# Phthalate exposure *in utero* causes epigenetic changes and impairs insulin signalling

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## Abstract

Di-(2-ethylhexyl)phthalate (DEHP) is an endocrine-disrupting chemical (EDC), widely used as a plasticiser. Developmental exposure to EDCs could alter epigenetic programming and result in adult-onset disease. We investigated whether DEHP exposure during development affects glucose homoeostasis in the $F_1$ offspring as a result of impaired insulin signal transduction in gastrocnemius muscle. Pregnant Wistar rats were administered DEHP (0, 1, 10 and 100 mg/kg per day) from embryonic days 9–21 orally. DEHP-exposed offspring exhibited elevated blood glucose, impaired serum insulin, glucose tolerance and insulin tolerance, along with reduced insulin receptor, glucose uptake and oxidation in the muscle at postnatal day 60. The levels of insulin signalling molecules and their phosphorylation were down-regulated in DEHP-exposed offspring. However, phosphorylated IRS1$^{Ser636/639}$, which impedes binding of downstream effectors and the negative regulator (PTEN) of $PIP_3$, was increased in DEHP-exposed groups. Down-regulation of glucose transporter 4 (*Glut4* (*Slc2a4*)) gene expression and increased GLUT4$^{Ser488}$ phosphorylation, which decreases its intrinsic activity and translocation towards the plasma membrane, were recorded. Chromatin immunoprecipitation assays detected decreased MYOD binding and increased histone deacetylase 2 interaction towards *Glut4*, indicative of the tight chromatin structure at the *Glut4* promoter. Increased DNMTs and global DNA methylation levels were also observed. Furthermore, methylation of *Glut4* at the MYOD-binding site was increased in DEHP-exposed groups. These findings indicate that, gestational DEHP exposure predisposes $F_1$ offspring to glucometabolic dysfunction at adulthood by down-regulating the expression of critical genes involved in the insulin signalling pathway. Furthermore, DEHP-induced epigenetic alterations in *Glut4* appear to play a significant role in disposition towards this metabolic abnormality.

**Key Words**
- di(2-ethylhexyl)phthalate (DEHP)
- insulin signalling
- glucose transporter 4
- DNA methylation

*Journal of Endocrinology* (2014) **223**, 47–66



## Introduction

Metabolic disorders such as type 2 diabetes (T2D) and obesity are being diagnosed in children and adolescents at an early stage and have reached epidemic rates in most developed and developing countries (Zimmet *et al.* 2001, Diamond 2003). An estimated of 342 million people have been affected by this disease worldwide (Danaei *et al.* 2011). Moreover, there is a considerable interest in understanding the contribution of 'non-traditional' risk factors, such as environmental chemicals, as causative factors in the diabetes epidemic. Insulin resistance can

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-14-0111

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arise independently from obesity. The onset of frank diabetes necessitates a deficit in β-cell insulin production, as either the primary defect or the failure to compensate for diminished insulin sensitivity. Therefore, the search for pollution-induced diabetes should include a specific focus on compounds, that have the capacity to induce insulin resistance and/or impair β-cell function (Neel & Sargis 2011). Historically, research on endocrine-disrupting chemicals (EDCs) has focused on the ability of exogenous chemicals to modulate the activity of classic nuclear hormone receptors of estrogens, androgens and thyroid hormone. Several of these pathways appear to be critically important for energy regulation in general and glucose homoeostasis in particular (Neel & Sargis 2011). Emerging evidence from population-based studies emphasises the link between the environmental exposure to persistent organic pollutants arsenic, bisphenol A, phthalates, organotins and non-persistent pesticides and the development of T2D (Thayer *et al*. 2012).

Di-(2-ethylhexyl)phthalate (DEHP), a plasticiser with endocrine-disrupting properties, is found as an ubiquitous environment pollutant in the form of consumer products such as those used in building construction, car and children's products, clothing, food packaging and medical devices made of polyvinyl chloride (PVC) (Hauser & Calafat 2005). It has been identified in human amniotic fluid, umbilical cord blood, milk, semen and saliva (Faniband *et al*. 2014). In order to add flexibility to PVC-derived plastics, DEHP is non-covalently bound to the PVC polymer (Kobayashi *et al*. 2006). This causes DEHP to easily leach into the environment. Annual DEHP production is approximately two million tonnes (Shelby 2006). Its widespread use and presence have resulted in constant human exposure through foetal development and postnatal life (Martinez-Arguelles *et al*. 2013). Current levels of exposure to DEHP are high enough to cause serious concern and adverse effects have triggered the interest of the public and government alike (Shelby 2006).

Maternal health, diet and chemical exposure during gestation are critical for predicting foetal outcomes, both immediately at birth and during adulthood. Recent advances in the field have indicated that numerous adulthood diseases, including those characteristic of metabolic syndrome, could be programmed *in utero* in response to maternal exposures, and that these 'programmable' diseases are associated with epigenetic modifications of vital genes (Strakovsky & Pan 2012). While little is currently known about the epigenetic changes induced by the endocrine disruptors, especially DEHP, studies on animals show that exposure to endocrine disruptors during a critical period of development (prenatal and postnatal) may influence the adult phenotype making it likely that the critical genes involved are epigenetically regulated, either by DNA methylation or by the modification of histone tails (Martinez-Arguelles *et al*. 2009, Wu *et al*. 2010*a*, Anderson *et al*. 2012). Evidence indicates the adverse effects of phthalate exposure during intrauterine life (Martinez-Arguelles *et al*. 2009, 2011, 2013). Exposure to a variety of pollutants appears to modify the epigenome (Anway *et al*. 2005), and evidence pertaining to this demonstrates that chemical-induced epigenetic changes can either be an expression memory or heritable (Anway & Skinner 2006, Jirtle & Skinner 2007, Wu *et al*. 2010*b*, Skinner *et al*. 2011, Singh & Li 2012).

DEHP is in the limelight because of its contribution to energy imbalance and metabolic disorders (Desvergne *et al*. 2009). DEHP interferes with carbohydrate metabolism by reducing blood glucose utilisation and hepatic glycogenesis and glycogenolysis in rat (Mushtaq *et al*. 1980). DEHP reduced the serum insulin and testosterone and increased the blood glucose, oestradiol, tri-iodothyronine and thyroxine levels in rats (Gayathri *et al*. 2004). DEHP-fed rats also showed a deficiency in muscle glucose and lactate transport, reductions in muscle hexokinase and hepatic glucokinase, and glycogen synthesis (Martinelli *et al*. 2006). It has been reported that developmental DEHP exposure disrupted the pancreas and altered whole-body glucose homoeostasis (Lin *et al*. 2011). Epidemiological studies also revealed a positive correlation between increased phthalate metabolites in urine and abdominal obesity as well as insulin resistance in adolescents and adult males (Stahlhut *et al*. 2007, Hatch *et al*. 2008, Trasande *et al*. 2013).

Results of previous studies at our laboratory have indicated that DEHP has a negative influence on the number of insulin receptors and glucose oxidation in cultured Chang liver cells and L6 myotubes (Rengarajan *et al*. 2007, Rajesh & Balasubramanian 2013). Furthermore, DEHP treatment of adult albino rats disrupts insulin signalling molecules, glucose uptake and oxidation in gastrocnemius muscle and adipose tissue (Srinivasan *et al*. 2011, Rajesh *et al*. 2013). Lactational exposure to DEHP impairs insulin signal transduction and glucose oxidation in the cardiac muscle of $F_1$ female albino rats (Mangala Priya *et al*. 2014).

Foetuses and neonates appear to be more sensitive than adults to DEHP, as they are more susceptible to endocrine disruption. Pertaining to the immaturity of the liver, neonates are not able to oxidise DEHP, making them more receptive to toxic chemicals (Dostal *et al*. 1987, Latini 2000). Additionally, DEHP has been found to be



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DOI: 10.1530/JOE-14-0111

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lipophilic and accumulates more in adipose tissue, breast milk and amniotic fluid. DEHP is able to cross the placenta and also pass into the breast milk (Latini *et al*. 2003, Calafat *et al*. 2004), resulting in a significant risk to the developing foetus and newborn. DEHP exposure during critical periods of development may induce epigenetic changes leading to a potential risk, at least in part, of the development of 'insulin resistance/T2D'.

The incidence of T2D is on the rise due to various factors including changes in life style (Hu 2011). There are epidemiological and experimental data demonstrating that exposure to DEHP has a negative influence on glucose homoeostasis. However, the possible effects of DEHP exposure during the critical period of development on glucose homoeostasis of the $F_1$ offspring have not been clarified so far. Based on these observations, it is suggested that DEHP exposure during gestation may impair glucose homoeostasis and insulin sensitivity. Furthermore, skeletal muscle plays a significant role in the maintenance of glucose homoeostasis and is the predominant site of peripheral glucose utilisation. Glucose transport in skeletal muscle is the rate-limiting step for glucose utilisation under physiological conditions (Sinacore & Gulve 1993). In view of this, we propose that DEHP exposure during gestation may affect insulin signal transduction in the skeletal muscle of rat $F_1$ offspring. Therefore, this study was designed to assess the effects of gestational DEHP exposure on insulin signalling molecules and glucose transporter 4 (GLUT4 (SLC2A4)) and its epigenome in gastrocnemius muscle of $F_1$ offspring.

## Materials and methods

### Chemicals and suppliers

All chemicals and reagents used in the study were of molecular and analytical grade and they were purchased from Sigma Chemical Company; Amersham Biosciences and Sisco Research Laboratories (Mumbai, India). Blood glucose strips were purchased from ACON Laboratories, Inc. (San Diego, CA, USA). $^{14}$C-glucose, $^{14}$C-2-deoxyglucose and iodine-125($[^{125}I]$) were purchased from the Board of Radiation and Isotope Technology (Mumbai, India). Antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), Santa Cruz Biotechnology, Inc. and Abcam (UK).

### Dose selection and treatment of animals

Animals were maintained as per the National Guidelines and Protocols approved by the Institutional Animal Ethical Committee (IAEC no. 01/01/2010). Nulliparous female albino (60 days old, Wistar strain (*Rattus norvegicus*), weighing 120±10 g) rats with regular cyclicity were caged with male rats at a proportion of 2:1. The following day, rats were examined for the presence of vaginal plug/vaginal lavage and microscopic examination for the presence of sperm in the vaginal smear was performed, and if mating was confirmed the day was considered as embryonic day 1 (ED1) and each pregnant rat was placed in an individual cage and provided with water and food and allowed to drink and eat *ad libitum*; pregnant rats received oral gavages of DEHP at three different dosages, 1, 10 and 100 mg/kg per day at 1000 h respectively. DEHP was dissolved in olive oil, and the dosage was adjusted daily for maternal body weight changes (2.0 ml/kg bw). Control animals received only vehicle (olive oil). Dose ranges used in this study correspond with normal to occupational human exposure. Each group consisted of six pregnant dams and the rats were treated for 12 days from ED9 to ED21 or until parturition. The day of birth was designated as postnatal day 1 (PND1). The litter size was culled to four male and four female offspring/rat to avoid suckling effects.

At PND60 female (diestrus phase) and male animals were anaesthetised with sodium thiopental (40 mg/kg bw, i.p.), blood was collected, and sera separated and stored at −80 °C until assay of hormones was performed and perfused with 20 ml of normal saline through the left ventricle, to clear blood from the liver and other organs. Skeletal muscle (gastrocnemius) was dissected out, snap frozen and stored at −80 °C until further use (six animals per treatment group belonging to different litters were used for various assays), whereas the other cohort offspring's tissues were fixed in buffered formalin solution for immunohistochemical analysis. Visceral adipose tissue deposits were excised and weighed as described previously (Krotkiewski & Bjorntorp 1976, Gauthier *et al*. 2004).

### Oral glucose tolerance test and insulin tolerance test

At PND60, oral glucose tolerance tests (OGTT) and insulin tolerance tests (ITT) were performed on six male and six female offspring from different litters (only one offspring was selected per sex per litter). For OGTT, offspring were fasted overnight for 16 h. Blood glucose was determined during and 60, 120 and 180 min after glucose administration (10 ml/kg; 50% w/v by oral gavage). For ITT, the different cohorts of animals were fasted for 6 h and received i.p. injections of human insulin (Novolin R; Novo Nordisk, Bagsvaerd, Denmark) at a dose of 0.75 U/kg

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DOI: 10.1530/JOE-14-0111

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bw. Blood glucose levels were measured before and 15, 30, 45 and 60 min after injection. Blood glucose was estimated using the On-Call Plus Blood Glucose Test Strips method (ACON Laboratories, Inc.). Blood sample for glucose estimation was collected from the tail tip. Results were obtained from the meter display as mg/dl.

### RIA

Serum fasting insulin was assessed using a commercially available $^{125}$I-labelled RIA Kit (Diasorin, Saluggia, Italy). The sensitivity of the assay was 3 μIU/ml. The percentage cross-reactivity of insulin antibody to human and rat insulin was 100% and to C-peptide was <0.01%. Intra- and inter-assay coefficient of variation (CV) values were 10.6 and 10.8% respectively. Results are expressed as μIU/ml.

### Real-time PCR

mRNA expression was examined using real-time PCR. Total RNA was extracted from gastrocnemius muscle using TRIzol reagent. RNA quantity was calculated by measuring the $A_{260/280\ nm}$. The purity of RNA obtained was 1.8–1.9 and the integrity of the RNA was validated by running samples on 1% formaldehyde agarose gels. The yield of RNA was expressed in μg. cDNA was synthesised from 2 μg of total RNA using M-MuLV Reverse Transcriptase according to the manufacturer's protocol. The lists of primer sequences are given in Table 1. Real-time PCR was carried out in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The reaction was performed using the MESA Green PCR Master Mix (which contains all the PCR components along with SYBR Green Dye). The specificity of the amplification product was determined by melting curve analysis for each primer pair. The relative amount of each mRNA was normalised to β-actin. Data were analysed by the comparative *CT* method and the fold change was calculated by the $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak 2008) using CFX Manager Version 2.1 (Bio-Rad).

