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 F1 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 IRS1Ser636/639, which impedes binding of downstream effectors and the negative regulator (PTEN) of PIP3, was increased in DEHP-exposed groups. Down-regulation of glucose transporter 4 (Glut4 (Slc2a4)) gene expression and increased GLUT4Ser488 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 F1 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

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
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 el al. 2009, Wu et al. 2010a, 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. 2010b, 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 F1 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 (Dosset et al. 1987, Latini 2000). Additionally, DEHP has been found to be
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 lifestyle (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 F₁ 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 F₁ 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 F₁ 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). 14C-glucose, 14C-2-deoxyglucose and iodine-125(1[125I]) 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...
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$_{260}$/A$_{280}$ 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 using BSA as a standard. INSRβ and pINSRβTyr1162/1163 levels were estimated for PM and GLUT4 and GLUT2 (SLC2A2) levels were estimated for both PM and cytosolic fractions. pGLUT4Ser488 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) and mature SREBP1c (SREBF1) were estimated using the MSP primer.

<table>
<thead>
<tr>
<th>Gene names</th>
<th>5'-Oligonucleotide</th>
<th>3'-Oligonucleotide</th>
<th>GenBank accession numbers</th>
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<td>Real-time PCR primers</td>
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<td>Dnmt1</td>
<td>CCAGATACTACCTCCGGTTATCG</td>
<td>TCCTTTAACGTGACGTAGGC</td>
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<td>Irs1</td>
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<td>ACACGGTTTGAGAGACAGAGG</td>
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<td>Akt</td>
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<td>AGTGGAAATCCAGTTCCAGAGTG</td>
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<td>Glut4</td>
<td>GGCGCTGTGAGTGGAGTCTCTT</td>
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<td>β-actin</td>
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<td>GCTCAGTAAACAGTCGGCTA</td>
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<td>MSP primer</td>
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<tr>
<td>Glut4 (methylated)</td>
<td>GATGGCTGTGAGTATTGTATCG</td>
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<td>ChiP assay primers</td>
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<td>Gapdh</td>
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<td>CACACCTTCAGGTGAGGATGTGGA-</td>
<td>X02231.1</td>
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</table>

Table 1  List of primers used in this study
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 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** 14C-2-deoxyglucose uptake in tissues was estimated by the method of Valverde et al. (1999). Results are expressed as c.p.m. of 14C-2-deoxyglucose taken up/10 mg tissue. 14C-glucose oxidation was estimated as per the standard method (Kraft & Johnson 1972). Results are expressed as c.p.m. of 14CO2 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 (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 MgCl2, 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; H2O 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).
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 MgCl2, 0.4 mM dNTPs and GoTaq 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 F1 offspring

