Early postnatal oestradiol exposure causes insulin resistance and signs of inflammation in circulation and skeletal muscle

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Abstract

Early postnatal events can predispose to metabolic and endocrine disease in adulthood. In this study, we evaluated the programming effects of a single early postnatal oestradiol injection on insulin sensitivity in adult female rats. We also assessed the expression of genes involved in inflammation and glucose metabolism in skeletal muscle and adipose tissue and analysed circulating inflammation markers as possible mediators of insulin resistance. Neonatal oestradiol exposure reduced insulin sensitivity and increased plasma levels of monocyte chemoattractant protein-1 (MCP-1) and soluble intercellular adhesion molecule-1. In skeletal muscle, oestradiol increased the expression of genes encoding complement component 3 (C3), MCP-1, retinol binding protein-4 (Rbp4) and transforming growth factor β1 (Tgfb1). C3 and MCP-1 are both related to insulin resistance, and C3, MCP-1 and TGFβ1 are also involved in inflammation. Expression of genes encoding glucose transporter-4 (Glut 4), carnitine-palmitoyl transferase 1b (Cpt1b), peroxisome proliferator-activated receptor δ (Ppard) and uncoupling protein 3 (Ucp3), which are connected to glucose uptake, lipid oxidation, and energy uncoupling, was down regulated. Expression of several inflammatory genes in skeletal muscle correlated negatively with whole-body insulin sensitivity. In s.c. inguinal adipose tissue, expression of Tgfb1, Ppard and C3 was decreased, while expression of Rbp4 and Cpt1b was increased. Inguinal adipose tissue weight was increased but adipocyte size was unaltered, suggesting an increased number of adipocytes. We suggest that early neonatal oestrogen exposure may reduce insulin sensitivity by inducing chronic, low-grade systemic and skeletal muscle inflammation and disturbances of glucose and lipid metabolism in skeletal muscle in adulthood.

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Introduction


One group of molecules of possible importance in this context is the sex steroids. In female rats, neonatal androgenisation thus increases visceral fat mass and leptin production by adipocytes, and reduces peripheral insulin sensitivity, in adulthood (Perello et al. 2003). In the same vein, prenatal androgenisation of female rhesus monkeys and sheep influences insulin sensitivity and other metabolic parameters (DeHaan et al. 1990, Gill & Hosking 1995, Hansen et al. 1995, Eisner et al. 2000, 2003, Recabarren et al. 2005, King et al. 2007). In a recent study, we showed that an early postnatal injection of oestradiol, testosterone or dihydrotestosterone causes insulin resistance in adult female rats, and that testosterone and oestradiol exposed rats also displayed increased mesenteric weight and/or adipocyte size. Oestradiol having the most pronounced effect suggests oestrogen receptors exert stronger metabolic programming effects than androgen receptors (Alexanderson et al. 2007). However, the mechanisms for the effects of early exposure to sex steroids on glucose metabolism in adulthood have not been studied as yet.

Skeletal muscle handles 75% of all insulin-mediated glucose disposal and is the most important tissue in insulin resistance (DeFronzo 1992). Although adipose tissue accounts for only a small portion of glucose disposal (DeFronzo 1992), it produces adipokines, which serve as endocrine mediators and influence the development of insulin resistance in skeletal muscle. Several adipokines are cytokines, suggesting a link between inflammation and dysfunctions in glucose and lipid metabolism associated with insulin resistance (Grimble 2002, et al. 1990, Gill & Hosking 1995, Hansen et al. 1995, Eisner et al. 2000, 2003, Recabarren et al. 2005, King et al. 2007). In a recent study, we showed that an early postnatal injection of oestradiol, testosterone or dihydrotestosterone causes insulin resistance in adult female rats, and that testosterone and oestradiol exposed rats also displayed increased mesenteric weight and/or adipocyte size. Oestradiol having the most pronounced effect suggests oestrogen receptors exert stronger metabolic programming effects than androgen receptors (Alexanderson et al. 2007). However, the mechanisms for the effects of early exposure to sex steroids on glucose metabolism in adulthood have not been studied as yet.

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Like adipose tissue, skeletal muscle secretes a multitude of signalling peptides and cytokines that have autocrine, paracrine or endocrine effects. These factors, some of which are immune-related and pro-inflammatory molecules, may be important in regulating glucose metabolism and may hence contribute to insulin resistance (Saghizadeh et al. 1996, Pedersen et al. 2007, Wei et al. 2008). Early life events may alter the immune system, thereby inducing insulin resistance in adulthood (Fernández-Real & Pickup 2008). However, the possible effects of perinatal exposure to sex hormones on circulating markers of inflammation or on skeletal muscle expression of immune-related genes have as yet not been studied.

In this study, we further investigated the effects of early postnatal exposure to oestradiol on the metabolism of adult female rats by assessing insulin sensitivity, adipose tissue distribution and adipocyte size. In addition, we explored if the effects of this treatment on metabolism were accompanied by a change in circulating markers of inflammation and/or in the expression of genes involved in inflammation and in lipid and glucose metabolism in skeletal muscle and adipose tissue.