### Western blot analysis

#### Isolation of plasma membrane and cytosolic fractions

Plasma membrane (PM) and cytosolic fractions from gastrocnemius muscles of control and experimental animals were prepared as described previously (Dombrowski *et al.* 1996). Protein concentration was estimated (Lowry *et al.* 1951) using BSA as a standard. INSRβ and pINSRβ$^{Tyr1162/1163}$ levels were estimated for PM and GLUT4 and GLUT2 (SLC2A2) levels were estimated for both PM and cytosolic fractions. pGLUT4$^{Ser488}$ levels in the cytosolic fraction were estimated. Results were normalised to β-actin (the phosphorylated form was normalised to the respective total protein).

**Nuclear lysate preparation** The nuclear lysate fraction from gastrocnemius muscle was prepared as described previously (Im *et al.* 2006), and protein concentrations were estimated. Mature SREBP1c (SREBF1), MYOD (MYOD1)

**Table 1** List of primers used in this study

| Gene names | 5′-Oligonucleotide | 3′-Oligonucleotide | GenBank accession numbers |
|---|---|---|---|
| Real-time PCR primers | | | |
| *Dnmt1* | CCAGATACCTACCGGTTATTCG | TCCTTTAACTGCAGCTGAGGC | NM_053354 |
| *Dnmt3a* | CTGAAATGGAAAGGGTGTTTGGC | CCATGTCCCTTACACACAAGC | NM_001003958 |
| *Dnmt3b* | CCAAGGCGTATTCGTCGCC | TACGTTTACTTGGGCCGCTT | NM_001003959.1 |
| *Dnmt3l* | AAGACCCATGAAACCTTGAACC | GTTGACTTCGTACCTGATGACCTC | NM_001003964.1 |
| *Insr* | GCCATCCCGAAAGCGAAGATC | TCTGGGGAGTCCTGATTGCAT | NM_017071.2 |
| *Irs1* | AAAGCACTGTGACACCGGAA | ACACGGTTTCAGAGCAGAGG | NM_012969.1 |
| *Akt* | GGAAGCCTTCAGTTTGGATCCCAA | AGTGGAAATCCAGTTCCGAGCTTG | NM_017093.1 |
| *Glut4* | GGGCTGTGAGTGAGTGCTTTC | CAGCGAGGCAAGGCTAGA | NM_012751.1 |
| β-actin | CTGTGTGGATTGGTGGCTCT | GCTCAGTAACAGTCCGCCTA | NM_031144.3 |
| MSP primer | | | |
| *Glut4* (methylated) | GATGGGTCGTAGATTGTGTATCG | ACCTTAAAAAATCCGCGACTCGC | L36125.1 |
| *Glut4* (unmethylated) | GGGATGGGTTGTAGATTGTGTATT | AACCTTAAAAAATCCACAAC | L36125.1 |
| ChIP assay primers | | | |
| *Glut4*-(*Myod–Mef2*) | CCAAAACAGGAGCTGACTCTG | AATGGCTATTTTTAGCTCCCA | L36125.1 |
| *Gapdh* | CCGGAATTCGAAGGTCGGTGTCAA-CGGATTTGG | CACACCTGCAGCCTGGAAGATGGTGA-TGGGTTTCC | X02231.1 |



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DOI: 10.1530/JOE-14-0111

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and histone deacetylase 2 (HDAC2) levels were estimated, TBP served as a loading control in the nuclear lysate.

**Preparation of tissue lysate** Total tissue lysate from gastrocnemius muscle and islets from control and experimental animals were prepared as described previously (Bennett & Tonks 1997), and protein concentration was estimated. Western blot was carried out to quantify IRS1, $IRS1^{Tyr632}$, $IRS1^{Ser636/639}$, PTEN, ARRB2, SRC, AKT1/AKT2/AKT3, $AKT^{Ser473}$, $AKT^{Thr308}$, $AKT^{Tyr315/316/312}$, AS160, $AS160^{Thr642}$, RAB8A, RAB13, ACTN4, DNMT1, DNMT3a/DNMT3b and DNMT3l. Rat β-actin was used as the invariant loading control.

### Insulin receptor assay

Insulin receptors were quantified as described previously (Torlinska *et al.* 2000). The receptor concentration is expressed as fmol/mg protein.

**Glucose uptake and oxidation** $^{14}$C-2-deoxyglucose uptake in tissues was estimated by the method of Valverde *et al.* (1999). Results are expressed as c.p.m. of $^{14}$C-2-deoxyglucose taken up/10 mg tissue. $^{14}$C-glucose oxidation was estimated as per the standard method (Kraft & Johnson 1972). Results are expressed as c.p.m. of $^{14}CO_2$ released/10 mg tissue.

**Estimation of glycogen** Glycogen was estimated using a standard method (Roe & Dailey 1966). The amount of glycogen is expressed as mg/g wet tissue.

### Immunohistochemistry

The gastrocnemius muscles were fixed in 4% paraformaldehyde, dehydrated using 30% sucrose solution and cryosectioned (10 μm thick). The sections were washed with 1× PBS twice (5 min/wash) followed by incubation in 1% BSA (in 1× PBS buffer) for 1 h. The blocked sections were then incubated with the primary GLUT4 antibody (at a dilution of 1:500) for 1 h at room temperature. The sections were washed with 1× PBS three times (5 min/wash) and then incubated with secondary antibodies using Alexafluor (568 nm) (at a dilution of 1:300) for 45 min at room temperature in the dark. The sections were washed with 1× PBS three times (5 min/wash) and were counterstained with mounting media containing 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. Sections were imaged under a Nikon fluorescent microscope using the NIS elements software at a magnification of 40×.

### Global DNA methylation level

Gastrocnemius muscle 5-methyl-2′-deoxycytidine level was assessed using the DNA Methylation EIA Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The sensitivity of the assay was ~3 ng/ml. The percentage cross-reactivity was 100% for 5-methyl-2′-deoxycytidine, 20% for 5-methylcytidine, 0.1% for 2′-deoxycytidine, 0.1% for cytidine and <0.01% for thymidine. Intra-and inter-assay CV values were 9.1 and 13.8%, respectively, at 150 ng/ml. Results are expressed as ng/ml.

### Methylation-specific PCR

CpG islands near the promoter area of *Glut4* (GenBank accession number L36125.1) were identified with a GC content of at least 50% and an observed CpG to expected CpG ratio >0.6 using the Methprimer program (http://www.urogene.org/methyprimer). The Methyl Primer Express Software v1.0 (Applied Biosystems) was used to design methylation-specific PCR (MSP) primers listed in Table 1. Briefly, genomic DNA was extracted from gastrocnemius muscles. The extracted DNA (1.5 μg) from animals of the control and experimental groups was subjected to bisulphite modification using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). The bisulphite-modified naked DNA served as the template in MSP. The PCR mix consisted of 0.2 mM deoxynucleotide triphosphate, 3 mM $MgCl_2$, 0.2 μM primers, 1 U HotStarTaq DNA Polymerase (Qiagen) and 2 μl bisulphite-treated DNA in 20 μl total volume. Rat DNA was hypermethylated *in vitro* by CpG methyltransferase (M.SssI) from New England Biolabs (Ipswich, MA, USA) as per the manufacturer's protocol, which served as a positive control; $H_2O$ was used as a negative control for MSP. The PCR conditions were as follows: initial activation at 95 °C (15 min), 40 cycles of 1 min at 94 °C denaturation, 1 min annealing at 57 °C, 1 min extension at 72 °C and 10 min final extension at 72 °C. PCR was performed with two primer pairs, which detected methylated and unmethylated DNA. After PCR, 10 μl of PCR mix were mixed with a loading dye and run on 2% agarose gel containing ethidium bromide. Stained gels were visualised and digitalised using the gel documentation system (Bio-Rad).



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### Chromatin immunoprecipitation assay

To assess both the binding of MYOD and HDAC2 to the *Glut4* promoter region, chromatin immunoprecipitation (ChIP) assay was performed using the EZ ChIP Chromatin Immunoprecipitation Kit, Upstate Biotech (Merck Millipore, Billerica, MA, USA), as recommended by the manufacturer's instructions. Briefly, powdered gastrocnemius muscle was fixed in 1% formaldehyde for 45 min at room temperature. The tissue pellet was resuspended in cell lysis buffer (5 mM Pipes (KOH), pH 8.0, 85 mM KCl and 0.5% Nonidet P-40) containing protease inhibitors (Sigma Chemical Co.) and homogenised with a Polytron-equipped homogeniser (Model PT 3000, Kinematica, Littau, Switzerland) at a precise low setting on ice. The separated nuclei were lysed in nuclear lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA and 1% SDS) containing protease inhibitors. The resultant chromatin was sonicated on ice by 20 pulses of 15 s each at setting four with a 1-min rest interval between pulses. The average length of sonicated chromatin was determined by resolving them on 1.5% agarose gel and found to be approximately 500 bp. The sample was then centrifuged at 4 °C (10 min at 14 000 ***g***) to remove cell debris from the crude chromatin lysate. Ten per cent of the lysate was used as the input control for PCR. To co-immunoprecipitate, the DNA, MYOD and HDAC2 antibodies were used. For negative controls, aliquots of cross-linked chromatin were immunoprecipitated with a normal rabbit IgG and a mouse IgG instead of MYOD and HDAC2. The mouse MAB to RNA Polymerase II served as a positive control for experiments. To confirm equal amounts of chromatins used in immunoprecipitation between groups, input chromatin was used. The eluted immunoprecipitated DNA, approximately 2–4 ng, was used as a template in each PCR. The PCR amplification of the *Glut4* promoter region from −836 to −452 bp was performed initially at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and then at 72 °C for 4 min. The PCR employed for GAPDH (amplification product targeted at the translational start site, 1–231 bp spanning exons 1–4) consisted of 25 μl of the reaction mix containing 2 μl of the DNA template, 0.5 μM forward primer, 0.5 μM reverse primer, 2× Master Mix with 3 mM $MgCl_2$, 0.4 mM dNTPs and Go-Taq DNA polymerase (50 U/ml), which were subjected to amplification in a Eppendorf master cycler. The PCR was performed as described above except for an annealing temperature of 60 °C for more than 60 s. The primers used in these PCRs are listed in Table 1. DNA from 10 μl of input sample that did not undergo ChIP, but was reverse cross-linked and purified as described above, was also PCR amplified using the same set of primers. Amplification products were analysed electrophoretically on 2.0% agarose gel containing 0.1 μg/ml ethidium bromide and photographed and the density of the bands quantified using the Quantity One Software (Bio-Rad).

### Statistical analysis

Statistical analyses were performed using the Prism 6.00 Software (GraphPad Software for Windows, La Jolla, CA, USA). All data are expressed as mean ± S.E.M. Data for males and females were analysed separately. Differences between groups were analysed by one-way ANOVA, followed by Duncan's multiple range test for multiple *post hoc* comparisons. In all cases, $P<0.05$ was considered statistically significant.

## Results

### *In utero* DEHP exposure induces glucose and insulin intolerance

The fasting glucose levels were increased in *in utero* DEHP-exposed groups compared with control rats (Table 2). After glucose challenge, blood glucose concentration of DEHP-exposed groups was persistently higher than that of the control group (Fig. 1A). DEHP causes significant dose-dependent decline in fasting serum insulin levels, lean body weight and gastrocnemius muscle glycogen concentration (Table 2), but increased fat weight at 10 and 100 mg doses was noted in both male and female rat offspring. After insulin load, blood glucose levels decreased slowly in control but remained high in the experimental groups (Fig. 1B), indicating that embryonic DEHP exposure reduced the insulin sensitivity in postnatal life irrespective of sex.

### DEHP exacerbated insulin signal transduction in $F_1$ offspring

Insulin binding was significantly decreased dose dependently in gastrocnemius muscle of $F_1$ offspring (PND60) of both sexes due to transient gestational (ED9–ED21) DEHP exposure when compared with control (Table 2). We also measured the key molecules involved in insulin signalling in insulin-sensitive gastrocnemius muscle. *Insr* mRNA (Fig. 1C), PM INSR protein (Fig. 1D) and its 1162/1163 tyrosine phosphorylated forms (Fig. 1E) were reduced significantly compared with control levels in a



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**Table 2** List of metabolic parameters

| Parameters | Male | | | | Female | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | 1 mg DEHP | 10 mg DEHP | 100 mg DEHP | Control | 1 mg DEHP | 10 mg DEHP | 100 mg DEHP |
| Lean body weight (g) | 108.21±1.22 | 104.19±1.09* | 94.69±1.20*,† | 87.80±1.57*,†,‡ | 105.60±1.56 | 97.22±1.25* | 88.17±1.55*,† | 83.81±1.65*,†,‡ |
| Fat weight (g) | 9.59±0.08 | 9.78±0.07 | 9.99±0.09* | 10.19±0.09*,† | 9.70±0.01 | 9.95±0.07 | 10.26±0.17* | 10.43±0.17*,† |
| Mes+RP+UG | | | | | | | | |
| Fasting blood glucose (mg/dl) | 81.16±2.07 | 94.33±2.41* | 104.5±2.40*,† | 115.66±2.24*,†,‡ | 83.16±2.32 | 100±2.92* | 120.16±2.86*,† | 123.66±2.40*,†,‡ |
| Insulin (μIU/ml) | 19.4±0.47 | 15.2±0.60* | 10±0.52*,† | 6.9±0.49*,†,‡ | 19.7±0.60 | 15.5±0.48* | 10.8±0.63*,† | 6±0.57*,†,‡ |
| Insulin-binding protein (fmol/mg) | 307±8.8 | 267±8.8* | 237±9.27*,† | 200±5.7*,†,‡ | 297±8.8 | 255±10.4* | 223±8.8*,† | 190±5.8*,†,‡ |
| c.p.m. of $^{14}$C-2-deoxyglucose uptake/10 mg tissue | 1183±44 | 941±29* | 832±21*,† | 683±15*,†,‡ | 1042±22 | 909±31* | 753±29*,† | 753±23*,† |
| c.p.m. of $^{14}CO_2$ released/10 mg tissue | 1660±31 | 1452±30* | 1266±43*,† | 1046±31*,†,‡ | 1619±42 | 1322±36* | 1157±23*,† | 1145±29*,† |
| Glycogen (mg/g wet tissue) | 11.5±0.29 | 9.3±0.20* | 8.4±0.20*,† | 6.8±0.39*,†,‡ | 10.1±0.24 | 8.9±0.17* | 7.8±0.44*,† | 7.5±0.32*,† |

Each value represents mean ± S.E.M. of six animals. Significance at $P<0.05$: *, compared with control; †, compared with 1 mg DEHP and ‡, compared with 10 mg DEHP. Sum of the relative to 100 g body weight of three visceral fat pads namely mesenteric (Mes), retroperitoneal (RP) and urogenital (UG).

dose-dependent manner upon transient gestational (ED9–ED21) exposure to DEHP in both male and female offspring.

