Journal of Steroid Biochemistry & Molecular Biology 122 (2010) 82–90

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

A single early postnatal estradiol injection affects morphology and gene expression of the ovary and parametrial adipose tissue in adult female rats☆

Camilla Alexanderson[a,*], Elisabet Stener-Victorin[a], Joel Kullberg[b], Staffan Nilsson[c], Max Levin[d], Stefan Cajander[e], Lars Lönn[f], Malin Lönn[d], Agneta Holmäng[a]

[a] Institute of Neuroscience and Physiology, Department of Physiology, Sahlgrenska Academy, University of Gothenburg, Sweden
[b] Department of Radiology, Uppsala University Hospital, Uppsala, Sweden
[c] Department of Mathematical Statistics, Chalmers University of Technology, Sweden
[d] Institute of Medicine, Wallenberg Laboratory, Sahlgrenska Academy, University of Gothenburg, Sweden
[e] Department of Pathology and Cytology, Akademiska sjukhuset, Uppsala, Sweden
[f] Faculty of Health Sciences, Radiology, Rigshospitalet, Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 24 June 2009
Received in revised form 16 October 2009
Accepted 19 October 2009

Keywords:
Estradiol
Programming
Ovary
Parametrial adipose tissue

ABSTRACT

Events during early life can affect reproductive and metabolic functions in adulthood. We evaluated the programming effects of a single early postnatal estradiol injection (within 3 h after birth) in female rats. We assessed ovarian and parametrial adipose tissue morphology, evaluated gene expression related to follicular development and adipose tissue metabolism, and developed a non-invasive volumetric estimation of parametrial adipose tissue by magnetic resonance imaging. Estradiol reduced ovarian weight, increased antral follicle size and number of atretic antral follicles, and decreased theca interna thickness in atretic antral follicles. Adult estradiol-injected rats also had malformed vaginal openings and lacked corpora lutea, confirming anovulation. Estradiol markedly reduced parametrial adipose tissue mass. Adipocyte size was unchanged, suggesting reduced adipocyte number. Parametrial adipose tissue lipoprotein lipase activity was increased. In ovaries, estradiol increased mRNA expression of adiponectin, complement component 3, estrogen receptor α, and glucose transporter 3 and 4; in parametrial adipose tissue, expression of complement component 3 was increased, expression of estrogen receptor α was decreased, and expression of leptin, lipoprotein lipase, and hormone-sensitive lipase was unaffected. These findings suggest that early postnatal estradiol exposure of female rats result in long-lasting effects on the ovary and parametrial adipose tissue at adult age.

1. Introduction

Events during a hormone-sensitive period in early life can affect physiological functions in adulthood, a process known as programming [1]. Thus, dysregulated reproductive or metabolic function in adulthood may originate from insults such as maternal food restriction, inflammation, and hormonal alterations during critical periods in pre- or postnatal life [2–4]. Hormonal alterations in early life may be exemplified by the exposure to numerous chemicals released into the environment. These molecules can disrupt endocrine function in animals and humans since some of them are known to exert estrogenic activity by binding to the estrogen receptor [5]. The developing organism seems to be extremely sensitive to perturbations by chemicals with hormone-like activity. Exposure to these chemicals during critical stages of differentiation have been suggested to have permanent long-lasting consequences, such as obesity [6] and reproductive tract problems [7], but the full extent of the health consequences of hormonal exposure is unknown.

Sex hormone programming in animals has been shown to affect adipose tissue, insulin sensitivity, and ovarian morphology and function [8–11]. We have recently reported that a single early postnatal injection of testosterone or estradiol benzoate, but not of dihydrotestosterone, resulted in insulin resistance and increased mesenteric adipose tissue mass and adipocyte size in adult female rats, suggesting that postnatal estrogen receptor activation exerts stronger programming effects on metabolic indices than androgen

Abbreviations: ASP, acylation-stimulating protein; C3, complement component 3; CL, corpora lutea; DXA, dual energy X-ray absorptiometry; ERα, estrogen receptor alpha; LPL, lipoprotein lipase; MRI, magnetic resonance imaging; TGFβ, transforming growth factor beta.

☆ This work was supported by grant from Novo Nordisk Foundation, the Swedish Medical Research Council (Project No. 12206, 2008-72VP-15445-01A, K2007-54X-20325-01-3), the Swedish Diabetes Association Research Foundation, Wilhelm and Martina Lundgrens's Science Fund, Hjalmar Svensson, Tore Nilson Foundation, Åke Wiberg Foundation, Adlerbert Research Foundation, and the Swedish federal government under the LUA/ALF agreement.

* Corresponding author at: Wallenberg Laboratory for Cardiovascular Research, Sahlgrenska Academy, University of Gothenburg, Box 434, SE-405 30 Gothenburg, Sweden. Tel.: +46 31 7863536; fax: +46 31 7863512.
E-mail address: camilla.alexanderson@wlab.gu.se (C. Alexanderson).

0960-0760/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2009.10.006

receptor activation [9]. Furthermore, we have extended these findings by showing that estradiol exposure affected gene expression in skeletal muscle; expression of inflammation markers was increased while expression of genes involved in glucose and lipid metabolism was decreased [11]. This suggests that estradiol may exert an early programming effect on insulin sensitivity in adult organism partly by inducing low-grade chronic inflammation. These rats also displayed an increased amount of subcutaneous adipose tissue without affecting adipocyte size, suggesting adipocyte hyperplasia. Since both human and animal preadipocytes and adipocytes express estrogen receptors, estradiol may influence adipose tissue mass and cellularity [12]. A recent publication shows that adipocyte number is set during childhood and adolescence [13], emphasizing the importance of elucidating different actors in early life able to influence adipose tissue development.

In adult humans and animals, estradiol has profound effects on adipose tissue distribution and metabolism, including lipoprotein lipase (LPL) activity [12,14]. Parametrial adipose tissue is of special interest in this context. Owing to its proximity to the ovary, it is exposed to locally produced ovarian hormones [15,16]. In parametrial adipose tissue, changes in adipocyte size or LPL activity due to modulations in ovarian steroid hormones seem to be more pronounced than in other fat depots [17–19]. Estrogen receptor gene expression and estradiol binding site concentrations are higher in parametrial adipose tissue compared with other depots [20,21]. Johnson et al. used magnetic resonance imaging (MRI) to quantify subcutaneous and visceral adipose tissue volume in rat [22], but the parametrial depot has not been evaluated using this technique.

In the rat ovary, cellular differentiation begins around the time of birth and is morphologically identified by the emergence of follicles [23,24]. In newborn rats, estrogen levels are normally low; high estrogen levels due to neonatal administration of synthetic estrogen (e.g., estradiol benzoate and estradiol valerate) cause a polycystic ovarian morphology without corpora lutea (CL) in rodents [25,26]. Several factors are thought to be involved [27,28], but the mechanisms and molecular events that control ovarian differentiation and development remain to be explained.

In this study, we investigated for the first time the effects of one early postnatal (within 3 h after birth) estradiol injection on ovarian morphology and parametrial adipose tissue mass, adipocyte size, and LPL activity in adulthood. We also assessed ovarian and parametrial adipose tissue expression of genes related to follicular development and adipose tissue metabolism. Finally, we developed and tested a technique for estimating rat parametrial adipose tissue volume using MRI.

2. Materials and methods

2.1. Animals

Time-mated Wistar female rats (Scanbur BK, Sollentuna, Sweden) were maintained under controlled noise-free conditions (light from 0700 to 1900 h; temperature 21 ± 2 °C; humidity 55–65%) with one rat per cage until parturition. Pups were raised with a lactating mother until 21 d of age. The rats were then housed four to five per cage and fed standard rat pellets *ad libitum*.

The local animal ethics committee at the University of Gothenburg, Gothenburg, Sweden approved the experiments.

