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Carbenoxolone exposure during late gestation in rats alters placental expressions of p53 and estrogen receptors

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ABSTRACT

Gestational carbenoxolone exposure inhibits placental 11β-hydroxysteroid dehydrogenase (11β-HSD), the physiological barrier for glucocorticoids, which increases fetal exposure to glucocorticoids and induces intrauterine growth restriction (IUGR). We hypothesized that carbenoxolone exposure influences the expression of placental estrogen receptors-α and β (ERα & ERβ) and p53 leading to inhibited fetal and placental growth. Pregnant Sprague-Dawley rats were injected twice daily with either carbenoxolone (10 mg/kg; s.c.) or vehicle (control group) from gestational days (dg) 12 onwards. Maternal blood and placentas were collected on 16 dg, 19 dg and 21 dg. The expression of ERα, ERβ and p53 were studied in placental basal and labyrinth zones by RT-PCR, Western blotting and immunohistochemistry. Carbenoxolone did not affect placental and fetal body weights, but ELISA showed decreased estradiol levels on 19 dg and 21 dg, and increased maternal luteinizing hormone levels on all dg. The follicle stimulating hormone levels decreased on 16 dg and 19 dg, and increased on 21 dg. Carbenoxolone decreased ERα mRNA levels on 16 dg in both zones and its protein level on 19 dg in the labyrinth zone. However, carbenoxolone increased ERβ mRNA levels on 19 dg and 21 dg and protein levels on 16 dg and 19 dg in the labyrinth zone. The p53 mRNA levels increased on all dg, but its protein levels increased on 21 dg in both zones. In conclusion, carbenoxolone exposure changes placental p53, ERα, ERβ expression in favor of cell death but these changes do not induce IUGR in rats.

1. Introduction

Human and rat placentas contain two distinct regions called basal (or junctional) and labyrinth zones (Matt and Macdonald, 1985). The basal zone functions as an endocrine organ, especially during late pregnancy (Ain et al., 2003; Matt and Macdonald, 1985; Waddell et al., 2000). The labyrinth zone is primarily involved in feto-maternal exchange of nutrients and waste products (Waddell et al., 2000). The labyrinth zone consists of trophoblast giant cells and syncytiotrophoblast cells, which transfer nutrients and wastes between fetal and maternal circulations (Soares et al., 1996). The basal zone contains trophoblast giant cells, spongiotrophoblasts and glycogen cells, which are involved in disparate placental functions including nutrition supply and endocrine secretions (Ain et al., 2003; Ain et al., 2006; Iwatsuki et al., 2000).

The placental growth, development and senescence are critical and paramount for normal fetal development (Waddell et al., 2000). The amount of glucocorticoids to which a fetus is exposed is crucial for its growth and development (Aurbach and McCormick, 1993). Thus, the availability of glucocorticoids to a fetus is tightly regulated by a placental enzyme called 11β-hydroxysteroid dehydrogenase (11β-HSD) (Sloboda et al., 2005). This enzyme functions as a biochemical/physiological placental barrier and protects the fetus from overexposure to the increased levels of Glucocorticoids (Moritz et al., 2005; Sloboda et al., 2005). Impairment of 11β-HSD functions in placentas results in low birth weight of fetuses, which indicates a significant role for the enzyme in maintaining normal fetal growth (Kajantie et al., 2003; Michael et al., 2003). Previous studies have shown that carbenoxolone, an inhibitor of 11β-HSD, stimulates placental apoptosis and reduces fetal growth (Waddell et al., 2000) suggesting that uncontrolled exposure to glucocorticoids results in intrauterine growth restriction (IUGR) (Aurbach and McCormick, 1993; Burton and Waddell, 1999; Mark et al., 2009).

Estrogens are required for the initiation and maintenance of pregnancy (Albrecht et al., 2000; Bartholomeusz et al., 1999; Matsuura et al., 2004) and for normal fetal development and growth (Handwerger, 1999). However, high levels of estrogens impede fetoplacental growth (Matsuura et al., 2004; Spencer et al., 1986) and decrease both fetal and placental weights (Dreisbach, 1959; Kühn et al., 1982; Ogle and George, 1995). Estrogens exert their actions by binding

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to the estrogen receptors (ERs) (Bazer, 1998), which have two isoforms, the ERα and ERβ (Falcone and Hurd, 2007; Fritz and Speroff, 2010). In rat placentas, both ER isoforms are expressed (Al-Bader, 2006), but with different expression patterns (Bukovsky et al., 2003a, 2003b).

A balance between cell proliferation and cell death should be maintained to ensure normal physiological placental growth (Heazell et al., 2011). p53, a tumor suppressor protein, plays a central role in execution of apoptosis (Fridman and Lowe, 2003; Heazell et al., 2011; Karen and Xin, 2002), an essential process for normal physiological development (Falco et al., 2005). Regulation of apoptosis is necessary to allow normal placental development and function through gestation (Falco et al., 2005). IUGR placentas show high p53 expression and enhanced apoptosis (Falco et al., 2005; Heazell et al., 2011; Levy et al., 2002).

Studies in various cancer cell lines have linked actions of estrogens and ERs to p53. Medina et al. indicated that estrogens activate p53, reduce cell proliferation and protect against breast cancer (Medina, 2004). Moreover, Hsu and colleagues showed that activation of the ERs inhibited human LoVo colon cancer cell proliferation by increasing p53 expression (Hsu et al., 2014). In contrast, others who studied leiomyoma cells showed that estradiol down-regulated p53 (Maruo et al., 2004). Thus, the link between proliferating cells, estrogens and its receptors and p53 differs among various cell types and tissues. Moreover, a putative relationship between p53 and the ERs in carbenoxolone-treated rat placentas has not been reported. The present study was designed to investigate carbenoxolone-induced alterations in expressions of p53 and ERs in rat placentas during late pregnancy.