*Irs1* mRNA in the gastrocnemius muscle of rat $F_1$ offspring at PND60 was unaltered upon transient gestational exposure to DEHP treatment (Fig. 2A). In contrast to mRNA, IRS1 protein levels in males exposed to 10 and 100 mg of DEHP showed a significant decrease. In contrast to the results found in males, all the doses of DEHP caused a significant reduction in IRS1 protein in females (Fig. 2B).

pIRS1$^{Tyr632}$ level was significantly reduced by all doses, but there was no difference among treatment groups compared with controls in male offspring. However, in female offspring, there was no change in the 1 mg group compared with controls but a significant decrease at doses of 10 and 100 mg was noted (Fig. 2C). Unlike tyrosine phosphorylation, pIRS1$^{Ser636/639}$ was significantly increased by the 100 mg dose in both male and female offspring, but no statistically significant effect was observed with the 1 and 10 mg doses (Fig. 2D). Cytosol HDAC2 protein level was increased dose-dependently in DEHP-treated muscle irrespective of sex compared with controls (Fig. 2E).

*Akt* (*Akt1*) mRNA expression in the gastrocnemius muscle of $F_1$ offspring at PND60 was reduced significantly in a dose-dependent manner upon *in utero* exposure to DEHP in both male and female offspring (Fig. 3A) compared with control. AKT protein also followed the same trend (Fig. 3B). pAkt$^{Ser473}$ level was significantly reduced by all doses, but there was no difference between 1 and 10 mg compared with controls in male offspring. However, in female offspring, there was no alteration in the 1 and 10 mg groups compared with controls, but a significant decrease at the 100 mg dose was prominent (Fig. 3C). pAkt$^{Tyr315/316/312}$ reflected the trend for total Akt level (Fig. 3E). The pAkt$^{Thr308}$ level was significantly decreased at 10 and 100 mg doses of DEHP treatment in both male and female offspring, but no significant alteration was observed in the 1 mg DEHP-treated group compared with controls (Fig. 3D).

PTEN protein level was significantly elevated in gastrocnemius muscle, but there was no dose-dependent difference among treatment groups when compared with controls (Fig. 4A). Transient gestational exposure to DEHP significantly decreased the ARRB2 protein level in the gastrocnemius muscle of pubertal $F_1$ male and female rat offspring at PND60 in 10 and 100 mg groups but was not affected in the 1 mg DEHP-treated group (Fig. 4D). A significant decline in the level of SRC protein (Fig. 4E) was observed in both sexes. The MTOR protein level was significantly decreased dose-dependently in the

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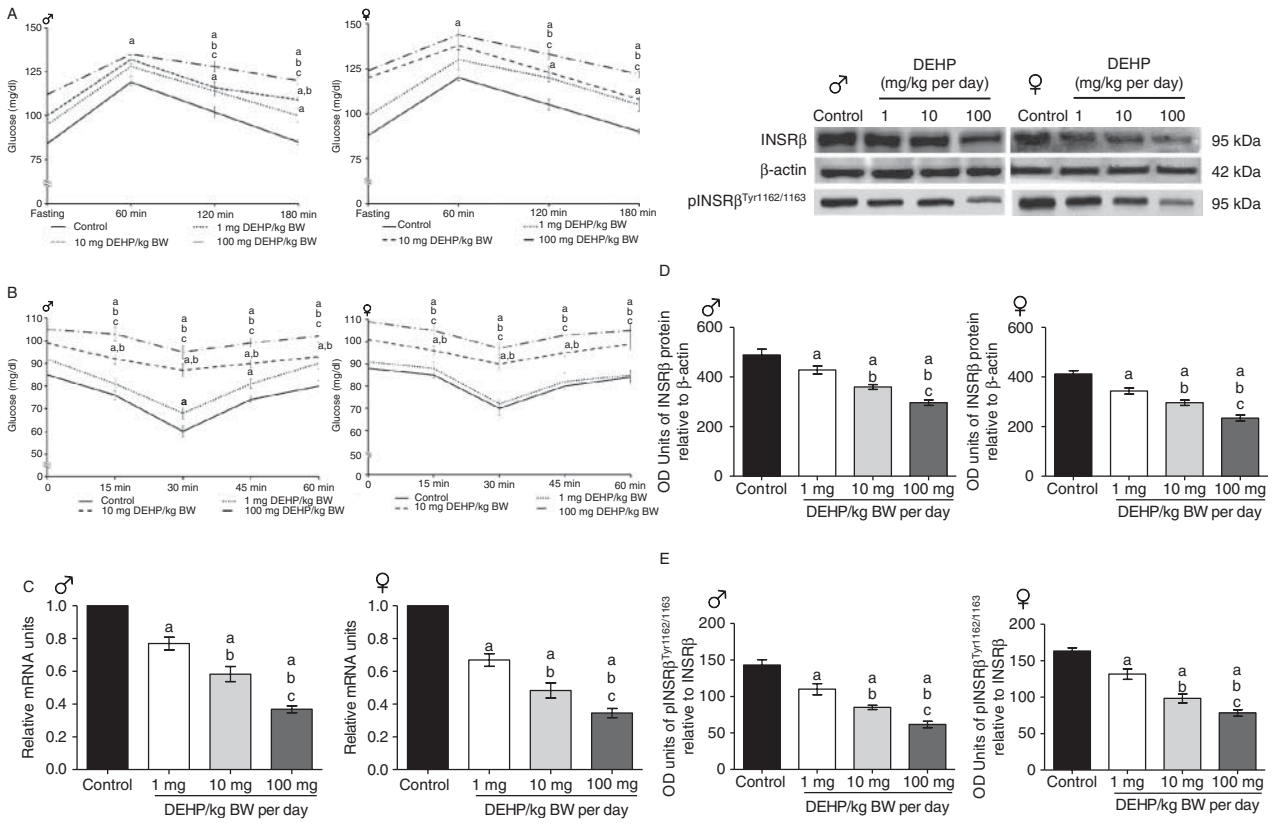


**Figure 1**

Effects of *in utero* DEHP exposure on oral glucose tolerance (A) and insulin tolerance (B) in male (♂) and female (♀) offspring at PND60; blood glucose level was checked before and after glucose and insulin administration. Gastrocnemius muscle total RNA was immediately extracted and converted into cDNA. The mRNA of the insulin receptor (*Insr*) gene was analysed by real-time PCR using SYBR Green Dye and protein expression by western blotting. Target gene expression was normalised to *Actb* and the results are expressed as fold change from control values (C). Protein levels were quantified using densitometry analysis and are expressed in OD units relative to INSRβ protein at plasma membrane (D). β-actin was used as an internal control. pINSRβ$^{Tyr1162/1163}$ was normalised to INSRβ protein (E). Immunoreactive bands were detected with an ECL reagent in chemidocumentation using the Chemi Doc XRS Imaging System, Bio-Rad. Values represent the mean ± S.E.M. of six male and six female offspring. Significance at $P<0.05$: a, compared with control; b, compared with 1 mg and c, compared with 10 mg DEHP kg per day.

DEHP-exposed groups (Fig. 4C). Surprisingly, no alteration was found in PDK1 protein levels (Fig. 4B).

Even though an effect of DEHP was not observed on AS160 (TBC1D4) protein level when compared with the control group (Fig. 5A), the pAS160$^{Thr642}$ level was significantly reduced in the 100 mg DEHP-treated group. No change was observed in the 1 and 10 mg DEHP-treated groups compared with controls in female offspring (Fig. 5B), but the pAS160$^{Thr642}$ level was dose-dependently decreased in gastrocnemius muscle of rat $F_1$ male offspring at PND60 (Fig. 5B). Male offspring had a significantly lower ACTN4 protein level in all groups compared with control. Female offspring showed no alteration in the 1 mg DEHP-treated group but a significant decrease in the 10 and 100 mg DEHP-treated groups was recorded (Fig. 5E). RAB13 protein level was markedly decreased in gastrocnemius muscle of $F_1$ offspring (PND60) due to transient gestational DEHP exposure in a dose-dependent manner compared with controls (Fig. 5D), whereas RAB8A showed a marked decrease in the 10 and 100 mg DEHP-treated groups only (Fig. 5C).

## Changes in expression, post-translational modification and localisation of GLUT4 upon *in utero* DEHP exposure

Among the isoforms of GLUT proteins, GLUT4 is the one which is insulin responsive/sensitive. Both male and female rat $F_1$ offspring showed a significant dose-dependent decline in *Glut4* mRNA expression compared with the control group (Fig. 6A). Cytosolic GLUT4 protein level (Fig. 6B) also followed the same trend as mRNA. However, pGLUT4$^{Ser488}$ was significantly increased in male and female offspring exposed to 10 and 100 mg DEHP but no significant alteration was observed in the 1 mg DEHP-treated group compared with the controls (Fig. 6C). PM GLUT4 protein level was significantly reduced in all the



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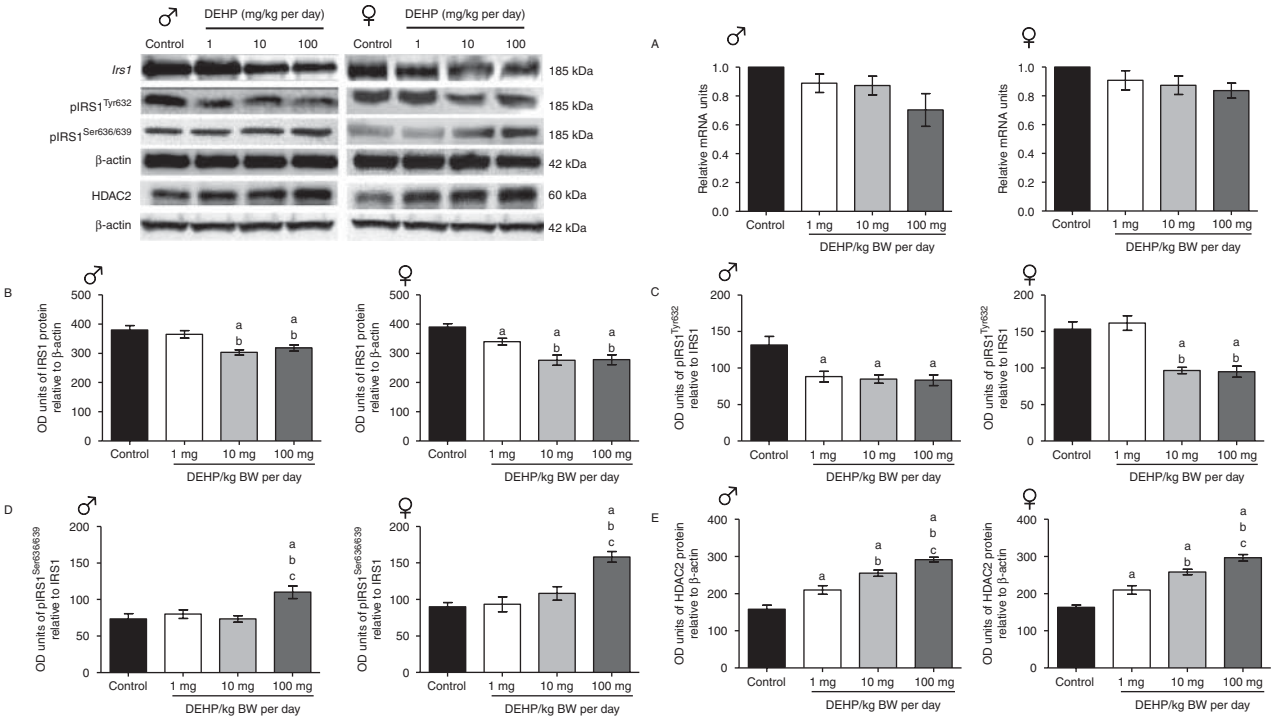


**Figure 2**

Effects of developmental DEHP exposure on insulin receptor substrate 1 (*Irs1*) mRNA (A), IRS1 protein (B), pIRS1$^{Tyr632}$ (C), pIRS1$^{Ser636/639}$ (D) and cytosol Histone deacetylase 2 (HDAC2; E) levels in the gastrocnemius muscle of male (♂) and female (♀) offspring at PND60. Gastrocnemius muscle total RNA was immediately extracted and converted into cDNA. The mRNA of *Irs1* was analysed by real-time PCR using SYBR Green Dye and protein expression by western blotting. Target gene expression was normalised to *Actb* and the results are expressed as fold change from control. Total protein concentration was determined before western blot analysis. Protein levels were quantified using densitometry analysis and are expressed in OD units relative to IRS1 protein and β-actin was used as an internal control. Phosphorylated forms were normalised to IRS1 protein. Immunoreactive bands were detected with an ECL reagent in chemi-documentation using the Chemi Doc XRS Imaging System, Bio-Rad. Values represent the mean ± S.E.M. of six male and six female offspring. Significance at $P<0.05$: a, compared with control; b, compared with 1 mg and c, compared with 10 mg DEHP kg per day.

experimental groups in a dose-dependent manner compared with the coeval control groups (Fig. 7A).

Figure 7B shows the effect of DEHP treatment on GLUT4 protein as observed by immunofluorescence. Intense GLUT4 staining was apparent in the PM of gastrocnemius muscle (Fig. 7B; magnification, 40×). GLUT4 staining was observed in the 1, 10 and 100 mg DEHP-exposed groups, but the intensity was decreased in a dose-dependent manner in the PM as well as cytosol region. These results are consistent with those of the immunoblotting analyses of GLUT4 protein in cytosol and PM fractions (Figs 6B and 7A).