2.2. Study procedures

Within 3 h after birth, female pups were weighed and injected once with 0.35 mg (modified from Ref. [9]) of estradiol benzoate (Apoteksbolaget, Stockholm, Sweden) dissolved in a sesame oil vehicle ($n = 14$). Controls ($n = 19$) received sesame oil only. Rats treated in early postnatal life with estradiol benzoate are hereafter called estradiol rats. Tail samples were collected for analyses of plasma testosterone, estradiol, and progesterone at 9 wk of age. At 12 wk, the estradiol rats were divided into two groups. In one group, the rats were decapitated and parametrial adipose tissue was dissected, weighed, snap-frozen in liquid nitrogen, and stored at −80 °C for analysis of LPL activity; in the other group, the rats underwent MRI examination of parametrial adipose tissue. When the controls were in estrus (age 13–16 wk), they were decapitated, and parametrial adipose tissue and ovaries were dissected and weighed. Part of the adipose tissue was placed in 4 v/v% formaldehyde and embedded in paraffin for analysis of adipocyte size. The rest was snap-frozen in liquid nitrogen and stored at −80 °C before RNA isolation. One ovary was fixed in neutral buffered 4 v/v% formaldehyde for 24 h, placed in 70 v/v% ethanol, dehydrated, and embedded in paraffin for morphological analysis. The other ovary was snap-frozen in liquid nitrogen and stored at −80 °C before RNA isolation.

2.3. Vaginal smears

Estrous status was determined from vaginal smears taken daily from 8 to 9 wk of age. An estrous cycle (estrus, diestrus 1, diestrus 2, and proestrus) lasting about 4–5 d with a characteristically clear ovulation—evinced as a rich amount of epithelial cells without leukocytes in the smears—was considered normal [29]. Blood samples for sex hormone analyses were taken in the estrus phase of controls.

2.4. Magnetic resonance imaging (MRI) of parametrial adipose tissue

Before scanning, rats were anesthetized by inhalation of isoflurane (2% in 1:1 mixture of oxygen and air; Abbott Scandinavia, Solna, Sweden). MRI was performed with a 7-Tesla Bruker BioSpec system (Bruker BioSpin MRI, Ettlingen, Germany). A T1-weighted spin echo sequence was used (TR 1000 ms, TE 10.25 ms, slice thickness 1 mm, field of view 65 mm × 65 mm, image matrix 256 × 256). Forty-six axial slices 1 mm apart were acquired from the proximal part of the tail to the liver. Image acquisition was triggered by a respiratory pressure sensor to minimize motion artifacts.

An experienced operator estimated adipose tissue volume in a blinded fashion on the MR images using manual segmentation and ImageJ software (version 1.32j, http://rsb.info.nih.gov/ij/index.html). Adipose tissue was segmented slice-wise per manually determined intensity thresholds. Slice-dependent thresholds compensated for signal intensity variation in image data. Visceral and subcutaneous adipose tissues were separated by manual delineation using slice-wise regions of interest.

To distinguish parametrial adipose tissue from the remaining intra-abdominal adipose tissue, an axial level was defined that served as a border between the two depots. After localization of the pelvic girdle, vertebrae L5 were set as the skeletal reference for this level. Visceral adipose tissue caudal to the axial level served as a proxy measure of parametrial adipose tissue. Motion artifacts were sometimes seen in the upper abdominal region. When seen, only every second slice was affected, so volume data from the previous slice were doubled. On average, 3.4 slices were affected per animal (maximum 8).

2.5. Dual energy X-ray absorptiometry (DXA)

Body composition was analyzed by dual-emission X-ray absorptiometry (DXA) at 12 wk of age with a whole-body DEXA instrument (QDR-1000/W, Hologic Inc., Waltham, MA). Rats were anesthetized by inhalation of isoflurane (Abbott Scandinavia AB, Solna, Sweden;

2% in 1:1 mixture of oxygen and air) before scanning. Calibration of the instrument was conducted with an aluminum/lucite phantom daily before measurements in animals. Animals were placed on the imaging-positioning tray in a prostrate fashion and all scans were analyzed using the software provided by the manufacturer.

2.6. Parametrial adipocyte size

One adipose tissue section from each rat was mounted on a glass slide and stained with hematoxylin and eosin. Mean cell size was determined by computerized image analysis (KS400 software, Carl Zeiss, Oberkochen, Germany) in a blinded fashion. In brief, the glass slide was transferred to the microscope stage and nine visual fields were photographed with a charge-coupled device (CCD) camera (Axiocam, Carl Zeiss). Three fields displaying intact cell morphology were selected, and adipocyte areas were measured by manual delineation.

2.7. LPL activity in parametrial adipose tissue

Food was withdrawn at 0700 a.m., 3–4 h before decapitation and adipose tissue dissection. LPL activity was measured in parametrial adipose tissue as described by Peterson et al. [30]. In brief, frozen tissue was homogenized in detergent-containing buffer. The homogenate was centrifuged, and the clear solution between the sediment and the floating fat layer was used to assay LPL activity. The substrate emulsion was Intralipid (10%, Kabi Pharmacia, Stockholm, Sweden) labeled with [^{3}H] triolein (gift of Dr. L. Krabisch, Lund University, Lund, Sweden); specific radioactivity was about 950 dpm/nmol fatty acid. The samples (5 μl) were incubated in triplicate in a total volume of 200 μl at 25 °C for 60 min. Fatty acids were extracted for counting, as described by Spooner et al. [31]. The intra-assay coefficient of variation was 5.4%. Bovine skim milk was used as a standard to correct for inter-assay variation. Activity was expressed as mU per g tissue (1 mU = 1 nmol fatty acid released/min).

2.8. Descriptive analysis of ovarian morphology

One ovary per rat was longitudinally and serially sectioned at 4 μm; six sections per ovary were mounted on a glass slide, stained with hematoxylin and eosin, and examined under a conventional birefringence microscope by two persons blinded to the origin of the sections. Each slide was scanned with ScanScope (Aperio Technologies, Vista, CA), measured and photographed, and further analyzed with ImageScope virtual microscopy software (Aperio Technologies). The diameter of the ovaries in the section with the largest ovarian cross-section was assessed with a calibrated scale tool in the virtual microscope. The diameter of the largest follicle was assessed similarly. Antral follicles, defined as follicles with an antrum and CL, were counted by two persons to avoid counting errors and classified as atretic or healthy. Primordial and primary follicles were not counted due to the risk of double-counting. Follicles were considered atretic if at least two pycnotic granulosa cells were observed or if the oocyte showed obvious signs of degeneration [32]. A calibrated scale tool was used to measure the thickness of the thickest theca interna cell layer in the largest healthy follicle and in the largest atretic follicle.

2.9. RNA isolation and cDNA synthesis

Total RNA was extracted from ovaries with an RNeasy Fibrous Tissue Mini Kit and from parametrial adipose tissue with an RNeasy Lipid Tissue Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). A DNase I (Qiagen) digestion step was included to eliminate DNA contamination. RNA concentration was determined spectrophotometrically (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE) and RNA integrity was checked with an Agilent Bioanalyzer 2100 and an RNA 6000 Nano LabChip Kit (Agilent Technologies, Santa Clara, CA). First-strand cDNA was synthesized from 1 μg of total RNA with a High-Capacity cDNA Reverse Transcription Kits (PE Applied Biosystems, Stockholm, Sweden) per the manufacturer's protocol.

2.10. Real-time RT-PCR

Real-time RT-PCR analysis was performed with custom TaqMan low density arrays (Applied Biosystems). Primers and probes for rat genes corresponding to TaqMan Gene Expression Assay numbers and GenBank accession numbers (Table 1) were spotted onto each array. Eight samples were randomly analyzed per card in one run. Duplicates were run on different cards to confirm the reproducibility of the method. cDNA (100 ng) was mixed with TaqMan Universal PCR Master Mix and No AmpErase UNG (Applied Biosystems) in a total volume of 100 μl and loaded into each sample loading port. Thermal cycling and fluorescence detection were performed with an ABI Prism 7900HT Sequence Detection System with ABI Prism 7900HT SDS Software 2.1 (Applied Biosystems). Thermal cycling conditions were 2 min at 50 °C and 10 min at 94.5 °C, followed by 40 cycles of 30 s at 97 °C and 1 min at 59.7 °C.

The NormFinder algorithm [33] was used to calculate expression stability of four putative reference genes—18S ribosomal RNA (*18S*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), peptidylprolyl isomerase A (*Ppia*), and hypoxanthine guanine phosphoribosyl transferase (*Hprt*)—for normalization in adipose tissue. According to the NormFinder algorithm, the reference gene that showed the lowest variability in parametrial adipose tissue was *Hprt*. In the ovaries, none of the traditional genes were stable, so a wider range of putative reference genes was analyzed with the NormFinder algorithm. Variability was lowest with the combination of *Ncoa2* and *Rela*. Gene expression values were calculated per the $2^{-\Delta\Delta Ct}$ method [34]. The ΔCt value of each sample was determined by subtracting the average Ct value of the reference gene from the average Ct value of the target gene. The ΔΔCt value was then calculated by subtracting the ΔCt value of the sample with highest expression (i.e., the sample with lowest ΔCt value) from the sample ΔCt value. The target gene expression level relative to the sample with highest expression was then estimated as $2^{-\Delta\Delta Ct}$.