Materials and Methods

Animals

Time-mated Wistar female rats (Scanbur BK AB, Sollentuna, Sweden) were maintained under controlled noise-free conditions (light from 0700 to 1900 h; temperature $21 \pm 2 ^\circ C$; humidity 55–65%) with one rat per cage until parturition. Pups were raised with a lactating mother until 21 days of age. The rats were then housed four to five per cage and had access to standard rat pellets ad libitum. The experiments were approved by the local animal ethics committee at Göteborg University, Göteborg, Sweden.

Study procedures

Within 3 h after birth, female pups were weighed and subcutaneously injected with 0.35 mg of oestradiol benzoate (Apoteksbolaget, Stockholm, Sweden) dissolved in vehicle ($n = 9$). Controls ($n = 11$) received vehicle only.

Tail blood samples were collected for analyses of plasma levels of monocyte chemoattractant protein-1 (MCP-1) at 9 weeks of age and retinol binding protein-4 (RBP4) and soluble intercellular adhesion molecule-1 (sICAM 1) at 12 weeks of age. At 14–16 weeks of age, insulin sensitivity was measured with a euglycemic-hyperinsulinemic clamp. At the end of the clamp experiment rats were decapitated and tibiales anterior, extensor digitorum longus and soleus muscles along with mesenteric and inguinal adipose tissues were removed and weighed. One part of each muscle and adipose tissue depot was snap frozen in liquid nitrogen for analysis of mRNA expression. Both mesenteric and inguinal adipose samples were taken from all rats for adipocyte size measurements.

Vaginal smears

The oestrous status was determined from vaginal smears taken daily at 8–9 weeks of age. The rat oestrous cycle (oestrus, diestrous 1, diestrous 2 and prooestrous) usually lasts about 4 d (Marcondes et al. 2002). Cycles of 4–5 days with a characteristically clear ovulation and a rich amount of epithelial cells without leukocytes in the smears were considered normal. Sampling for clamp measurements in controls were performed in the oestrus phase.

Euglycemic-hyperinsulinemic clamp

At 13–16 weeks of age, euglycemic-hyperinsulinemic clamp studies were performed as described (Holmang et al. 1990). Briefly, the rats were anesthetised with an i.p. injection of thiobutabarbitual sodium (Inactin, Sigma; 130 mg/kg body weight). Catheters were inserted into the left carotid artery for blood sampling and into the right jugular vein for infusion of glucose and insulin. Body temperature was maintained at $37 ^\circ C$ with a heating blanket. Insulin was given as a bolus injection (100 U/ml; human Actrapid, Novo Nordisk Pharma, Copenhagen, Denmark) and then continuously infused at a rate of 8 mU/min per kilogram. To maintain the plasma glucose concentration at 6 mmol/l, a 20% glucose solution was infused at a rate guided by the glucose concentration in $10 \mu l$ blood samples obtained every 5 min for 40 min and then every 10 min. The mean glucose infusion rate (GIR) was calculated from values during the last 90–120 min. At 110 and 120 min of infusion, 50 $\mu l$ blood samples were taken for determination of insulin concentration. A total of less than 1 $\mu l$ blood was used for the determinations; this was compensated for by the infusion volumes.

Adipocyte size

Approximately, 0.4 g inguinal and mesenteric adipose tissue respectively, from each rat were cut into small pieces and treated with 1 $\mu g$/ml collagenase (Type A, Roche) in MEM (Invitrogen) containing 5.5 mM glucose, 25 mM Hepes, 4% bovine albumin (Fraction V, Sigma) and 0.15 mM adenosine, pH 7.4, for 60 min at $37 ^\circ C$ as described (Smith et al. 1972). After filtration through a 250 $\mu m$ nylon mesh, the adipocytes were washed three times and suspended in fresh medium. The mean size and the size distribution of the adipocytes were determined by computerised image analysis (KS400 software, Carl Zeiss, Oberkochen, Germany; Bjornheden et al. 2004). In brief, the cell suspension was...
placed between a siliconised glass slide and a cover slip and transferred to the microscope stage. Nine random visual fields were photographed with a CCD camera (AxioCam, Carl Zeiss). The surface of the relevant areas was measured automatically, and the diameter of the corresponding circles was calculated. Uniform microspheres 98 μm in diameter (Bangs Laboratories, Fishers, IN, USA) were used as a reference.

RNA isolation and cDNA synthesis

Total RNA was extracted from skeletal muscles with RNeasy Fibrous Tissue Mini Kit and from adipose tissues using RNeasy Lipid Tissue Mini Kit according to the manufacturer’s protocol (Qiagen). A DNase I (Qiagen) digestion step was included to eliminate DNA contamination. The RNA concentration was determined spectrophotometrically with a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was checked with an Agilent Bioanalyzer 2100 and an RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA, USA).