## Expression and binding of transactivating nuclear factors MYOD and HDAC2 towards *Glut4*

Nuclear concentration of MYOD (Fig. 8A) and SREBP1c proteins were significantly decreased (Fig. 8B) in experimental groups, but HDAC2 showed an increase (Fig. 8C). ChIP assay demonstrated a significant increase in the binding of HDAC2 (repressor) to *Glut4* (−836 to −452 bp distal promoter region) in DEHP-exposed groups compared with control (Fig. 8E) and the same was observed in both sexes. In contrast, the same region of *Glut4*, which has the MYOD (enhancer)-binding site, exhibited low level binding of the MYOD nuclear factor in the DEHP-exposed male offspring in a dose-dependent manner compared with coeval controls (Fig. 8D). Female offspring showed reduced MYOD interaction towards *Glut4* in all the experimental groups at PND60 (Fig. 8D).

## Global DNA methylation and gene-specific *Glut4* promoter region methylation in gastrocnemius muscle are altered by developmental DEHP exposure

To further evaluate the role of epigenetic alterations in the modulation of insulin signalling, global DNA methylation changes were assessed using a DNA Methylation EIA Kit.



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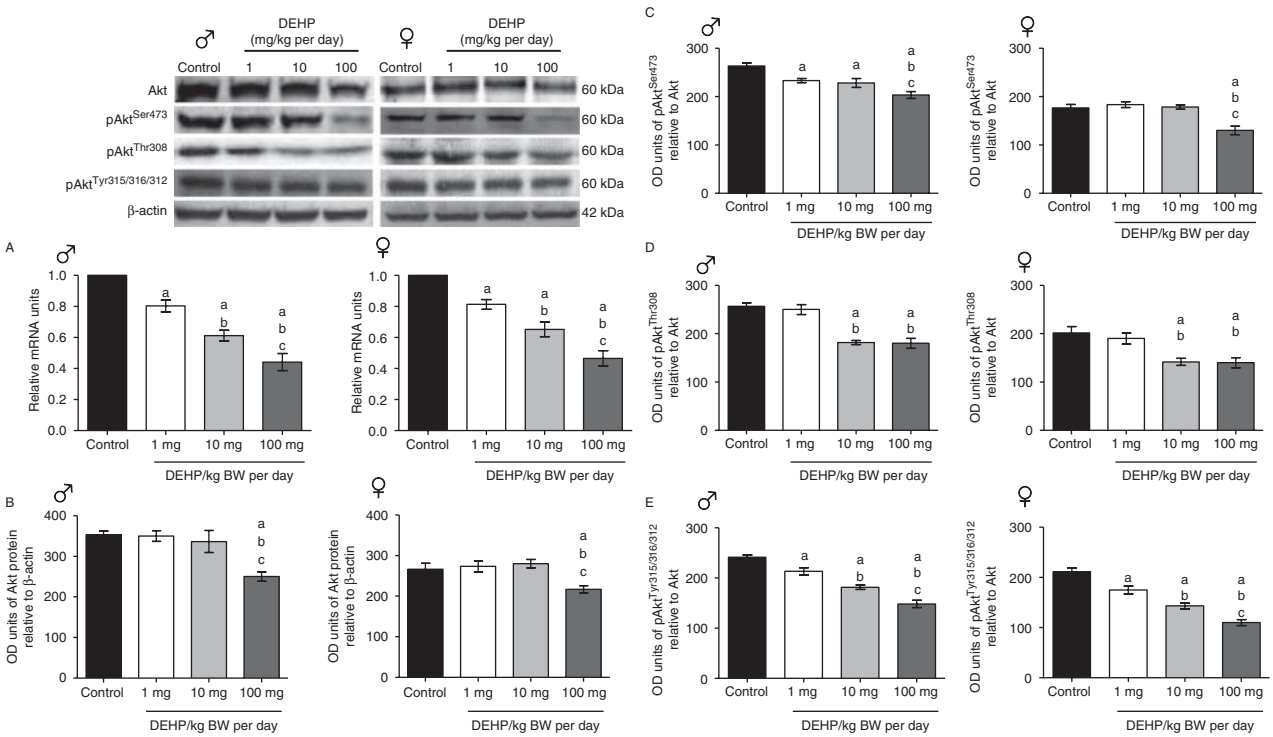


**Figure 3**

Effects of gestational DEHP exposure on *Akt* mRNA (A); AKT protein (B); $pAkt^{Ser473}$ (C); $pAkt^{Thr308}$ (D) and $pAkt^{Tyr315/316/312}$ (E) levels in the gastrocnemius muscle of male (♂) and female (♀) offspring at PND60. Gastrocnemius muscle total RNA was immediately extracted and converted into cDNA. The expression of *Akt* mRNA was analysed by real-time PCR using SYBR Green Dye and protein expression by western blotting. Target gene mRNA was normalised to *Actb* expression. Results are expressed as fold change from control values. Total protein concentration was determined before western blot analysis. Protein levels were quantified using densitometry analysis and are expressed in OD units of AKT protein relative to β-actin. Phosphorylated forms were normalised with AKT protein. Immunoreactive bands were detected with an ECL reagent in chemidocumentation using the Chemi Doc XRS Imaging System, Bio-Rad. Values represent mean ± S.E.M. of six male and six female offspring. Significance at $P<0.05$: a, compared with control; b, compared with 1 mg and c, compared with 10 mg DEHP kg per day.

5-Methyl-2′-deoxycytidine level in gastrocnemius muscle was significantly increased in a dose-dependent manner upon transient DEHP exposure compared with controls (Fig. 9B). To evaluate the levels of methylation of the CpG island, MSP was conducted with primers listed in Table 1 to screen for possible methylation changes in *Glut4* in the gastrocnemius muscle. Methylation was increased in the *Glut4* MYOD-binding site in response to DEHP exposure irrespective of doses and sex at PND60 (Fig. 9A).

## Developmental DEHP exposure up-regulates expression of DNMTs in the gastrocnemius muscle

*De novo* DNMTs are responsible for the addition of new methyl groups to DNA. To determine whether the DEHP-induced gene-specific and global DNA hypermethylation is associated with increased DNMT levels, the mRNA and protein levels of *Dnmt1*, *Dnmt3a*, *Dnmt3b* and *Dnmt3l* in the gastrocnemius muscle of DEHP-exposed $F_1$ rat offspring at PND60 were studied. The level of *Dnmt1* mRNA was increased in both males and females when compared with controls (Fig. 10A). Unlike mRNA levels, a dose-dependent significant increase in DNMT1 protein was observed in both male and female DEHP-exposed offspring (Fig. 10E). Interestingly, *Dnmt3a*/*Dnmt3b* mRNA and protein levels were elevated dose dependently (Fig. 10B, C and F). However, *Dnmt3l* mRNA and protein levels were unaltered compared with the control group (Fig. 10D and G).

## Gastrocnemius muscle glucose uptake and oxidation were impaired by developmental DEHP exposure

The eventual drive of insulin signalling is stimulation of glucose uptake from the circulation and subsequent oxidation at target tissues. To gain insight into the



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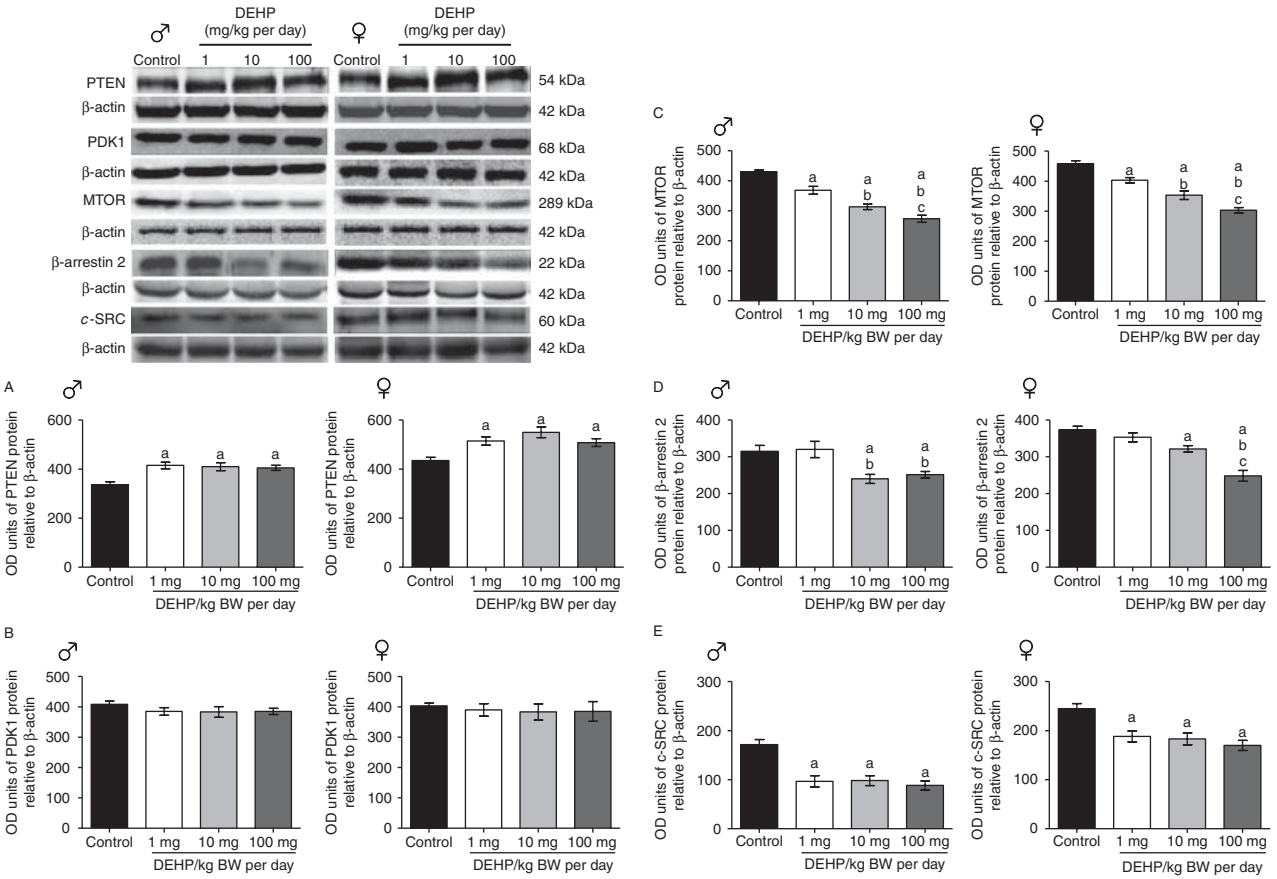


**Figure 4**

Effects of gestational DEHP exposure on PTEN (A), PDK1 (B), MTOR (C), ARRB2 (D) and c-SRC (E) protein levels in the gastrocnemius muscle of male (♂) and female (♀) offspring at PND60. Total protein concentrations were determined before western blot analysis. Protein levels were quantified using densitometry analysis and are expressed as relative OD units of protein normalised against β-actin. Immunoreactive bands were detected with an ECL reagent in chemidocumentation using the Chemi Doc XRS Imaging System, Bio-Rad. Values represent the mean ± S.E.M. of six male and six female offspring. Significance at $P<0.05$: a, compared with control; b, compared with 1 mg and c, compared with 10 mg DEHP kg per day.

influence of developmental DEHP exposure on these processes, $^{14}$C-2-deoxyglucose uptake and $^{14}$C-glucose oxidation were studied. DEHP-exposed male and female $F_1$ offspring showed a significant dose-dependent decline in glucose uptake and oxidation (Table 2). This observation is in line with the decreased PM GLUT4.

## Discussion

In response to elevated blood glucose, insulin has a pleiotropic biological effect in virtually all tissues in order to control glucose homoeostasis. In this study, we observed a decrease in insulin and elevated fasting blood glucose level along with impaired glucose and insulin tolerances and reduced glycogen concentrations at PND60 of $F_1$ offspring exposed to DEHP. Results from previous studies have indicated that DEHP impairs blood glucose regulation (Gayathri *et al.* 2004, Stahlhut *et al.* 2007, Srinivasan *et al.* 2011, Svensson *et al.* 2011, Rajesh *et al.* 2013) in rats and humans. In addition, we found significantly lower lean body weight with higher fat mass at PND60. It has been shown previously that developmental DEHP exposure maintained relatively lighter body weight up to PND190 (Lin *et al.* 2011). Furthermore, mono-(2-ethylhexyl)phthalate, the primary metabolite of DEHP, promotes adipogenesis (Hao *et al.* 2012). This might be one of the reasons for the increased fat mass observed in this study. The current data do support our primary hypothesis that *in utero* exposure to DEHP affects the glucose metabolism and insulin sensitivity of the $F_1$ offspring.