2.11. Analytical methods

Plasma testosterone, estradiol, and progesterone were determined with RIA kits (Testosterone RIA kit, DSL-4100; 3rd Generation Estradiol RIA, DSL-39100; Progesterone RIA kit, DSL-3400, Diagnostic Systems Laboratories, Webster, TX). Intra- and inter-assay coefficients of variation and sensitivity were, respectively, 7.5%, 8.1%, and 0.05 ng/ml (testosterone); 3.6%, 6.0%, and 0.6 pg/ml (estradiol); and 5.1%, 2.5%, and 0.1 ng/ml (progesterone).

2.12. Statistical analyses

Results are expressed as mean ± SEM. Intergroup differences in parametrial adipose tissue volume, weight, and adipocyte size and in ovarian weight, area, and morphology were analyzed with unpaired *t*-tests. Differences in weight development were analyzed with General Linear Model with repeated-measures ANOVA. Differences in gene expression between groups were tested with the Mann–Whitney test. Relationships between numeric variables were investigated by partial correlation analysis. $p < 0.05$ was considered significant.

Table 1
Genes presented on the TaqMan low density array with their respective TaqMan gene expression assay numbers and GenBank accession numbers.

Gene symbol	Gene description	TaqMan gene expression assay no.	GenBank accession no.
18S (reference gene)	18S ribosomal RNA	Hs99999901_s1	X03205
Gapdh (reference gene)	Glyceraldehyde-3-phosphate dehydrogenase	Rn99999916_s1	NM_017008.3
Hprt (reference gene)	Hypoxanthine guanine phosphoribosyl transferase	Rn01527840_m1	NM_012583.2
Ncoa2 (reference gene)	Nuclear receptor coactivator 2	Rn00582729_m1	NM_031822.1
Ppia (reference gene)	Peptidylprolyl isomerase A	Rn00690933_m1	NM_017101.1
Rela (reference gene)	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	Rn01502266_m1	NM_199267.2
Adipoq	Adiponectin	Rn00595250_m1	NM_144744.2
AR	Androgen receptor	Rn00560747_m1	NM_012502.1
C3	Complement component 3	Rn00566466_m1	NM_016994.1
Esr1/*ERα*	Estrogen receptor 1/Estrogen receptor alpha	Rn00562166_m1	NM_012689.1
Igf1r	Insulin-like growth factor 1 receptor	Rn00583837_m1	NM_052807.1
Lep	Leptin	Rn00565158_m1	NM_013076.1
Lipe/*Hsl*	Hormone stimulating lipase	Rn00563444_m1	NM_012859.1
Lpl	Lipoprotein lipase	Rn00561482_m1	NM_012598.1
Slc2a3/*Glut3*	Solute carrier family 2 (facilitated glucose transporter), member 3/glucose transporter 3	Rn00567331_m1	NM_017102.1
Slc2a4/*Glut4*	Solute carrier family 2 (facilitated glucose transporter), member 4/glucose transporter 4	Rn00562597_m1	NM_012751.1
Tgfβ1	Transforming growth factor beta 1	Rn00572010_m1	NM_021578.1

Table 2
Proxy MRI-estimated parametrial adipose tissue volume at age 12 wk and dissected parametrial adipose tissue weight and adipocyte size at age 13–16 wk in early postnatal estradiol-exposed and control female rats.

Group	Volume (cm^3)	Weight (g)	Weight (g/kg BW)	Adipocyte size (μm^2)
Controls	5.5 ± 0.9 (n = 7)	5.5 ± 0.4 (n = 11)	21.4 ± 1.5 (n = 11)	4.3×10^3 ± 478 (n = 10)
Estradiol	3.1 ± 0.5[a] (n = 9)	1.2 ± 0.2[b] (n = 9)	4.0 ± 0.5[b] (n = 9)	4.4×10^3 ± 510 (n = 9)

Values are mean ± SEM. BW, body weight.
[a] $p < 0.05$ vs. control (unpaired t-test).
[b] $p < 0.001$ vs. control (unpaired t-test).

3. Results

3.1. Growth and body composition

There were no difference in weight development during the first 12 wk of the study period or in body composition (lean body mass and fat body mass) measured with DXA at 12 wk of age between the two groups (results not shown).

3.2. Parametrial adipose tissue weight, volume, adipocyte size, and LPL activity

Parametrial fat depots were smaller in the estradiol group than in controls, as shown by dissection, weighing, and volumetric MRI estimation (Table 2). MRI estimates of adipose tissue volume correlated positively with dissected tissue weights (Fig. 1). Parametrial adipocyte size was similar in the two groups (Table 2). In each sample, 130–240 adipocytes (mean 187.8 ± 5.9) were analyzed. LPL activity in parametrial adipose tissue was higher in the estradiol rats ($p < 0.01$) (Fig. 2).

3.3. Vaginal smears

All control rats had a normal estrous cycle of 4 d. Estradiol rats were acyclic and had malformed vaginal openings.

3.4. Ovarian morphology

Ovarian weight and area were lower in the estradiol group than in the controls (Table 3). Morphologically, control rat ovaries presented follicles in various stages of development, from primordial follicles to mature preovulatory follicles (Fig. 3A), and several CL, many of which had resulted from recent ovulation (Fig. 3B and C). Some follicles were moderately atretic with pycnotic granulosa cells. But in the ovarian sections (six sections per ovary and rat) of the estradiol rats, CL were absent and antral follicle area was increased (Table 3), owing to large, fluid-filled follicles (Fig. 3D). Although the number of antral follicles was similar in the two groups, the estradiol group had a higher proportion of atretic antral follicles (Fig. 3E and F), in which the thickness of the theca interna cell layer was decreased (Fig. 3F, Table 3).

3.5. Gene expression in the ovary and parametrial adipose tissue

Fig. 4A and B shows the relative mRNA expression levels of selected genes in the ovary and parametrial adipose tissue, respectively. Estradiol exposure increased adiponectin (*Adipoq*) expression in the ovary but not in parametrial adipose tissue. Estra-

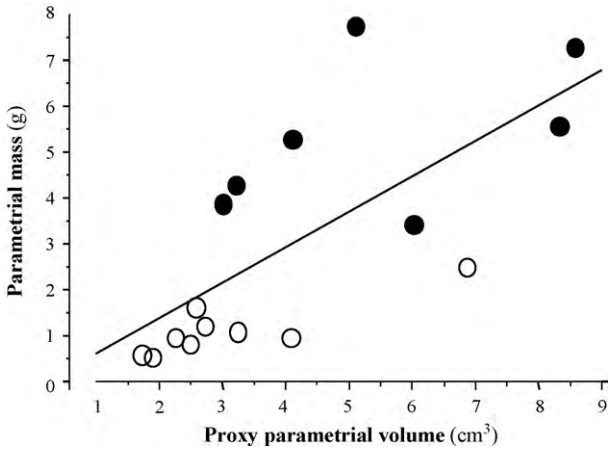

Fig. 1. Correlation between proxy MRI-estimated parametrial adipose tissue volume and dissected parametrial adipose tissue mass. Correlation between adipose tissue volume at 12 wk of age and dissected adipose tissue mass at 13–16 wk of age in early postnatal estradiol-exposed (n = 9, open circles) and control (n = 7, filled circles) female rats. $p < 0.01$, $R = 0.70$ (simple regression).