First-strand cDNA was synthesised from 1 μg total RNA using high-capacity cDNA reverse transcription kits (PE Applied Biosystems, Stockholm, Sweden), according to the manufacturer’s protocol.

Real-time RT-PCR

Real-time RT-PCR analysis was performed with custom TaqMan low-density array cards (Applied Biosystems). Primers and probes for rat genes corresponding to the TaqMan Gene Expression Assay numbers and GenBank accession numbers listed in Table 1 were spotted onto a 384-well card. Eight samples were randomly analysed per card in one run. Duplicates were run on different cards to guarantee the reproducibility of the method. Each port was loaded with cDNA (100 ng) mixed with TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) in a total volume of 100 μl. Thermal cycling and fluorescence detection was performed with an ABI Prism 7900HT Sequence Detection System and ABI Prism 7900HT SDS Software 2.1 (Applied Biosystems). The 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 was used to calculate the expression stability of four putative reference genes – 18S rRNA (18S), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), peptidylprolyl isomerase A (Ppipa) and hypoxanthine guanine phosphoribosyl transferase (Hprt) – for normalisation in adipose tissues and skeletal muscles (Andersen et al. 2004). According to the NormFinder algorithm, the genes that showed the lowest variability were 18S in tibialis anterior, the combination of Hprt and Gapdh in extensor digitorum longus, and the combination of 18S and Ppipa in soleus. The most suitable reference genes in mesenteric and inguinal adipose tissues were the combinations of Hprt and Gapdh in extensor digitorum longus, and the combination of 18S and Ppipa in soleus. Gene expression values were calculated with the 2−ΔΔCt method (Livak & Schmittgen 2001). Gene expression values were calculated using the 2−ΔΔCt method because Applied Biosystems TaqMan

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

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene description</th>
<th>TaqMan gene expression assay number</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>18S rRNA (reference gene)</td>
<td>Hs99999901_s1</td>
<td>X03205</td>
</tr>
<tr>
<td>Hprt</td>
<td>Hypoxanthine guanine phosphoribosyl transferase (reference gene)</td>
<td>Rn01527840_m1</td>
<td>NM_012583.2</td>
</tr>
<tr>
<td>Ppipa</td>
<td>Peptidylprolyl isomerase A (reference gene)</td>
<td>Rn00690933_m1</td>
<td>NM_017101.1</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase (reference gene)</td>
<td>Rn99999916_s1</td>
<td>NM_017008.3</td>
</tr>
<tr>
<td>Adipoq</td>
<td>Adiponectin</td>
<td>Rn00595250_m1</td>
<td>NM_144744.2</td>
</tr>
<tr>
<td>C3</td>
<td>Complement component 3</td>
<td>Rn00566466_m1</td>
<td>NM_016994.1</td>
</tr>
<tr>
<td>Cpt1b</td>
<td>Carnitine palmitoyltransferase 1b, muscle</td>
<td>Rn00566242_m1</td>
<td>NM_013200.1</td>
</tr>
<tr>
<td>Glut4/Slc2a4</td>
<td>Glucose transporter 4/solute carrier family 2 (facilitated glucose transporter), member 4</td>
<td>Rn00562597_m1</td>
<td>NM_012751.1</td>
</tr>
<tr>
<td>Lep</td>
<td>Leptin</td>
<td>Rn00565158_m1</td>
<td>NM_013076.1</td>
</tr>
<tr>
<td>Lipe/Hsl</td>
<td>Hormone stimulating lipase</td>
<td>Rn00563444_m1</td>
<td>NM_012859.1</td>
</tr>
<tr>
<td>Lipl</td>
<td>Lipoprotein lipase</td>
<td>Rn00561482_m1</td>
<td>NM_012598.1</td>
</tr>
<tr>
<td>Mcp-1/Ccl2</td>
<td>Monocyte chemoattractant protein 1/chemokine (C–C motif) ligand 2</td>
<td>Rn00580555_m1</td>
<td>NM_031530.1</td>
</tr>
<tr>
<td>Ppard</td>
<td>Peroxisome proliferator-activated receptor δ</td>
<td>Rn00565707_m1</td>
<td>NM_013141.1</td>
</tr>
<tr>
<td>Pparg</td>
<td>Peroxisome proliferator-activated receptor γ</td>
<td>Rn00440945_m1</td>
<td>NM_013124.1</td>
</tr>
<tr>
<td>Rbp4</td>
<td>Retinol binding protein 4</td>
<td>Rn01451318_m1</td>
<td>XM_215285.4</td>
</tr>
<tr>
<td>Tgfb1</td>
<td>Transforming growth factor β-1</td>
<td>Rn00572010_m1</td>
<td>NM_021578.1</td>
</tr>
<tr>
<td>Ucp2</td>
<td>Uncoupling protein 2</td>
<td>Rn00571166_m1</td>
<td>NM_019354.1</td>
</tr>
<tr>
<td>Ucp3</td>
<td>Uncoupling protein 3</td>
<td>Rn00565874_m1</td>
<td>NM_013167.2</td>
</tr>
</tbody>
</table>
low-density array cards meet the assumptions necessary to use this equation (Applied Biosystems 2006). The \( \Delta C_t \) value of each sample was determined by subtracting the average \( C_t \) value of the reference gene from the average \( C_t \) value of the target gene. The \( \Delta \Delta C_t \) value was then calculated by subtracting the \( \Delta C_t \) value of the sample with the highest expression (i.e. the lowest \( \Delta C_t \) value) from the sample \( \Delta C_t \) value. The target gene expression level relative to the sample with the highest expression was then estimated as \( 2^{-\Delta \Delta C_t} \).