Subsequently, we measured the expression of important molecules involved in skeletal muscle insulin signalling, which showed an alteration at PND60 due to



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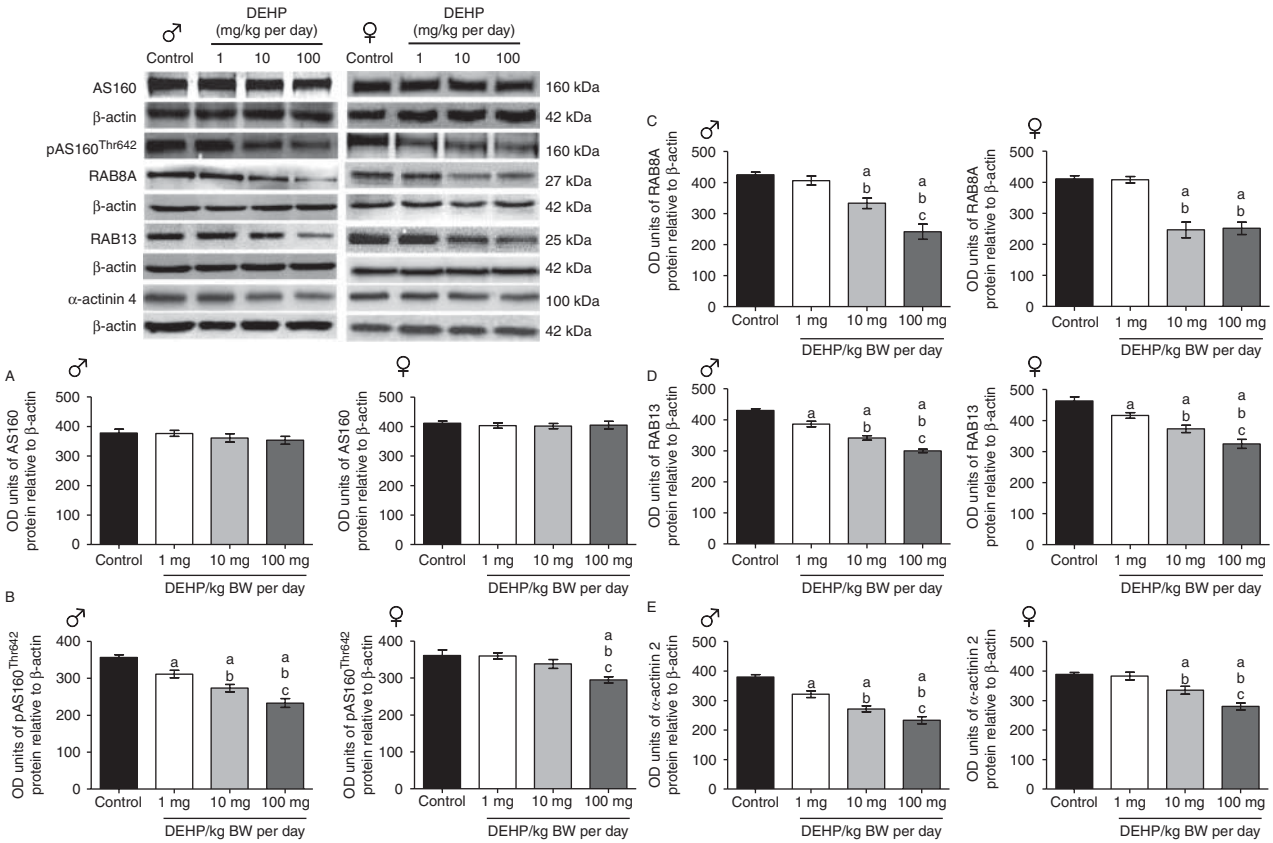


**Figure 5**

Effects of *in utero* DEHP exposure on AS160 (A), pAS160$^{Thr642}$ (B), RAB8A (C), RAB13 (D) and ACTN4 (E) protein levels in the gastrocnemius muscle of male (♂) and female (♀) offspring at PND60. Total protein concentration was determined before western blot analysis. Protein levels were quantified using densitometry analysis and are expressed in relative OD units of protein normalised against β-actin. The phosphorylated form was normalised to as160 protein. Immunoreactive bands were detected with an ECL reagent in chemidocumentation using the Chemi Doc XRS Imaging System, Bio-Rad. Values represent the mean ± S.E.M. of six male and six female offspring. Significance at $P<0.05$: a, compared with control; b, compared with 1 mg and c, compared with 10 mg DEHP kg per day.

*in utero* DEHP exposure. InsR is the master switch for insulin signal transduction and, therefore, alterations of the INSR expression and kinase activity account for the insulin-resistant phenotype (Pessin & Saltiel 2000). In the current investigation, the DEHP-exposed groups showed significantly reduced *Insr* mRNA levels and PM INSR protein and its phosphorylation at Tyr1162/1163 sites. This may be due to impaired *Insr* gene expression.

IRS1 is a major docking substrate for InsR and other tyrosine kinases. It plays a vital role in eliciting many of insulin's actions, including binding and activation of phosphatidylinositol (PI) 3-kinase and the subsequent increase in glucose transport (Rondinone *et al.* 1997). Unaltered *Irs1* mRNA was observed but the decrease in IRS1 protein levels indicates that the site of action of DEHP may be elsewhere at the translational or post-translational level. Acetylation of IRS1 is permissive for tyrosine phosphorylation and facilitates insulin-stimulated signal transduction (Kaiser & James 2004). Interestingly, *in utero* DEHP treatment elevated HDAC2 levels in the cytosol with diminished IRS1$^{Tyr632}$ phosphorylation levels when compared with controls irrespective of sex. However, phosphorylated IRS1$^{Ser636/639}$, which impedes binding of downstream effectors, and the negative regulator (PTEN) of intracellular levels of $PIP_3$ were increased in DEHP-exposed groups. The unaltered *Irs1* mRNA indicates that changes observed in protein may be an outcome of specific changes at the level of translational/post-translational modifications. Rather, the decrease in IRS1 protein may also be the result of increased degradation of IRS1. Ser336/639/307 is a well-recognised phosphorylation site in IRS1, and the preponderance of evidence indicates that it can negatively influence insulin signalling via increased ubiquitin–proteasome



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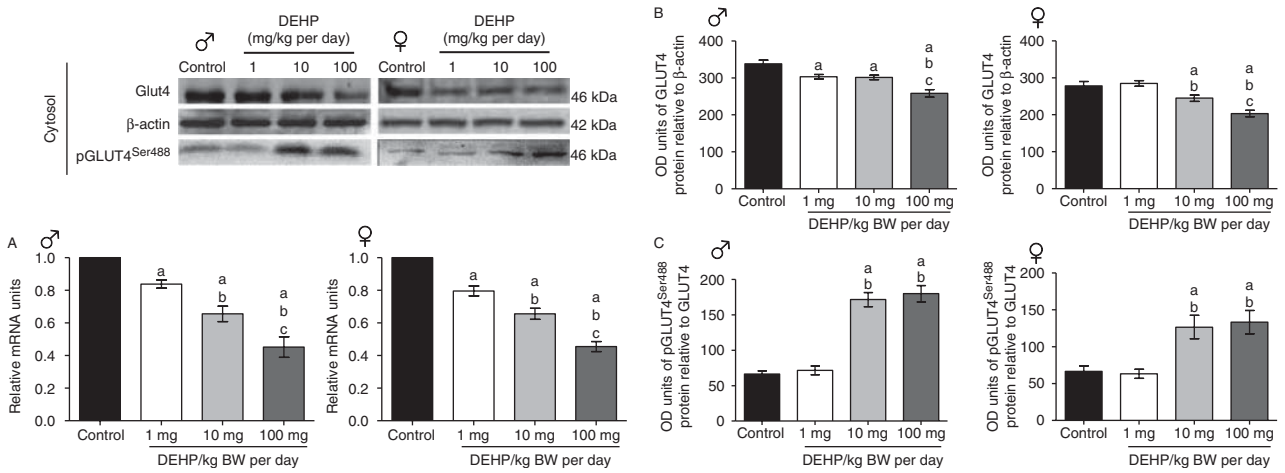


**Figure 6**

Effects of gestational DEHP exposure on *Glut4* mRNA (A), cytosol GLUT4 protein (B) and pGLUT4$^{Ser488}$ (C) levels in the gastrocnemius muscle of male (♂) and female (♀) offspring at PND60. Gastrocnemius muscle total RNA was immediately extracted and converted into cDNA. *Glut4* mRNA was analysed by real-time PCR using SYBR Green Dye and protein expression by western blotting. *Glut4* mRNA was normalised to *Actb*. Results are expressed as fold change from control values. Cytosol protein concentration was determined before western blot analysis. Protein levels were quantified using densitometry analysis and are expressed in OD units relative to GLUT4 protein normalised against β-actin. The phosphorylated form was normalised to cytosol GLUT4 protein. Immunoreactive bands were detected with an ECL reagent in chemidocumentation using the Chemi Doc XRS Imaging System, Bio-Rad. Values represent the mean ± S.E.M. of six male and six female offspring. Significance at $P<0.05$: a, compared with control; b, compared with 1 mg and c, compared with 10 mg DEHP kg per day.

degradation of IRS1, reduced tyrosine phosphorylation and subsequent alteration of insulin-induced PI3-kinase activation (Bouzakri *et al*. 2003).

Significant decreases in ARRB2 and c-SRC protein levels were observed in DEHP-exposed groups. In this regard, it has been shown *in vivo* that *Arrb2* down-regulation/knockdown contributes to the development of insulin resistance and progression of T2D by disturbing Akt and c-Src recruitment to the insulin receptor (Luan *et al*. 2009).

*Akt* mRNA levels were down-regulated in DEHP-exposed groups in both the sexes. Surprisingly, *in utero* DEHP treatment significantly decreased the levels of total AKT protein and activity-dependent Ser473 phosphorylation in a dose-dependent manner and increased the miRNA143 levels. Furthermore, phosphorylation at Thr308 and Tyr315/316/312 residues in DEHP-exposed offspring was significantly reduced compared with controls. Phosphorylation of Akt at Tyr315/326 by Src enhances Akt serine/threonine phosphorylation and is a prerequisite for full Akt activation (Jiang & Qiu 2003). The reduction in Akt phosphorylation may be due to deficiency of β-arrestin 2, c-Src and mTOR. AS160, an Akt substrate of 160 kDa, contains a RAB GTPase-activating protein (GAP) domain. Unaltered total AS160 but diminished pAS160$^{Thr642}$ levels in gestational DEHP-exposed $F_1$ offspring were observed in the current study, indicating that phosphorylation of AS160 is dependent on the PI3K/Akt pathway. It has been proposed that Akt-induced phosphorylation of AS160 inhibits its GAP activity, leading to an increase in the active GTP-bound form of the AS160-targeting RAB (AGFG1) proteins for vesicle trafficking (Miinea *et al*. 2005). As insulin-induced translocation of GLUT4 needs a RAB in its active GTP-bound form, insulin-stimulated phosphorylation of AS160 is required for GLUT4 translocation (Sano *et al*. 2003). This observation is consistent with the reduced intensity of PM-bound GLUT4 immunofluorescence in DEHP-exposed groups.

GLUT4 exists in insulin-sensitive tissues, mainly skeletal muscles, and is thus the major transporter protein responsible for insulin-mediated whole-body glucose uptake. Translocation of GLUT4 is mediated through the insulin signalling pathway and any abnormality in this pathway leads to insulin resistance and in turn T2D (Watson *et al*. 2004). In this study, *Glut4* mRNA levels were down-regulated in developmental DEHP-exposed $F_1$ offspring. Furthermore, we examined epigenetic mechanisms responsible for changes in *Glut4* expression underlying cellular memory retention. The two processes that underlie epigenesis are DNA methylation and histone N-tail post-translational modification. SREBP1c, which activates *Glut4* expression by directly binding to the sterol response element in the *Glut4* promoter region



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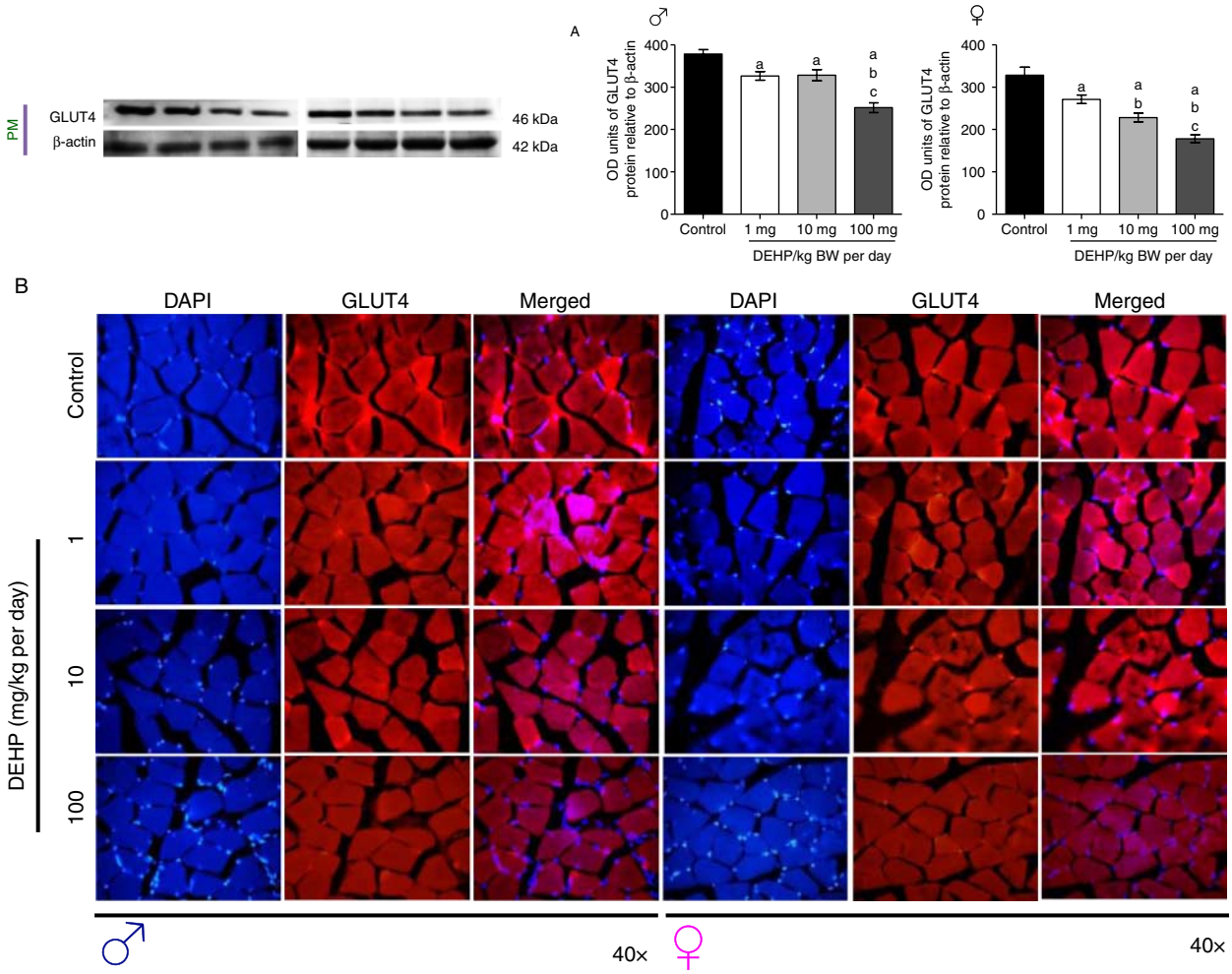


**Figure 7**

Effects of gestational DEHP exposure on plasma membrane (PM) GLUT4 (A) level in the gastrocnemius muscle of male (♂) and female (♀) offspring at PND60. Fluorescence microscopy of gastrocnemius muscle sections from DEHP-exposed (ED9–ED21) offspring resulted in reduced GLUT4 immunostaining in both cytosol and PM, stained for GLUT4 (red) and DAPI (blue) shown at 40× magnification (B). PM protein concentration was determined before western blot analysis. Protein levels were quantified using densitometry analysis and are expressed in relative OD units of PM GLUT4 protein normalised against β-actin. Immunoreactive bands were detected with an ECL reagent in chemidocumentation using the Chemi Doc XRS Imaging System, Bio-Rad. Values represent the mean ± S.E.M. of six male and six female offspring. Significance at $P<0.05$: a, compared with control; b, compared with 1 mg and c, compared with 10 mg DEHP kg per day. A full colour version of this figure is available via http://dx.doi.org/10.1530/JOE-14-0111.