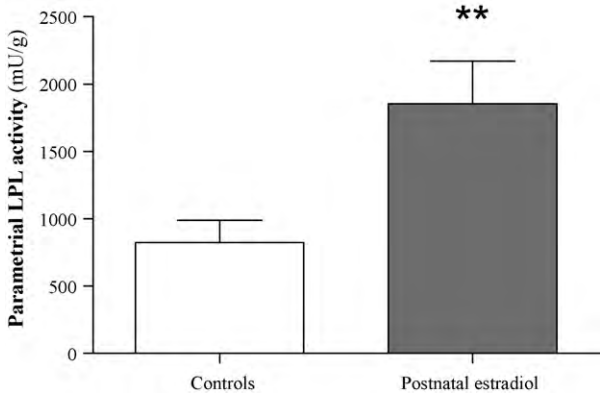

Fig. 2. Parametrial adipose tissue LPL activity. Comparison between early postnatal estradiol-exposed ($n = 5$) and control ($n = 8$) female rats aged 12 wk. Values are mean ± SEM. $^{**}p < 0.01$ vs. controls (unpaired t-test).

Table 3
Ovarian morphology in early postnatal estradiol-exposed and control female rats at age 13–16 wk.

Parameter	Controls ($n = 9$)	Estradiol ($n = 8$)
Ovarian area (mm^2)	23.9 ± 1.6	8.2 ± 0.8[c]
Ovarian weight (mg)	81.5 ± 4.9	25.7 ± 1.6[c]
Corpora lutea (n)	20.2 ± 2.3	0 ± 0[c]
Antral follicles (n)	17.3 ± 3.0	17.5 ± 2.2
No. of antral follicles		
Atretic	1.9 ± 0.6	6.3 ± 1.3[b]
Healthy	15.4 ± 2.9	11.1 ± 1.8
Ratio healthy/atretic follicles	10.1 ± 2.4	2.3 ± 0.6[b]
Antral follicle area (mm^2)	0.28 ± 0.06	0.75 ± 0.06[c]
Theca interna thickness (μm)		
Healthy antral follicle	24.5 ± 1.2	23.0 ± 1.4
Atretic antral follicle	26.8 ± 1.9	21.3 ± 0.8[a]

Values are mean ± SEM.
[a] $p < 0.05$ vs. controls (unpaired t-test).
[b] $p < 0.01$ vs. controls (unpaired t-test).
[c] $p < 0.001$ vs. controls (unpaired t-test).

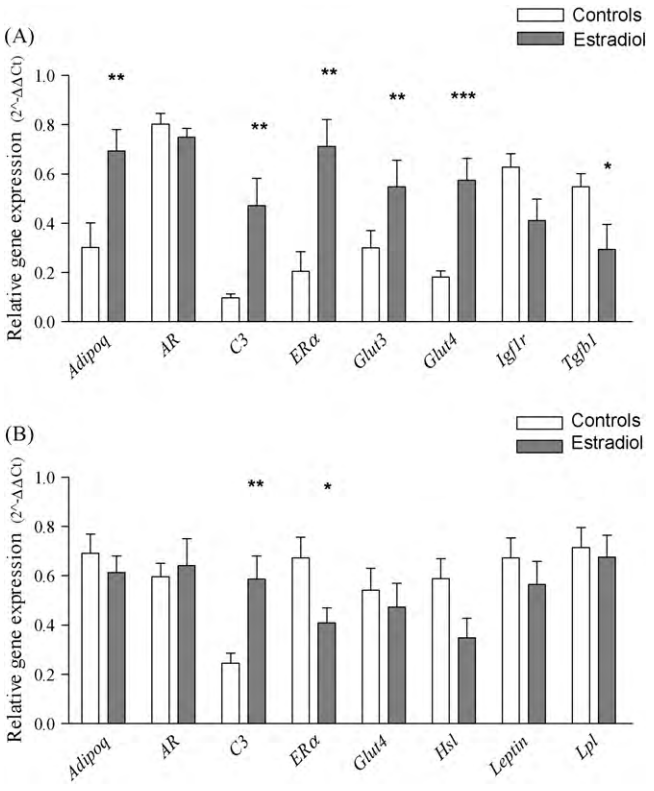

Fig. 4. (A) Ovarian mRNA expression. Expression of *Adipoq*, *AR*, *C3*, *ERα*, *Glut 3*, *Glut 4*, *Igf1r* and *Tgfβ1* mRNA in early postnatal estradiol-exposed ($n = 7$) and control ($n = 9$) female rats aged 13–16 wk. Values are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. controls (Mann–Whitney). (B) Parametrial adipose tissue mRNA expression. Expression of *Adipoq*, *AR*, *C3*, *ERα*, *Glut 4*, *Hsl*, *Lep* and *Lpl* mRNA in early postnatal estradiol-exposed ($n = 7$) and control ($n = 9$) female rats aged 13–16 wk. Values are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. controls (Mann–Whitney).

diol also increased expression of complement component 3 (*C3*) in ovary and parametrial adipose tissue.

Postnatal estradiol exposure had no effect on androgen receptor (*AR*) expression in ovary and parametrial adipose tissue. But in the estradiol rats, *ERα* expression was increased in the ovary and decreased in parametrial adipose tissue. Expression of glucose

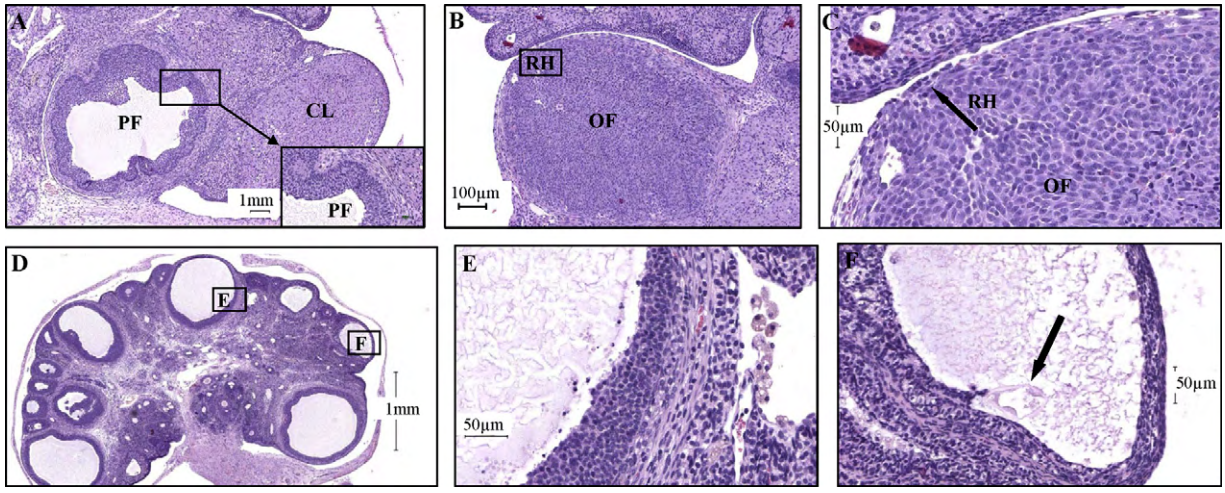

Fig. 3. Ovarian morphology. (A–C) Ovary from a normal cycling control rat, aged 13–16 wk. (A) Ovary slice showing a healthy, slightly wrinkled preovulatory follicle (PF) and a fresh corpora lutea (CL); the magnified boxed inset shows healthy granulosa and theca interna cell layer. (B) Slice of a recently ovulated follicle (OF); the box depicts a rupture hole (RH). (C) Magnified box from (B) with arrow indicating the RH clogged by granulosa cells. (D–F) Ovary from an acyclic early postnatal estradiol-exposed rat, aged 13–16 wk. (D) Ovary with large fluid-filled follicles and no corpora lutea. (E) Magnified box from (D) displays early follicle atresia with scattered pycnotic cells in the antrum. (F) Magnified box from (D) displays more advanced atresia with a degenerated oocyte (arrow) and a thin theca interna cell layer.

Table 4
Estrus phase, plasma sex steroid levels in early postnatal estradiol-exposed and control female rats at age 9 wk.

Group	Testosterone (nmol/l)	Progesterone (nmol/l)	Estradiol (nmol/l)
Controls ($n = 12$)	0.48 ± 0.09	84.7 ± 6.8	0.13 ± 0.03
Estradiol ($n = 9$)	0.20 ± 0.02[a]	16.7 ± 3.3[c]	0.10 ± 0.02

Values are mean ± SEM.
[a] $p < 0.05$ vs. controls (unpaired t-test).
[c] $p < 0.001$ vs. controls (unpaired t-test).

transporter 3 (*Glut 3*) and 4 (*Glut 4*) was increased in estradiol rat ovaries, but *Glut 4* was unaffected in parametrial adipose tissue. Ovarian *Adipoq* expression correlated positively with both ovarian *Glut 3* expression ($R = 0.67$, $p < 0.01$; $n = 16$) and ovarian *Glut 4* expression ($R = 0.57$, $p < 0.05$; $n = 16$) in the pooled groups. Estradiol reduced *Tgfβ1* expression in the ovary. Gene expressions of ovarian insulin-like growth factor 1 receptor (*Igf1r*) and parametrial adipose tissue leptin (*Lep*), hormone-sensitive lipase (*Hsl*), and *Lpl* were unaltered.