Insulin binding was significantly decreased dose dependently in gastrocnemius muscle of F1 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 INSNR protein (Fig. 1D) and its 1162/1163 tyrosine phosphorylated forms (Fig. 1E) were reduced significantly compared with control levels in a
Table 2 List of metabolic parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean body weight (g)</td>
<td>Control: 104.1 ± 1.22 mg, DEHP 1 mg: 94.69 ± 1.20 mg, DEHP 10 mg: 90.99 ± 0.07 mg, DEHP 100 mg: 78.70 ± 0.07 mg</td>
<td>Male: 105.60 ± 1.56 mg, Female: 97.22 ± 1.22 mg</td>
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<tr>
<td>Fat weight (g)</td>
<td>Control: 10.19 ± 0.07 mg, DEHP 1 mg: 9.77 ± 0.07 mg, DEHP 10 mg: 9.59 ± 0.07 mg, DEHP 100 mg: 10.70 ± 0.01 mg</td>
<td>Male: 9.70 ± 0.01 mg, Female: 9.95 ± 0.07 mg</td>
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<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>Control: 81.16 ± 2.07 mg/dl, DEHP 1 mg: 94.33 ± 2.40 mg/dl, DEHP 10 mg: 104.05 ± 2.65 mg/dl, DEHP 100 mg: 115.66 ± 2.46 mg/dl</td>
<td>Male: 83.16 ± 2.24 mg/dl, Female: 83.81 ± 1.93 mg/dl</td>
</tr>
<tr>
<td>Insulin (mIU/ml)</td>
<td>Control: 8.8 267, DEHP 1 mg: 8.8* 237, DEHP 10 mg: 9.27* 200, DEHP 100 mg: 10.4* 223</td>
<td>Male: 8.8 255, Female: 10.4* 223</td>
</tr>
<tr>
<td>Glycogen (mg/g wet tissue)</td>
<td>Control: 11.5 ± 0.29 mg/g, DEHP 1 mg: 9.1 ± 0.22 mg/g, DEHP 10 mg: 10.49 ± 0.07 mg/g, DEHP 100 mg: 15.06 ± 0.24 mg/g</td>
<td>Male: 11.5 ± 0.29 mg/g, Female: 9.1 ± 0.29 mg/g</td>
</tr>
<tr>
<td>c.p.m. of 14C-deoxyglucose uptake/10 mg tissue</td>
<td>Control: 1160 ± 3.1 mg, DEHP 1 mg: 1600 ± 3.1 mg, DEHP 10 mg: 2166 ± 3.1 mg, DEHP 100 mg: 2540 ± 3.1 mg</td>
<td>Male: 1160 ± 3.1 mg, Female: 1600 ± 3.1 mg</td>
</tr>
<tr>
<td>In vitro insulin binding protein</td>
<td>Control: 1183 ± 4.4 fmol/mg protein, DEHP 1 mg: 941 ± 2.9 fmol/mg protein, DEHP 10 mg: 1045 ± 2.6 fmol/mg protein, DEHP 100 mg: 1266 ± 2.3 fmol/mg protein</td>
<td>Male: 1183 ± 4.4 fmol/mg protein, Female: 941 ± 2.9 fmol/mg protein</td>
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<tr>
<td>Glycogen (mg/g wet tissue)</td>
<td>Control: 11.5 ± 0.29 mg/g, DEHP 1 mg: 9.1 ± 0.22 mg/g, DEHP 10 mg: 10.49 ± 0.07 mg/g, DEHP 100 mg: 15.06 ± 0.24 mg/g</td>
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<tr>
<td>In vitro insulin binding protein</td>
<td>Control: 1183 ± 4.4 fmol/mg protein, DEHP 1 mg: 941 ± 2.9 fmol/mg protein, DEHP 10 mg: 1045 ± 2.6 fmol/mg protein, DEHP 100 mg: 1266 ± 2.3 fmol/mg protein</td>
<td>Male: 1183 ± 4.4 fmol/mg protein, Female: 941 ± 2.9 fmol/mg protein</td>
</tr>
</tbody>
</table>

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).

In utero DEHP exposure and insulin signalling

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In utero DEHP exposure

Table 2

P remarkably decreased the ARRB2 protein level in the 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 F1 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 MXR protein level was significantly decreased dose-dependently in the
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 INSRI protein at plasma membrane (D). β-actin was used as an internal control. pINSR\(^{\text{β}}\) Tyr1162/1163 was normalised to INSRI protein (E). Immunoreactive bands were detected with an ECL reagent in chemidocumentation using the Chemi Doc XR 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 DEHP kg per day; c, compared with 10 mg DEHP kg per day.

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 F1 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, pGLUT4Ser488 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

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
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.
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 were 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 DEHP/kg per day; c, compared with 10 mg DEHP/kg per day.

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
F1 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 glycolysis concentrations at PND60 of F1 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 PND90 (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 F1 offspring.

Subsequently, we measured the expression of important molecules involved in skeletal muscle insulin signalling, which showed an alteration at PND60 due to the influence of developmental DEHP exposure on these processes, 14C-2-deoxyglucose uptake and 14C-glucose oxidation were studied. DEHP-exposed male and female F1 offspring showed a significant dose-dependent decline in glucose uptake and oxidation (Table 2). This observation is in line with the decreased PM GLUT4.
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 Ser336/639, which impedes binding of downstream effectors, and the negative regulator (PTEN) of intracellular levels of PIP3 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

Figure 5
Effects of in utero DEHP exposure on AS160 (A), pAS160Thr642 (B), RABBA (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.
Effects of gestational DEHP exposure on Glut4 mRNA (A), cytosol GLUT4 protein (B) and pGLUT4Ser488 (C) levels in the gastrocnemius muscle of male (d) and female (f) 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.

Figure 6

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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 pAS160Thr642 levels in gestational DEHP-exposed F1 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 F1 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...
(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
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,
the Glut4 promoter in DEHP-exposed F1 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 GLUT4Ser488 was increased significantly in DEHP-exposed F1 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).
RAB8A and RAB13 were under the direct control of AS160 in muscle cells. Consistent with this, gestational DEHP-exposed F1 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
processes declines 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 Thr473, 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 F1 offspring to glucometabolic 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.

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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.

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