(Im *et al*. 2006), was down-regulated in the DEHP-exposed groups. MYOD is a DNA-binding protein which acts as a co-regulator of MEF2 (MEF2A) involved in *Glut4* transcription (Im *et al*. 2007). ChIP assay results indicated that the DEHP-exposed groups had decreased interaction between MEF2A and MYOD with diminished binding of MYOD; increased HDAC2 interaction with *Glut4* DNA in a dose-dependent manner indicates a stage of repressed gene transcription or tight chromatin structure. HDAC2 interaction with *Glut4* gene promoter results in co-repressor complex formation, which interferes the formation of co-activator complex. We further tested whether expression of DNMTs would contribute to global and gene-specific methylations, which impede the binding of MYOD to *Glut4*. DNMT1 and DNMT3A/DNMT3B were increased in the DEHP-exposed offspring along with global DNA methylation levels. Global DNA hypermethylation is associated with an increased risk of insulin resistance independent of established risk factors (Zhao *et al*. 2012).

There is also evidence that the DNA methylation memory is involved in maintaining gene expression patterns associated with insulin resistance in T2D; several genes involved in (glucose) metabolism have been shown to exhibit differential DNA methylation in their



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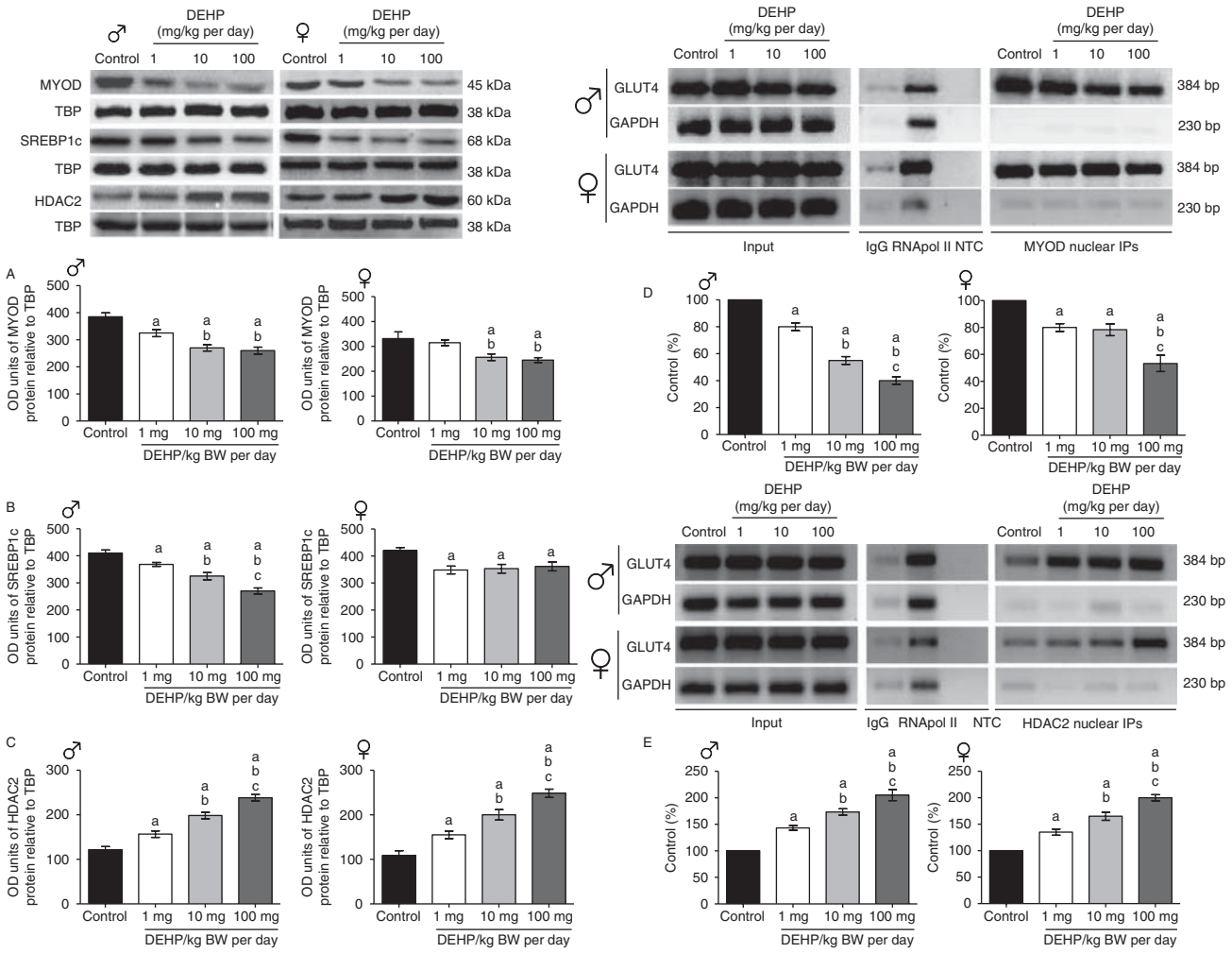


**Figure 8**

Effects of gestational DEHP exposure on MYOD (A), SREBP1c (B) and HDAC2 (C) protein levels in the gastrocnemius muscle of male (♂) and female (♀) offspring at PND60. Nuclear protein concentration was determined before western blot analysis. Protein levels were quantified using densitometry analysis and are expressed in relative OD units of protein normalised against TBP. Immunoreactive bands were detected with an ECL reagent in chemidocumentation using the Chemi Doc XRS Imaging System, Bio-Rad. MYOD (D) and HDAC2 (E) interaction towards *Glut4* 5′ upstream region (−836 to −452 bp). A representative 2% agarose gel (inverted image) was quantified by densitometric scanning (Bio-Rad), which demonstrates the input PCR *Glut4* and *Gapdh* control without an antibody (left panels), in the presence of non-specific (−) and anti-polymerase II (+) IgGs (middle panels), and ChIP assay demonstrating the 384-bp PCR *Glut4* DNA amplification product, which contains the MEF2 and MYOD-binding sites and the 230-bp PCR *Gapdh* DNA amplification product (serving as an internal control) obtained from MYOD (upper)/HDAC2 (lower) nuclear immunoprecipitates (IPs) (right panels). Quantification of the amplified 384-bp *Glut4* DNA product as a ratio to that of *Gapdh*, corrected for the input control and expressed as percentages of the control value. Differences among groups were assessed by the ANOVA followed by the SNK *post hoc* test. Values represent the mean±S.E.M. of six male and six female offspring. Significance at $P<0.05$: a, compared with control; b, compared with 1 mg and c, compared with 10 mg DEHP kg per day.

promoters, e.g. facilitative *Glut4*, the major GLUT in adipose and muscle tissues (Yokomori *et al.* 1999), and hypermethylation of the *Glut2* promoter suppressing its gene expression leading to reduced consumption of glucose (Ban *et al.* 2002). Expression of *Ins* gene is closely related to the level of methylation at its promoter (Kuroda *et al.* 2009) and an uncoupling protein (Carretero *et al.* 1998), a major candidate gene for the development of T2D. *In utero* glucose and insulin levels influence the risk of developing T2D later in life, independent of the maternal type of diabetes and therefore independent of genetic predisposition (Dabelea *et al.* 2000). This indicates the presence of a cellular memory in insulin target tissues. The results of these studies indicate that DNA methylation correlates with gene silencing and is consistent with this study of *Glut4* expression. In the current investigation,



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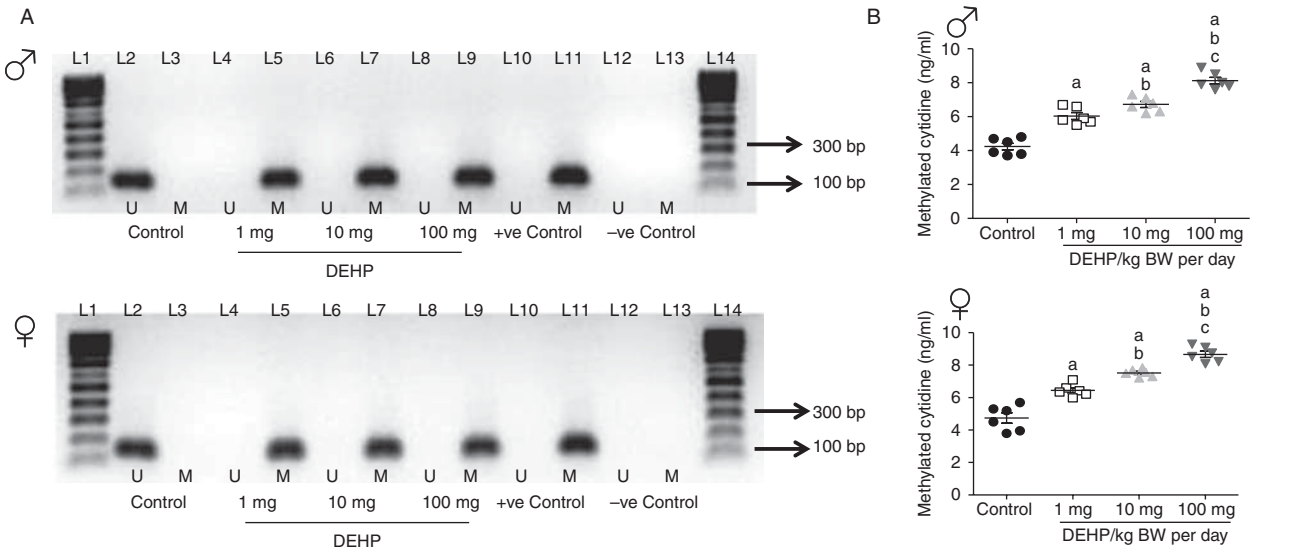


**Figure 9**

Effects of gestational DEHP exposure on methylation of CpG sites in *Glut4* in nucleotides −706 to −564 bp of the promoter region (in which the start codon of *Glut4* is defined as +1) (A) and global methylation level (B) in the gastrocnemius muscle of male (♂) and female (♀) offspring at PND60. Methylation-specific PCR (MSP) after bisulphite conversion of genomic DNA was performed with methylated-DNA-specific primers and non-methylated-DNA-specific primers. PCR products were run on 2% agarose gel pre-stained with ethidium bromide. PCR product size was 142 bp. L1 and L14 – 100 bp DNA ladder; L2, L4, L6, L8, L10 and L12 – unmethylated GLUT4 (U) and L3, L5, L7, L9, L11 and L13 – methylated GLUT4 (M). L2 and L3 – control; L4 and L5 – 1 mg; L6 and L7 – 10 mg; L8 and L9 – 100 mg DEHP *in utero*-exposed groups; L10 and L11 – *in vitro* methylated (*Sss*I methylase), bisulphite-treated rat gastrocnemius DNA was used as a positive control for PCR; L12 and L13 – Monk ($H_2O$) was used as a negative control for PCR. Global DNA methylation level was assayed using an EIA Kit and results are expressed as methylated cytidine level (ng/ml). Values represent the mean±S.E.M. of six male and six female offspring. Significance at $P<0.05$: a, compared with control; b, compared with 1 mg and c, compared with 10 mg DEHP kg per day.

the *Glut4* promoter in DEHP-exposed $F_1$ offspring was hypermethylated at MYOD-binding sites while GLUT4 protein expression was decreased, indicating a negative correlation between *Glut4* expression and methylation level of the CpG islands. It is inferred from this study that hypermethylation of the *Glut4* promoter leads to impaired *Glut4* expression.

This imprint of reduced *Glut4* expression may be due to recruitment of DNMT1/DNMT3A/DNMT3B enzymes into a co-repressor complex, which attracts HDAC2, resulting in histone modifications. Histone modifications consisting of de-acetylation of H3.K14 with a hierarchical progression into di-methylation of H3.K9 contribute to heterochromatin formation. This further recruits repressor proteins, such as the chromodomain-containing HP1α (CBX5; Zhang *et al*. 2002) and MEF2D, into the co-repressor complex that associates with the *GLUT4* promoter. In addition, heterochromatin precludes DNA binding of activators (MYOD and MEF2A) to the *Glut4* promoter. These epigenetic changes collectively diminished *Glut4* transcription at adulthood.

Subsequently, we explored the possible mechanism behind the defective GLUT4 translocation towards the PM. Phosphorylation of GLUT4 decreases its intrinsic activity whereas under normal circumstances, insulin promotes dephosphorylation of GLUT4, which may be stimulating its intrinsic activity (Lawrence *et al*. 1990). An increase in phosphorylation of GLUT4 was associated with a decrease in the ability of insulin to stimulate glucose uptake in adipocytes (Begum *et al*. 1993). In this study, phosphorylated $GLUT4^{Ser488}$ was increased significantly in DEHP-exposed $F_1$ offspring at 10 and 100 mg doses. This may be one of the factors responsible for the decreased PM GLUT4.