2. Materials and methods

2.1. Animals

Adult male and female Sprague-Dawley rats (n=4–8; 2–3 month-old) were procured from the Animal Resources Center at the Health Sciences Center of Kuwait University. All rats were housed in plastic cages with sawdust bedding under controlled temperature and humidity (22–23 °C; 40–50% humidity). The rats were given food and water *ad libitum*. Maintenance, experimental procedures, and euthanization of the rats and disposal of animal remains were in accordance with the regulations of Animal Ethics Committee of the Health Sciences Center of the Kuwait University and conformed to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

2.2. Carbenoxolone administration

The female rats were mated overnight with age-matched male rats and the presence of sperm in vaginal smears was confirmed the next day; this day was designated as day 0 of pregnancy. The pregnant rats were segregated into six groups (n=4–8). Three groups served as control groups, which received s.c. injection of 0.1 ml of 4% ethanol in saline, twice daily. The remaining three groups received carbenoxolone ((3β)−3-[(3-carboxypropanoyl)oxy]−11-oxoolean-12-en-30-oic acid; Cat# C4790; Sigma-Aldrich, USA) twice daily (s.c., injections; 10 mg in 0.1 ml of 4% ethanol in saline) starting from 12th gestational day (dg) to 21st dg. The dose selection and treatment protocol were based on earlier reports (Smith and Waddell, 2000). In this study, we used twice daily injections of 10 mg of the drug each, which is a slight modification of the treatment regimen followed in our earlier study in which 20 mg drug was given throughout the gestation period (Mouihate and Al-Bader, 2013). The reason for this modification was that we wanted to investigate the effects of the drug when given to pregnant rats during latter half of the gestation period. In addition, by dividing 20 mg dose into two doses of 10 mg each, we were able to maintain steady level of drug in the circulation.

2.3. Animal euthanization and tissue collection

One group each of control and carbenoxolone-treated pregnant rats was killed by cervical dislocation on 16 dg, 19 dg and 21 dg. Maternal blood was collected from the right ventricle and the serum was separated. Uterine horns containing conceptuses were removed and immediately placed on ice. Fetuses and placentas were separated and weighed. Two placentas per rat were fixed in 10% normal buffered saline and embedded in paraffin for immunohistochemistry. The remaining placentas were dissected on ice into basal and labyrinth zones. The weight of each zone was recorded and frozen. 10% DMSO (Dimethyl sulfoxide) was added to samples before freezing for protein analysis, and samples for RNA analysis were frozen in liquid nitrogen. All samples, except paraffin blocks, were stored at −70 °C, until use.

2.4. Enzyme-linked immunosorbent assay (ELISA) for hormones

The ELISA for LH (luteinizing hormone), FSH (follicle stimulating hormone) and estradiol was conducted by using commercially available kits (Cat# E0441r, E0830r and E0461r, respectively; Cusabio Biotech Co., Ltd.). Briefly, to each well in the respective antibody-coated 96 well microtitre plate, 50 µl of sample/standard was added. Then, to each well, except to the blank wells, 50 µl of horseradish peroxidase-conjugate and 50 µl of antibody was added, and incubated for 1 h at 37 °C. The liquid was removed from each well and the wells were washed thrice with 200 µl of wash buffer and the liquid was completely removed. To each well, 50 µl of substrate A was added followed by 50 µl of substrate B. The plate was incubated for 15 min at 37 °C. Then, 50 µl of stop solution was added and development of yellow color was observed. The absorbance of the mixture was read at 450 nm within 10 min. The data were fitted into four parameter logistic (4P-L) curve by using Expert 1.3 software.

2.5. Real-Time PCR (RT-PCR)

Total RNA was extracted, DNAse treated and reverse transcribed as described previously (Al-Bader, 2006; Al-Bader et al., 2015, 2011). RT-PCR was performed to quantify the expression of ERs and p53 genes by using Taqman probes [ERα (Catalog # Rn01430446_m1with amplicon length of 73 bp, Applied Biosystems, U.S.A.), ERβ (Catalog # Rn00562610_ml with amplicon length of 73 bp, Applied Biosystems, U.S.A.) and p53 (Catalog # Rn00755717_m1with amplicon length of 94 bp, Applied Biosystems, U.S.A.)]. The 18 S house-keeping gene was used as a reference control (Catalog # Rn03928990_g1with amplicon length of 61 bp, Applied Biosystems, U.S.A).

Aliquots of 20 µl master mixture (Taqman universal mix, DEPC water, 18 S primer and gene specific primers) were pipetted into 96-well plate and 5 µl of the cDNA was added to the appropriate wells in duplicates. The 96-well plate was loaded into the RT-PCR ABI 7500 system (Applied Biosystems) and PCR cycles were run as follows: 2 min at 50 °C for 1 cycle; 10 min at 95 °C for 1 cycle; 15 s at 95 °C and 1 min at 60 °C for 40 cycles. The fold change in expression was calculated as described previously (Al-Bader and Kilarkaje, 2015).

2.6. Western blotting for p53 and estrogen receptors

Tissue samples were processed as described previously (Al-Bader, 2006; Al-Bader et al., 2015). Twenty µg of protein was loaded into each well including a high range molecular weight marker (12–225 kDa; GE Healthcare, UK). Precise Tris-HEPES-SDS Precast Polyacrylamide Mini Gels 4–20% (Thermo Scientific, USA) were used for the protein electrophoresis. Samples (20 µl) and the molecular weight markers were loaded into designated wells and electrophoresed at 250 V in room temperature. Proteins were transferred onto PVDF (Polyvinylidene difluoride) membranes and the cassettes were assembled and inserted into the transfer apparatus and connected to a

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