3.6. Plasma testosterone, estradiol, and progesterone concentrations

Table 4 shows hormone levels. Testosterone concentrations were significantly lower in estradiol-exposed rats than in controls, as were progesterone concentrations. Estradiol concentrations did not differ significantly between control and estradiol rats.

4. Discussion

This study shows for the first time that a single estradiol injection within 3 h after birth alters ovarian morphology associated with alterations in the ovarian expression of genes involved in follicle development. Estradiol exposure also reduced the weight of parametrial adipose tissue and its volume—as shown by a novel MRI approach—increased parametrial adipose tissue LPL activity, and altered the parametrial adipose tissue gene expression of *C3* and *ERα*.

The dose of 0.35 mg estradiol benzoate used in this study has previously been applied by us [11]. We have earlier also evaluated imprinting effects of a single dose of 0.5 mg estradiol benzoate [9]. Since both doses affected insulin sensitivity in adult female rats to a similar extent, the lower dose was chosen in the present study. There is no standardized protocol for how to mimic early life estrogen exposure. Instead, several different doses, time points of administration, and administration regimes are described in the literature [26,35,36].

Investigation of the cross-talk between the ovary and parametrial adipose tissue was not the focus of this study. However, adipose tissue and the ovary interplay in both directions [17–19,37], why it is important to bear in mind that some of the effects seen in the present study might be the result of such cross-talk.

4.1. Programming effects on ovarian morphology and gene expression, and circulating sex steroid levels

The estradiol rats showed decreased ovarian size, increased antral follicle area and number of atretic antral follicles, thinning of the theca interna of atretic antral follicles, and a total absence of CL, determined by descriptive analysis and disrupted cyclicity. Since no CL were found (no ovulation occurs), all follicles will eventually become atretic in subsequent follicular development, which Fig. 3D shows.

Previous studies showed that when female neonates are administered estrogen during the first few days of life, the animals, as adults, enter persistent vaginal estrus with loss of estrous cyclicity and the cyclic release of pituitary gonadotropin [38]. Although not assessed in our study, effects on the hypothalamic-pituitary-gonadal axis might have contributed to the ovarian phenotype in our estradiol-exposed rats.

The ovarian weight of the estradiol rats was decreased by 68.5% compared to controls. Calculating an approximate value of the ovarian volume by using the formula for a perfect circle showed that estradiol rats had an ovarian reduction of 79.5% compared with controls. This suggests that there is no major alteration in ovarian density or shape. We also found decreased testosterone and progesterone levels at 9 wk of age and unaltered estradiol levels. Adrenal production of androgens is low in rodents, and the major source of testosterone in the female rat is the ovary [39]. Since androgens are produced in ovarian theca interna cells [40,41], the reduced thickness of the theca interna may partially explain the lower testosterone levels in the estradiol rats. The increased ovarian expression of *Adipoq* mRNA might also have contributed, because adiponectin may inhibit theca cell production of progesterone and androstenedione, which is regulated by luteinizing hormone and IGF-1 [42]. Since CL produce large quantities of progesterone during the luteal phase, the low progesterone levels in the estradiol rats likely reflect their anovulatory state. The estradiol result is consistent with the findings of another study of neonatal estrogenization [26], which also showed unchanged estradiol levels measured during the estrus phase of adult control rats. Because no significant differences were found when hormone-exposed rats were compared to controls, which were all sampled in the estrus phase, agrees with the notion of neonatally androgenized or estrogenized, anovulatory female rats being in a state of constant estrus [25,38]. This does not rule out the possibility that differences between estradiol rats and controls in our studies could have been found if the controls had been sampled in other phases of the cycle. Furthermore, since this hormone varies during the cycle, it is important to point out that to be able to determine whether serum estradiol levels are actually affected, repeated measurements during the cycle need to be done.

Interestingly, ovarian expression of *Tgfβ1* was down-regulated in estradiol rats. TGFβ is a key autocrine and paracrine modulator produced by theca and granulosa cells in humans and rodents and plays important intra-ovarian roles during follicular development [43,44]. Studies on several mammalian species, principally rodents, indicate that several ligands, receptors, signaling intermediaries, and binding proteins associated with the TGFβ superfamily are expressed by oocytes and ovarian somatic cells in various developmental stages [28,45–48]. A growing body of experimental evidence supports TGFβ involvement in multiple aspects of follicle development, including primordial follicle recruitment, granulosa and theca cell proliferation and atresia, steroidogenesis, gonadotropin receptor expression, oocyte maturation, ovulation, luteinization, and corpus luteum formation. The results of functional *in vitro* studies involving ovarian explants indicate positive effects [49,50] but there are also some studies that report an inhibitory effect of TGFβ on primary follicle survival and/or progression to later stages [51].

TGFβ has also been shown to (1) stimulate FSH receptor expression [52] and (2) amplify FSH-induced aromatase activity, inhibin production, and progesterone production by theca cells and LH receptor induction [53–58]. In addition, TGFβ is able to suppress theca P450c17 expression and androgen production in a similar manner as activin A [59,60]. In mice, estradiol exposure on postnatal days 1–5 reduces ovarian weight, inhibits follicular development, and decreases expression of *Tgfβ* superfamily genes at day 19 [61]. Thus, decreased *Tgfβ1* expression might explain the

absence of CL and the anovulatory state in our estradiol rats, but not the low testosterone levels. Since TGFβ seems to suppress androgen production, reduced theca interna thickness is probably a better explanation of the low plasma testosterone levels we observed in our postnatal estradiol rats.

Our estradiol rats also had increased mRNA expression of *ERα* in the ovaries. Ovaries from adult ERα knock-out mice are characterized by the accumulation of enlarged, cystic follicles accompanied by anovulation [62], and mice that over-express *ERα* during prenatal and postnatal life have increased numbers of preantral and antral follicles at postnatal day 90 [63]. These results are evidence for key involvement of this receptor in ovarian function.

The effects of estradiol benzoate on the developing ovary seem to be mediated by ERα in theca and interstitial cells. Ikeda et al. treated rats with estradiol benzoate for 5 d—starting within 24 h after birth—and found *ERα* expression decreased in ovarian theca and interstitial cells at postnatal day 6 but recovered by day 14 [64]. Discrepancies between this study and ours, such as time point of exposure and age of *ERα* expression analysis, might explain the difference found in *ERα* expression. A follow-up of gene expression in the Ikeda et al. study [64] might have revealed an increase in *ERα* expression, which occurred in our estradiol rats.

Both *Tgfβ* and *ERα* seem to be targets for estrogen in the early development of the rodent ovary. Thus, changes in their expression and signaling might mediate, at least in part, some effects of early estrogen exposure. The altered expression of *Tgfβ* and *ERα* might have contributed to dysregulated ovarian development in our estradiol rats.

Upregulated expression levels of *Adipoq* occurred in the estradiol rats. There is abundant evidence that adiponectin has beneficial effects on reproductive processes, and it seems to be involved in ovulation. In rats, adiponectin is produced in theca, granulosa, and luteal cells of the ovary [65]. In granulosa cells *in vitro*, recombinant adiponectin induces ovarian gene expression and expression of proteins associated with ovulation and steroid hormone synthesis [66]. Further, adiponectin interacts with other hormones, in particular with insulin, to increase glucose uptake and lipid oxidation [67] but also to induce target gene expression in ovarian granulosa cells [66]. This might therefore be a compensatory response, an attempt to stimulate follicular development.

Glucose transporters may play a vital part in supplying energy to the developing follicle and oocyte by facilitating glucose uptake [68,69]. Through its insulin-like action, adiponectin is known to increase skeletal muscle *Glut4* translocation [70]. Speculatively, this might also be the case in the ovary since we found a positive correlation between ovarian *Adipoq* expression and expression of the two glucose transporters.