RAB proteins are small G proteins, which serve as important regulators of insulin-stimulated GLUT4 translocation to the PM. It interacts with myosin-Vb to mediate the final steps of insulin-stimulated GLUT4 storage vesicle (GSV) translocation to the PM (Ishikura & Klip 2008). Results from previous studies have indicated that Akt phosphorylation of AS160, a GAP for RAB proteins is required for GLUT4 translocation. Based on their presence in GLUT4 vesicles and activity as AS160 GAP substrates, RAB8A and RAB13 are candidate RABs. Among those RABs, only the knockdown of *Rab8A* or *Rab13* inhibited GLUT4 translocation (Ishikura & Klip 2008, Sun *et al*. 2010).



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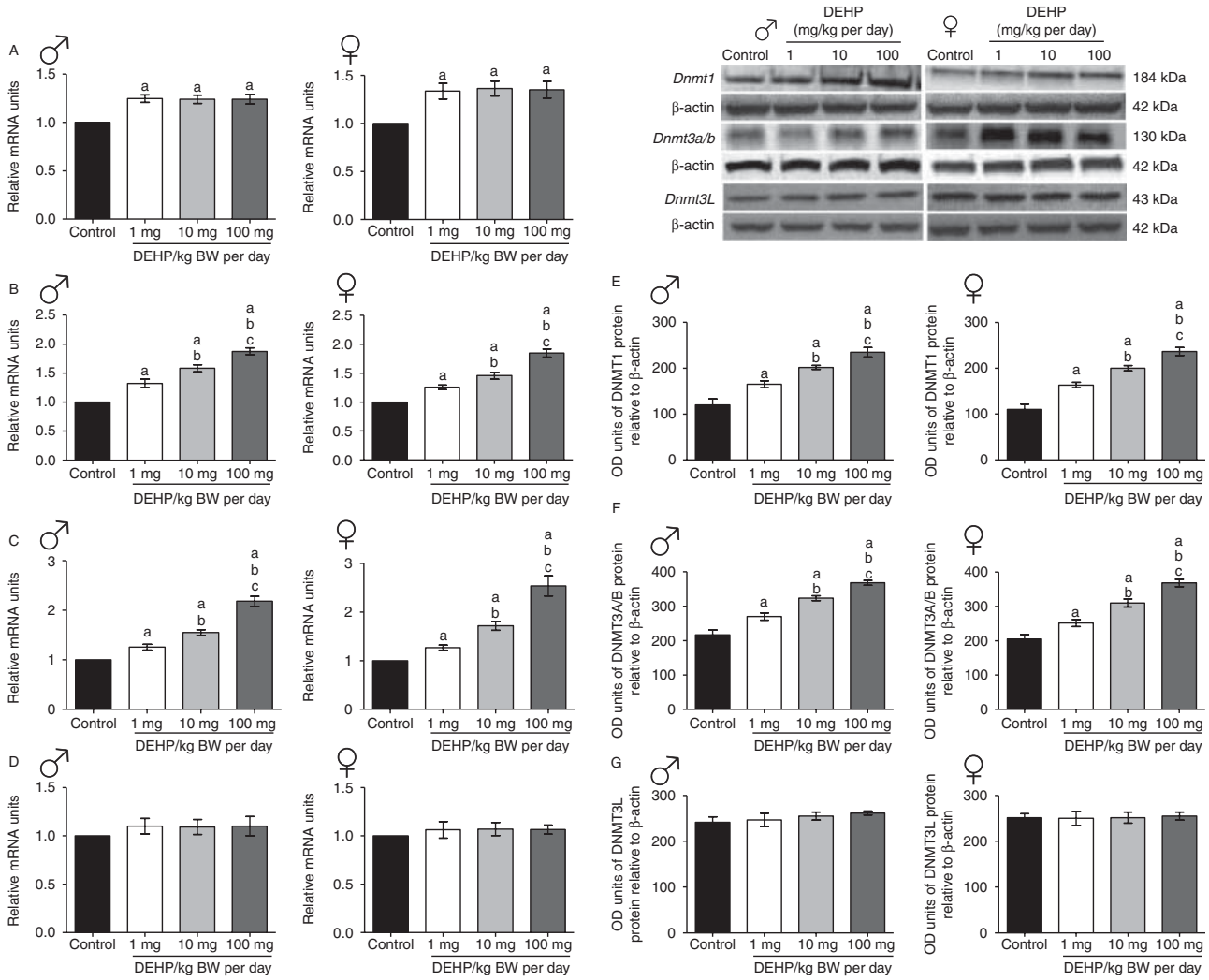


**Figure 10**

Effects of gestational DEHP exposure on *Dnmt1* (A), *Dnmt3a* (B), *Dnmt3b* (C) and *Dnmt3l* (D) mRNA levels and DNMT1 (E), DNMT3A/DNMT3B (F) and DNMT3L (G) protein levels in the gastrocnemius muscles of male (♂) and female (♀) offspring at PND60. Gastrocnemius muscle total RNA was immediately extracted and converted into cDNA. The expression of mRNA was analysed by real-time PCR using SYBR Green Dye and protein expression by western blotting. Target gene mRNA was normalised to *Actb*. Results are expressed as fold change from control values. Total protein concentration was determined before western blot analysis. Protein levels were quantified using densitometry analysis and are expressed in OD units of protein relative to β-actin. Immunoreactive bands were detected with an ECL reagent in chemidocumentation using the Chemi Doc XRS Imaging System, Bio-Rad. Values represent the mean ± S.E.M. of six male and six female offspring. Significance at $P<0.05$: a, compared with control; b, compared with 1 mg and c, compared with 10 mg DEHP kg per day.

RAB8A and RAB13 were under the direct control of AS160 in muscle cells. Consistent with this, gestational DEHP-exposed $F_1$ offspring showed a significant decline in RAB8A and RAB13 proteins, and this might have contributed to impaired translocation of GSVs. Furthermore, we observed a reduction in ACTN4 protein levels in the DEHP-treated groups. Results from a previous study have indicated that GLUT4 was colocalised with ACTN4 (Talior-Volodarsky *et al*. 2008). Results from *Actn4* knockdown studies have shown that GLUT4–actin colocalisation was prevented and GLUT4 localised in a tight perinuclear location. This emphasises the role of α-actinin 4 in contributing to GLUT4 traffic, probably by tethering GLUT4 vesicles to the cortical actin cytoskeleton (Talior-Volodarsky *et al*. 2008).

GLUT4-dependent glucose uptake and oxidation are essential functional processes, which supply energy to cells to execute diverse functions (Huang & Czech 2007). The rate of glucose oxidation in a cell depends on the rate of entry of glucose into the cell. In this study, both



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processes declined in a dose-dependent manner. Reduced PM GLUT4 levels lead to impaired glucose uptake and subsequent oxidation. It has been previously shown that DEHP exposure alters carbohydrate-metabolising enzymes (Martinelli *et al.* 2006).

Most of the parameters displayed a similar trend in both sexes. However, in few parameters (IRS1, IRS1$^{Tyr632}$, Akt$^{Ser473}$, AS160$^{Thr642}$ and MYOD), though the protein levels and its phosphorylation were decreased, the dose-dependent reduction was not similar in both sexes.

At the molecular level, insulin resistance results from defects in insulin signalling in peripheral tissues. Altogether, these results clearly indicate that the gestational DEHP exposure predisposes $F_1$ offspring to gluco-metabolic dysfunction at adulthood by down-regulating the expression of critical genes involved in the insulin signalling pathway in both sexes. Furthermore, DEHP-induced epigenetic alteration of *Glut4* appears to play a significant role in disposition towards such metabolic abnormalities.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**
This work was supported by the Government of India, Department of Science and Technology, New Delhi, India (Award letter no.: DST/AORC-IF/UPGRD/2013/110 dated 18-06-2013), in the form of a Junior/Senior Research Fellowship (SRF) to Mr P R under the DST-INSPIRE scheme and financial assistance to the Department of Endocrinology in the form of infrastructural facilities through the UGC-SAP-DRS, UGC-ASIST and DST-FIST programmes.

**Author contribution statement**
P R and K B conceived and designed the experiments, analysed the data, contributed reagents/materials/analysis tools and wrote the manuscript. P R performed the experiments.

**Acknowledgements**
The authors acknowledge Dr. A. Anne Williams, Examinations Customer Services Executive, British Council, Chennai, India for critical proof reading of the manuscript. The authors also acknowledge Dr. V. Sankar, Head, Department of Anatomy, University of Madras for continued support (cryosections).

## References

Anderson AM, Carter KW, Anderson D & Wise MJ 2012 Coexpression of nuclear receptors and histone methylation modifying genes in the testis: implications for endocrine disruptor modes of action. *PLoS ONE* **7** e34158. (doi:10.1371/journal.pone.0034158)

Anway MD & Skinner MK 2006 Epigenetic transgenerational actions of endocrine disruptors. *Endocrinology* **147** S43–S49. (doi:10.1210/en.2005-1058)

Anway MD, Cupp AS, Uzumcu M & Skinner MK 2005 Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* **308** 1466–1469. (doi:10.1126/science.1108190)

Ban N, Yamada Y, Someya Y, Miyawaki K, Ihara Y, Hosokawa M, Toyokuni S, Tsuda K & Seino Y 2002 Hepatocyte nuclear factor-1α recruits the transcriptional co-activator p300 on the GLUT2 gene promoter. *Diabetes* **51** 1409–1418. (doi:10.2337/diabetes.51.5.1409)

Begum N, Leitner W, Reusch JE, Sussman KE & Draznin B 1993 GLUT-4 phosphorylation and its intrinsic activity. Mechanism of $Ca^{2+}$-induced inhibition of insulin-stimulated glucose transport. *Journal of Biological Chemistry* **268** 3352–3356.

Bennett AM & Tonks NK 1997 Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinases. *Science* **278** 1288–1291. (doi:10.1126/science.278.5341.1288)

Bouzakri K, Roques M, Gual P, Espinosa S, Guebre-Egziabher F, Riou JP, Laville M, Le Marchand-Brustel Y, Tanti JF & Vidal H 2003 Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes. *Diabetes* **52** 1319–1325. (doi:10.2337/diabetes.52.6.1319)

Calafat AM, Needham LL, Silva MJ & Lambert G 2004 Exposure to di-(2-ethylhexyl) phthalate among premature neonates in a neonatal intensive care unit. *Pediatrics* **113** e429–e434. (doi:10.1542/peds.113.5.e429)

Carretero MV, Torres L, Latasa U, Garcia-Trevijano ER, Prieto J, Mato JM & Avila MA 1998 Transformed but not normal hepatocytes express UCP2. *FEBS Letters* **439** 55–58. (doi:10.1016/S0014-5793(98)01335-0)

Dabelea D, Hanson RL, Lindsay RS, Pettitt DJ, Imperatore G, Gabir MM, Roumain J, Bennett PH & Knowler WC 2000 Intrauterine exposure to diabetes conveys risks for type 2 diabetes and obesity: a study of discordant sibships. *Diabetes* **49** 2208–2211. (doi:10.2337/diabetes.49.12.2208)

Danaei G, Finucane MM, Lu Y, Singh GM, Cowan MJ, Paciorek CJ, Lin JK, Farzadfar F, Khang YH, Stevens GA *et al.* 2011 National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. *Lancet* **378** 31–40. (doi:10.1016/S0140-6736(11)60679-X)

Desvergne B, Feige JN & Casals-Casas C 2009 PPAR-mediated activity of phthalates: a link to the obesity epidemic? *Molecular and Cellular Endocrinology* **304** 43–48. (doi:10.1016/j.mce.2009.02.017)

Diamond J 2003 The double puzzle of diabetes. *Nature* **423** 599–602. (doi:10.1038/423599a)

Dombrowski L, Roy D, Marcotte B & Marette A 1996 A new procedure for the isolation of plasma membranes, T tubules, and internal membranes from skeletal muscle. *American Journal of Physiology* **270** E667–E676.

Dostal LA, Jenkins WL & Schwetz BA 1987 Hepatic peroxisome proliferation and hypolipidemic effects of di(2-ethylhexyl)phthalate in neonatal and adult rats. *Toxicology and Applied Pharmacology* **87** 81–90. (doi:10.1016/0041-008X(87)90086-X)

Faniband M, Lindh CH & Jonsson BA 2014 Human biological monitoring of suspected endocrine-disrupting compounds. *Asian Journal of Andrology* **16** 5–16. (doi:10.4103/1008-682X.122197)

Gauthier MS, Couturier K, Charbonneau A & Lavoie JM 2004 Effects of introducing physical training in the course of a 16-week high-fat diet regimen on hepatic steatosis, adipose tissue fat accumulation, and plasma lipid profile. *International Journal of Obesity and Related Metabolic Disorders* **28** 1064–1071. (doi:10.1038/sj.ijo.0802628)

Gayathri NS, Dhanya CR, Indu AR & Kurup PA 2004 Changes in some hormones by low doses of di (2-ethyl hexyl) phthalate (DEHP), a



http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-14-0111

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commonly used plasticizer in PVC blood storage bags & medical tubing. *Indian Journal of Medical Research* **119** 139–144.