Analytical methods

Commercial ELISA kits were used to measure plasma levels of MCP-1 (KRC1011; Invitrogen), RBP4 (RB0642EK; Adipogen, Seoul, South Korea) and sICAM-1 (RIC100; Quantikine, Minneapolis, MN, USA). Human insulin given during the clamp was measured with a human insulin ELISA kit (10-1113-01; Mercodia, Uppsala, Sweden).

Statistical analyses

Results are expressed as mean \( \pm \) S.E.M. Differences in skeletal muscle weight and adipose tissue volume and weight between the groups were analysed with unpaired \( t \)-tests. Differences in gene expression and mesenteric and inguinal adipocyte size were analysed with the Mann–Whitney test. Simple regression was used for correlations analysis. \( P<0.05 \) was considered significant.

Results

Body weight

At the end of the study, oestradiol-exposed rats were significantly heavier than controls \((290 \pm 20 \text{ g} \text{ vs } 260 \pm 10 \text{ g}, \ P<0.05)\).

Vaginal smears

Oestradiol-exposed rats had dysfunctional vaginal openings and were acyclic. All control rats had a normal oestrous cycle of 4–5 days.

Glucose infusion rate

During euglycemic–hyperinsulinemic clamp studies, the GIR was significantly lower in oestradiol-exposed rats than in controls \((6.8 \pm 1.68 \text{ mg/kg per minute, } P<0.001; \text{ Fig. 1})\), indicating peripheral insulin resistance. At 60–120 min (steady state), the plasma glucose level was \( \sim 6 \text{ mmol/l} \). The mean plasma insulin concentration was \( 192 \pm 14 \text{ mU/l} \).

Skeletal muscle weights

At 14–16 weeks of age, the tibialis anterior muscle was heavier in oestradiol-treated rats than in controls \((0.56 \pm 0.03 \text{ g} \text{ vs } 0.45 \pm 0.11 \text{ g}, \ P<0.001)\), as was the extensor digitorum longus \((0.12 \pm 0.01 \text{ g} \text{ vs } 0.10 \pm 0.01 \text{ g}, \ P<0.05)\). The weight of the soleus and muscle weights in relation to body weight did not differ between groups.

Weights of adipose tissues and adipocyte size

Oestradiol exposure increased the weight of inguinal adipose tissue at the end of the study (Table 2). There were no differences in the weight of the mesenteric depot. In the inguinal and mesenteric fat depot, adipocyte size was unaltered in oestradiol-exposed rats (Table 2). In each adipocyte sample, a mean of 1431.5 \( \pm \) 89.7 adipocytes \((\text{range, } 1049–2227)\) was analysed. The mean diameter of the reference microspheres was 97.8 \( \pm \) 0.04 \( \mu \text{m} \) \((\text{range, } 97.5–98.1 \mu \text{m}; \ n=3)\).

Plasma analyses

In oestradiol-exposed rats, plasma MCP-1 levels \((\text{oestradiol rats: } 158.5 \pm 16.8 \text{ pg/ml}, \text{ controls: } 105.1 \pm 9.7 \text{ pg/ml}, \ n=8–9, \ P<0.05 \text{ unpaired } t\text{-test})\) were increased at 9 weeks of age and siCAM-1 levels \((\text{oestradiol rats: } 2.5 \pm 0.2 \text{ ng/\mu l}, \text{ controls: } 1.9 \pm 0.1, \ n=9–10, \ P<0.05, \text{ unpaired } t\text{-test})\) were increased at 12 weeks. Plasma RBP4 levels at 12 weeks did not differ between groups.

Gene expression in skeletal muscle and adipose tissue

The mRNA levels of studied genes in skeletal muscle and the correlations between tibialis anterior gene expression and GIR are shown in Table 3. Inguinal gene expressions are shown in Table 4. Oestradiol did not affect gene expression in mesenteric adipose tissue (data not shown). In tibialis anterior and extensor digitorum longus, Mcp-1 expression was increased after oestradiol exposure and found to correlate

![Figure 1](https://via.placeholder.com/150)
with plasma MCP-1 levels (pooled groups; tibialis anterior: \(R = 0.62, P < 0.05, n = 16\); extensor digitorum longus: \(R = 0.64, P < 0.05, n = 16\)). Moreover, in oestradiol-exposed rats, expression of complement component 3 (C3) and transforming growth factor \(\beta 1\) (Tgf\(\beta 1\)) was increased in tibialis anterior and decreased in inguinal adipose tissue. Rbp4 expression was increased in both tissues.