Interestingly, *C3* was also upregulated in the ovaries. Several cells synthesize C3 [71], but to our knowledge, C3 synthesis has not been reported in ovarian cells. C3 is the most central protein of the complement system, which is important to immune defense, and provides a link between innate and adaptive immunity. Cleavage of C3 is needed to produce acylation-stimulating protein (ASP). Increased *C3* mRNA expression might increase production of ASP [72].

C3 and ASP were also associated with obesity, coronary sclerosis, the metabolic syndrome, fasting, and postprandial triacylglycerols (TAGs) [73]. Moreover, chylomicrons are the strongest stimulators of adipocyte C3 production via activation of the alternative complement cascade [74]. A postprandial C3 increment was observed in healthy subjects, in patients with coronary artery disease, and in patients with familial combined hyperlipidemia [75].

ASP was shown to increase triglyceride synthesis in fat-storing cells by stimulating (1) re-esterification of free fatty acids and (2) glucose transport [72]. In human adipose tissue, ASP stimulates glucose uptake [76] through translocation of glucose transporters, such as Glut 1, 3, and 4, to the cell surface [77,78]. Not only does ASP increase lipogenesis in adipocytes, but ASP also inhibits hormone-sensitive lipase-mediated lipolysis [72]. It is therefore tempting to speculate that C3 and ASP play a key role in supplying energy to developing follicles.

4.2. Programming effects on parametrial adipose tissue

In parametrial adipose tissue, estradiol exposure markedly reduced weight and volume and increased LPL activity while adipocyte size was unaltered. Further, *C3* gene expression was increased while *ERα* expression was decreased. *Lep*, *Lpl*, and *Hsl* gene expression were unaffected by estradiol exposure. MRI was used to evaluate parametrial adipose tissue volume. The MRI-estimated volume correlated well with the weight of dissected tissue, indicating reliability of this method. Thus, in future studies, changes in parametrial adipose tissue volume may be followed non-invasively.

Neonatal exposure of rats and mice with synthetic estrogens results in large decreases in adipose tissue mass in adulthood without alteration in adipocyte size, suggesting a reduction in number of adipocytes [12]. In our study, estradiol exposure decreased the weight and volume of the parametrial adipose tissue depot without affecting adipocyte size, which is consistent with a reduced number of adipocytes. Consequently, altering normal estradiol or ER signaling, during development seems to induce large changes in adipocyte number. Estrogen may act at multiples stages of adipogenesis to alter adipocyte number, and depending on the stage of adipogenesis, its effect could vary [12].

Hamosh et al. exposed adult female rats to estrogen and observed a direct, inhibitory effect on adipose tissue lipogenesis through inhibition of LPL activity [79]. Contrary to this, we found that early postnatal estrogen exposure increased parametrial adipose tissue LPL activity in adult age, favoring triglyceride storage. Interestingly, this suggests that age of estradiol exposure is an important determinant for the effect of estradiol on adipose tissue LPL activity. Parametrial adipose tissue *Lpl* mRNA expression was not influenced by early postnatal estrogen exposure in the present study. Regulation of LPL activity involves both transcriptional and post-transcriptional mechanisms [80]. Ottosson et al. showed that adipose tissue LPL activity can be affected despite unchanged LPL mRNA levels [81].

Parametrial adipose tissue *C3* expression was increased in the estradiol group. In human adipose tissue, ASP (the C3 cleavage product) stimulates glucose uptake and nonesterified fatty acid storage [76,82]. In female mice lacking ASP, adiposity is decreased [83]. The increases in LPL activity and *C3* mRNA expression may therefore be consistent with a compensatory mechanism in postnatal estradiol-exposed rats to inhibit further decreases in parametrial adipose tissue.

In this study we found several interesting alterations in ovarian and adipose tissue gene expression. It is important however to bear in mind that changes in specific mRNA expression levels are not always accompanied by corresponding changes at the protein level.

4.3. Conclusions

For the first time, we show that a single dose of estradiol benzoate within 3 h after birth disrupts cyclicity and profoundly affects ovarian morphology, parametrial adipose tissue weight, and LPL activity in adult female rats. It also alters ovarian and adipose tissue expression of genes related to follicle development and adipose tissue metabolism. The decrease in parametrial adipose tissue mass without alteration in adipocyte size is consistent with a reduced number of adipocytes. Furthermore, we developed an MRI approach for studying the volume of parametrial adipose tissue.

The morphological, metabolic and endocrine alterations observed after early postnatal estradiol exposure reflect a complex interplay between many different components and must be further explained. Our data from female rats advance knowledge on the long-standing effects of a single estradiol injection directly after birth. This knowledge contributes to further understanding of the sex steroid environment in early life and its role in adult reproduction and metabolism.

Funding

This study was supported by grants from Novo Nordisk Foundation, the Swedish Medical Research Council (Project No. 12206, 2005-72VP-15445-01A, 2005-72VX-15276-01A, K2007-54X-20325-01-3), the Swedish Diabetes Association Research Foundation, Wilhelm and Martina Lundgrens's Science Fund, Hjalmar Svensson, Tore Nilson Foundation, Åke Wiberg Foundation, Adlerbert Research Foundation, and the Swedish federal government under the LUA/ALF agreement.**Declaration of interest**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgments

We thank Britt-Mari Larsson, Birgitta Odén, Robert Jakubowicz, Jessica Hansson, and Sophia Svensson-Zetterlund for excellent technical assistance. We also thank the SWEGENE Göteborg Genomics Core Facility platform and Michael Horn at the Center for Mouse Physiology and Bio-Imaging.

