0) was used. The time of microwave exposure at full power was different for various types of tissues, for umbilical cord slides it was about 2 min (because of the delicate structure) for other tissues 2 × 3 min. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in TBS (Tris buffered saline, pH 7.4) for 25 min. After cooling, sections were incubated with 5% normal goat serum (Sigma–Aldrich, St. Louis, MO, USA) for 20 min, to prevent nonspecific reactions.

The primary rabbit polyclonal antibody anti-AR (1:1000, Santa Cruz Biotechnology) was used for immunodetection of the AR protein. Incubation continued overnight at 4 °C. Next day sections were washed in TBS buffer and incubated for 1.5 h with the secondary goat biotinylated antibody (1:400, Vector Labs, Burlingame, CA, USA). After visualization of the product reaction slides were dehydrated and mounted on DPX (Fluka Chemie GmbH, Buchs, Switzerland).

Photomicrographs were taken using a Nikon Eclipse E200 microscope with an attached Nikon Coolpix 5400 digital camera system and then quantified by a videodensitometric analysis using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The intensity of AR staining was expressed as relative optical density (ROD) of diaminobenzidine (DAB) brown reaction products (Smolen, 1990). Statistical differences between experimental and control groups were estimated using Student's t test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Western blot analysis

Tissue fragments collected for Western blot analysis were frozen at −80 °C. Tissues were thawed and mechanically homogenized on ice with Tris–EDTA buffer (pH 7.5). Homogenates were then sonicated and centrifuged at 10,000 × g for 20 min at 4 °C as previously described. Protein levels were measured using a NanoDrop 2000 UV–vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) with Tris–EDTA as a standard. Supernatants containing 20 μg of protein were solubilized in a sample buffer consisting of 62.5 mM Tris–HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue and 5% β-mercaptoethanol (Bio-Rad Laboratories, Munich, Germany). After heating at 99.9 °C for 5 min samples were run on SDS polyacrylamide gel electrophoresis. Taking into account the size of the AR protein (110 kDa), 5% stacking and 12% separating gels were selected. Electrophoresis was conducted under denaturing conditions according to Laemmli (1970). Transfer to nitrocellulose membranes was at 250 mA using a wet blotter in the Genie Transfer Buffer (20 mM Tris, 150 mM glycine in 20% methanol, pH 8.4) and then membranes were incubated with non-fat milk in TBS, 0.1% Tween 20 at 4 °C with shaking all night to block nonspecific reactions. Next day membranes were incubated for 1.5 h with the primary rabbit polyclonal antibody anti-AR (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After the first incubation and thorough washing the second incubation of membranes was conducted with a secondary horseradish peroxidase-conjugated, anti-rabbit IgG (1:3000, Vector Labs, Burlingame, CA, USA). Time of incubation was 1 h at room temperature. To visualize the presence of the AR Western blotting luminol reagent solution (Santa Cruz Biotechnology) was used for 1 min and then images of membranes were taken by ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA). The homogenate of porcine prostate was used as a positive control. Molecular masses were estimated by reference to standard proteins (Standards High Range, Bio-Rad). To obtain quantitative results, blots were densitometrically scanned and analyzed using Image Lab™ 2.0 (Bio-Rad Labs). Quantitative analysis was performed for three separately repeated experiments. Protein level within the control group was arbitrarily set as 1, against which statistical significance was analyzed.