\text{Glut} \, 4 \, \text{expression was down regulated in tibialis anterior and soleus of oestradiol-exposed rats. In tibialis anterior, Glut} \, 4 \, \text{expression correlated negatively with \(Mcp-1\) expression (pooled groups; \(R = -0.67, P < 0.01, n = 16\)). In extensor digitorum longus and soleus from oestradiol-exposed rats, carnitine-palmitoyl transferase 1b (Cpt1b) expression was decreased and inguinal expression was increased. In soleus, only GIR and Cpt1b expression correlated positively (pooled groups; \(R = 0.54, P < 0.05, n = 16\)). In extensor digitorum longus, no correlations were found between the altered gene expression levels and GIR. Oestradiol also decreased peroxisome proliferator-activated receptor \(\delta\) (Ppard) expression in extensor digitorum longus and inguinal adipose tissue and uncoupling protein 3 (Ucp3) expression in extensor digitorum longus. No correlations were found between the altered inguinal adipose tissue gene expression levels and GIR.

**Inflammatory signs after postnatal oestradiol exposure**

**Discussion**

This study confirms that a single oestradiol injection within 3 h after birth reduces insulin sensitivity at adult age and shows that this effect is accompanied by an increase in serum levels of two markers of inflammation, MCP-1 and sICAM-1. Expression of immune-related genes and genes involved in the regulation of metabolism and insulin sensitivity (C3, Mcp-1, Tgf\(\beta 1\) and Rbp4) were increased in skeletal muscle; for some of these genes (C3, Mcp-1, Tgf\(\beta 1\)) the expression was decreased in inguinal adipose tissue. Muscle expression of

**Table 2** Inguinal and mesenteric absolute and relative weights and adipocyte size in female rats 14–16 weeks of age

<table>
<thead>
<tr>
<th>Group</th>
<th>Inguinal adipose tissue</th>
<th>Mesenteric adipose tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute weight (g)</td>
<td>Relative weight (g/kg BW)</td>
</tr>
<tr>
<td></td>
<td>Relative weight (g/kg BW)</td>
<td>(m)</td>
</tr>
<tr>
<td>Controls</td>
<td>1.86±0.17</td>
<td>7.22±0.61</td>
</tr>
<tr>
<td></td>
<td>(n=11)</td>
<td>(n=11)</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>2.59±0.32*</td>
<td>8.63±0.70</td>
</tr>
<tr>
<td></td>
<td>(n=9)</td>
<td>(n=9)</td>
</tr>
<tr>
<td></td>
<td>3.73±0.24</td>
<td>14.52±0.90</td>
</tr>
<tr>
<td></td>
<td>(n=11)</td>
<td>(n=11)</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. BW, body weight. *P<0.05 versus controls (unpaired t-test).

**Table 3** Skeletal muscle relative gene expression and correlations between tibialis anterior relative gene expression and GIR in pooled groups of female rats

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tibialis anterior</th>
<th>Extensor digitorum longus</th>
<th>Soleus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=8)</td>
<td>Oestradiol (n=8)</td>
<td>Control (n=8)</td>
</tr>
<tr>
<td>C3</td>
<td>0.04±0.02</td>
<td>0.46±0.12*</td>
<td>0.15±0.12</td>
</tr>
<tr>
<td>Cpt1b</td>
<td>0.75±0.06</td>
<td>0.71±0.05</td>
<td>0.86±0.04</td>
</tr>
<tr>
<td>Glut4</td>
<td>0.82±0.04</td>
<td>0.69±0.04*</td>
<td>0.88±0.03</td>
</tr>
<tr>
<td>Mcp-1</td>
<td>0.37±0.08</td>
<td>0.65±0.09*</td>
<td>0.36±0.05</td>
</tr>
<tr>
<td>Ppard</td>
<td>0.76±0.05</td>
<td>0.64±0.06</td>
<td>0.81±0.05</td>
</tr>
<tr>
<td>Rbp4</td>
<td>0.05±0.01</td>
<td>0.46±0.12*</td>
<td>0.15±0.12</td>
</tr>
<tr>
<td>Tgf(\beta 1)</td>
<td>0.65±0.05</td>
<td>0.86±0.04*</td>
<td>0.52±0.02</td>
</tr>
<tr>
<td>Ucp3</td>
<td>0.62±0.08</td>
<td>0.44±0.09</td>
<td>0.75±0.07</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. Correlations were performed using simple regression (n=16). *P<0.05; †P<0.01 versus controls (Mann–Whitney test).
Mcp-1 was found to correlate with plasma MCP-1 levels. The expression of genes involved in glucose and lipid metabolism was altered in muscle (Glut4, Cpt1b, Ppard and Ucp3) in a direction corresponding to the observed insulin resistance. Taken together, these findings suggest that early postnatal oestrogen exposure may regulate insulin sensitivity by inducing chronic, low-grade systemic and skeletal muscle inflammation in adulthood.