References

[1] A. Lucas, Programming by early nutrition in man, Ciba Foundation Symposium 156 (1991) 38–50 (discussion 50-35).
[2] M. Davies, R. Norman, Programming and reproductive functioning, Trends in Endocrinology and Metabolism 13 (9) (2002) 386–392.
[3] D. Barker, C. Hales, C. Fall, C. Osmond, K. Phipps, P. Clark, Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth, Diabetologia 36 (1) (1993) 62–67.
[4] D. Phillips, Birth weight and the future development of diabetes. A review of the evidence, Diabetes Care 21 (Suppl. 2) (1998) B150–B155.
[5] T. Colborn, F. vom Saal, A. Soto, Developmental effects of endocrine-disrupting chemicals in wildlife and humans, Environmental Health Perspectives 101 (5) (1993) 378–384.
[6] R.R. Newbold, E. Padilla-Banks, W.N. Jefferson, J.J. Heindel, Effects of endocrine disruptors on obesity, International Journal of Andrology 31 (2) (2008) 201–208.
[7] R. Newbold, Cellular and molecular effects of developmental exposure to diethylstilbestrol: implications for other environmental estrogens, Environmental Health Perspectives 103 (Suppl. 7 (0091-6765 (Print))) (1995) 83–87.
[8] C. Nilsson, M. Niklasson, E. Eriksson, P. Bjorntorp, A. Holmang, Imprinting of female offspring with testosterone results in insulin resistance and changes in body fat distribution at adult age in rats, Journal of Clinical Investigations 101 (1) (1998) 74–78.
[9] C. Alexanderson, E. Eriksson, E. Stener-Victorin, T. Lystig, B. Gabrielsson, M. Lonn, A. Holmang, Postnatal testosterone exposure results in insulin resistance, enlarged mesenteric adipocytes, and an atherogenic lipid profile in adult female rats: comparisons with estradiol and dihydrotestosterone, Endocrinology 148 (11) (2007) 5369–5376.
[10] D. Abbott, V. Padmanabhan, D. Dumesic, Contributions of androgen and estrogen to fetal programming of ovarian dysfunction, Reproductive Biology and Endocrinology 4 (2006) 17.
[11] C. Alexanderson, E. Eriksson, E. Stener-Victorin, M. Lonn, A. Holmang, Early postnatal oestradiol exposure causes insulin resistance and signs of inflammation in circulation and skeletal muscle, Journal of Endocrinology 201 (1) (2009) 49–58.
[12] P. Cooke, A. Naaz, Role of estrogens in adipocyte development and function, Experimental Biology and Medicine 229 (11) (2004) 1127–1135.
[13] K. Spalding, E. Arner, P. Westermark, S. Bernard, B. Buchholz, O. Bergmann, L. Blomqvist, J. Hoffstedt, E. Naslund, T. Britton, H. Concha, M. Hassan, M. Ryden, J. Frisen, P. Arner, Dynamics of fat cell turnover in humans, Nature 453 (7196) (2008) 783–787.
[14] G. Wade, J. Gray, T. Bartness, Gonadal influences on adiposity, International Journal of Obesity 9 (Suppl. 1) (1985) 83–92.
[15] A. Brodie, W. Schwarzel, A. Shaikh, H. Brodie, The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer, Endocrinology 100 (6) (1977) 1684–1695.
[16] A. Shaikh, S. Shaikh, Adrenal and ovarian steroid secretion in the rat estrous cycle temporally related to gonadotropins and steroid levels found in peripheral plasma, Endocrinology 96 (1) (1975) 37–44.
[17] M. Krotkiewski, The effects of estrogens on regional adipose tissue cellularity in the rat, Acta Physiologica Scandinavica 96 (1) (1976) 128–133.
[18] M. Krotkiewski, P. Bjorntorp, The effect of progesterone and of insulin administration on regional adipose tissue cellularity in the rat, Acta Physiologica Scandinavica 96 (1) (1976) 122–127.
[19] L. Steingrimsdottir, J. Brasel, M. Greenwood, Hormonal modulation of adipose tissue lipoprotein lipase may alter food intake in rats, American Journal of Physiology-Endocrinology Metabolism 239 (2) (1980) E162–E167.
[20] S. Rodriguez-Cuenca, M. Monjo, A. Proenza, P. Roca, Depot differences in steroid receptor expression in adipose tissue: possible role of the local steroid milieu, American Journal of Physiology-Endocrinology Metabolism 288 (1) (2005) E200–E207.
[21] G. Wade, J. Gray, Cytoplasmic 17 beta-[3H]estradiol binding in rat adipose tissues, Endocrinology 103 (5) (1978) 1695–1701.
[22] D. Johnson, C. Flask, P. Ernsberger, W.C.K. Wong, D. Wilson, Reproducible MRI measurement of adipose tissue volumes in genetic and dietary rodent obesity models, Journal of Magnetic Resonance Imaging 28 (4) (2008) 915–927.
[23] A. Hirshfield, Development of follicles in the mammalian ovary, International Review of Cytology 124 (1991) 43–101.
[24] R. Rajah, E. Glaser, A. Hirshfield, The changing architecture of the neonatal rat ovary during histogenesis, Developmental Dynamics 194 (3) (1992) 177–192.
[25] M. Aihara, S. Hayashi, Induction of persistent diestrus followed by persistent estrus is indicative of delayed maturation of tonic gonadotropin-releasing systems in rats, Biology of Reproduction 40 (1) (1989) 96–101.
[26] R. Sotomayor-Zarate, M. Dorfman, A. Paredes, H. Lara, Neonatal exposure to estradiol valerate programs ovarian sympathetic innervation and follicular development in the adult rat, Biological Reproduction 78 (2007) 673–680.
[27] A. Gougeon, Regulation of ovarian follicular development in primates: facts and hypotheses, Endocrine Reviews 17 (2) (1996) 121–155.
[28] K. McNatty, A. Fidler, J. Juengel, L. Quirke, P. Smith, D. Heath, T. Lundy, A. O'Connell, D. Tisdall, Growth and paracrine factors regulating follicular formation and cellular function, Molecular and Cellular Endocrinology 163 (1–2) (2000) 11–20.
[29] F. Marcondes, F. Bianchi, A. Tanni, Determination of the estrous cycle phases of rats: some helpful considerations, Brazilian Journal of Biology 62 (2002) 609–614.
[30] J. Peterson, T. Olivecrona, G. Bengtsson-Olivecrona, Distribution of lipoprotein lipase and hepatic lipase between plasma and tissues: effect of hypertriglyceridemia, Biochimica et Biophysica Acta 837 (3) (1985) 262–270.
[31] P. Spooner, M. Garrison, R. Scow, Regulation of mammary and adipose tissue lipoprotein lipase and blood triacylglycerol in rats during late pregnancy. Effect of prostaglandins, Journal of Clinical Investigations 60 (3) (1977) 702–708.
[32] A. Hirshfield, A. Midgley Jr., Morphometric analysis of follicular development in the rat, Biology of Reproduction 19 (3) (1978) 597–605.
[33] C. Andersen, J. Jensen, T. Orntoft, Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets, Cancer Research 64 (15) (2004) 5245–5250.
[34] K. Livak, T. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta]CT method, Methods 25 (4) (2001) 402–408.
[35] Y. Arai, Persistent-estrous and -diestrous conditions induced by early postnatal administration of estrogen in female rats, Endocrinologia Japonica 11 (3) (1964) 204–208.
[36] L. Pinilla, M. Barreiro, L. Gonzalez, M. Tena-Sempere, E. Aguilar, Comparative effects of testosterone propionate, oestradiol benzoate, ICI 182,780, tamoxifen and raloxifene on hypothalamic differentiation in the female rat, Journal of Endocrinology 172 (3) (2002) 441–448.
[37] L. Ghizzoni, A. Barreca, G. Mastorakos, M. Furlini, A. Vottero, B. Ferrari, G. Chrousos, S. Bernasconi, Leptin inhibits steroid biosynthesis by human granulosa-lutein cells, Hormone and Metabolic Research 33 (June (6)) (2001) 323–328.
[38] C. Barraclough, Sex differentiation of cyclic gonadotropin secretion, in: K.M. Kaye, A.M. Kaye (Eds.), Advances in the Biosciences, Pergamon Press, Oxford, 1979, p. 433.
[39] R. Falvo, A. Buhl, A. Nalbandov, Testerone concentrations in the peripheral plasma of adrogenized female rats and in the estrous cycle of normal female rats, Endocrinology 95 (1) (1974) 26–29.
[40] C. Gilling-Smith, D. Willis, R. Beard, S. Franks, Hypersecretion of androstenedione by isolated thecal cells from polycystic ovaries, Journal of Clinical Endocrinology and Metabolism 79 (4) (1994) 1158–1165.
[41] K. McNatty, A. Makris, V. Reinhold, C. De Grazia, R. Osathanondh, K. Ryan, Metabolism of androstenedione by human ovarian tissues in vitro with particular reference to reductase and aromatase activity, Steroids 34 (4) (1979) 429–443.
[42] D. Lagaly, P. Aad, J. Grado-Ahuir, L. Hulsey, L. Spicer, Role of adiponectin in regulating ovarian theca and granulosa cell function, Molecular and Cellular Endocrinology 284 (1–2) (2008) 38–45.
[43] P. Knight, C. Glister, Local roles of TGF-[beta] superfamily members in the control of ovarian follicle development, Animal Reproduction Science 78 (3–4) (2003) 165–183.