Hao C, Cheng X, Xia H & Ma X 2012 The endocrine disruptor mono-(2-ethylhexyl) phthalate promotes adipocyte differentiation and induces obesity in mice. *Bioscience Reports* **32** 619–629. (doi:10.1042/BSR20120042)

Hatch EE, Nelson JW, Qureshi MM, Weinberg J, Moore LL, Singer M & Webster TF 2008 Association of urinary phthalate metabolite concentrations with body mass index and waist circumference: a cross-sectional study of NHANES data, 1999–2002. *Environmental Health* **7** 27. (doi:10.1186/1476-069X-7-27)

Hauser R & Calafat AM 2005 Phthalates and human health. *Occupational and Environmental Medicine* **62** 806–818. (doi:10.1136/oem.2004.017590)

Hu FB 2011 Globalization of diabetes: the role of diet, lifestyle, and genes. *Diabetes Care* **34** 1249–1257. (doi:10.2337/dc11-0442)

Huang S & Czech MP 2007 The GLUT4 glucose transporter. *Cell Metabolism* **5** 237–252. (doi:10.1016/j.cmet.2007.03.006)

Im SS, Kwon SK, Kang SY, Kim TH, Kim HI, Hur MW, Kim KS & Ahn YH 2006 Regulation of GLUT4 gene expression by SREBP-1c in adipocytes. *Biochemical Journal* **399** 131–139. (doi:10.1042/BJ20060696)

Im SS, Kwon SK, Kim TH, Kim HI & Ahn YH 2007 Regulation of glucose transporter type 4 isoform gene expression in muscle and adipocytes. *IUBMB Life* **59** 134–145. (doi:10.1080/15216540701313788)

Ishikura S & Klip A 2008 Muscle cells engage Rab8A and myosin Vb in insulin-dependent GLUT4 translocation. *American Journal of Physiology. Cell Physiology* **295** C1016–C1025. (doi:10.1152/ajpcell.00277.2008)

Jiang T & Qiu Y 2003 Interaction between Src and a C-terminal proline-rich motif of Akt is required for Akt activation. *Journal of Biological Chemistry* **278** 15789–15793. (doi:10.1074/jbc.M212525200)

Jirtle RL & Skinner MK 2007 Environmental epigenomics and disease susceptibility. *Nature Reviews. Genetics* **8** 253–262. (doi:10.1038/nrg2045)

Kaiser C & James SR 2004 Acetylation of insulin receptor substrate-1 is permissive for tyrosine phosphorylation. *BMC Biology* **2** 23. (doi:10.1186/1741-7007-2-23)

Kobayashi K, Miyagawa M, Wang RS, Suda M, Sekiguchi S & Honma T 2006 Effects of *in utero* and lactational exposure to di(2-ethylhexyl)phthalate on somatic and physical development in rat offspring. *Industrial Health* **44** 652–660. (doi:10.2486/indhealth.44.652)

Kraft LA & Johnson AD 1972 Epididymal carbohydrate metabolism. II. Substrates and pathway utilization of caput and cauda epididymal tissue from the rabbit, rat and mouse. *Comparative Biochemistry and Physiology. B, Comparative Biochemistry* **42** 451–461. (doi:10.1016/0305-0491(72)90261-1)

Krotkiewski M & Bjorntorp P 1976 The effect of progesterone and of insulin administration on regional adipose tissue cellularity in the rat. *Acta Physiologica Scandinavica* **96** 122–127. (doi:10.1111/j.1748-1716.1976.tb10177.x)

Kuroda A, Rauch TA, Todorov I, Ku HT, Al-Abdullah IH, Kandeel F, Mullen Y, Pfeifer GP & Ferreri K 2009 Insulin gene expression is regulated by DNA methylation. *PLoS ONE* **4** e6953. (doi:10.1371/journal.pone.0006953)

Latini G 2000 Potential hazards of exposure to di-(2-ethylhexyl)-phthalate in babies. A review. *Biology of the Neonate* **78** 269–276. (doi:10.1159/000014278)

Latini G, De Felice C, Presta G, Del Vecchio A, Paris I, Ruggieri F & Mazzeo P 2003 *In utero* exposure to di-(2-ethylhexyl)phthalate and duration of human pregnancy. *Environmental Health Perspectives* **111** 1783–1785. (doi:10.1289/ehp.6202)

Lawrence JC Jr, Hiken JF & James DE 1990 Phosphorylation of the glucose transporter in rat adipocytes. Identification of the intracellular domain at the carboxyl terminus as a target for phosphorylation in intact-cells and *in vitro*. *Journal of Biological Chemistry* **265** 2324–2332.

Lin Y, Wei J, Li Y, Chen J, Zhou Z, Song L, Wei Z, Lv Z, Chen X, Xia W *et al.* 2011 Developmental exposure to di(2-ethylhexyl) phthalate impairs endocrine pancreas and leads to long-term adverse effects on glucose homeostasis in the rat. *American Journal of Physiology. Endocrinology and Metabolism* **301** E527–E538. (doi:10.1152/ajpendo.00233.2011)

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ 1951 Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193** 265–275.

Luan B, Zhao J, Wu H, Duan B, Shu G, Wang X, Li D, Jia W, Kang J & Pei G 2009 Deficiency of a β-arrestin-2 signal complex contributes to insulin resistance. *Nature* **457** 1146–1149. (doi:10.1038/nature07617)

Mangala Priya V, Mayilvanan C, Akilavalli N, Rajesh P & Balasubramanian K 2014 Lactational exposure of phthalate impairs insulin signaling in the cardiac muscle of F1 female albino rats. *Cardiovascular Toxicology* **14** 10–20. (doi:10.1007/s12012-013-9233-z)

Martinelli MI, Mocchiutti NO & Bernal CA 2006 Dietary di(2-ethylhexyl) phthalate-impaired glucose metabolism in experimental animals. *Human & Experimental Toxicology* **25** 531–538. (doi:10.1191/0960327106het651oa)

Martinez-Arguelles DB, Culty M, Zirkin BR & Papadopoulos V 2009 *In utero* exposure to di-(2-ethylhexyl) phthalate decreases mineralocorticoid receptor expression in the adult testis. *Endocrinology* **150** 5575–5585. (doi:10.1210/en.2009-0847)

Martinez-Arguelles DB, Guichard T, Culty M, Zirkin BR & Papadopoulos V 2011 *In utero* exposure to the antiandrogen di-(2-ethylhexyl) phthalate decreases adrenal aldosterone production in the adult rat. *Biology of Reproduction* **85** 51–61. (doi:10.1095/biolreprod.110.089920)

Martinez-Arguelles DB, McIntosh M, Rohlicek CV, Culty M, Zirkin BR & Papadopoulos V 2013 Maternal *in utero* exposure to the endocrine disruptor di-(2-ethylhexyl) phthalate affects the blood pressure of adult male offspring. *Toxicology and Applied Pharmacology* **266** 95–100. (doi:10.1016/j.taap.2012.10.027)

Miinea CP, Sano H, Kane S, Sano E, Fukuda M, Peranen J, Lane WS & Lienhard GE 2005 AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. *Biochemical Journal* **391** 87–93. (doi:10.1042/BJ20050887)

Mushtaq M, Srivastava SP & Seth PK 1980 Effect of di-2-ethylhexyl phthalate (DEHP) on glycogen metabolism in rat liver. *Toxicology* **16** 153–161. (doi:10.1016/0300-483X(80)90045-1)

Neel BA & Sargis RM 2011 The paradox of progress: environmental disruption of metabolism and the diabetes epidemic. *Diabetes* **60** 1838–1848. (doi:10.2337/db11-0153)

Pessin JE & Saltiel AR 2000 Signaling pathways in insulin action: molecular targets of insulin resistance. *Journal of Clinical Investigation* **106** 165–169. (doi:10.1172/JCI10582)

Rajesh P & Balasubramanian K 2013 Di(2-ethylhexyl)phthalate exposure impairs insulin receptor and glucose transporter 4 gene expression in L6 myotubes. *Human & Experimental Toxicology* **33** 685–700. (doi:10.1177/0960327113506238)

Rajesh P, Sathish S, Srinivasan C, Selvaraj J & Balasubramanian K 2013 Phthalate is associated with insulin resistance in adipose tissue of male rat: role of antioxidant vitamins. *Journal of Cellular Biochemistry* **114** 558–569. (doi:10.1002/jcb.24399)

Rengarajan S, Parthasarathy C, Anitha M & Balasubramanian K 2007 Diethylhexyl phthalate impairs insulin binding and glucose oxidation in Chang liver cells. *Toxicology In Vitro* **21** 99–102. (doi:10.1016/j.tiv.2006.07.005)

Roe JH & Dailey RE 1966 Determination of glycogen with the anthrone reagent. *Analytical Biochemistry* **15** 245–250. (doi:10.1016/0003-2697(66)90028-5)

Rondinone CM, Wang LM, Lonnroth P, Wesslau C, Pierce JH & Smith U 1997 Insulin receptor substrate (IRS) 1 is reduced and IRS-2 is the main docking protein for phosphatidylinositol 3-kinase in adipocytes from subjects with non-insulin-dependent diabetes mellitus. *PNAS* **94** 4171–4175. (doi:10.1073/pnas.94.8.4171)

Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW & Lienhard GE 2003 Insulin-stimulated phosphorylation of a Rab GTPase-



http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-14-0111



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activating protein regulates GLUT4 translocation. *Journal of Biological Chemistry* **278** 14599–14602. (doi:10.1074/jbc.C300063200)

Schmittgen TD & Livak KJ 2008 Analyzing real-time PCR data by the comparative $C_T$ method. *Nature Protocols* **3** 1101–1108. (doi:10.1038/nprot.2008.73)

Shelby MD 2006 *NTP-CERHR monograph on the potential human reproductive and developmental effects of di (2-ethylhexyl) phthalate (DEHP)*. Research triangle Park, North Carolina: National Toxicology Program–Center For The Evaluation Of Risks To Human Reproduction.

Sinacore DR & Gulve EA 1993 The role of skeletal muscle in glucose transport, glucose homeostasis, and insulin resistance: implications for physical therapy. *Physical Therapy* **73** 878–891.

Singh S & Li SS 2012 Epigenetic effects of environmental chemicals bisphenol a and phthalates. *International Journal of Molecular Sciences* **13** 10143–10153. (doi:10.3390/ijms130810143)

Skinner MK, Manikkam M & Guerrero-Bosagna C 2011 Epigenetic transgenerational actions of endocrine disruptors. *Reproductive Toxicology* **31** 337–343. (doi:10.1016/j.reprotox.2010.10.012)

Srinivasan C, Khan AI, Balaji V, Selvaraj J & Balasubramanian K 2011 Diethyl hexyl phthalate-induced changes in insulin signaling molecules and the protective role of antioxidant vitamins in gastrocnemius muscle of adult male rat. *Toxicology and Applied Pharmacology* **257** 155–164. (doi:10.1016/j.taap.2011.08.022)

Stahlhut RW, van Wijngaarden E, Dye TD, Cook S & Swan SH 2007 Concentrations of urinary phthalate metabolites are associated with increased waist circumference and insulin resistance in adult U.S. males. *Environmental Health Perspectives* **115** 876–882. (doi:10.1289/ehp.9882)

Strakovsky RS & Pan YX 2012 *In utero* oxidative stress epigenetically programs antioxidant defense capacity and adulthood diseases. *Antioxidants & Redox Signaling* **17** 237–253. (doi:10.1089/ars.2011.4372)

Sun Y, Bilan PJ, Liu Z & Klip A 2010 Rab8A and Rab13 are activated by insulin and regulate GLUT4 translocation in muscle cells. *PNAS* **107** 19909–19914. (doi:10.1073/pnas.1009523107)

Svensson K, Hernandez-Ramirez RU, Burguete-Garcia A, Cebrian ME, Calafat AM, Needham LL, Claudio L & Lopez-Carrillo L 2011 Phthalate exposure associated with self-reported diabetes among Mexican women. *Environmental Research* **111** 792–796. (doi:10.1016/j.envres.2011.05.015)

Talior-Volodarsky I, Randhawa VK, Zaid H & Klip A 2008 α-Actinin-4 is selectively required for insulin-induced GLUT4 translocation. *Journal of Biological Chemistry* **283** 25115–25123. (doi:10.1074/jbc.M801750200)

Thayer KA, Heindel JJ, Bucher JR & Gallo MA 2012 Role of environmental chemicals in diabetes and obesity: a National Toxicology Program workshop review. *Environmental Health Perspectives* **120** 779–789. (doi:10.1289/ehp.1104597)

Torlinska T, Mackowiak P, Nogowski L, Hryniewiecki T, Witmanowski H, Perz M, M dry E & Nowak KW 2000 Age dependent changes of insulin receptors in rat tissues. *Journal of Physiology and Pharmacology* **51** 871–881.

Trasande L, Spanier AJ, Sathyanarayana S, Attina TM & Blustein J 2013 Urinary phthalates and increased insulin resistance in adolescents. *Pediatrics* **132** e646–e655. (doi:10.1542/peds.2012-4022)

Valverde AM, Navarro P, Teruel T, Conejo R, Benito M & Lorenzo M 1999 Insulin and insulin-like growth factor I up-regulate *GLUT4* gene expression in fetal brown adipocytes, in a phosphoinositide 3-kinase-dependent manner. *Biochemical Journal* **337** 397–405. (doi:10.1042/0264-6021:3370397)

Watson RT, Kanzaki M & Pessin JE 2004 Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes. *Endocrine Reviews* **25** 177–204. (doi:10.1210/er.2003-0011)

Wu S, Zhu J, Li Y, Lin T, Gan L, Yuan X, Xu M & Wei G 2010*a* Dynamic effect of di-2-(ethylhexyl) phthalate on testicular toxicity: epigenetic changes and their impact on gene expression. *International Journal of Toxicology* **29** 193–200. (doi:10.1177/1091581809355488)

Wu S, Zhu J, Li Y, Lin T, Gan L, Yuan X, Xiong J, Liu X, Xu M, Zhao D *et al*. 2010*b* Dynamic epigenetic changes involved in testicular toxicity induced by di-2-(ethylhexyl) phthalate in mice. *Basic & Clinical Pharmacology & Toxicology* **106** 118–123. (doi:10.1111/j.1742-7843.2009.00483.x)

Yokomori N, Tawata M & Onaya T 1999 DNA demethylation during the differentiation of 3T3-L1 cells affects the expression of the mouse GLUT4 gene. *Diabetes* **48** 685–690. (doi:10.2337/diabetes.48.4.685)

Zhang CL, McKinsey TA & Olson EN 2002 Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation. *Molecular and Cellular Biology* **22** 7302–7312. (doi:10.1128/MCB.22.20.7302-7312.2002)

Zhao J, Goldberg J, Bremner JD & Vaccarino V 2012 Global DNA methylation is associated with insulin resistance: a monozygotic twin study. *Diabetes* **61** 542–546. (doi:10.2337/db11-1048)

Zimmet P, Alberti KG & Shaw J 2001 Global and societal implications of the diabetes epidemic. *Nature* **414** 782–787. (doi:10.1038/414782a)

Received in final form 30 July 2014
Accepted 8 August 2014



http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-14-0111



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