The dose of 0.35 mg oestradiol benzoate used in this study is based on our previous study (Alexanderson et al. 2007) since this dose is known to affect insulin sensitivity and metabolism in adult female rats. There is no standardised dose or administration procedure when studying the postnatal effects of oestrogenisation, several different doses and administration regimes have been used (Arai 1964, Pinilla et al. 2002, Sotomayor-Zarate et al. 2008). However, the injected dose used in our study concurs with other studies where 0.1 mg oestradiol benzoate (Pinilla et al. 2002) or 0.1 mg oestradiol valerate (Sotomayor-Zarate et al. 2008) has been injected into 1-day-old female rats. The oestradiol-exposed rats in our study displayed acyclicity, a well-known effect from early life oestradiol exposure (Barralough 1979).

The euglycemic-hyperinsulinemic clamp technique measures mainly insulin sensitivity in muscle (DeFronzo 1988). Our previous observation that postnatal oestradiol reduces insulin sensitivity as assessed using this method hence prompted us to speculate that altered muscle-derived molecules, for example, inflammatory markers, could act in an autocrine/paracrine manner, thereby contributing to insulin resistance. The effects of oestradiol treatment on the expression of inflammation-related genes in skeletal muscle observed in this study lends support for this theory, as does the observation of increased plasma levels of two inflammatory markers, sICAM-1 and MCP-1 in these animals (van de Stolpe & van der Saag 1996, Rollins 1997, Baggiolini 1998, Libby 2002, Szmitko et al. 2003). Notably, MCP-1 levels are also increased in diabetic patients (Nomura et al. 2000) and sICAM-1 has been linked to insulin resistance, suggesting that measures of inflammatory status, including sICAM-1, may be useful as clinical indicators of the metabolic syndrome and type 2 diabetes (Kent et al. 2004).

MCP-1 is an adipokine produced by adipose tissues and macrophages, however, is also expressed in murine and human skeletal muscle cells (Confolonieri et al. 2000, Boyd et al. 2006). It mediates insulin resistance through negative crosstalk between adipose tissue and skeletal muscle, exerting a strong influence on insulin sensitivity in skeletal muscle; at concentrations lower than those found in the circulation, it thus impairs insulin signalling and glucose uptake (Sell et al. 2006). MCP-1 was recently designated as a myokine – a molecule produced, expressed and released by muscle fibres – and therefore might have autocrine, paracrine or endocrine effects (Pedersen et al. 2007). Our observation that Mcp-1 mRNA expression was upregulated in tibialis anterior and extensor digitorum longus in oestradiol-exposed rats supports this view, and suggests that it may play a role in oestradiol-induced insulin resistance. This possibility is supported by the associations (albeit modest) between Mcp-1 and Glut4 expression in tibialis anterior, and between tibialis anterior, Mcp-1 expression and whole-body insulin sensitivity. It is also noteworthy that the increased plasma levels of MCP-1 correlated positively with Mcp-1 expression in both tibialis anterior and extensor digitorum longus.

RBP4 is a adipocyte-secreted molecule that is associated with impaired insulin signalling in skeletal muscle. Serum RBP4 levels are elevated in insulin-resistant mice and in humans with obesity and type 2 diabetes (Yang et al. 2005). Although information is sparse, human skeletal muscle has been reported to express Rbp4 (Yao-Borengasser et al. 2007). Since expression of Rbp4 in tibialis anterior muscle was increased in oestradiol-exposed rats, and since muscle Rbp4 expression correlated negatively with GIR, it is tempting to speculate that Rbp4 in skeletal muscle has autocrine/paracrine effects on insulin signalling and anterior, like adipose-derived RBP4, thereby may reduce insulin sensitivity.

TGFβ1 is a cytokine that acts as a chemoattractant for monocytes/macrophages in adipose tissue (Letterio & Roberts 1998). Circulating TGFβ1 levels are increased in patients with impaired insulin sensitivity and obesity (Pfeiffer et al. 1996, Romano et al. 2003). In various animal systems, skeletal muscle ontogeny and early postnatal physiology are exquisitely sensitive to the superfamily of TGFβ cytokines (Kollias & McDermott 2008) which profoundly influence cellular proliferation, differentiation and growth of skeletal muscles. Programming effects of TGFβ during early life may thus have consequences for the function and physiology of adult skeletal muscle. In the present study, whole-body insulin sensitivity correlated negatively with TGFβ1 expression, which was upregulated in tibialis anterior after oestradiol exposure.