[44] P. Knight, C. Glister, TGF-{beta} superfamily members and ovarian follicle development, Reproduction 132 (2) (2006) 191–206.
[45] S. Bristol, T. Woodruff, Follicle-restricted compartmentalization of transforming growth factor {beta} superfamily ligands in the feline ovary, Biology of Reproduction 70 (3) (2004) 846–859.
[46] A. Drummond, M. Dyson, M. Tan Le, J.-F. Ethier, J. Findlay, Ovarian follicle populations of the rat express TGF-[beta] signalling pathways, Molecular and Cellular Endocrinology 202 (1–2) (2003) 53–57.
[47] G. Erickson, S. Shimasaki, The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle, Reproductive Biology and Endocrinology 1 (1) (2003) 9.
[48] S. Shimasaki, R. Zachow, D. Li, H. Kim, S.-I. Iemura, N. Ueno, K. Sampath, R. Chang, G. Erickson, A functional bone morphogenetic protein system in the ovary, Proceedings of the National Academy of Sciences of the United States of America 96 (1999) 7282–7287.
[49] J. Fortune, The early stages of follicular development: activation of primordial follicles and growth of preantral follicles, Animal Reproduction Science 78 (3–4) (2003) 135–163.
[50] J. Juengel, K. McNatty, The role of proteins of the transforming growth factor-{beta} superfamily in the intraovarian regulation of follicular development, Human Reproductive Update 11 (2) (2005) 144–161.
[51] D. Trombly, T. Woodruff, K. Mayo, Roles for transforming growth factor beta superfamily proteins in early folliculogenesis, Seminars in Reproductive Medicine 27 (2009) 14–23.
[52] L. Dunkel, J. Tilly, T. Shikone, K. Nishimori, A. Hsueh, Follicle-stimulating hormone receptor expression in the rat ovary: increases during prepubertal development and regulation by the opposing actions of transforming growth factors beta and alpha, Biology of Reproduction 50 (4) (1994) 940–948.
[53] J. Dorrington, J. Bendell, S. Khan, Interactions between FSH, estradiol-17 beta and transforming growth factor-beta regulate growth and differentiation in the rat gonad, Journal of Steroid Biochemistry and Molecular Biology 44 (4–6) (1993) 441–447.
[54] A. Drummond, M. Dyson, E. Thean, N. Groome, D. Robertson, J. Findlay, Temporal and hormonal regulation of inhibin protein and subunit mRNA expression by post-natal and immature rat ovaries, Journal of Endocrinology 166 (2) (2000) 339–354.
[55] E. Hernandez, A. Hurwitz, D. Payne, A. Dharmarajan, A. Purchio, E. Adashi, Transforming growth factor-beta 1 inhibits ovarian androgen production: gene expression, cellular localization, mechanisms(s), and site(s) of action, Endocrinology 127 (6) (1990) 2804–2811.
[56] L. Hutchinson, J. Findlay, F. de Vos, D. Robertson, Effects of bovine inhibin, transforming growth factor-[beta] and bovine activin-A on granulosa cell differentiation, Biochemical and Biophysical Research Communications 146 (3) (1987) 1405–1412.
[57] I. Kim, D. Schomberg, The production of transforming growth factor-beta activity by rat granulosa cell cultures, Endocrinology 124 (0013-7227 (Print)) (1989) 1345–1351.
[58] J. Zhang, R. Findlay, A. Carson, H. Herington, H. Burger, Transforming growth factor beta enhances basal and FSH-stimulated inhibin production by rat granulosa cells in vitro, Molecular and Cellular Endocrinology 58 (1988) 161–166.
[59] M. Demeter-Arlotto, W. Rainey, E. Simpson, Maintenance and regulation of 17 alpha-hydroxylase expression by bovine thecal cells in primary culture, Endocrinology 132 (3) (1993) 1353–1358.
[60] N. Fournet, S. Weitsman, R. Zachow, D. Magoffin, Transforming growth factor-beta inhibits ovarian 17 alpha-hydroxylase activity by a direct noncompetitive mechanism, Endocrinology 137 (1) (1996) 166–174.
[61] J. Kipp, S. Kilen, S. Bristol-Gould, T. Woodruff, K. Mayo, Neonatal exposure to estrogens suppresses activin expression and signaling in the mouse ovary, Endocrinology 148 (5) (2007) 1968–1976.
[62] J. Couse, K. Korach, Contrasting phenotypes in reproductive tissues of female estrogen receptor null mice, Annals of the New York Academy of Sciences 948 (1) (2001) 1–8.
[63] D. Tomic, M. Frech, J. Babus, D. Symonds, P. Furth, R. Koos, J. Flaws, Effects of ER[alpha] overexpression on female reproduction in mice, Reproductive Toxicology 23 (3) (2007) 317–325.
[64] Y. Ikeda, A. Nagai, M. Ikeda, S. Hayashi, Neonatal estrogen exposure inhibits steroidogenesis in the developing rat ovary, Developmental Dynamics 221 (4) (2001) 443–453.
[65] C. Chabrolle, L. Tosca, J. Dupont, Regulation of adiponectin and its receptors in rat ovary by human chorionic gonadotrophin treatment and potential involvement of adiponectin in granulosa cell steroidogenesis, Reproduction 133 (4) (2007) 719–731.
[66] S. Ledoux, D. Campos, F. Lopes, M. Dobias-Goff, M. Palin, B. Murphy, Adiponectin induces periovulatory changes in ovarian follicular cells, Endocrinology 147 (11) (2006) 5178–5186.
[67] M. Trujillo, P. Scherer, Adiponectin—journey from an adipocyte secretory protein to biomarker of the metabolic syndrome, Journal of Internal Medicine 257 (2) (2005) 167–175.
[68] S. Kol, I. Ben-Shlomo, K. Ruutiainen, M. Ando, T. Davies-Hill, R. Rohan, I. Simpson, E. Adashi, The midcycle increase in ovarian glucose uptake is associated with enhanced expression of glucose transporter 3. Possible role for interleukin-1, a putative intermediary in the ovulatory process, Journal of Clinical Investigations 99 (9) (1997) 2274–2283.
[69] R. Roberts, J. Stark, A. Iatropoulou, D. Becker, S. Franks, K. Hardy, Energy substrate metabolism of mouse cumulus-oocyte complexes: response to follicle-stimulating hormone is mediated by the phosphatidylinositol 3-kinase pathway and is associated with oocyte maturation, Biology of Reproduction 71 (1) (2004) 199–209.
[70] R. Ceddia, R. Somwar, A. Maida, X. Fang, G. Bikopoulos, G. Sweeney, Globular adiponectin increases GLUT4 translocation and glucose uptake but reduces glycogen synthesis in rat skeletal muscle cells, Diabetologia 48 (1) (2005) 132–139.
[71] S.R. Barnum, J.E. Volanakis, Structure and function of C3, Year in Immunology 6 (1989) 208–228.
[72] K. Cianflone, Z. Xia, L. Chen, Critical review of acylation-stimulating protein physiology in humans and rodents, Biochimica et Biophysica Acta (BBA)-Biomembranes 1609 (2) (2003) 127–143.
[73] A. van Oostrom, A. Alipour, T. Plokker, A. Sniderman, M. Cabezas, The metabolic syndrome in relation to complement component 3 and postprandial lipemia in patients from an outpatient lipid clinic and healthy volunteers, Atherosclerosis 190 (1) (2007) 167–173.
[74] C. Verseyden, S. Meijssen, H. van Dijk, H. Jansen, M. Cabezas, Effects of atorvastatin on fasting and postprandial complement component 3 response in familial combined hyperlipidemia, Journal of Lipid Research 44 (11) (2003) 2100–2108.
[75] C. Halkes, H. van Dijk, P. de Jaegere, H. Plokker, Y. van der Helm, D. Erkelens, M. Castro Cabezas, Postprandial increase of complement component 3 in normolipidemic patients with coronary artery disease: effects of expanded-dose simvastatin, arteriosclerosis, Thrombosis and Vascular Biology 21 (9) (2001) 1526–1530.
[76] K. Cianflone, M. Maslowska, A. Sniderman, Acylation stimulating protein (ASP), an adipocyte autocrine: new directions, Seminars in Cell & Developmental Biology 10 (1) (1999) 31–41.
[77] R. Germinario, A. Sniderman, S. Manuel, S. Lefebvre, A. Baldo, K. Cianflone, Coordinate regulation of triacylglycerol synthesis and glucose transport by acylation-stimulating protein, Metabolism 42 (5) (1993) 574–580.
[78] Y. Tao, K. Cianflone, A. Sniderman, S. Colby-Germinario, R. Germinario, Acylation-stimulating protein (ASP) regulates glucose transport in the rat L6 muscle cell line, Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism 1344 (3) (1997) 221–229.
[79] M. Hamosh, P. Hamosh, The effect of estrogen on the lipoprotein lipase activity of rat adipose tissue, Journal of Clinical Investigations 55 (5) (1975) 1132–1135.
[80] S. Enerback, J. Gimble, Lipoprotein lipase gene expression: physiological regulators at the transcriptional and post-transcriptional level, Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism 1169 (2) (1993) 107–125.
[81] M. Ottosson, K. Vikman-Adolfsson, S. Enerback, A. Elander, P. Bjorntorp, S. Eden, Growth hormone inhibits lipoprotein lipase activity in human adipose tissue, Journal of Clinical Endocrinology and Metabolism 80 (3) (1995) 936–941.
[82] Z. Yasruel, K. Cianflone, A. Sniderman, M. Rosenbloom, M. Walsh, M. Rodriguez, Effect of acylation stimulating protein on the triacylglycerol synthetic pathway of human adipose tissue, Lipids 26 (7) (1991) 495–499.
[83] I. Murray, P. Havel, A. Sniderman, K. Cianflone, Reduced body weight, adipose tissue, and leptin levels despite increased energy intake in female mice lacking acylation-stimulating protein, Endocrinology 141 (3) (2000) 1041–1049.