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### Table 4 Inguinal adipose tissue relative gene expression in female rats

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control (n=8)</th>
<th>Oestradiol (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipoq</td>
<td>0.34 ± 0.07</td>
<td>0.60 ± 0.10</td>
</tr>
<tr>
<td>Cit</td>
<td>0.69 ± 0.10</td>
<td>0.22 ± 0.04</td>
</tr>
<tr>
<td>Cpt1b</td>
<td>0.28 ± 0.05</td>
<td>0.55 ± 0.09</td>
</tr>
<tr>
<td>Glut4</td>
<td>0.33 ± 0.06</td>
<td>0.53 ± 0.10</td>
</tr>
<tr>
<td>Lep</td>
<td>0.17 ± 0.04</td>
<td>0.45 ± 0.13</td>
</tr>
<tr>
<td>Lipe/HSL</td>
<td>0.37 ± 0.08</td>
<td>0.55 ± 0.10</td>
</tr>
<tr>
<td>Lpl</td>
<td>0.19 ± 0.04</td>
<td>0.48 ± 0.13</td>
</tr>
<tr>
<td>Mcp-1</td>
<td>0.17 ± 0.04</td>
<td>0.27 ± 0.13</td>
</tr>
<tr>
<td>Ppard</td>
<td>0.72 ± 0.07</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>Ppard</td>
<td>0.35 ± 0.08</td>
<td>0.55 ± 0.10</td>
</tr>
<tr>
<td>Rbp4</td>
<td>0.21 ± 0.06</td>
<td>0.48 ± 0.11</td>
</tr>
<tr>
<td>Tgfβ1</td>
<td>0.58 ± 0.08</td>
<td>0.38 ± 0.03</td>
</tr>
<tr>
<td>Ucp2</td>
<td>0.64 ± 0.06</td>
<td>0.59 ± 0.10</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m. *P<0.05; †P<0.01 versus controls (Mann–Whitney test).

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[Table 4](#) Inguinal adipose tissue relative gene expression

(2−ΔΔCt)
C3 is the most versatile molecule in the complement system, which upon activation generates a cascade of inflammatory mediators (Barnum & Volanakis 1989). It is produced and expressed by normal human myoblasts in vitro and is up regulated by inflammatory cytokines (Legoe dec et al. 1995). Interestingly, plasma C3 levels correlate positively with insulin and glucose levels and negatively with glucose disposal rate (Cianflone et al. 2003), suggesting a role for this molecule in insulin resistance. Thus, increased local expression of cytokines in muscle might further reduce insulin sensitivity by up regulating muscle C3 expression. In line with this, C3 expression in tibialis anterior was up-regulated in oestriadiol-exposed rats and found to correlate negatively with the GIR.

Selective ablation of PPARD in skeletal muscle diminishes the oxidative capacity of this tissue, leading to obesity and glucose intolerance (Schuler et al. 2006). Activation of Ppard represses several inflammatory genes, such as Mip-1 in macrophages, atherosclerotic lesions and liver (Li et al. 2004, Graham et al. 2005, Nagasawa et al. 2006, Schuler et al. 2006). Extensor digitorum longus displayed decreased expression of Ppard after oestriadiol exposure. The up regulation of Mip-1 might reflect the reduced expression of Ppard. Expression of Cpt1b and Ucp3 was reduced in the extensor digitorum longus, as was Cpt1b expression in the soleus.

PPARD, UCP3 and CPT1b are all markers and initiators of mitochondrial biogenesis, which could enhance the lipid and glucose oxidation capacity of skeletal muscle (Kerner & Hoppel 2000, Jones et al. 2003, Shen et al. 2008). PPARD downstream signalling involves both Ucp3 and Cpt1b (Reilly & Lee 2008), which are key genes in mitochondrial fatty acid oxidation (Muoio et al. 2002, Dressel et al. 2003). Mitochondrial dysfunction contributes to insulin resistance in the elderly (Petersen et al. 2003) and in the children of patients with type 2 diabetes (Petersen et al. 2004). Ucp3 is mainly expressed in skeletal muscle (Pecqueur et al. 2001) and up regulation of Ucp3 increases fatty acid oxidation and protects against insulin resistance (Bezaire et al. 2007). In addition, expression of Ucp3 and Cpt1b in skeletal muscle is down regulated in type 2 diabetes (Krook et al. 1998, Schrauwen et al. 2001, Carey et al. 2006). CPT1b is a key enzyme in controlling fatty acid oxidation (Manco et al. 2004). In soleus, Cpt1b expression correlated negatively with GIR. Thus, the insulin resistance in oestriadiol-exposed rats might reflect dysfunctional muscle lipid metabolism that promotes insulin resistance. To study skeletal muscle lipid metabolism was, however, beyond the scope of this study.

A number of interesting correlations between gene expression levels and measures of metabolic and inflammatory status were observed in the present study. However, it is important to bear in mind that correlations do not necessarily imply that the studied parameters are causally related. Furthermore, changes in a specific mRNA expression level are not always followed by corresponding changes at the protein level.

In s.c. inguinal adipose tissue of oestriadiol–exposed rats, expression of Tgfβ1, Ppard and C3 was decreased and expression of Rbp4 and Cpt1b was increased. The weight of this tissue was also increased, but adipocyte size was unaltered, suggesting an increased number of adipocytes. Oestriadiol exposure had no effect on weight, adipocyte size or gene expression in mesenteric adipose tissue. Previously, we found that postnatal oestriadiol exposure increases mesenteric adipocyte size (Alexanderson et al. 2007). This discrepancy likely reflects the lower dose of oestriadiol in the present study. Indeed, the outcome of programming depends on several factors, including the dose of the stimulus (Newbold et al. 2004, Lombardo et al. 2005). Although, adipocyte size is reported to have a strong influence on insulin sensitivity, the effects on glucose tolerance were similar although the adipocyte size outcome differs in our two studies. Moreover, gene expression was unaltered in the mesenteric adipose depot. Thus, mesenteric adipose tissue probably has a minor influence on the development of insulin resistance in this programming model.

Inguinal absolute weight was increased in oestriadiol-exposed rats while the adipocyte size was unaltered, suggesting an increased adipocyte number in this depot. In another study, the number of adipocytes was altered after neonatal oestriadiol exposure of mice and rats (Cooke & Naaz 2004). In conjunction, these findings indicate a critical role for oestriadiol during the neonatal period in the establishment of adult adipocyte number.

Rbp4 expression was increased in the inguinal adipose tissue of oestriadiol–exposed rats. RBP4 is an adipokine produced in rat and human adipose tissue (Janke et al. 2006, Kang et al. 2007). Increased serum levels of this molecule derived from adipose tissue impair insulin signalling in skeletal muscle (Yang et al. 2005). However, in our study circulatory RBP4 was unaltered. This discrepancy could reflect different time-points between the circulatory and local adipose tissue measurements or a lack of endocrine effects of the increased Rbp4 levels in inguinal adipose tissue.

C3 and Tgfβ were down-regulated in inguinal adipose tissue. Notably, TGFβ potently inhibits adipocyte differentiation in preadipocyte cell lines and primary cultures from various animals (Gregoire et al. 1998), such as rat inguinal adipocyte precursors (Serrero & Mills 1991). Thus, the reduced expression of Tgfβ might have contributed to increased differentiation, and consequently to increased adipocyte number and fat mass. Acylation-stimulating protein (ASP), the cleavage product of C3, increases during the differentiation of preadipocytes (Cianflone & Maslowska 1995) and has anabolic effects on human adipose tissue (Yasruel et al. 1991, Germinario et al. 1993, Tao et al. 1997, Cianflone et al. 1999). Thus, the down-regulation of C3 and the increased weight of the inguinal fat depot in the oestriadiol-exposed rats may seem contradictory. However, factors related to ASP production, including C3, are reported to be down regulated in obesity, perhaps to limit further expansion of the adipose tissue (Xia & Cianflone 2003).
Ppard was also down-regulated in inguinal adipose tissue. Activation of Ppard in adipocytes may promote the oxidation and utilisation of fatty acids (Wang et al. 2003), consistent with a role as a broad regulator of lipid metabolism. Targeted activation of Ppard in mouse adipose tissue specifically induces the expression of genes required for fatty acid oxidation and dissipation, leading to reduced adiposity (Wang et al. 2003). Thus, the down regulation of Ppard might explain the increased inguinal fat mass. Since PPARD signals through CPT1b, which increases fatty acid transport and oxidation (Manco et al. 2004), it may seem inconsistent that Cpt1b was up regulated in inguinal adipose tissue, despite its increased weight after oestradiol exposure. Reportedly, Cpt1b expression is decreased in skeletal muscle and increased in adipose tissue in patients with type 2 diabetes (Carey et al. 2006). This is in agreement with our findings in these tissues.

Thus, oestradiol exposure resulted in increased s.c. adipose tissue weight without affecting adipocyte size, suggesting adipocyte hyperplasia. Several inguinal genes involved in adipose metabolism were altered and inflammatory markers were down-regulated. Consequently, we found no direct evidence that adipose tissue metabolism or adipokine expression contributes to the insulin resistance in adult female mice exposed to oestradiol shortly after birth.

To conclude, this study confirms our earlier finding that postnatal exposure to oestradiol results in insulin resistance in adult female rats. It also revealed increased mRNA expression of inflammation markers in the skeletal muscle and alterations in genes involved in glucose and lipid metabolism. Circulatory inflammation markers were increased and plasma MCP-1 associated with muscle gene expression, suggesting that muscle-derived molecules act in an endocrine, paracrine and/or autocrine way. We suggest that oestradiol may exert an early programming effect on insulin sensitivity in adult organism partly by inducing low-grade chronic inflammation.

Declaration of interest

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

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